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CD300e as a novel biomarker in obesity: crosstalk between immune system and adipose tissue

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1. SUMMARY

Obesity is a multifactorial pathology associated with metabolic dysfunction, whose incidence has reached epidemic proportions in the last decades. Most obese patients (OPs) develop insulin resistance (IR) and have an increased risk of developing type 2 diabetes (T2D). IR is defined as decreased cellular response to insulin, which is a well-established precursor of T2D. Obesity and IR are closely associated with a state of chronic, low-grade inflammation. Recent studies have identified many cellular and molecular players that participate in the development of obesity-induced inflammation and IR, including adipose tissue macrophages (ATMs). OPs have been described with increased infiltration of pro-inflammatory ATMs, which have been identified as the primary source of many of the circulating inflammatory molecules (such as TNF-α, IL-6 and IL-1β) that are detected in the obese state and are postulated to be the main player of the systemic low-grade inflammation and thus the development of IR, leading to T2D.

Recently, it has been demonstrated that both Type 1 (T1D) and Type 2 (T2D) diabetes patients are seropositive for the self-antigen CD300e. CD300e, also called IREM-2 (Immune Receptor Expressed by Myeloid cells), is a monomeric 32 kDa glycosylated surface receptor expressed by myeloid cells (monocytes, macrophages, and peripheral blood myeloid dendritic cells). Since the ligand of CD300e is still unknown, its function in human cells has been studied by using an agonistic anti-CD300e monoclonal antibody (clone UP-H2).

Data obtained in our laboratory revealed that OPs were seropositive for CD300e and the anti-CD300e antibody titre in their sera declined after weight loss, following bariatric surgery. In addition, the decline of anti-CD300e titre after weight loss correlated positively with the improvement of insulin sensitivity. Published studies suggested that CD300e might participate to the obesity-induced IR. A study performed on a cohort of 49 monozygotic twin pairs, discordant for body mass index (BMI), revealed that the mRNA encoding CD300e was significantly upregulated in the adipose tissue (AT) of the heavier subjects than their leaner co-twins. Another research showed that, in the subcutaneous adipose tissue (SAT) of healthy adults with obesity, CD300e gene expression was significantly downregulated following surgery-induced weight loss. Lastly, it is noteworthy the *in vivo* observation that CD300e *knockout* mice showed a lower glycemia than their wild-type counterpart. CD300e engagement was reported to provide the cells with survival signals, to trigger the expression of activation markers and to induce the release of pro-inflammatory cytokines. Hence, CD300e was ascribed to the group of activating immune receptors. However, the finding that the engagement of

CD300e in human monocytes by the agonistic monoclonal antibody hampered the expansion of T cell-mediated responses raised doubts about the role of this molecule as immune activating receptor *sensu stricto.*

Given these premises, since the signalling pathway triggered by CD300e had not yet been investigated, the objective of the present project was to study the intracellular cascade initiated by CD300e and to evaluate the possible role of this immune receptor in glucose homeostasis, shedding light on whether it could impact the insulin pathway.

We chose monocytes purified from buffy coat of healthy donors as our first cell model. Indeed, although monocytes are not the primary target of insulin, they strongly express CD300e, and they also expose GLUT4 on the cell membrane after insulin stimulation. We revealed that the activation of CD300e hindered the insulin-stimulated phosphorylation of AKT, a serine/threonine protein kinase which plays a pivotal role in glucose uptake triggered by the hormone. In accordance, insulin-induced glucose internalization was hampered. These results support the notion that the receptor CD300e negatively affects the insulin pathway through the hindrance of AKT phosphorylation.

We found that the negative modulation of the insulin cascade triggered by CD300e engagement was recapitulated in cell models which are the physiological target of insulin, such as hepatocytes and skeletal muscle cells.

Subsequently, we revealed that circulating monocytes, which are precursors of tissue macrophages that abundantly infiltrate the AT of OPs, displayed a higher expression of the receptor in obese individuals if compared with monocytes from normal weight subjects. Interestingly, the surface expression of the receptor in circulating monocytes of OPs correlated positively with their BMI, supporting again our hypothesis that CD300e might take part in the systemic IR.

Aware that several immune receptors are released in the bloodstream in their soluble form after proteolytic cleavage of the surface ones, we wonder whether a soluble form of CD300e (sCD300e) may exist. We have analysed by ELISA the level of human CD300e in the sera of OPs before and after weight loss. Surprisingly, we found that the level of sCD300e was higher in normal weight subjects than in OPs, both before and after weight loss. We could speculate that the lower level of sCD300e in the sera of OPs mirrored the increased expression of the receptor on the cell membranes of their circulating monocytes, as we have previously found. We cannot confirm this hypothesis since the level of sCD300e and the surface expression of the receptor on circulating monocytes were not measured in the same healthy subjects.

Since human CD300e is still an orphan receptor, we tried to identify its possible ligand(s). It has been demonstrated that other members of the CD300 family recognize sphingolipids, and sphingomyelin (SM) was already shown to be the endogenous ligand of the CD300e orthologue in mice. Interestingly, adipocyte-derived extracellular vesicles (EVs), whose release increase during obesity, are enriched of SM, and the increased number of circulating EVs is correlated with the onset of obesity-related complications such as IR. Utilizing liposomes consisting of cholesterol and three distinct types of phospholipids, we have demonstrated that SM could potentially act as the ligand for human CD300e.

Collectively, our findings support the notion that the immune receptor CD300e might be a critical modulator of the insulin-mediated pathways, and its activation could be promoted by sphingolipids carried by EVs abundantly released by the AT under obesity. In this context, the immune receptor might become a target for therapies aimed at ameliorating the condition of subjects suffering from IR and T2D through counteracting its negative modulation on insulin-triggered cascade.

2. INTRODUCTION

2.1. Metabolic syndrome as an inflammatory disorder

Metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance (IR), visceral obesity, hypertension, hyperglycaemia, and dyslipidaemia (P. L. Huang, 2009). These conditions increase the risk of developing atherosclerotic cardiovascular diseases (CVD), non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes (T2D). T2D is considered a consequence of IR, which is a frequent metabolic condition that affects most obese patients (OPs). In IR, the loss of sensitivity to the hormone insulin affects the metabolism of specific target cells, primarily adipocytes, hepatocytes, and myocytes. The biological action of insulin to maintain glucose and lipid homeostasis is compromised, and this condition is initially compensated by over-activity of the pancreatic β cells. However, in the long-term such compensatory enhanced insulin secretion exhausts the β cells population, which ultimately ceases to function, affecting hormone-induced metabolic responses and glucose uptake in peripheral tissues (Guria et al., 2023). It is recognized that a chronic low-grade inflammation and an activation of the immune system are involved in the pathogenesis of obesity-related IR and T2D (Esser et al., 2014; Liu & Nikolajczyk, 2019). Recent studies have identified many cellular and molecular players that participate in the development of obesity-induced inflammation and IR (B.-C. Lee & Lee, 2014), although increased monocyte/macrophage activity appears to be pivotal in promoting inflammation. Lipid-laden hypertrophic adipocytes release various chemotactic signals that induce macrophage infiltration into AT and their skewing towards a pro-inflammatory profile (Guria et al., 2023). The primary sources of adipose tissue macrophages (ATMs) are circulating monocytes recruited from the blood into the AT during obesity (X. Li et al., 2023).

AT is the main site of lipid storage and is primarily involved in the modulation of lipid pathways to regulate systemic lipid homeostasis. Another important function of AT is to act as an endocrine organ that regulates the secretion of cytokines/chemokines, collectively called adipokines. The hormones produced by AT include leptin, adiponectin, resistin, and cytokines such as TNF-α and IL-6. Some of these adipokines can function as chemotactic signals for macrophages, including Monocyte Chemoattractant Protein-1 (MCP-1) and Stromal cell-derived factor 1 (SDF-1) that are extensively secreted by hypertrophic adipocytes. While AT is composed of many different cell types, including adipocytes and pre-adipocytes (adipocyte progenitors), endothelial cells, and immune cells, recent

studies have showed that the regulation of inflammatory mediators is mainly mediated by the stromal vascular fraction (SVF) that contains the immune cells, further supporting the role of the AT immune cells in the obesity-induced inflammation (Xu et al., 2003). During obesity, adipocytes first expand becoming hypertrophic to accumulate more lipids, but when adipose mass reaches the limit of expansion and cannot store more triglycerides, ectopic lipid accumulation occurs. Such lipid accumulation in non-adipose tissues gives rise to lipotoxicity and, consequently, to systemic IR (Santoro et al., 2021). Lipotoxicity is defined as the harmful effect of high concentrations of lipids and lipid derivatives in the cells of non-fatty tissues, causing alterations in their metabolism and/or apoptosis (Lipke et al., 2022). In recent years, ceramides, a subgroup of sphingolipids, have received increased attention for their involvement in numerous pathophysiologic mechanisms, including obesity, diabetes, CVD (atherosclerosis, heart failure) and hepatic steatosis (Field et al., 2020). Many studies have shown that plasma, AT, and skeletal muscle ceramides are elevated in patients with obesity and T2D (Kolak et al., 2007; Haus et al., 2009; Field et al., 2020). In addition, the increased bioavailability of free fatty acids (FFAs) in obese subjects induce an increase of ceramide production via *de novo* pathway (Torretta et al., 2019). In this context, a study by Holland et al. demonstrated that saturated fatty acids induce the biosynthesis of sphingolipids (i.e., ceramide) through the toll-like receptor 4 (TLR-4) pathway (Holland et al., 2011), demonstrating that the increased production and accumulation of sphingolipids during obesity is highly dependent upon inflammatory events controlled by TLR4.

2.2. Heterogeneity of adipose tissue macrophages in obesity

Macrophages represent a very heterogeneous population that are morphologically and functionally different in various tissues. Furthermore, even in the same tissue there are many different subpopulations of macrophages. Despite this diversity, they are traditionally classified into M1 and M2 phenotype. M1 macrophages are considered "classically activated" and are generated *in vitro* by treating bone marrow-derived macrophages (BMDMs) with LPS and IFN-γ; the resulting M1 cells are believed to be pro-inflammatory macrophages since they release factors such as TNF-α, IL-6, IL-1β and nitric oxide (NO). By contrast, M2 macrophages are "alternatively activated" and are differentiated by treating BMDMs with IL-4/IL-13 or IL-10; they secrete anti-inflammatory cytokines e.g., IL-4, IL-10, IL-13, and TGF-β; they are also involved in tissue remodelling and wound healing

through the production of growth factors (VEGF, PDGF, EGF), matrix metalloproteinases (MMPs) and arginase 1 (K. Y. Lee, 2019) (Figure 1).
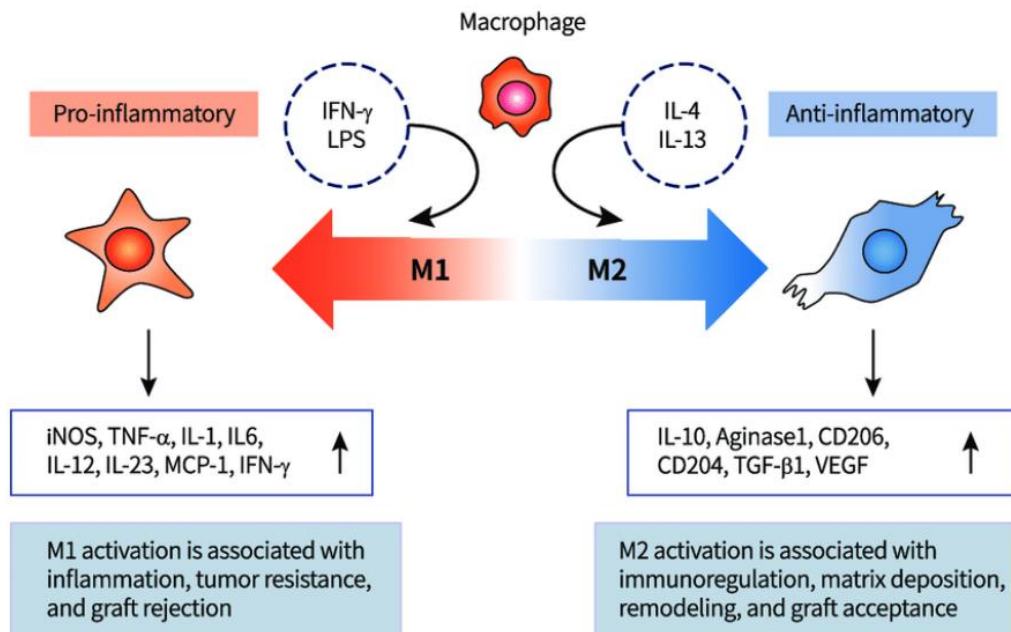

Figure 1. Pro-inflammatory M1 and anti-inflammatory M2 polarization of macrophages (K. Y. Lee, 2019).

Many studies agree that obesity induces the metabolic reprogramming of infiltrating AT macrophages (ATMs), consequently inducing their polarization from M2 to M1 phenotype. In this context, Zhou et al. have recently demonstrated that the oscillation of mitochondrial Ca^{2+} levels is directly involved in promoting the polarization of pro-inflammatory M1 macrophages in AT through connexin 43, a ubiquitous gap junction protein able to interact with mitochondrial Ca^{2+} uniporter (MCU), consequently mediating mitochondrial Ca^{2+} overload, which resulted in over-production of ROS (Zhou et al., 2023). Macrophages use different pathways to meet energy demands depending upon their phenotypes and functional needs. In M1-like subtypes, aerobic glycolysis is the principal source for energy production. The tricarboxylic acid (TCA) cycle is consequently inhibited at different steps owing to down-regulation of specific enzymes, resulting in greater accumulation of TCA cycle intermediates, primarily citrate and succinate. The accumulated succinate promotes the stabilization of hypoxia-inducible factor 1α (HIF-1α), resulting in enhanced expression of pro-inflammatory genes. On the other hand, accumulated mitochondrial citrate is exported to the cytosol and cleaved into acetyl-CoA and oxaloacetate, the latter used to generate pyruvate along with NADPH that serves as cofactor for inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX) to produce nitric oxide

(NO) and ROS, respectively. Moreover, increased fatty acids uptake and triglycerides biosynthesis are observed in M1 macrophages, explaining the great infiltration of this cell subtype in the AT over-loaded with lipids. Conversely, in M2-like macrophages the main source of energy is the mitochondrial respiration (TCA along with oxidative phosphorylation) and fatty acid β-oxidation (Guria et al., 2023).

2.3.   CD300 immune receptor family

CD300, originally termed "Immune Receptor Expressed by Myeloid cells" (IREM), represents a multigenic family of leukocyte surface glycoproteins belonging to the Immunoglobulin (Ig) superfamily, which is conserved in different mammalian species. The human CD300 gene cluster is located on chromosome 17 (17q25.1) and includes eight members, named alphabetically according to the order of their location in the chromosome, which modulate a broad array of immune cell processes via their paired activating and inhibitory functions (Borrego, 2013). CD300 inhibitory receptors (i.e., CD300a and CD300f) have a long cytoplasmic tail characterized by the presence of an ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif) domain. Ligand-receptor complex formation results in tyrosine phosphorylation of ITIMs that function as docking sites for phosphatases such as SHP-1/2 and SHIP. Upon recruitment, tyrosine phosphatases become activated and dephosphorylate key signalling mediators of activating pathways, consequently downregulating the intracellular signalling cascade. By contrast, immune activating receptors have a short cytoplasmic domain lacking ITIM but containing a positively charged amino acid residue in the transmembrane region. This residue is involved in the association with adaptor transmembrane proteins bearing an ITAM (Immunoreceptor Tyrosine-based Activating Motif) domain, such as CD3ζ, FcεRIγ, DAP10 and DAP12. Receptor engagement promotes tyrosine phosphorylation of ITAM-bearing molecules and the recruitment and activation of protein kinases, such as Syk and Zap-70, to propagate the downstream activating signals (Aguilar et al., 2004) and stimulating a series of intracellular events inducing cell differentiation, growth and survival, adhesion, migration, phagocytosis, cytokine production and/or cytotoxicity (Figure 2). All CD300 members were found to interact with each other, forming both homo- and heterodimers. The combination of CD300 receptors in a complex differentially modulates the signalling outcome, and this distinctiveness of CD300 receptors may represent a mechanism by which myeloid cells are able to precisely control immune responses in terms of intensity and duration (Martínez-Barriocanal et al., 2010).

