The CD163-expressing macrophages recognize and internalize TWEAK. Potential consequences in atherosclerosis.

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Abstract

Background: CD163 is a new potential scavenger receptor of Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) which elicits diverse biologic actions involved in atherosclerosis. We have analyzed the importance of TWEAK-CD163 interaction in atherosclerosis.

Methods: TWEAK and CD163 expression was studied in cultured human macrophages. Moreover, TWEAK and CD163 expression was analyzed in carotid atherosclerotic plaques (immunohystochemistry) and plasma (ELISA). We have also assessed their potential association with intima/media thickness (IMT) in asymptomatic subjects.

Results: In vitro studies revealed that CD163-expressing macrophages can bind and internalize TWEAK protein exogenously added from supernatants. Accordingly, we observed an inverse correlation between the expression of CD163 and TWEAK ($r=-0.51$; $p=0.008$) in the shoulder region of atherosclerotic plaques obtained from twenty-five patients undergoing carotid endarterectomy. The same trend was observed when we analyzed the plasma concentration of both proteins in 90 subjects free from clinical cardiovascular disease ($r=-0.25$; $p=0.016$) in which carotid ultrasonography was performed to determine IMT. In these subjects, we found a positive correlation between sCD163 and IMT ($r=0.36$; $p<0.001$) and between sCD163/sTWEAK ratio and IMT ($r=0.51$; $p<0.001$). This association remained significant after adjusting for traditional cardiovascular risk factors and inflammatory markers explaining 39% (sCD163) or 48% (sCD163/sTWEAK ratio) of IMT variance.

Conclusions: Our results suggest that TWEAK-CD163 interaction take place in vivo, probably decreasing TWEAK plasma concentration. Furthermore, we have observed that CD163/TWEAK plasma ratio is a potential biomarker of clinical and subclinical atherosclerosis.

1. Introduction

CD163 is a 130-kDa member of the scavenger receptor cysteine rich (SRCR) family exclusively expressed on the surface of monocytes/macrophages. It has been identified as the receptor which uptakes haptoglobin–haemoglobin (Hp–Hb) complexes for the removal and metabolism of the potent oxidant haemoglobin (1). A soluble form of CD163 (sCD163) is a normal constituent in plasma (2) and is generated by proteolytic cleavage (shedding) of CD163 at the cell surface (3). This receptor is now recognized as an immunomodulator of the atherosclerotic plaque, with pivotal anti-inflammatory and antiatherogenic properties (4-5).

Recently, CD163 has been identified as a new potential scavenger receptor for tumor necrosis factor-like weak inducer of apoptosis (TWEAK) (6). TWEAK is a novel member of the TNF superfamily of structurally-related cytokines (7). The first reported TWEAK receptor was fibroblast growth factor-inducible 14 (Fn14) (8-9). TWEAK is expressed in normal non-atherosclerotic arteries whereas Fn14 expression is almost absent. In contrast, both Fn14 and TWEAK are expressed in atherosclerotic plaques and these proteins colocalize with smooth muscle cells (SMCs) and macrophages (10). In this context, the interaction between TWEAK and Fn14 has several potential proatherogenic effects in cultured cells which may be important in the pathogenesis of atherosclerosis. TWEAK induces production of proinflammatory cytokines, proliferation and migration of cells present in atherosclerotic plaques and increases the expression of metalloproteinases that degrade the extracellular matrix (11).

Since all these processes play an important role in the pathogenesis of atherosclerosis, we would expect that soluble TWEAK (sTWEAK) plasma concentration should be elevated in this disease. In contrast, sTWEAK plasma levels are diminished in patients with carotid atherosclerosis (12), or atherosclerosis associated diseases, such as type 2 diabetes or end-stage renal disease (13). Furthermore, sTWEAK is negatively associated with carotid artery intima/media thickness (IMT) in subjects free from clinical cardiovascular disease (12). The mechanisms leading to lower sTWEAK plasma levels in subjects with atherosclerosis remain undefined. In this work, we have analyzed the importance of TWEAK-CD163 interaction in human macrophages in vitro and in human atherosclerotic plaque. Furthermore, sCD163 plasma

levels were evaluated in 90 asymptomatic subjects in whom IMT and sTWEAK had been previously measured (12).

2. Patients and Methods

2.1. In Vitro Studies:

2.1.1 Reagents

RPMI-1640, penicillin and streptomycin were obtained from BioWhittaker. Fetal bovine serum was from Gibco. Recombinant soluble human TWEAK (r-HuTWEAK) was from Alexis. Mouse anti-human Fn14 blocking antibody (ITEM-2) was from eBioscence. r-HuTWEAK was labelled with Cy5 labelling kit (PA35001; Amershan) following the manufacter´s instructions. The remaining reagents were obtained from Sigma unless specified otherwise.

2.1.2. Cell Culture

Human monocytic cell line THP-1 (ATCC; CRL-1999) were cultured in RPMI supplemented with 10% decomplemented fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, at 37°C in 5% CO2. Cells were differentiated to macrophages using PMA (10^{-7} M) for 48 hours. After that, THP-1 macrophages cells were treated for 24 hours with dexamethasone (2.5×10^{-7} M) and CD163, TWEAK and Fn14 expression was then assessed by Western blot analysis.