Elucidating the function of these receptors has been complicated by difficulties in developing specific monoclonal antibodies (mAb) for each of the CD300 molecules. Firstly, the amino acid sequences of the Ig domain of each family member share significant sequence similarity, and some of the mAb generated bind more than one family member. Secondly, the ability of CD300 molecules to form a variety of homo- and hetero-dimers on the cell surface adds a further potential layer of complexity (Gasiorowski et al., 2013).

Several groups have used CD300-Fc chimeric proteins as a tool to search for the ligand of the CD300 molecules. Most of the members of the CD300 family are able to recognize lipids, such as extracellular ceramide, phosphatidylserine, and phosphatidylethanolamine, that are exposed on the outer leaflet of the plasma membrane of dead and activated cells (Choi et al., 2011; Izawa et al., 2012; Nakahashi-Oda et al., 2012; Simhadri et al., 2012a).
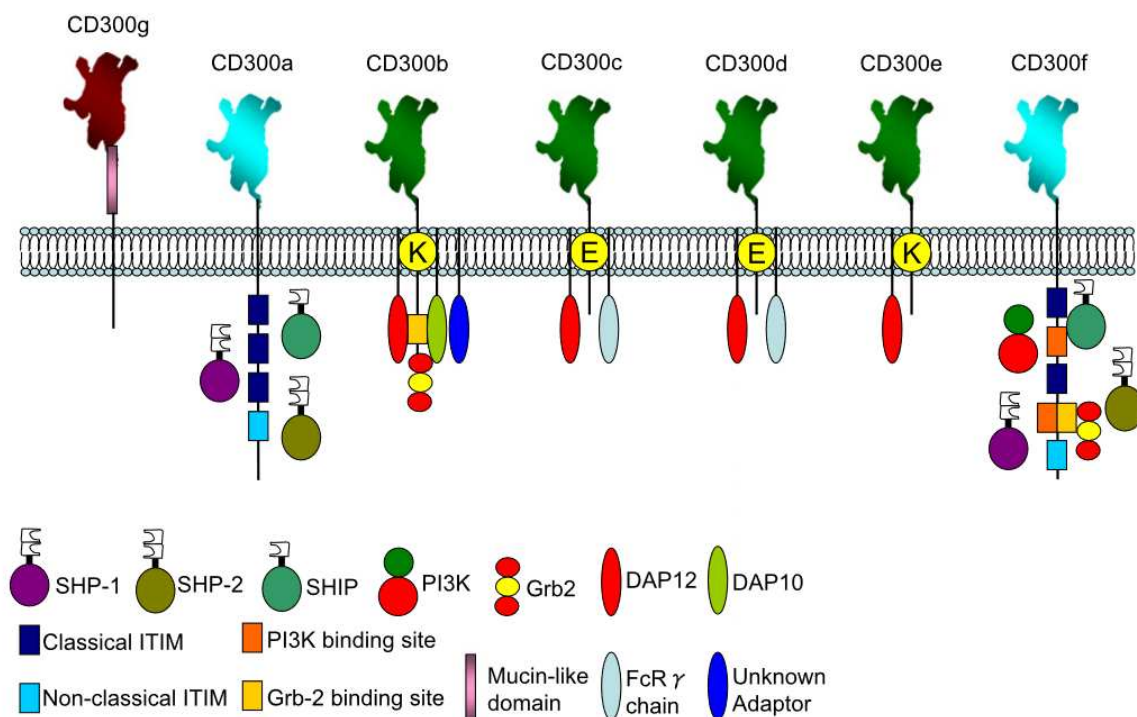
Figure 2. Schematic representation of the CD300 receptors in human. The extracellular domain of the ITIM-containing receptors is coloured in turquoise, and that of the non–ITIM containing receptors is coloured in green. The extracellular domain of CD300g is coloured in brown. The signalling motifs of each receptor are indicated, and the interaction with phosphatases (SHP-1, SHP-2, SHIP), adaptor molecules (DAP10, DAP12, Grb2 and FcRγ chain), and kinases (PI3K) is shown (Borrego, 2013).

2.4. The immune receptor CD300e

CD300e is a monomeric 32 kDa glycosylated surface receptor with a single extracellular Ig-like domain, a transmembrane lysine residue allowing the receptor to associate with the adaptor protein DAP12 and a short cytoplasmic tail. It is a type I transmembrane glycoprotein, and the transmembrane domain shows an α-helix structure. The extracellular domain has an unstructured region near the cell membrane and a structured β-sheet region more distal (*CD300E - CMRF35-like molecule 2 - Homo sapiens (Human) | UniProtKB | UniProt*, s.d.) (Figure 3). CD300e is expressed on the cell surface of monocytes/macrophages and circulating myeloid dendritic cells (mDCs) *in vivo*. CD300e engagement was reported to provide the cells with survival signals, to trigger the expression of activation markers and to induce the release of pro-inflammatory cytokines. Hence, CD300e was ascribed to the group of activating immune receptors (Brckalo et al., 2010). In accordance, CD300e was shown to associate with the ITAM-bearing adaptor protein DAP12 in COS-7 transfected cells (Aguilar et al., 2004). However, a recent study highlighted that the ligation of CD300e by an agonistic monoclonal antibody (clone UP-H2) in human monocytes hampered the expression of the human leukocyte antigen (HLA) class II, thereby affecting its synthesis. Importantly, the decreased expression of HLA-II on the surface of CD300e-activated monocytes negatively impacted their capacity to activate T cells in an antigen-specific manner (Coletta et al., 2020). These data raised doubts about the role of this molecule as immune activating receptor *sensu stricto*. This opens the intriguing possibility that CD300e, although displaying characteristics of an activating receptor, might also elicit an inhibitory signalling cascade.
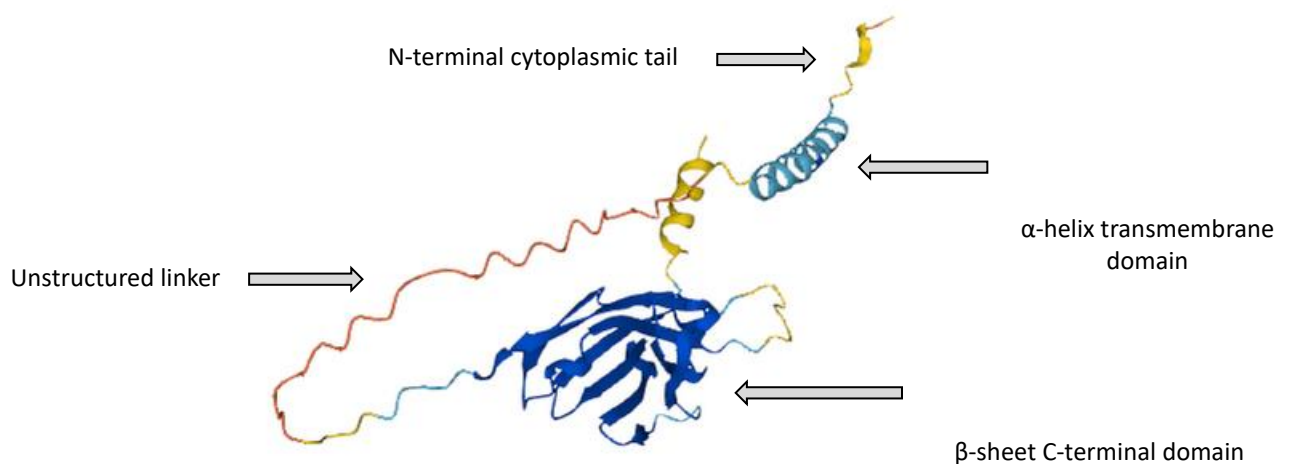

N-terminal cytoplasmic tail

α-helix transmembrane domain

Unstructured linker

β-sheet C-terminal domain

Figure 3. Predicted three-dimensional structure of CD300e. The short cytoplasmic tail is in the N-terminal region (coloured in yellow), while the transmembrane domain shows an α-helix structure (in light blue). The extracellular region shows an unstructured portion and a structured β-sheet region in the C-terminal domain (in dark blue) (*CD300E - CMRF35-like molecule 2 - Homo sapiens (Human) | UniProtKB | UniProt*, s.d.).

In mouse, CD300e is expressed only in non-classical (CD14^{low}CD16$^+$) and intermediate (CD14$^+$CD16$^+$) monocytes, while the human counterpart is expressed in all subsets of human monocytes, including the classical one (CD14$^+$CD16$^-$). However, intermediate and non-classical monocytes exhibit slightly higher expression levels (Zenarruzabeitia et al., 2016). I*n vitro* differentiation of monocytes to macrophages is accompanied by the down-regulation of CD300e expression (Aguilar et al., 2004). However, recently it has been reported that the expression of the immune receptor in monocyte-derived macrophages can be rescued by the down-modulation of miR-4270 (Pagliari et al., 2017). Furthermore, immunohistochemical analysis demonstrated CD300e expression in tissue macrophages, albeit with varying receptor positivity among macrophages depending on the tissue type. Noteworthy, the immune receptor CD300e has been shown to be expressed by macrophages infiltrating the AT of patients with obesity (Coletta et al., 2020) and by monocyte-derived macrophages from HIV patients, which contributed to increased ROS production and oxidative stress in these subjects (Bowman et al., 2020). A study performed on a cohort of 49 monozygotic twin pairs, discordant for body mass index (BMI), revealed that the mRNA encoding CD300e was significantly upregulated in AT of the heavier subjects than their co-twin (van der Kolk, Saari, et al., 2021) and its expression was significantly downregulated after weight loss (van der Kolk, Muniandy, et al., 2021).

To date, the endogenous ligand of human CD300e is not yet known, even though Isobe et al. demonstrated that sphingomyelin (SM) is a ligand of the CD300e ortholog in mice (Isobe et al., 2018). Since the ligand of CD300e is still unknown, its function in human cells has been studied by using an agonistic anti-CD300e monoclonal antibody (clone UP-H2) (Aguilar et al., 2004).

2.5.   Insulin signalling pathway

Insulin is an anabolic peptide hormone secreted by pancreatic β cells, which controls a myriad of cellular and metabolic events, including nutrients transport into cells, cellular growth and differentiation, transcription of metabolic genes, vesicle trafficking and its own clearance. Insulin

receptor is a glycosylated, disulphide-linked $\alpha_2\beta_2$ tetramer belonging to the subfamily of receptor tyrosine kinases (RTKs). β-subunits cross the membrane through a helical structure, while the hormone binds to the α-subunits. As a result of insulin binding, the tyrosine kinase activity of the β-subunits is activated, and one subunit phosphorylates the other, causing a conformational change that further increases the kinase activity, leading to the activation of downstream intracellular substrates. Among the direct substrates of the insulin receptor there are insulin/IGF-1 receptor substrate (IRS) protein family, Gab-1, DOK-1, SH2B2, SHP2, and the various isoforms of SHC family of adaptor proteins (Saltiel, 2021). IRS family consists of six proteins (IRS1-6), among which IRS1 and IRS2 are crucial in insulin signalling transduction. It seems that IRS1 plays the main role in myocytes and adipose tissue, while IRS2 is a key player in hepatocytes and pancreatic islets. Their regulation occurs mainly by multiple serine and tyrosine residues, which may be phosphorylated by different kinases. Generally, the phosphorylation of serine residues inhibits insulin signalling by blocking tyrosine phosphorylation, which is necessary for signal transduction (Świderska et al., 2018). Activated IRS proteins can trigger two major signalling pathways, the first one is the Ras/MAPK pathway, which is involved in the expression of genes regulating cell survival, growth, proliferation, and differentiation; the second one is the PI3K/AKT pathway, which is responsible for the metabolic actions of insulin. Considering the latter, the tyrosine phosphorylated IRS proteins activate phosphoinositide 3-kinase (PI3K) through the phosphorylation of the src homology 2 (SH2) domain of its regulatory subunit p85. The subsequent activation of the catalytic subunit of PI3K, p110, leads to the cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to form phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$). PIP$_3$ recruits the serine/threonine kinase phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane, which in turn phosphorylates AKT (also named PKB) on the threonine 308 (Thr308) in the activation loop of the kinase domain. To maintain AKT in its active conformation, a secondary phosphorylation on serine 473 (Ser473) within the regulatory domain is necessary. This process is facilitated by mTOR complex 2 (mTORC2), although the precise regulatory mechanism remains unclear. AKT activation is terminated through the action of protein phosphatase 2 (PP2) and PH domain leucine rich repeat phosphatase (PHLRRP), which dephosphorylate Thr308 and Ser473, respectively (Świderska et al., 2018). In mammals, there are three AKT isoforms with different expression patterns: AKT1 is found everywhere in the body, AKT2 is primarily in insulin-sensitive tissues like muscle, fat, and liver, while AKT3 is mainly in the testes and brain. AKT regulates many cellular processes, including cell survival, cell cycle progression, apoptosis, and metabolism. Considering the latter, glucose and lipid

metabolism must be finely tuned to maintain energy homeostasis in normal physiology (Figure 4). By contrast, the imbalance of PI3K/AKT pathway in various insulin-sensitive tissues leads to hyperglycaemia and insulin resistance, pivotal conditions for the development of obesity and T2D.

2.6.   Insulin action on its target tissues

Skeletal muscle is responsible for about 90% of glucose homeostasis by promoting glucose transport and glycogen synthesis. AKT directly phosphorylates AKT substrate of 160 kDa (AS160), which is a Rab GAP (GTPase-activating protein) required for the translocation of the vesicles containing the glucose transporter type 4 (GLUT4) to the plasma membrane to allow glucose internalization by the cells. Indeed, AS160 phosphorylation via AKT relieves its inhibitory effect on targets Rab8 and Rab14 in muscle cells, triggering GLUT4 exocytosis in response to insulin (Saltiel, 2021). Moreover, in skeletal muscle AKT stimulates glycogen synthesis since it directly inhibits through phosphorylation glycogen synthase kinase 3 (GSK3), whose activity consists of inhibiting the glycogen synthase (GS). In this context, insulin acts by stimulating glycogen synthesis through the activation of AKT and consequently the activity of GS.

In adipose tissue, insulin regulates the utilization of about 10% of glucose and *de novo* lipogenesis from excessive fatty acids. In normal physiology, activated AKT stimulates mTORC1 to induce sterol regulatory element-binding proteins (SREBPs) transcription, which in turn promotes the expression of fatty acid synthase and acetyl-CoA carboxylase, the two major enzymes of lipogenesis. FoxO proteins regulate lipolysis and fatty acids β-oxidation by inducing the expression of the rate-limiting lipolytic enzyme adipose triglyceride lipase (ATGL). FoxO proteins are other targets of AKT, resulting in insulin-mediated inhibition of lipolysis (X. Huang et al., 2018).

In the obesity condition, the expansion of adipose tissue exceeds its storage capacity, which causes lipotoxicity, a type of cellular stress induced by the accumulation of lipid intermediates and leads to reduced oxygen supply. This condition triggers the infiltration of immune cells and the activation of stress-induced kinases, responsible for PI3K/AKT pathway impairment. Hypertrophic adipocytes also begin to secrete pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, which hamper AKT-mediated inhibition of lipolysis and activation of lipid synthesis, resulting in defective response to insulin (da Silva Rosa et al., 2020; Świderska et al., 2018).

The liver plays a pivotal role to control glucose homeostasis through a coordinated regulation between *de novo* glucose production (gluconeogenesis) and glycogen breakdown (glycogenolysis).

As in the skeletal muscle, also in the liver AKT triggers the phosphorylation and inhibition of GSK3, which leads to the stimulation of glycogen synthesis (Świderska et al., 2018). In humans, the main substrates of gluconeogenesis are lactate, glycerol, alanine, glutamine, and citric cycle intermediates, from which pyruvate can be generated to start the formation of glucose. It is widely accepted that insulin inhibits gluconeogenesis by modulating the transcription of two key hepatic genes involved in this process, phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6P), the first and the last enzymes of the pathway, respectively. FoxO proteins, along with peroxisome proliferator-activated receptor-coactivator 1α (PGC1α), concurrently induce the expression of PEPCK and G6PC. Insulin-mediated AKT activation directly inhibits FoxO proteins through the phosphorylation of three conserved residues, leading to their nuclear export following the binding to 14-3-3 proteins, with subsequent inhibition of gluconeogenic gene expression (Hatting et al., 2018). Insulin can also regulate PGC1α activity; indeed, AKT directly phosphorylates CDC-like kinase 2 (CLK2), a protein kinase which in turn phosphorylates PGC1α, impairing its ability to function as transcriptional coactivator of FoxO proteins. Along with direct control of gluconeogenesis by transcriptional regulation of hepatic enzymes, insulin also affects the process indirectly by acting on other tissues, firstly by reducing gluconeogenic substrates release from adipose tissue and skeletal muscle. Since insulin has inhibitory effects on lipolysis and proteolysis, plasma levels of free fatty acids derived from the adipose tissue and amino acids released from the skeletal muscle decrease, with subsequent reduction of their availability for the liver. In the liver, insulin also regulates *de novo* lipogenesis, the conversion of excess sugar into fatty acids that are then esterified to storage triacylglycerols (TGs). These TGs could later provide energy via β-oxidation. This process occurs through the activation of AKT/mTORC1/SREBP axis (da Silva Rosa et al., 2020).

In a chronic over-nutrition state, even though activation of SREBP should be hampered by insulin pathway dysregulation, *de novo* lipogenesis is increased since mTORC1 activation is mediated by other inputs such as amino acids (S. Li et al., 2010). Intrahepatic lipid accumulation triggers the activation of DAG/PKC pathway, impairing insulin signalling. As a result, gluconeogenesis is increased, further fueling the hyperglycemia condition.
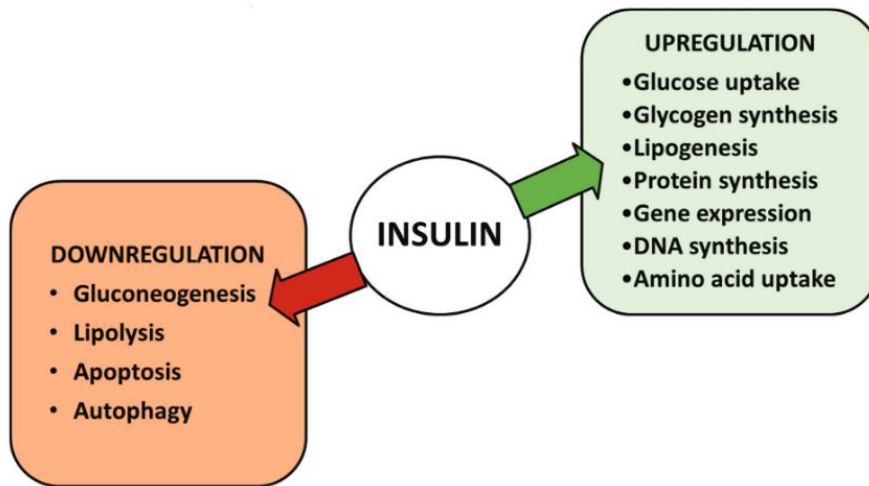
Figure 4. Critical cellular events and metabolic pathways controlled by insulin. In general, it promotes cellular events leading to energy storage and represses processes of energy release (Świderska et al., 2018).

2.7.   Adipocyte-derived extracellular vesicles in obesity

Adipose tissue (AT) is not only an energy storage organ, but it also performs endocrine functions that regulates the metabolic homeostasis of other tissues throughout the body. The pleiotropic effect of AT relies on its ability to synthesize and release many hormones, cytokines, extracellular matrix proteins and growth factors, collectively termed adipokines, that influence a variety of physiological and pathological processes. In the obese state, excessive lipids accumulation causes adipocyte hypertrophy and hyperplasia, leading to severe AT dysfunctionality and consequently changes in the secretion profile of adipokines. Specifically, pro-inflammatory cytokines are upregulated and anti-inflammatory adipokines are downregulated (Unamuno et al., 2018). This scenario can lead to the onset of obesity-related complications such as IR and T2D (Figure 5). In addition to adipokines, adipocyte-derived extracellular vesicles (EVs) have been found to play important roles in metabolic disorders (Mei et al., 2022). Adipocytes secrete two different types of EVs, namely small EVs (sEVs) and large EVs (lEVs). The first ones range from 30 to 100 nm in size, and they are endosome-derived vesicles released after they fuse with the plasma membrane. The second ones range from 100 nm to 1 µm in size and they are shed from plasma membrane due to cytoskeleton reorganization. The EVs are transported through various biofluids, carrying a broad array of biological cargoes including mRNAs, long non-coding RNAs, microRNA, DNA fragments, lipids and proteins, hence mediating intercellular communication (Kwan et al., 2021). Many studies have highlighted that the release of adipocyte-derived EVs is increased during obesity. In addition, obesity alters the type of molecules

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carried by EVs, which probably contribute to the onset of obesity-related metabolic disorders, including inflammation, IR and T2D (Dang et al., 2019; Eguchi et al., 2016).
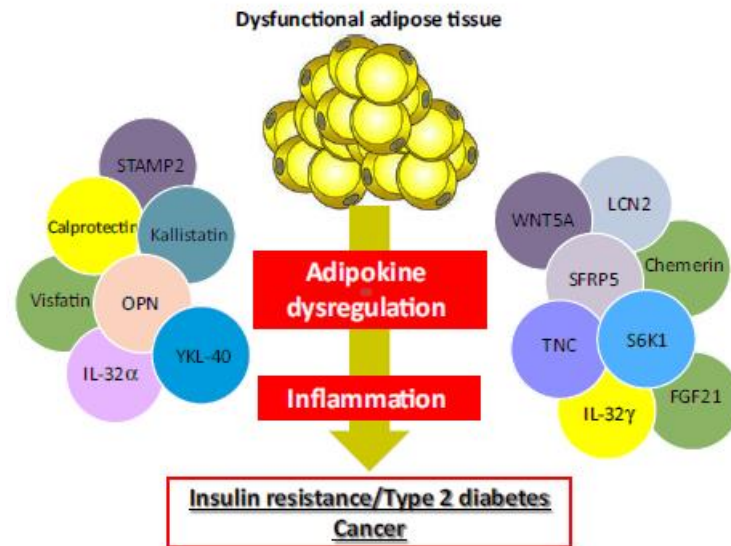

Figure 5. Dysregulated adipokine profile during obesity. In the obese state, adipocytes turn hypertrophic and hyperplastic since they are overloaded with lipids and become severely dysfunctional. Consequently, the secretion of pro-inflammatory cytokines (such as Calprotectin, Kallistatin, Visfatin, Osteopontin, IL-32α) is upregulated, while the release of anti-inflammatory adipokines (e.g., Lipocalin 2, WNT-5A, SFRP5, Chemerin, Tenascin C, IL-32γ and FGF-21) is downregulated (Unamuno et al., 2018).

Considering the membrane lipids composition of EVs of endosomal origin secreted by cells, they are enriched in cholesterol, sphingomyelin, ceramide and phosphatidylserine (Kowal et al., 2014). Durcin et al. identified a specific signature for large and small EVs able to distinguish these two subpopulations (Durcin et al., 2017). In this study, phospholipids represent the sum of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Sphingolipids include ceramides (Cer), glycosylceramides (GlyCer) and sphingomyelin (SM). Glycerolipids are uniquely represented by diacylglycerols (DGs) (Figure 6).

The biosynthesis of sphingomyelin is catalyzed by the enzyme sphingomyelinase, and it involves the transfer of phosphorylcholine from phosphatidylcholine to ceramide, with the formation of diacylglycerol. Ceramide was proposed to induce inward curvature inside an intracellular endosome, leading to the formation of a multivesicular body (MVB), which could then fuse with the plasma

membrane and release outside its internal vesicles (Kowal et al., 2014). Sphingomyelin is a building block of membranes, forming a stable and chemically resistant lipid bilayer. Along with other sphingolipids, it can interact with cholesterol and proteins through hydrogen bonds and strong van der Waals interactions. Blandin et al. found out considerable increases in phosphatidylcholine (PC), lysoPC species (LPC), sphingomyelin (SM), ceramide (Cer), as well as cholesterol esters and free cholesterol (FC) in ob/ob and high-fat diet (HFD) obese mice plasma compared with their lean respective controls (Blandin et al., 2023). Many studies agree that increased ceramide and sphingomyelin correlate with pathogenesis of metabolic diseases, including obesity, insulin resistance, hepatic steatosis, type 2 diabetes, non-alcoholic steatohepatitis (NASH) and cardiovascular diseases (Green et al., 2021). Based on this evidence, it is plausible that sphingolipids associated to adipocyte-derived EVs might be considered crucial mediators for adipose tissue remodelling and inflammation, and for intercellular communication correlated with the onset of obesity-related dysmetabolic diseases.
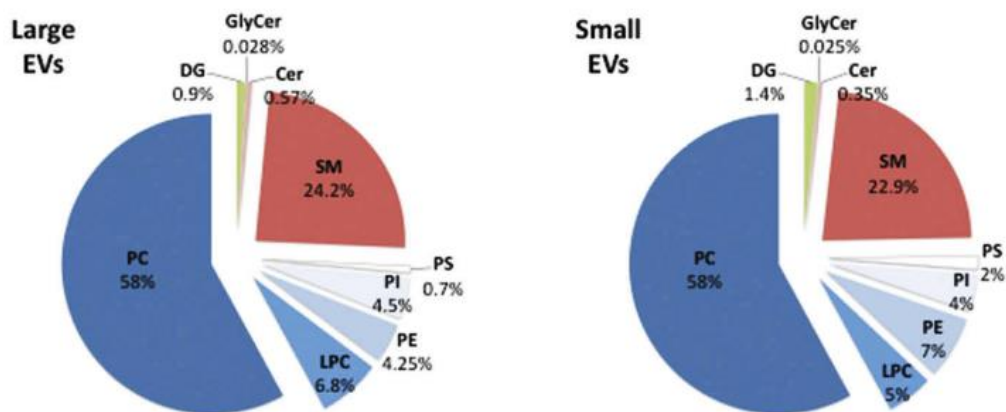
Figure 6. Composition of large EV and small EV membrane lipids according to lipid classes in a non-obese condition. Data are expressed as a percentage of the total lipid content (Durcin et al., 2017).

3. MATERIALS and METHODS

3.1. Monocytes isolation

Monocytes were purified from buffy coats derived from healthy donors, kindly provided by the blood transfusion centre of the University Hospital of Padova. The bag of blood, containing about

45 mL, is divided into 3 aliquots of equal volume, then made up to volume of 40 mL with sterile PBS 1x without Ca^{2+} and Mg^{2+}. After adding 10 ml of 5% dextran solution (Sigma-Aldrich), each aliquot was mixed by inversion and allowed to settle for 30 minutes with the cap partially open to allow the precipitation of erythrocytes. Indeed, dextran is a branched polymer of glucose which induces the aggregation of red blood cells and their consequent selective sedimentation. The supernatant containing white blood cells was collected and centrifuge at 100 g for 10 minutes. Cells pellet was resuspended in 30 ml of sterile PBS 1x without Ca^{2+} and Mg^{2+}, stratified on the same volume of Ficoll-Paque (GE Healthcare) and centrifuged for 20 minutes at 400 g without brake and accelerator. Lymphocytes and monocytes were harvested and centrifuged for 5 minutes at 300 g. Cell pellet was resuspended in RPMI 1640 supplemented with 10% FBS (v/v, Sigma-Aldrich) and then was stratified on Percoll (GE Healthcare) gradient (15.71 ml RPMI 1640 with 10% FBS + 15.54 ml of 7.5% sterile PBS 10x in Percoll) and centrifuged 20 minutes at 400 g without brake and accelerator. Percoll solution is composed of colloidal silica nanoparticles coated with polyvinylpyrrolidone and with different sizes, thus having different sedimentation coefficients. The density gradient centrifugation allows the separation of lymphocytes, which sediment, from monocytes, which remain in the interphase to form a ring between the medium and the underlying medium. Monocytes were collected and centrifuged 5 minutes at 300 g, then the cells were resuspended in RPMI 1640, 10% FBS, 4 mM HEPES (Gibco) and 50 µg/ml gentamycin (Gibco). Cell count was performed using a Burker chamber (Blaubrand®). The purity of monocytes was assessed by the flow cytometer BD LSRFortessa X-20 (Becton Dickinson) and it was approximately 40-50% compared to the percentage of lymphocytes. The desired number of monocytes was sorted to proceed with subsequent treatments.

3.2. Treatment of monocytes in suspension

To evaluate the impact of the activation through CD300e, a total of $1x10^6$ freshly isolated monocytes were used for each condition. Cells were aliquoted in 1.5 ml tubes, pelleted by centrifugation at 300 g for 5 minutes and resuspended in 30 µl sterile PBS 1x containing 1 µg mouse monoclonal anti-CD300e antibody (clone UP-H2, Abcam) or the same amount of isotype-matched control (IgG1, clone MOPC-21, Millipore), with or without the addition of 100 nM human insulin solution (Sigma-Aldrich). Cells were incubated at 37°C for 30 sec, 1 min, 5 min, 10 min, 15 min, 30 min, to allow the activation of the intracellular signalling. After the incubation time, each tube containing monocytes was placed on ice and 1 ml cold PBS 1x was added. A centrifuge at 17000 g for 10 minutes was performed,

supernatant was discarded, and each pellet was frozen in liquid nitrogen before being stored at -80°C until its use for subsequent experiments.

3.3. Cell lines

The cell lines HepG2 (human hepatocellular carcinoma) and HEK-293 (human embryonic kidney cells) were cultured in DMEM supplemented with 10% FBS, 10 mM HEPES, 10,000 Units/ml penicillin and 10 mg/ml streptomycin (Microgem) at 37°C in a 5% CO_2 atmosphere.

hTERT/cdk4 immortalized myogenic human cell lines LHLMN2 and 1190 (kindly provided by Professor Vincent Mouly, Center of Research in Myology of the Pitié-Salpétrière hospital in Paris) were grown in Ham's F-12 Nutrient Mix (Gibco) with the addition of 10% FBS, 5 ng/ml hEGF, 0.5 ng/ml hFGF, 5 μg/ml insulin, 0.2 μg/ml dexamethasone, 10,000 Units/ml penicillin and 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were used at 80-90% confluency for subsequent experiments.

3.4. Treatment of cell lines in suspension

HepG2 cells and 1190 myoblasts were treated as previously described for freshly isolated monocytes. Briefly, cells in cultures were detached with 0.25% trypsin (Corning), counted with the LUNA-II™ automated cell counter (Labtech) and a total of 1×10^6 cells for each condition were aliquoted in 1.5 ml tubes. Cells were pelleted by centrifugation at 300 g for 5 minutes and resuspended in 30 μl sterile PBS 1x containing 1 μg mouse monoclonal anti-CD300e antibody (clone UP-H2, Abcam) or the same amount of isotype-matched control (IgG1, clone MOPC-21, Millipore), with or without the addition of 100 nM insulin solution human (Sigma-Aldrich). Cells were incubated at 37°C for 30 sec, 1 min, 5 min, 10 min, 15 min, 30 min, to allow the activation of the intracellular signalling. After the incubation time, each tube containing stimulated cells was placed on ice and 1 ml cold PBS 1x was added. A centrifuge at 17000 g for 10 minutes was performed, supernatant was discarded, and each pellet was frozen in liquid nitrogen before being stored at -80°C until its use for subsequent experiments.

3.5. SDS-PAGE and western blot

Monocytes and cell lines were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, NP-40 1%, Na-deoxycholate 0.5%, SDS 0.1%, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 2% PMSF) with protease inhibitor cocktail (Millipore), and proteins were quantified by BCA protein assay kit (ThermoFisher), according to the manufacturer's instructions. Thirty μg of proteins from each sample were resuspended in NuPAGE LDS sample buffer (ThermoFisher) supplemented with 50 mM DTT and denaturated for 5 minutes at 100°C. Proteins were separated electrophoretically in NuPAGE Bis-Tris 4-12% polyacrylamide gel (ThermoFisher), and subsequently transferred on nitrocellulose membrane, 0.45 μm (ThermoFisher). Membranes were blocked for 1 hour with 5% non-fat dry milk in Tris-buffered saline (TBS, 50 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 0.1% Tween 20 (Sigma-Aldrich), and antigens were revealed using the following antibodies: rabbit anti-phospho-AKT (Ser473) monoclonal antibody (1:1000, clone D9E, Cell Signaling Technology), rabbit anti-AKT monoclonal antibody (1:1000, clone 11E7, Cell Signaling Technology), rabbit anti-phospho-SHP1 (Tyr536) polyclonal antibody (1:1000, ECM Biosciences), rabbit anti-SHP1 polyclonal antibody (1:1000, Merck Millipore), rabbit anti-DAP12 monoclonal antibody (1:1000, clone D7G1X, Cell Signaling Technology), rabbit anti-CD300e polyclonal antibody (1:200, Sigma-Aldrich), mouse anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) monoclonal antibody (1:2000, clone E10, Cell Signaling Technology), rabbit anti-p44/42 MAPK (Erk1/2) polyclonal antibody (1:1000, Cell Signaling Technology), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) polyclonal antibody (1:1000, Cell Signaling Technology), rabbit anti-p38 MAPK polyclonal antibody (1:1000, Cell Signaling Technology), rabbit anti-vinculin monoclonal antibody (1:500, clone 42H89L44, Invitrogen), mouse anti-β-actin monoclonal antibody (1:10000, Sigma-Aldrich). Blots were washed three times with TBS plus 0.1% Tween 20 and incubated for 1 hour at RT with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Merck Millipore) or horseradish peroxidase-conjugated donkey anti-mouse IgG secondary antibody (Southern Biotech). Membranes were developed with extra sensitive chemiluminescent substrate (LiteAblot® TURBO, EuroClone), and the protein bands were detected using the digital imaging system ImageQuant LAS 4000 mini (GE Healthcare Life Science). The ImageJ software (version 1.8.0) was used to evaluate quantitatively intensity's differences of the bands, in comparison to the house-keeping protein, by performing a densitometric analysis.

3.6.   Immunoprecipitation

Immunoprecipitation of proteins was performed by using Dynabeads Protein G (ThermoFisher) according to the manufacturer's protocol. Briefly, 50 µl of Dynabeads magnetic beads were transferred to a tube, which was placed on a magnet to separate the beads from the solution and the supernatant was removed. To bind antibody to the beads, 2.5 µg of monoclonal anti-CD300e antibody (clone UP-H2) or the same quantity of isotype-matched control (clone MOPC-21) diluted in 200 µl PBS with 0.02% Tween 20 were added to the beads and incubated with rotation for 2 hours at 4°C. To avoid co-elution of antibodies, covalent crosslinking of antibodies to the beads was performed by using the membrane-insoluble reagent BS^3 (ThermoFisher). According to the protocol, the antibody bound Dynabeads were resuspended in 250 µl of 5 mM BS^3 diluted in Conjugation Buffer (20 mM Sodium Phosphate, 0.15 M NaCl, pH 8) and incubated at room temperature for 30 minutes with rotation. The cross-linking reaction was quenched by adding 12.5 µl of Quenching Buffer (1 M Tris-HCl, pH 7.5). After the incubation at room temperature for 15 minutes with rotation, cross-linked beads were washed 3 times before proceeding with the IP protocol. Incubation of the beads with 200 µg of monocyte-derived protein lysates was performed with rotation over-night at 4°C. The next day, after 3 washes with PBS plus Tween 20, elution of the target antigen was carried out by adding 20 µl of Elution Buffer (50 mM glycine, pH 2.8). Samples were denatured by adding 10 µl of NuPAGE LDS sample buffer and heated for 10 minutes at 70°C. Supernatants were stored at -20°C until gel loading.

3.7. Patients

To analyze the expression of CD300e on peripheral blood monocytes, a total of 37 obese adult patients were recruited at the Center for the Study and Integrated Treatment of Obesity of the Padua Hospital. The patients initially underwent a visit to the bariatric surgery clinics for an evaluation aimed at identifying the pathology, any comorbidities and the subject's possible candidacy for bariatric surgery (laparoscopic sleeve gastrectomy). Blood samples were collected from patients before bariatric surgery-induced weight loss. Written informed consent was obtained from all participants after the approval of the study protocol by the University Hospital Ethical Committee (Comitato Etico per la Sperimentazione Clinica della Provincia di Padova). Measures of anthropometric and clinical data were taken according to the study protocol and to the Good Clinical Practice (GCP) guidelines. For patients' recruitment, the following inclusion criteria were considered:

- age between 18 and 65 years;

- BMI > 35.0 kg/m^2 or BMI between 30.0 kg/m^2 and 34.9 kg/m^2;
- presence of at least one comorbidity among the following: ischemic heart disease, T2D, obstructive sleep apnea syndrome, NAFLD, hypertension, dyslipidemia, asthma, venous stasis).

Nine normal weight healthy adult subjects were considered as a control group.

To evaluate the level of soluble CD300e (sCD300e), sera of 19 normal weight subjects and 23 obese patients before and after bariatric surgery-induced weight loss were collected and stored at -80°C for subsequent analysis.

3.8. Flow cytometry

Flow cytometry is a powerful technique for characterizing and/or sorting heterogeneous suspended cell populations based on their physical and fluorescence characteristics. It is well suited for experiments where quantitative information is desired at a single-cell level for large amounts of cells simultaneously. This can provide information on the number of cells of a given type and the amount of a specific protein in the cell. The flow cytometry process works by relying on hydrodynamic focusing of a cell suspension sample to create a single-cell stream. Cells are crossed by a laser beam, perturbing their direction and intensity depending on their size and composition. A detector placed in front of the radiation source measures its forward scatter (FSC), related to cell size, while other detectors measure its side scatter (SSC), related to cytoplasmic granularity and complexity. These data, displayed with a dot plot, are used to identify the cell population of interest on which to conduct subsequent analyses, based on the morphological characteristics. The light signals pass through a series of filters and mirrors to discriminate between the different emission wavelengths, thus obtaining information on the presence of the fluorophore through which the sample has been labeled. When several antigens are labeled, it is necessary to use different fluorophores whose emission peaks do not overlap. Since cells themselves have a certain level of emission at different wavelengths, defined as autofluorescence, a representative sample of the analyzed cell population is maintained unlabeled and is used to determine the basal level of fluorescence, whose value of median fluorescence intensity (MFI) should be subtracted from that of samples of interest.

3.8.1. CD300e expression in circulating monocytes

To evaluate the expression of CD300e on the cell surface of monocytes from normal weight subjects and obese individuals, blood samples were collected at the Centre for the Study and Integrated Treatment of Obesity, Department of Medicine, University Hospital of Padova. Lysis of red blood cells was performed by incubating 150 µl of each blood sample with 3 ml Lysis Buffer 1x (BD Biosciences) for 5 minutes at room temperature on a roller mixer. Cells were pelleted by centrifugation at 300 g for 5 minutes and washed with 1 ml PBS 1x. Subsequently each pellet was incubated with 5 µl of Human BD Fc Block (BD Pharmingen) for 10 minutes to saturate Fc receptors and reduce potential non-specific antibody staining. Samples were then stained with combinations of the following antibodies: FITC-conjugated anti-CD3 (clone UCHT1, BioLegend), FITC-conjugated anti-CD19 (clone HIB19, eBioscience), FITC-conjugated anti-CD56 (clone TULY56, eBioscience), BV786-conjugated anti-CD16 (clone 3G8, BD Biosciences), PerCP-Cyanine5.5-conjugated anti-CD14 (clone 63D3, BioLegend), PE-conjugated anti-CD300e (clone UP-H2, BioLegend), APC-conjugated anti-HLA-DR (clone L243, BioLegend). The fixable cell viability dye eFluor780 (eBiosciences) was used to exclude dead cells from the analysis. Cells were resuspended in PBS 1x and analysed by the flow cytometer BD LSRFortessa X-20 (Becton Dickinson). Values were expressed as n-fold change of median fluorescence intensity (MFI) vs T_0 or untreated cells. Data were analysed using FlowJo software, version 14 (Tree Star Inc). In figure 7, it is shown the gating strategy applied to evaluate CD300e expression on monocytes among all cell populations of a random subject's blood. Briefly, in the first graph it has been considered the population of monocytes based on their dimension (as identified by the FSC) and cytoplasmic granularity (shown by the SSC). In the second graph, the single cells were selected, which were distributed along the diagonal, to exclude any cell cluster. The third graph represented live cells, which were negative to the viability marker indicated by the fluorophore APC-Cy7. Next, it has been selected the FITC$^-$ cells to exclude T cells (CD3$^+$), B cells (CD19$^+$) and NK cells (CD56$^+$). The L-shape in the next graph included the whole population of monocytes divided in three major subsets: classical (CD14$^+$ / CD16$^-$), intermediate (CD14$^+$ / CD16$^+$), and non-classical (CD14^{dim} / CD16$^+$). Thus, any contaminating cells have been excluded. In this graph, the fluorophore PerCP-Cy5.5 indicated the antigen CD14, while the fluorophore BV785 showed the marker CD16. Subsequently, it has been excluded the cells CD16$^+$ / MHC-II$^-$, which were neutrophils and NK cells. Major histocompatibility complex class II molecules (MHC-II) were marked by the fluorophore APC. The penultimate graph was used to exclude any remaining B cells, which were CD14$^-$ / MHC-II$^+$. The final graph again indicated the three subpopulations of monocytes, from which it was possible to

obtain the MFI in PE, indicating the expression of CD300e, both on the entire population of monocytes and on each individual subtype.
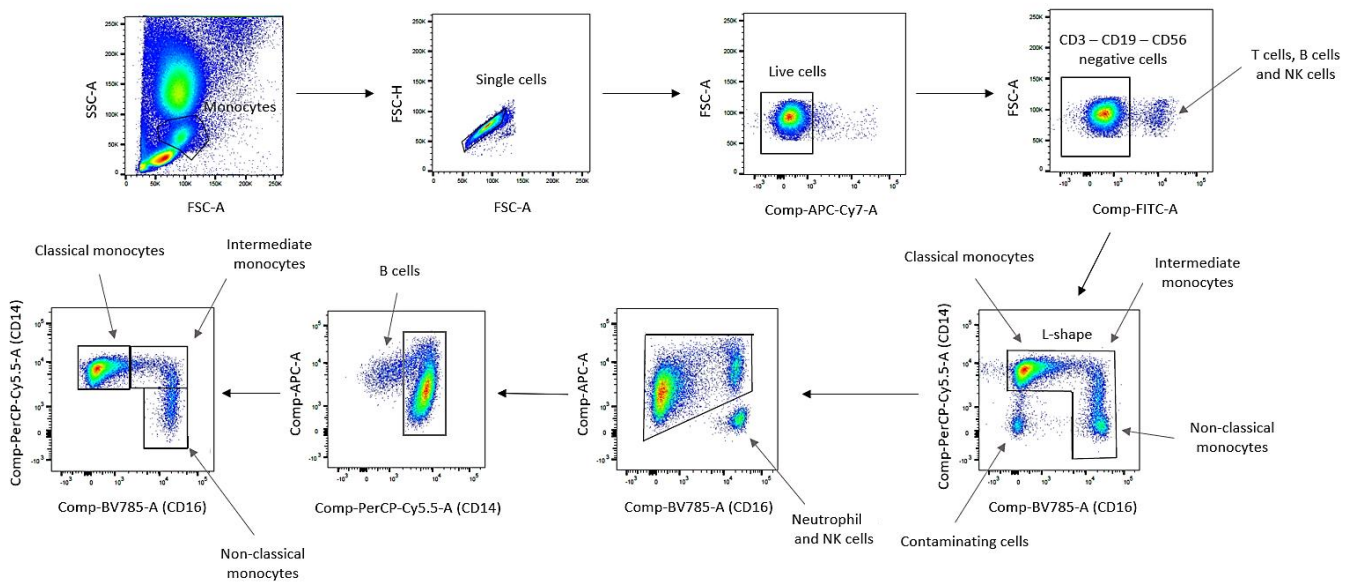

Figure 7. Schematic representation of the gating strategy used to evaluate the surface expression of CD300e in circulating monocytes from blood samples of normal weight subjects and OPs. Each step had the aim of excluding any cellular contaminant to include in the analysis only the three subpopulations of monocytes (classical, intermediate, non-classical).

3.8.2. Glucose uptake assay

To investigate the impact of the activation of CD300e on the functional insulin pathway, specifically glucose uptake by cells, we employed 2-NBDG (Invitrogen), a fluorescent glucose analogue used to monitor its internalization in live cells. Freshly isolated monocytes were aliquoted at the desired concentration ($1x10^6$ cells) and incubated for 60 minutes at 37°C in a buffer (140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, and 5.5 mM glucose, pH 7.4) containing 50 µM 2-NBDG resuspended in DMSO, in presence or absence of 100 nM insulin with or without the co-stimulation by the agonistic monoclonal antibody anti-CD300e (clone UP-H2, kindly provided by M. López-Botet, Barcelona, Spain). Termination of incubation was achieved by placing each tube on ice. A wash with 1 ml cold PBS 1x was performed and all tubes were analysed by the BD LSRFortessa X-20 Cell Analyzer at the wavelength of 488 nm.

3.9. ELISA

Sera of normal weight subjects and obese patients were collected and stored at -80°C for subsequent quantification of the level of CD300e. ELISA kit specific for human CD300e/LMIR6 (Invitrogen) was used following the manufacturer's instructions.

3.10. Transduction of cell lines

HEK-293 cell line was transduced with an adenovirus produced in our laboratory to induce the expression of the receptor CD300e. Briefly, $0.1x10^6$ cells were seeded in 24-well plates to reach 70-80% confluency and were transduced the next day. The amount of adenovirus employed was proportional to the cell density in each well and it was equal to 1 μL/well in a 24-well plate. HEK-293 cells were used after 24 hours of transduction while THP-1 cells were used after 3-4 days of transduction for subsequent experiments involving treatment with liposomes.

3.11. Production of liposomes

Liposomes are hollow microspheres with variable dimensions between 25 nm and 1 μm in diameter, formed by one or more lipid bilayers. The outer layers have a remarkable affinity for plasma membranes since their compositions are similar (phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters). In this way, the water-soluble substances contained within the liposomal microspheres can be easily carried inside the cells. At the same time, liposomes can also incorporate pharmacologically active lipophilic molecules into their outer phospholipid bilayer. To produce liposomes, the Thin Layer Hydration Technique (TLH) was used. Briefly, lipids dissolved in ethanol in suitable volumes were added inside a round bottom flask and were subjected to a vacuum evaporation process at 35°C for about 20 minutes by using a rotatory evaporator (Rotavapor, Büchi). Thus, a lipid film was created inside the flask, which was then rehydrated with 1 mL of PBS 1x (10 mM) and sonicated for 15 minutes (Fisherbrand Sonicator). This process led to the formation of multilamellar vesicles (MLVs) or structures of aggregated liposomes. For this reason, the suspension was extruded 11 times through 200 nm pore size polycarbonate membranes (Nucleopore™) to obtain unilamellar structures with smaller dimensions. For the extrusion, a specific instrument with two Hamilton syringes inserted inside was used (Avanti® Polar

Lipids, Sigma-Aldrich). The final suspension was then analyzed by Dynamic Light Scattering (DLS, Malvern), a tool to characterize the size of particles in suspension. The stock of produced liposomes had a concentration of 1 mg/mL, but for subsequent cell treatments we have used a final concentration of 0,1 mg/mL. Liposome composition was 59% cholesterol, 1% fluorescein and 40% sphingomyelin (SM) or phosphatidylcholine (PC) or dioleoyl phosphatidylethanolamine (DOPE).

3.12. Cell treatment with liposomes

HEK-293 cells were transduced for 24 hours and subsequently subjected to serum starvation for 2 hours. Then, liposomes dissolved in DMEM without serum were added and cells were incubated for 4 hours at 37°C. Cells were harvested in tubes and stained with PE-conjugated anti-CD300e (clone UP-H2, BioLegend). The fixable cell viability dye eFluor780 (eBiosciences) was used to exclude dead cells from the analysis. Cells were resuspended in PBS 1x and analysed by the flow cytometer BD LSRFortessa X-20 (Becton Dickinson). Acquisitions were conducted considering three fluorophores: FITC to evaluate the binding/internalization of liposomes, PE to analyze CD300e expression on the cell surface and APC-Cy7 to include only live cells in the analysis.
Human monocytes were purified from buffy coat as previously described. They were resuspended in RPMI with no FBS and plated at a density of 1.2×10^6 cells/well in a 24-well plate. After 1 hour of adhesion and two consecutive washes, liposomes dissolved in RPMI without serum were added and cells were incubated for 3 hours at 37°C. At the end of the treatment, monocytes were detached with sterile 5 mM Na-EDTA in PBS 1x (pH = 7.4) and collected in tubes. Cells were labelled with anti-CD300e antibody and subjected to FACS analysis as stated above.

3.13. Statistical analysis

All statistical analyses were carried out using GraphPad Prism software version 5.0.3. Significance was determined by two tailed Student's t-test or by one-way ANOVA when appropriate. Non-parametric Mann-Whitney test or Kruskal-Wallis test was carried out to assess differences between continuous variables with Bonferroni correction for multiple comparison. Correlation analyses were performed using simple linear regression, which estimates the relationship between one independent variable and one dependent variable using a straight line.

4. RESULTS

4.1. CD300e hampers the insulin pathway through the hindrance of AKT phosphorylation

Obesity and IR are inextricably linked through the actions of specific inflammatory immune cells. The development of IR is thought to occur in response to increased production of pro-inflammatory cytokines by AT during obesity, that, in turn, have an inhibitory effect on insulin signalling pathways in its target tissues. OPs have been described with increased infiltration of macrophages in AT, whose activation and secretion of pro-inflammatory cytokines certainly play an important role in local and systemic inflammation with consequent development of IR (Strand et al., 2022). Several studies have suggested the involvement of the immune receptor CD300e, primarily expressed by myeloid cells such as monocytes and macrophages, in obesity-induced insulin resistance (IR). This is supported by evidence, including the discovery that CD300e is expressed by macrophages infiltrating the adipose tissue of obese individuals (Coletta et al., 2020) and a study involving a cohort of 49 discordant monozygotic twin pairs with varying body mass indices (BMI). This study revealed a significant upregulation of CD300e mRNA in the adipose tissue of the heavier subjects compared to their co-twins (van der Kolk, Saari, et al., 2021).

These observations prompted us to investigate whether CD300e was involved in the hindrance of the insulin-triggered cascade contributing to the onset of systemic inflammation and IR in OPs.

To test the hypothesis that the activation of the immune receptor CD300e interferes with the insulin pathway, we stimulated human monocytes with the agonistic anti-CD300e monoclonal antibody (clone UP-H2), or the corresponding isotype-matched control (clone MOPC-21), both in absence and in presence of insulin. As said before, AKT activation plays a central role in insulin-stimulated glucose uptake by the cells. Considering that AKT phosphorylation at serine 473 is necessary for maximum activation of the kinase, we evaluated the level of phosphorylation of AKT (pAKT) in lysates derived from treated human monocytes by western blot. We tested different times of activation, and we found that the level of pAKT decreased significantly after 15 and 30 minutes following CD300e activation (Figure 8a). Notably, the latter inhibited the increased AKT phosphorylation also in cells stimulated by insulin (Figure 8b).
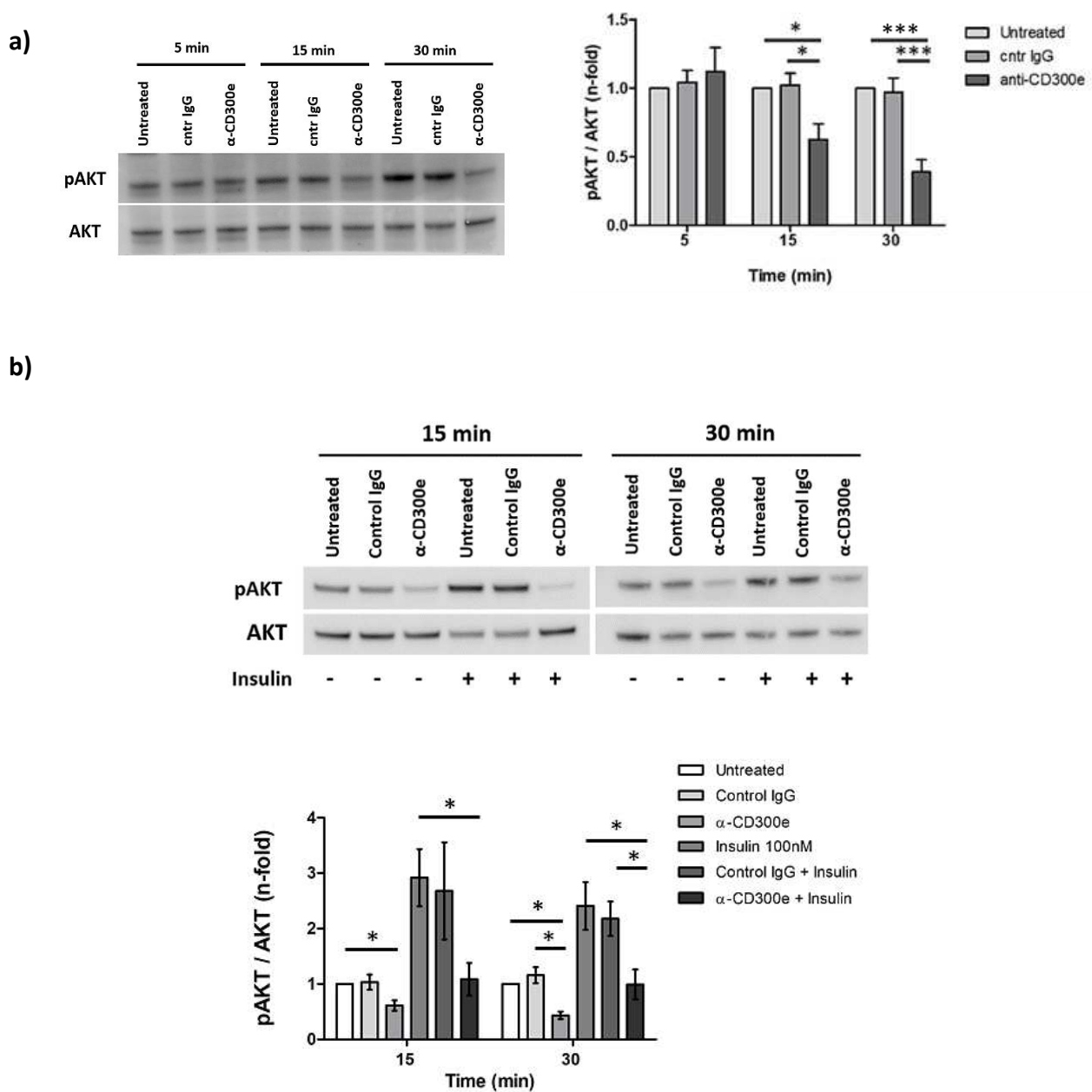
Figure 8. CD300e activation interferes with AKT phosphorylation, both in absence and in presence of insulin. (a) Level of phosphorylation of AKT evaluated by western blot in monocytes stimulated with anti-CD300e monoclonal antibody for 5, 15, 30 minutes. Data are shown as mean ± SEM of 7 independent experiments. Quantification of pAKT was performed by densitometry and normalized to total AKT. Significance was determined by two tailed Student's t-test (*$p \leq 0.05$; ***$p \leq 0.001$). **(b)** Level of phosphorylation of AKT evaluated by western blot in monocytes stimulated with anti-CD300e monoclonal antibody for 15 and 30 minutes, both in absence and in presence of 100 nM of insulin. Data are shown as mean ± SEM of 7 independent experiments. Quantification of pAKT was performed by densitometry and normalized to total AKT. Significance was determined by two tailed Student's t-test (*$p \leq 0.05$).

4.2. Possible involvement of the phosphatase SHP-1 in the negative modulation exerted by CD300e on the insulin pathway

In glucose homeostasis, the phosphatase SHP-1 is also involved (Dubois et al., 2006). For instance, it has been demonstrated that SHP-1, in its phosphorylated form, negatively modulates the action of insulin in skeletal muscle cells through down-regulation of both AKT activation and GLUT4 translocation on the cell membrane (Bergeron et al., 2011). Moreover, it is well known that SHP-1 is expressed broadly throughout the hematopoietic system and most studies agree that it down-regulates signalling from TLRs in monocytes and macrophages, reducing the production of pro-inflammatory cytokines (Abram & Lowell, 2017). To explain how CD300e could interfere with AKT phosphorylation, we wondered whether the engagement of the receptor induced the activation of SHP-1, which in turn could de-phosphorylate AKT and consequently interfere with the translocation of GLUT4 on the cell membrane after insulin stimulation. SHP-1 itself is also regulated by phosphorylation, in particular phosphorylation at tyrosine 536 (Y536) in the C-terminal tail engages the N-terminal SH2 domain to relieve basal inhibition, contributing to phosphatase activation, and it is more important for binding downstream signalling molecules. After stimulating monocytes with the agonistic anti-CD300e monoclonal antibody, or the corresponding isotype-matched control, we have evaluated the level of phosphorylation of SHP-1 by western blot. The engagement of the receptor resulted in a significant increase in the level of pSHP-1 (Y536) when compared to isotype IgG after half an hour (Figure 9). This result did not align with the previously observed time course of decreased pAKT levels, indicating that the phosphatase SHP-1 may only play a partial role in the negative modulation induced by CD300e on the insulin-triggered cascade.
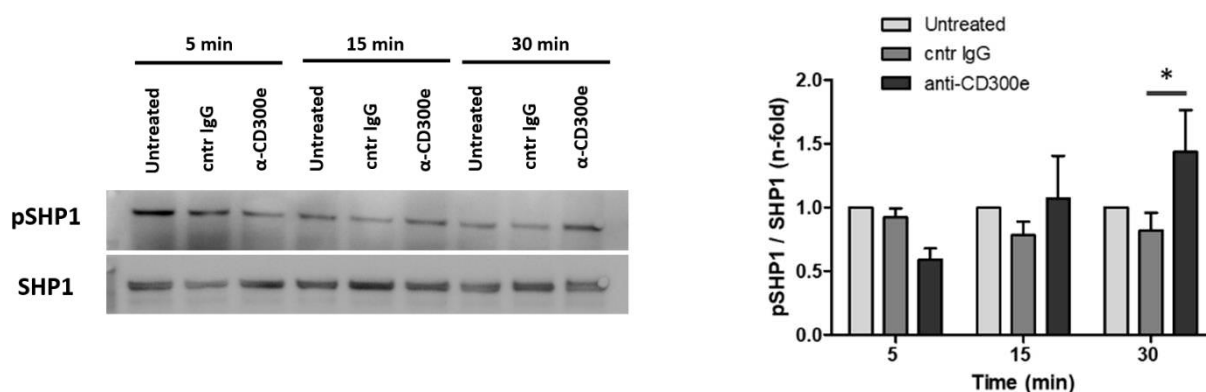
Figure 9. CD300e engagement induces the activation of the phosphatase SHP-1. Level of phosphorylation of SHP-1 evaluated by western blot in monocytes stimulated with anti-CD300e monoclonal antibody for 5, 15 and 30 minutes. Data are shown as mean ± SEM of 7 independent experiments. Quantification of pSHP-1 was performed by densitometry and normalized to total SHP-1. Significance was determined by two-way ANOVA (*$p \leq 0.05$).

4.3. CD300e hampers the insulin-induced glucose uptake

We proceeded to investigate whether the interference of CD300e in monocytes with the insulin pathway translated into decreased glucose internalization by the cells. Indeed, even though monocytes are not the primary target of insulin, it is well known that they expose GLUT4 on the cell membrane after insulin stimulation (Mavros et al., 2009). Monocytes were resuspended in a buffer containing 2-NBDG, a fluorescent analogue of glucose, and stimulated with the anti-CD300e monoclonal antibody, both in absence and in presence of insulin. As shown in Figure 10, the activation of the receptor significantly reduced glucose uptake triggered by insulin.
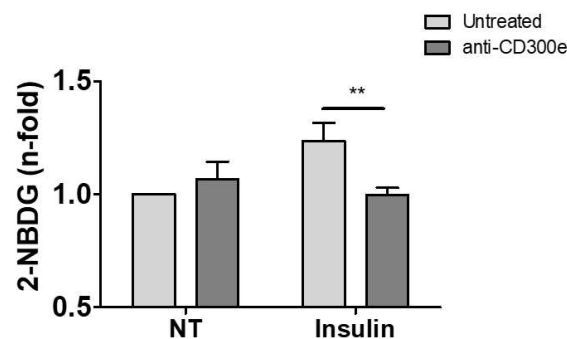

Figure 10. CD300e activation interferes with glucose uptake in presence of insulin. Monocytes were resuspended in a buffer containing 50 µM of 2-NBDG and then stimulated with the anti-CD300e antibody for 1 h, both in absence and in presence of 100 nM of insulin. Fluorescence was measured by flow cytometry at an excitation wavelength of 488 nm. Data are shown as mean ± SEM of 5 independent experiments. Significance was determined by two tailed Student's t-test (**$p \leq 0.01$).

4.4. CD300e does not associate with DAP12 in human monocytes

To investigate which proteins are recruited by CD300e at the plasma membrane for downstream signalling cascade after the activation of the receptor, we attempted immunoprecipitation using beads previously conjugated with the anti-CD300e antibody or the isotype-matched control. Antibodies were covalently crosslinked to the beads before immunoprecipitation to increase the efficiency of the antigen elution. Monocyte lysates were incubated with the beads and the immunoprecipitated material was collected for subsequent gel loading. Blots were probed for CD300e and DAP12. Even though we managed to immunoprecipitate the receptor (Figure 11a), we failed to reproduce the constitutive association between CD300e and DAP12 (Figure 11b). To improve our protocol, we performed an intracellular crosslinking on isolated monocytes by using the lipophilic and membrane-permeable crosslinker disuccinimidyl suberate (DSS), but also in this case it appeared that DAP12 was not associated with CD300e (data not shown). On the other hand, this result is consistent with previous research, which demonstrated that it was necessary to overexpress CD300e and DAP12 *in vitro* to replicate their association, as it had not been observed with the endogenous counterparts (Isobe et al., 2018).
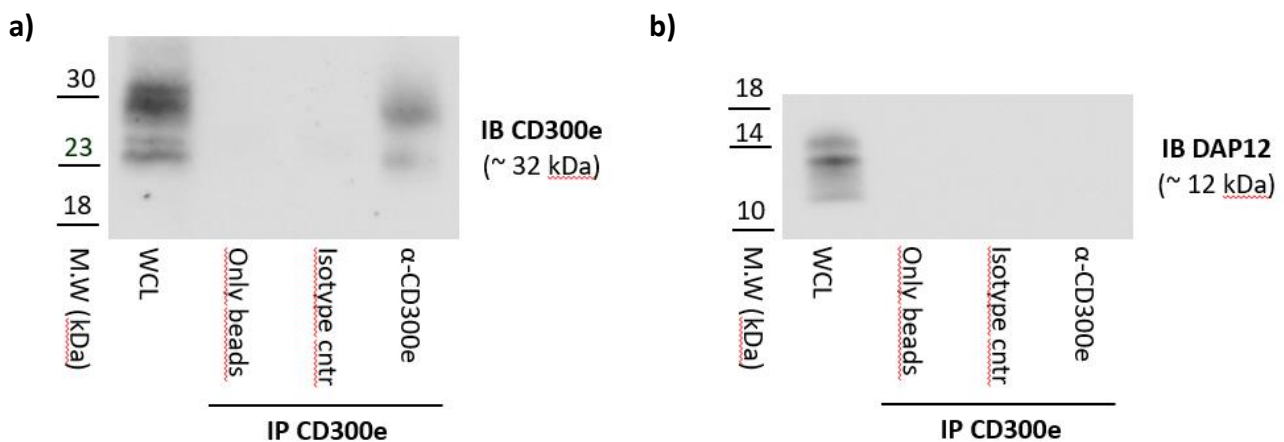

Figure 11. CD300e does not associate with DAP12 in human monocytes. Human monocytes were harvested, and total lysates were quantified by using the bicinchoninic acid (BCA) method. Two hundred μg of protein lysates were subjected to immunoprecipitation (IP) using magnetic beads (only beads) or beads previously crosslinked with the isotype control or with the anti-CD300e antibody. Immunoprecipitated material was probed for the presence of CD300e **(a)** and DAP12 **(b)** by immunoblot (IB). Whole cell lysates (WCL) were loaded as positive controls.

4.5. CD300e hampers AKT phosphorylation triggered by insulin in hepatocytes

Subsequently, we moved to verify whether the negative modulation of the insulin pathway triggered by CD300e activation could be replicated in cell models mimicking the physiological targets of the hormone. Considering HepG2 cell line as our *in vitro* cell model to mimic human hepatocytes, first we confirmed the surface expression of CD300e by flow cytometry (Figure 12) and then we evaluated AKT phosphorylation by western blot after the activation of the receptor, both in absence and in presence of insulin. As shown in figure 13, the anti-CD300e monoclonal antibody reduced the phosphorylation of AKT after 15 and 30 minutes, both in the absence and, most importantly, in the presence of insulin, thus confirming the previous result obtained in monocytes.
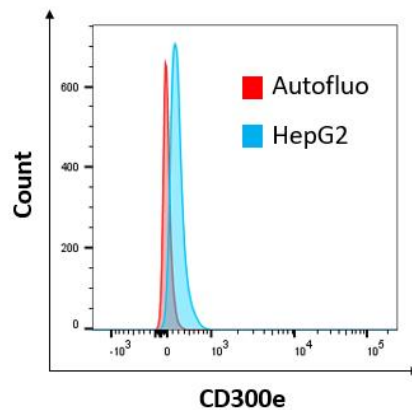

Figure 12. Surface expression of CD300e in HepG2 cell line. HepG2 cells were harvested in tubes and stained with human PE-conjugated anti-CD300e antibody. A representative sample of the analyzed cell population, defined as autofluorescence, was maintained unlabeled and was used to determine the basal level of fluorescence.
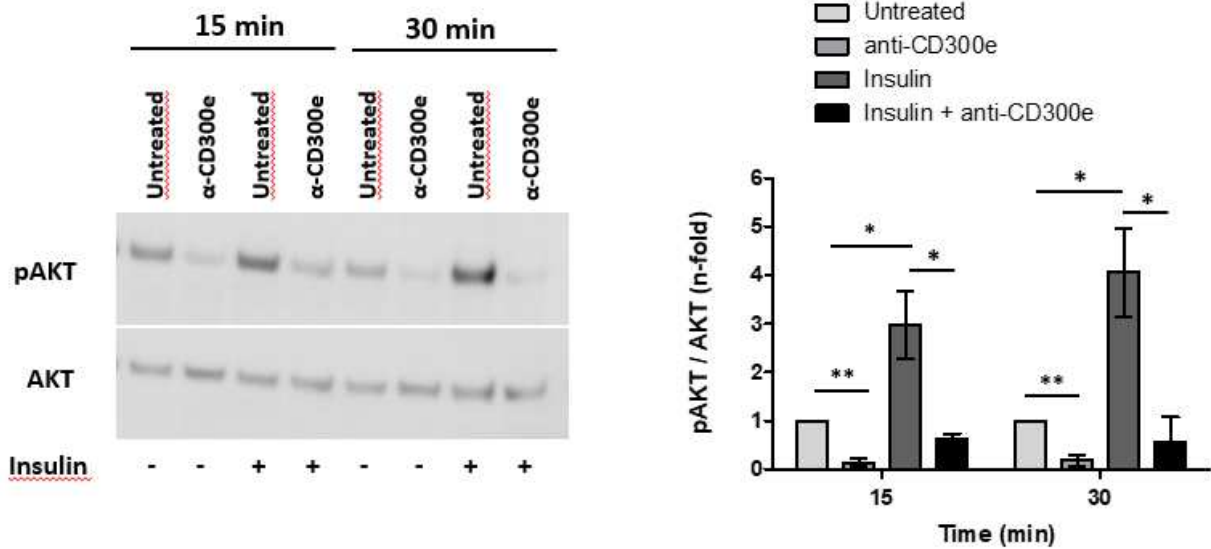
Figure 13. CD300e activation interferes with AKT phosphorylation, both in the absence and in the presence of insulin, in HepG2 cell line. The level of AKT phosphorylation was assessed by western blot in HepG2 cell line following stimulation with anti-CD300e antibody, both with and without 100 nM of insulin, for 15 and 30 minutes. Data are shown as mean ± SEM of 3 independent experiments. Quantification of pAKT was performed by densitometry and normalized to total AKT. Significance was determined by two tailed Student's t-test (*$p \leq 0.05$; **$p \leq 0.01$).

4.6. CD300e hampers AKT phosphorylation triggered by insulin in skeletal muscle cells

Regarding the skeletal muscle, which is the paramount tissue for insulin-stimulated glucose disposal, we used two hTERT/cdk4 immortalized myogenic human cell lines, named LHLMN2 and 1190. After evaluating the surface expression of CD300e by flow cytometry (figure 14), decided to use the 1190 cell line because it expressed the receptor more prominently. Cells were stimulated with the anti-CD300e antibody with or without co-stimulation by insulin and the amount of pAKT was evaluated by western blot. Even though the data were not statistically significant due to the variability of the results, the trend observed recapitulated that seen in the HepG2 cell line (Figure 15).
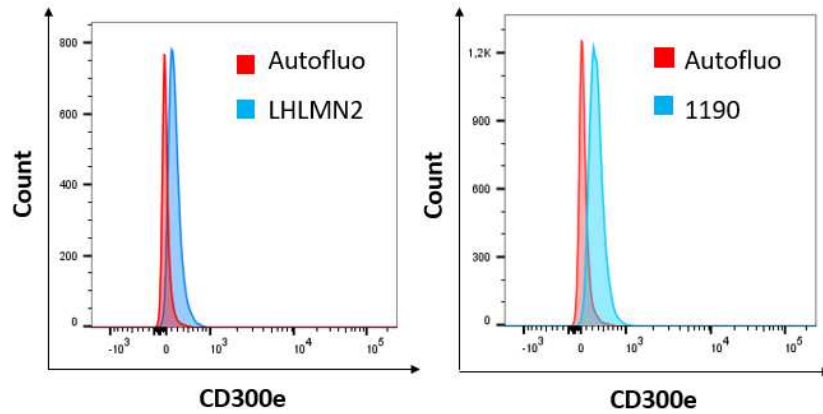
Figure 14. Surface expression of CD300e in myoblast cell lines. LHLMN2 and 1190 cells were harvested in tubes and stained with human PE-conjugated anti-CD300e antibody. A representative sample of the analyzed cell populations, defined as autofluorescence, was maintained unlabeled and was used to determine the basal level of fluorescence.
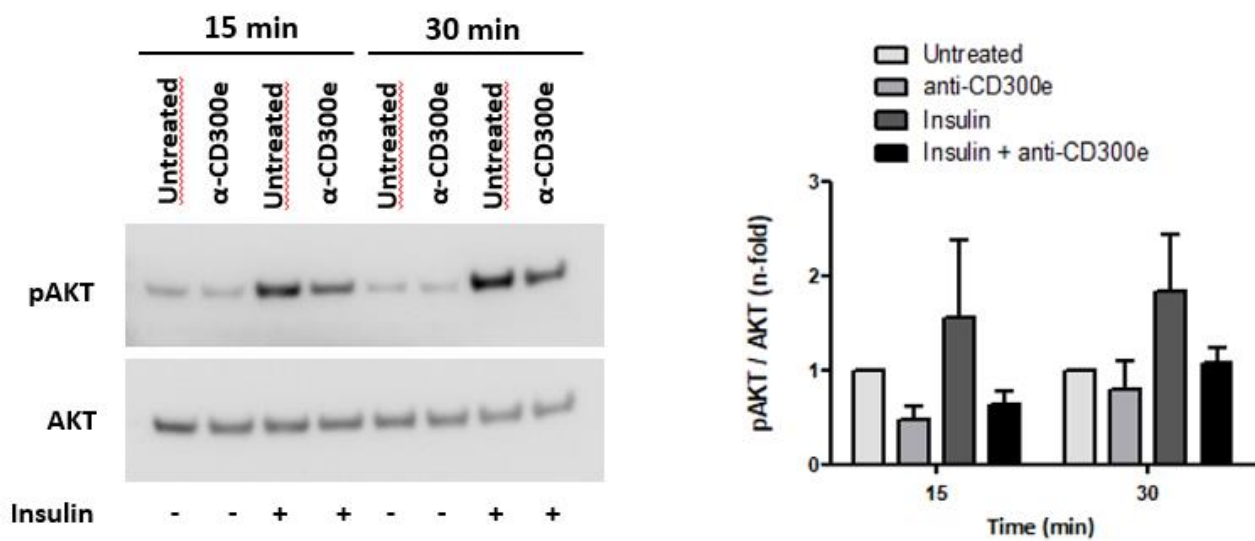

Figure 15. Evidence of a possible interference of CD300e activation on AKT phosphorylation in primary human myoblasts. The level of AKT phosphorylation was assessed by western blot in 1190 myoblasts following stimulation with anti-CD300e antibody, both with and without 100 nM of insulin, for 15 and 30 minutes. Data are shown as mean ± SEM of 3 independent experiments. Quantification of pAKT was performed by densitometry and normalized to total AKT.

4.7. CD300e engagement hampers phosphorylation of p38 MAPK

To study the intracellular signalling cascade triggered by CD300e activation, we leveraged the knowledge of another family of receptors known as Triggering Receptor Expressed on Myeloid Cells (TREM), which are important regulators of the mammalian immune response (Konishi & Kiyama, 2018). As CD300e, the engagement of the over-expressed receptor TREM2 upon stimulation with the anti-TREM2 monoclonal antibody in human monocyte-derived dendritic cells induces the recruitment and association of the intracellular adaptor protein DAP-12, as demonstrated by immunoprecipitation (Bouchon et al., 2001). Subsequent tyrosine phosphorylation of DAP-12 within its immunoreceptor tyrosine-based activation motifs (ITAMs) by Src family kinases leads to recruitment of Syk kinase to activate downstream signalling molecules, such as ERK, PI3K and PLCγ (Konishi & Kiyama, 2018). It is worth keeping in mind that IRS proteins, which are activated downstream of insulin binding to its receptor, can trigger two major signalling pathways. The first one is the PI3K/AKT pathway, responsible for the metabolic actions of the hormone, and the second one is the Ras/MAPK pathway, which is involved in the expression of genes regulating cell survival, growth, proliferation, and differentiation. Among the members of the Mitogen-Activated Protein Kinase (MAPK) superfamily there is p38, whose activation occurs through phosphorylation at threonine 180 (Thr180) and tyrosine 182 (Tyr182). Since we had previously demonstrated that CD300e activation hampered the insulin pathway by decreasing pAKT, we wonder whether the receptor could also interfere with the p38 MAPK phosphorylation after stimulation of human monocytes with the anti-CD300e monoclonal antibody. In a preliminary set of experiments, we found that in monocyte lysates, the level of phosphorylation of p38 increased up to 30 minutes, but it sharply decreased after 1 hour (Figure 16).

Collectively, our results shows that the engagement of CD300e hinders not only the PI3K/Akt cell signalling, as previously demonstrated, but also the p38 MAPK pathway. The latter is known to play important roles in a huge variety of biological processes, including mRNA translation, cell proliferation and survival, nuclear genomic responses to mitogens and cellular stresses and, most importantly, it is an essential secondary branch of the insulin signalling cascade.
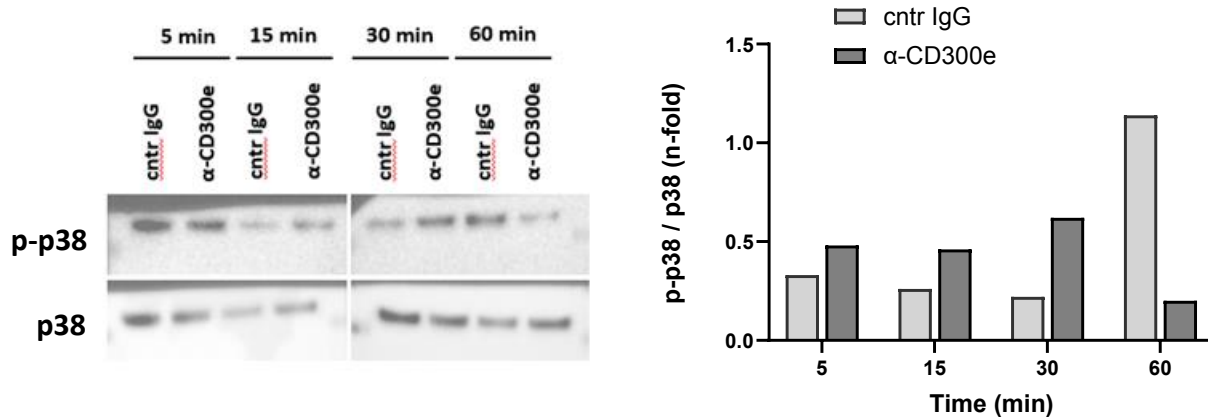
Figure 16. CD300e engagement interferes with the phosphorylation of p38 MAPK. Level of phosphorylation of p38 MAPK evaluated by western blot in monocytes stimulated with anti-CD300e monoclonal antibody, or with the corresponding isotype-matched control, for 5', 15', 30' and 60'. Quantification of phospho-p38 was performed by densitometry and normalized to total p38.

4.8. Monocytes from obese patients display a higher expression of CD300e, which positively correlates with the BMI

Next, we decided to assess whether circulating monocytes, which are precursors of tissue macrophages that abundantly infiltrate the AT of obese individuals, displayed a higher expression of the receptor in OPs if compared with monocytes from normal weight subjects. A FACS analysis, performed with a monoclonal antibody specific for CD300e, revealed that monocytes from OPs expressed significantly more CD300e than monocytes from normal weight subjects (Figure 17a). Interestingly, simple linear regression analysis revealed a positive correlation between the expression of CD300e in monocytes of obese subjects and their BMI (Figure 17b). Another study demonstrated that higher baseline BMI and waist circumference were associated with higher CD300e expression across all monocyte subsets (classical, intermediate, and non-classical) (van der Valk et al., 2022). In this study, patients with obesity were enrolled in the lifestyle intervention program, involving healthy nutrition, increased exercise, and behavioural changes. They were monitored after 10 weeks and at the end of the intervention (1.5 years). The authors observed that, although CD300e levels had significantly decreased when patients initially lost weight, they increased to a greater extent than the initial expression by the end of the program. They speculated that the higher expression of the receptor in OPs was indicative of a pro-inflammatory status during

obesity, which was relieved following the decline in BMI. In accordance, we have previously displayed in isolated human monocytes that the activation of the receptor negatively affects the insulin-triggered signalling, a well-known prerequisite for the onset of obesity-induced IR and low-grade systemic inflammation. On the other hand, the authors guessed that the next increase of CD300e during the final phase of the program (i.e., after 1.5 years) might be due to the development of a different inflammatory response in which the receptor could be involved again.
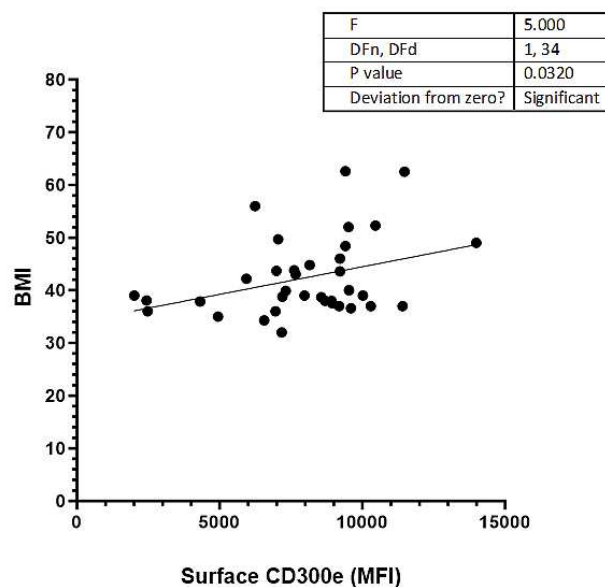
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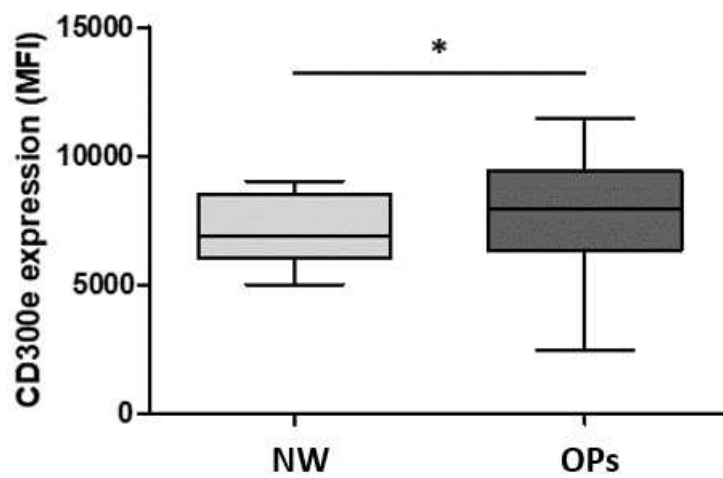
b)

Figure 17. Monocytes from obese patients displays a higher expression of CD300e, which is positively correlated with the BMI. (a) CD300e expression in circulating monocytes from normal weight subjects (NW) and from obese patients (OPs). The expression level of CD300e was determined by flow cytometry and expressed as mean fluorescence intensity (MFI). Data are shown as mean ± SEM. Significance was determined by Student's *t*-test (*$p \leq 0.05$). **(b)** Positive correlation between CD300e surface expression on monocytes (expressed as MFI) and BMI of obese patients. Simple linear regression analysis (F-test) was used to find a linear relationship to describe the correlation between two continuous quantitative variables. ($p = 0.0320$). DFn = degrees of freedom in the numerator; DFd = degrees of freedom in the denominator. The DFn is the number of degrees of freedom that the estimate of variance used in the numerator is based on. The DFd is the number of degrees of freedom that the estimate used in the denominator is based on.

4.9. Sera from obese patients display a lower level of soluble CD300e

It has been demonstrated that the sera of OPs with IR contain autoantibodies specific for intracellular or cell-associated proteins, suggesting that under obesity conditions most of these autoantibodies detected in the OPs' sera are against adipocyte-derived antigens released by cells dying in the AT (Frasca et al., 2020). It is also known that a soluble form of TREM2 (sTREM2) derived from proteolytic cleavage of the cell surface receptor is detected in human cerebrospinal fluid, whose levels are elevated in several neurological inflammatory diseases such as Alzheimer's disease and multiple sclerosis (Zhong et al., 2017). Considering our finding that anti-CD300e antibodies were present in the sera of OPs (Coletta et al., 2022), in addition to the increased expression of the surface receptor on circulating monocytes and macrophages infiltrating the expanded adipose tissue in OPs, we contemplated the existence of a soluble form of CD300e. To investigate this, we analysed by ELISA the level of human CD300e in the sera of subjects with obesity, before and after weight loss, and compared them to those of individuals with normal weight. Surprisingly, we observed that the level of sCD300e was higher in individuals with a normal weight compared to those with obesity, and this pattern remained consistent regardless of weight loss (see Figure 18). At present, we lack a definitive explanation for this outcome. We could speculate that the lower levels of sCD300e in the sera of individuals with obesity corresponded to the heightened expression of the receptor on the cell membranes of their circulating monocytes, as previously observed. In this context, the reduction in sCD300e levels in the sera of individuals with obesity could potentially serve as an indicator of the increased receptor expression on the cell membranes of their circulating monocytes. Unfortunately, we do not have the data concerning both the surface expression of CD300e on circulating monocytes

and the level of sCD300e of the same individuals, thus our hypothesis requires further investigations. In addition, the exact physiological role of sCD300e remains an area for future studies. We could guess it might act as a decoy receptor for specific ligands to dampen an inflammatory response in normal weight subjects; alternatively, the proteolytic cleavage of the transmembrane receptor might be a way to prevent the propagation of the intracellular signal interfering with the insulin pathway in healthy individuals.
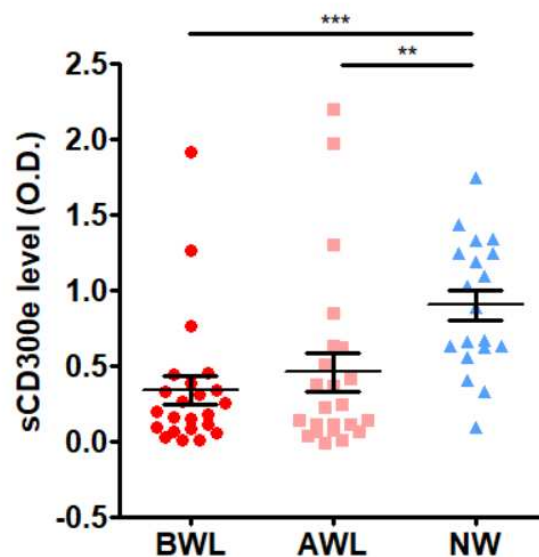

Figure 18. The level of soluble form of CD300e (sCD300e) is higher in normal weight subjects than in obese patients, regardless of weight loss. Sera of normal weight subjects (NW) and obese patients, before (BWL) and after (AWL) weight loss, were evaluated by ELISA. Data are expressed as optical density (O.D.). Significance was determined by one-way ANOVA test (**$p < 0.01$; ***$p < 0.001$).

4.10. Sphingomyelin-containing liposomes interact with CD300e

Previous studies evidenced that the synthesis of adipocytes-derived sphingolipids in subcutaneous AT from obese type 2 diabetic patients increased compared to non-diabetic, BMI-matched individuals and the AT-specific deletion of serine palmitoyl transferase (*Sptlc*), the first enzyme involved in the sphingolipid biosynthesis cascade, increased insulin sensitivity and reduce inflammation as a result of the conversion of inflammatory M1 macrophages into anti-inflammatory M2 cells (Chaurasia et al., 2016). In addition, in the obesity context, it has been demonstrated a significant increase of plasma adipocyte-derived EVs, which are enriched in ceramide, SM and

phosphatidylglycerol (PG) compared with the lean state (Blandin et al., 2023). As other members of the CD300 family are known to recognize sphingolipids (Nakahashi-Oda et al., 2012; Simhadri et al., 2012b), we were curious to explore whether sphingolipids could serve as potential ligands for the human CD300e receptor. We synthetized liposomes carrying three different phospholipids in their membranes: SM, phosphatidylcholine (PC) and dioleoyl phosphatidylethanolamine (DOPE). Liposomes are vesicles that enable the maintenance of lipids in solution. They are also highly similar to extracellular vesicles (EVs) identified in the plasma of obese individuals (Blandin et al., 2023), making them a suitable model for testing our hypothesis that CD300e binds to vesicles released from AT in OPs.

We treated HEK-293 cells that we had transduced to express the receptor CD300e, as well as the corresponding non-transduced control cells, with liposomes for 4 hours at 37°C. Flow cytometry analysis was performed to evaluate the surface expression of CD300e (fluorescence in PE) and/or the binding of liposomes (fluorescence in FITC).

As seen in Figure 19, cells were efficiently transduced, confirming that HEK-293 is a suitable model.
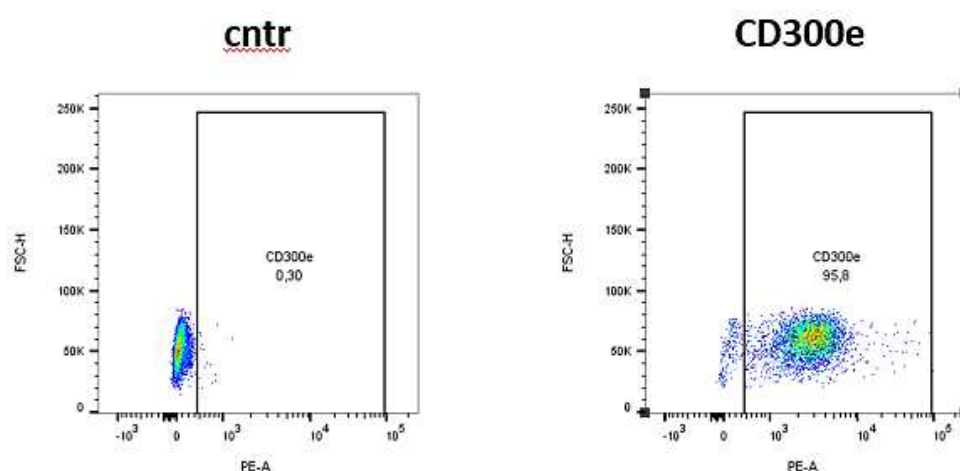
Figure 19. HEK-293 cells transduced for 24 hours to express the CD300e receptor. As can be seen in the cytograms, the transduction efficiency is 96%.

After performing the FACS analysis, we adopted the following gating strategy: we considered all live cells and their capability in binding liposomes, and we expressed the data in terms of percentage of live cells FITC[+]. The treatment with liposomes revealed that all three phospholipids considered in our analysis can interact with HEK-293 cells. However, the results did not achieve statistical significance, likely because of the inherent variability in liposome preparation across repeated

experiments (Figure 20). For this reason, we chose not to average the 3 separate experiments we conducted. This approach allowed us to better highlight the results obtained, as shown in Figure 20b and 20c. We observed that the binding capacity of liposomes loaded with SM and PC was higher in transduced cells compared to non-transduced ones (Figure 20b). On the other hand, when considering DOPE, while there is a trend showing increased binding in transduced cells, it's important to note that it is widely used as a component of lipofectamine, a common transfection reagent. DOPE is a neutral phospholipid that forms complexes with negatively charged nucleic acid molecules, allowing them to overcome the electrostatic repulsion of cell membranes. Given its strong affinity for binding to plasma membranes, it seems more plausible that DOPE binds to cells in a receptor-independent manner rather than acting as a specific ligand for CD300e. The same conclusions can be drawn by normalizing the percentage of live cells FITC[+] of the transduced cells with the percentage of live cells FITC[+] of the non-transduced ones for each treatment (Figure 20c). The binding capability of liposomes made of SM and PC seemed to be CD300e-dependent, although to a different extent. Indeed, in one experiment it is comparable considering both phospholipids, in another it is higher in the case of PC, and in the third it is higher in the case of SM. The same result was not found in the non-normalized graphs where instead in 2 out of 3 experiments the binding was greater with the SM-loaded liposomes. This is probably since the number of non-transduced and transduced cells was different for each treatment and this was considered when performing normalization. By contrast, in just 1 out of 3 experiments, DOPE seemed to interact with the cells in a receptor-dependent manner.
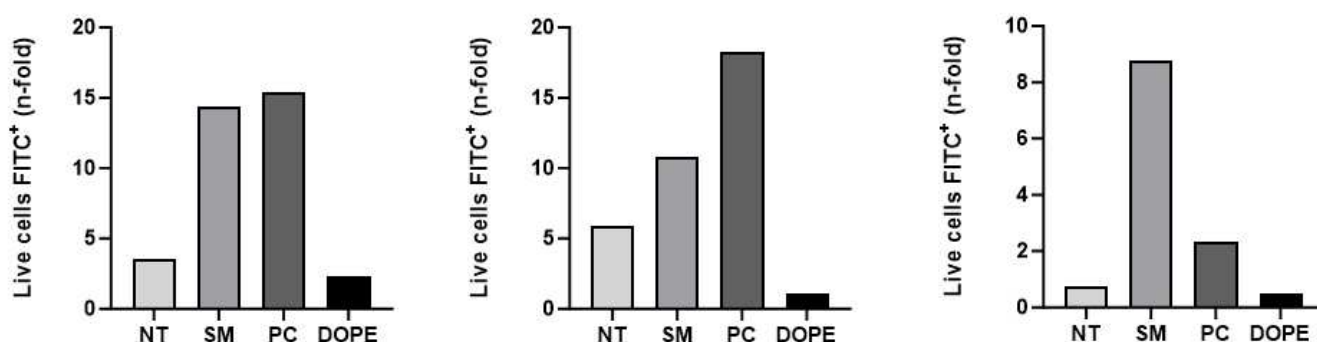
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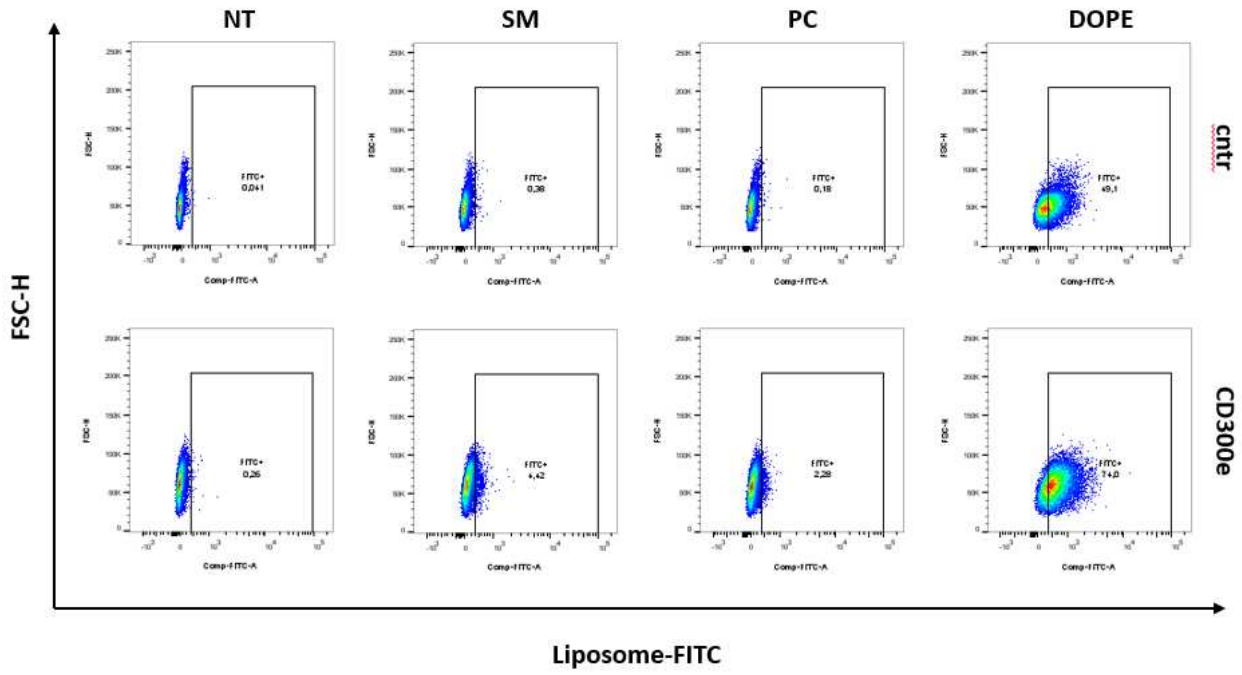
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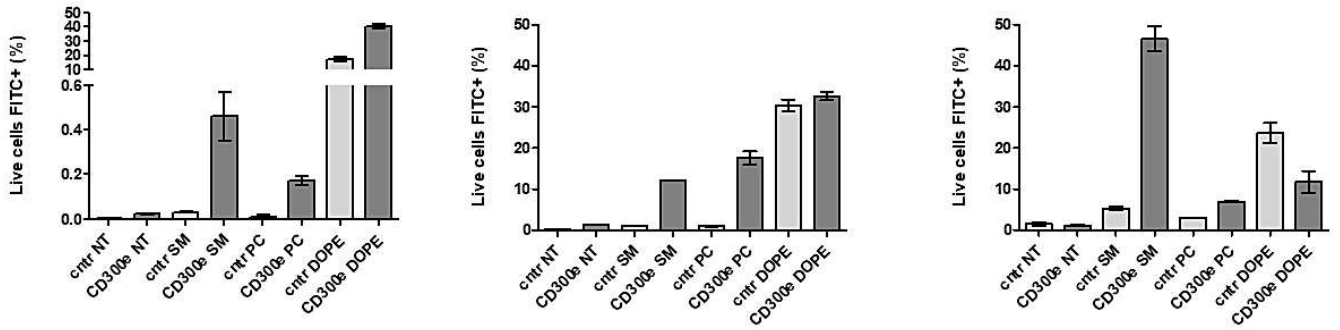
c)

Figure 20. Liposomes interaction with HEK-293 cells expressing CD300e. Cells transduced to express the CD300e receptor, or the relative non-transduced control cells (cntr), were treated with liposomes carrying sphingomyelin (SM) or phosphatidylcholine (PC) or dioleoyl phosphatidylethanolamine (DOPE). Untreated cells (NT) were incubated in medium with no serum and no liposomes. After 4 h, the fluorescence in FITC was measured to assess the liposomes' binding to the cells. **(a)** Representative cytograms showing the percentage of live cells FITC+ in all treated groups. **(b)** The percentage (%) of live cells FITC+ of 3 independent experiments represented by 3 separate histograms. Data are represented as mean ± SEM of 2 technical replicates. **(c)** For each treatment, the percentage (%) of live cells FITC+ of the transduced cells was normalized to the percentage (%) of live cells FITC+ of the corresponding non-transduced cells. The data are expressed as fold-change, and the three bar charts are derived from the same three independent experiments shown in panel B.

4.11. Liposomes interact with human monocytes

After using transduced HEK-293 cells to conduct our experiments on liposome binding, which provided a valuable negative control due to their lack of CD300e expression, we proceeded to perform the same treatments on freshly isolated human monocytes. This cell type exhibits a strong expression of the receptor, making it a more suitable representation of a physiological scenario. Although we didn't have a negative control lacking CD300e expression in this case, we observed a significant binding capacity of SM and DOPE to the live cells compared to untreated monocytes (Figure 21). As mentioned earlier, DOPE cannot be considered due to its high affinity for binding to cell membranes. On the other hand, we have demonstrated that monocytes interact with liposomes carrying SM.

a)
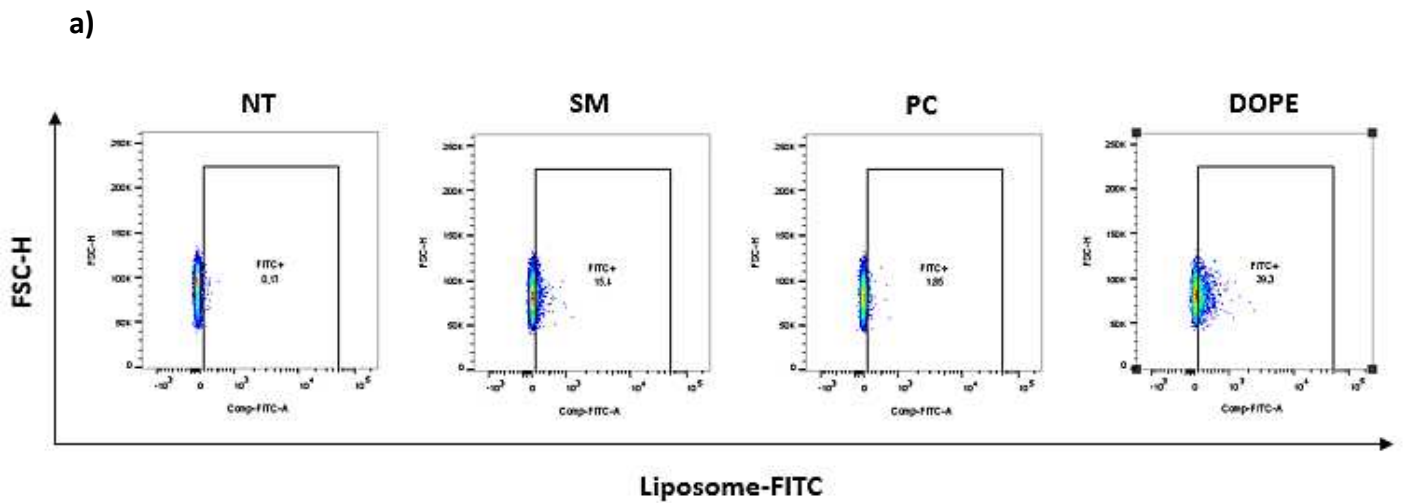
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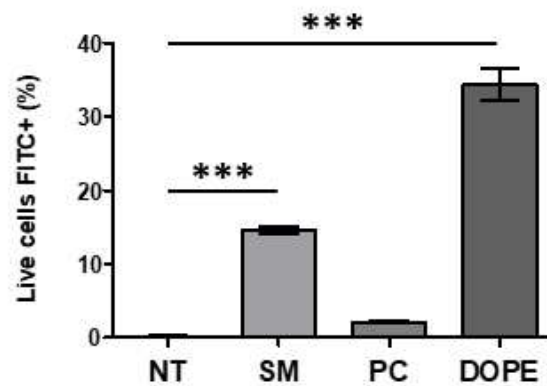
Figure 21. Liposomes interaction with human monocytes. Freshly isolated monocytes were treated for 3 h with liposomes carrying sphingomyelin (SM) or phosphatidylcholine (PC) or dioleoyl phosphatidylethanolamine (DOPE). Untreated cells (NT) were incubated in the medium with no serum and no liposomes. FACS analysis was performed and the fluorescence in FITC was used to evaluate the binding ability of liposomes to the cells. **(a)** Representative cytograms showing the percentage of live cells FITC[+] in all treated groups. **(b)** Percentage (%) of live cells FITC[+] ± SEM of 3 independent experiments were calculated. Significance was determined by one-way ANOVA test (***$p \leq 0.001$).

Collectively, our findings provide the initial evidence that the human receptor CD300e, whose ligand remains unidentified, can indeed bind phospholipids that have previously been shown to interact with other members of the same receptor family (Cannon et al., 2012). Particularly noteworthy is

sphingomyelin (SM), that is already established as the ligand for the CD300e ortholog in mice (Isobe et al., 2018).

5. DISCUSSION

Metabolic disorders, including obesity, type 2 diabetes (T2D) and insulin resistance (IR), have attracted great attention from biomedical researchers and clinicians due to the worrying increase in their prevalence. Although specific public health policies and treatment efforts have been developed to counteract the obesity epidemic, this social and economic burden has nearly tripled between 1975 and 2016. According to the World Health Organisation (WHO), 39% of adults aged 18 years and over are overweight and 13% are obese. In addition, the prevalence of overweight and obesity among children and adolescents aged 5-19 has risen dramatically from just 4% in 1975 to over 18% in 2016 (*Prevalence of Obesity*, s.d.). Chronically obese patients (OPs) have a greater chance of developing T2D which is characterized by hyperglycaemia, along with other comorbidities affecting multiple organs such as cardiovascular disease, microangiopathies, diabetic neuropathies and renal failure. Both obesity and T2D are associated with IR, defined as decreased response to insulin stimulation, resulting in the failure of target tissues to properly dispose of blood glucose (Smith & Kahn, 2016). Current treatments and therapies include healthy eating, regular exercise, weight loss surgery, hypoglycaemic drugs (among which metformin is the primary choice) and the assumption of insulin itself. Unfortunately, these interventions cannot ensure long-term glycaemic control or reverse the progress of the disease.

It is now widely appreciated that chronic low-grade inflammation plays a key role in the initiation, propagation and development of metabolic diseases (Baker et al., 2011). Recent research findings evidenced that the immune receptor CD300e might be involved in the development of IR. First evidence was given by Haseda et al., who detected that the titre of the anti-CD300e antibody was higher in the sera from patients with type 1 and type 2 diabetes compared to healthy controls (Haseda et al., 2016). In addition, CD300e has been shown to be expressed by macrophages infiltrating the adipose tissue (AT) of OPs (Coletta et al., 2020), and its gene expression was significantly upregulated in the heavier subjects compared to their leaner siblings in a cohort of BMI-discordant monozygotic twin pairs (van der Kolk, Saari, et al., 2021). CD300e is mainly expressed by myeloid cells (monocytes and macrophages), which widely infiltrate the adipose tissue (AT) of obese individuals, and thus they have been identified as the primary source of circulating inflammatory

molecules, contributing to the IR-associated systemic inflammation (Guria et al., 2023; Lauterbach & Wunderlich, 2017; B.-C. Lee & Lee, 2014). The evidence that insulin shows anti-inflammatory effects (Sun et al., 2014) prompted us to speculate that CD300e is a piece of the puzzle involved in the onset of systemic IR and inflammation through its negative modulation of the insulin pathway. Our first aim was to unravel the signalling cascade elicited by CD300e that hijacks insulin-triggered pathway by using *in vitro* approaches. We revealed that the stimulation of monocytes through CD300e decreased the basal level of the active form of AKT (phospho-AKT, pAKT) and, most importantly, hampered the insulin-induced phosphorylation of the kinase. In accordance, the insulin-induced glucose uptake was hindered in these cells. Further investigations are needed to identify other upstream/downstream molecules and pathways involved. It is known that the MAPK pathway is an essential secondary branch of the insulin signalling cascade, and it is activated independently of the PI3K/AKT pathway. Crosstalk between MAPK and insulin pathways involves transcriptional control of the insulin-like growth factor-1 receptor (IGF-1R) gene, which is employed to maintain circulating glucose at appropriate levels (Zhang et al., 2011). Our first data evidenced that the p38 MAPK pathway was also downregulated, as indicated by the decrease of its level of phosphorylation after 1 hour following CD300e engagement. This preliminary result confirmed our previous data regarding the negative modulation of the insulin pathway by CD300e. It will be necessary to investigate if the phosphorylation of p38 MAPK is also hampered in presence of insulin, as has already been demonstrated for the AKT pathway.

Based on the evidence that patients with both T1D and T2D are seropositive for CD300e (Haseda et al., 2016), we revealed that OPs were also seropositive to CD300e and the anti-CD300e antibody titre in their sera declined after weight loss following bariatric surgery. Moreover, it has been demonstrated that the anti-CD300e titre in OPs before weight loss can be considered an immune marker able to predict the beneficial effect of the bariatric surgery-induced weight loss on the improvement of insulin sensitivity (Coletta et al., 2022). To date, it is not known whether the improvement in insulin sensitivity in these patients will last in the long term or there will be a relapse. In addition, the reason for producing anti-CD300e antibodies needs to be clarified. Antibodies are released by plasma cells derived from B lymphocytes activated by CD4^{+} T cell, which act as helpers to promote the humoral response. A first study pinpointed that a significant proportion of CD4^{+} T cell clones derived from peripheral blood of OPs, but not from lean metabolically healthy subjects, were activated by CD300e, proliferated, and showed a typical Th1 profile (Coletta et al., 2022). This evidence suggested that in the peripheral blood of OPs there was

an anti-CD300e-specific T cells response. The pro-inflammatory Th1 phenotype, which is the most represented in obesity, could fuel systemic inflammation and obesity-associated IR (Liu & Nikolajczyk, 2019). Another point to clarify is whether these autoantibodies may act as ligands for CD300e. We can guess that these anti-CD300e antibodies are ligands for the transmembrane receptor itself, which we have found to be expressed more on circulating monocytes from OPs compared to normal weight individuals. This antibody/receptor binding might result in the hindrance of the insulin pathway, leading to a systemic inflammation and IR under obesity condition. As mentioned above, we found that circulating monocytes from OPs displayed a higher surface expression of CD300e compared to normal weight subjects, even though the input responsible for this over-expression requires further studies. Indeed, it is not known if there is a transcriptional regulation or a vesicular exocytosis underlying this effect, and which are the intracellular molecules and signals involved. From a clinical point of view, we have noticed that the increased expression of the surface receptor in OPs positively correlated with their BMI.

This finding aligns with another study showing that individuals with higher initial BMI and waist circumference had increased CD300e expression in all three types of monocytes. This expression decreased when patients participated in a lifestyle program focused on healthy nutrition, increased exercise, and cognitive behavioural therapy to enhance their mental well-being (van der Valk et al., 2022).

It is known that several immune receptors are released into the blood circulation in their soluble form after proteolytic cleavage of the transmembrane protein. For instance, we can cite CD163, a circulating biomarker of macrophage activation (Semnani-Azad et al., 2021), and CD300b, which belongs to the same family of CD300e (Yamanishi et al., 2012), About this, Yamanashi et al. showed that a soluble form of CD300b was constitutively released by neutrophils through matrix metalloproteinases (MMPs)-mediated proteolytic cleavage of the surface receptor. The release was augmented by TLR agonists, including LPS stimulation, and its interaction with macrophages resulted in the production of pro-inflammatory cytokines. In this context, sCD300b was an amplifier of LPS-induced inflammation (Yamanishi et al., 2012). By contrast, another study focused on the inflammatory bowel disease (IBD) demonstrated that sCD300b was increased in the colons of DSS-treated mice, but in this case its binding to epithelial cells triggered reduced expression of epithelial cell adhesion molecules (EpCAMs), promoting their motility and migration with subsequent wound healing (Avlas et al., 2023). In addition, the innate immune receptor TREM2 undergoes proteolytic cleavage by ADAM proteases releasing its ectodomain into the extracellular space as a soluble form

(sTREM2). TREM2 is mainly expressed in microglia in the central nervous system, and it shows many similarities with CD300e due to its ability to recruit and activate the ITAM-bearing adaptor protein DAP12 to the cell membrane to propagate the downstream signal. It has been found that sTREM2 has dual roles, both promoting the production of inflammatory cytokines and stimulating microglial survival (Zhong et al., 2017).

We detected that sCD300e was present in the sera of both healthy donors and OPs, with the latter having lower levels. This result could mirror our previous finding that monocytes from OPs expressed significantly more CD300e on their cell surface than monocytes from normal weight subjects. In this context, the decrease of the sCD300e level in the sera of OPs could be an indicator of the increased expression of the receptor on the cell membranes of their circulating monocytes.

Since the ligand of CD300e is still unknown, making it an orphan receptor right now, its function in human cells has been studied by using an agonistic anti-CD300e monoclonal antibody (clone UP-H2) (Brckalo et al., 2010). With the purpose of discovering possible ligand(s) of CD300e, we synthetised liposomes using 3 different phospholipids, including sphingomyelin (SM), phosphatidylcholine (PC) and dioleoyl phosphatidylethanolamine (DOPE). FACS analysis revealed that monocytes bound both SM and, to a greater extent, DOPE. DOPE is a neutral phospholipid belonging to the class of phosphatidylethanolamines and displays sensitivity towards pH change. Indeed, at acidic pH, it is an inverted hexagonal micelle, which turns into spherical micelle at alkaline pH. It is widely used as a component of lipofectamine, a common transfection reagent, since it complexes with negatively charged nucleic acid molecules to allow them to overcome the electrostatic repulsion of the cell membrane. In this context, DOPE acts as a neutral co-lipid (helper lipid). The DNA-containing liposomes (positively charged on their surface) can fuse with the negatively charged cell membranes, due to the neutral co-lipid mediating fusion of the liposome with the cell membrane, allowing negatively charged nucleic acid cargo molecules to cross into the cytoplasm (Abbasi et al., 2023). In the light of these properties, it is more plausible that DOPE binds to the cell membrane in a receptor-independent manner rather than as a specific ligand for CD300e. This hypothesis is supported by the results obtained using HEK-293 cells, which demonstrated the same binding capacity to DOPE both in the absence of CD300e and in cells transduced to express the receptor.

Under obese condition, the imbalance between energy intake and expenditure causes accumulation of excess lipids in the form of triglycerides into the adipocytes, causing their hypertrophy and hyperplasia with subsequent inflammation of the AT (Camino et al., 2023). Dysfunctional adipocytes dysregulate the secretion of adipokines, further fueling the systemic inflammation and giving rise to

obesity-related diseases, including IR and T2D. Recently, adipocyte-derived extracellular vesicles (EVs) are emerging as alternative communication pathways mediating intercellular and interorgan crosstalk. EVs might be considered potentially good biomarkers of diseases since they carry membrane and luminal biomolecules, whose identity changes under physiological or pathological conditions, affecting the signaling and metabolic responses in recipient cells (Durcin et al., 2017). Multiple studies identified a significant increase of plasma EVs concentrations in OPs. Furthermore, lipidomic analysis evidenced that adipocyte-derived EVs are enriched in ceramide, SM and phosphatidylglycerol (PG) in the obesity context compared with lean state and with secreting visceral AT (VAT) (Blandin et al., 2023). Given these premises and based on our evidence, we could speculate that CD300e acts as a sensor for SM-carrying vesicles released abundantly by AT during obesity. In this context, SM could behave as a ligand for the receptor, starting the intracellular signaling cascade that interferes with pathways elicited by insulin and, consequently, giving rise to systemic IR which affects different target tissues located throughout the body. The significance of this discovery lies in the fact that CD300e, previously regarded solely as an immune receptor, appears to take on a role as a metabolic modulator. This finding reveals its potential as a therapeutic target for obesity and related metabolic disorders.

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