2.1.2 Western Blot

Cells from different experimental conditions were collected and pelleted. Western blots were performed as previously described (14). The blots were incubated with mouse anti-human Fn14 antibody (ITEM-4; eBioscence), goat anti-TWEAK polyclonal antibody (AF1090; R&D Systems) or mouse anti-CD163 monoclonal antibody (EDhu-1; Serotec) and rehybridated with anti-tubulin monoclonal antibody (B-5 to 1–2; Sigma) to confirm equal loading and transfer of proteins. Quantification was expressed as arbitrary densitometric units (ADU).

2.1.3 RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA was obtained by Trizol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. Real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *Taq*DNA polymerase (Amplitaq Gold). After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. 18S rRNA served as

housekeeping gene and was amplified in parallel with the genes of interest. The expression of target gene was normalized to housekeeping transcripts. Target gene, forward and reverse primers, and probes were designed using Primer Express 1.5 software (Applied Biosystems). All primers, probes, and reagents were obtained from Applied Biosystems. All measurements were performed in duplicate. Values of each sample were obtained as fold to their baseline values.

2.2. Patients with carotid atherosclerosis.

Twenty five consecutive patients (carotid stenosis >70%, 20 men, 5 women; age, 70±7 years; 88% with hypertension, 28% with diabetes, 36% with hyperlipidemia) undergoing carotid endarterectomy at our institutions were included. Informed consent was obtained before enrolment. The study was approved by the local Ethical Committees in accordance with institutional guidelines.

2.3. Subjects free from clinical cardiovascular disease.

The population studied consisted of 90 asymptomatic subjects (69 men, age 56.6±12.3 years) in whom global risk assessment was performed in the course of a general health check-up by Internal Medicine Department (University Clinic of Navarra, Spain). The clinical characteristics as well as the prevalence of cardiovascular risk factors had been previously described (12). In all subjects, absence of history of coronary disease, stroke or peripheral arterial disease was recorded; additional exclusion criteria were the presence of severely impaired renal function, arteritis, connective tissue diseases, alcohol abuse, or use of nonsteroidal anti-inflammatory drugs in the 2 weeks before entering the study. Conventional cardiovascular risk factors hypertension, dyslipidemia, diabetes, obesity and smoking were recorded in every patient and were defined as previously described (15, 16). Patients were considered to be hypertensive if they had systolic blood pressure >139 mmHg and/or diastolic pressure >89 mmHg and/or use of antihypertensive drugs. Dyslipidemia was diagnosed in the presence of total cholesterol ≥200 mg/dL, LDL cholesterol ≥130 mg/dL, HDL cholesterol <50 mg/dL, triglycerides ≥150 mg/dL

and/or use of cholesterol-lowering drugs. Obesity was estimated by the body mass index (BMI ≥30 kg/m^2). Diabetes mellitus was defined by fasting glucose levels >126 mg/dL, or by the use of glucose-lowering agents.

The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. In all subjects, carotid ultrasonography was performed to determine IMT, as previously described (15-16). Subjects were examined by the same 2 sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination (17). The between-observer intraclass correlation coefficient was 0.76 ($p<0.001$) and the between subject repeatability was 0.82 ($p<0.001$). The corresponding coefficients of variance were 5% and 10%, respectively.

2.4. Enzyme-Linked Immunosorbent Assay

Venous blood samples from different subjects were collected on EDTA. The whole-plasma samples were stored at -80°C until analysis was performed. Plasma concentrations of sTWEAK and sCD163 were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (BMS2006INST; Bender MedSystems; S-1015; Bachem; respectively). Plasma samples were assayed in parallel to known standard concentrations of recombinant TWEAK or recombinant CD163. Intra- and inter-assay coefficients of variation were 6.2% and 8.3% (sTWEAK) and 4.2% and 7.3% (sCD163), respectively.

2.5. Immunohistochemistry

Carotid atherosclerotic plaques were fixed in paraformaldehyde for 24 hours and stored in ethanol until paraffin-embedded. Immunohistochemistry was performed on 4-μm-thick sections as previously described (18). Primary antibodies were goat anti-TWEAK polyclonal antibody, mouse anti-CD163 monoclonal antibody and anti-human macrophages (HAM-56; Dako).

Sections were counterstained with Carazzi´s hematoxylin. Negative controls using the corresponding IgG were included to check for nonspecific staining.

2.6. Quantification

Computer-assisted morphometric analysis with the Olympus semiautomatic image analysis system Micro Image software (version 1.0 for Windows) was performed by a pathologist (L.O.) as previously described (18-19). Results are expressed as percentage of positive staining per mm^2.

2.7. Statistical analysis

Statistical analysis was performed with SPSS for Windows software package version 11.0 (SPSS Inc, Chicago, Ill). In vitro experiments were performed at least three times. Results are expressed as mean±SEM or mean±SD and were analyzed by ANOVA and Student's *t* test. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT as dependent variable, including in the model the traditional cardiovascular risk factors and inflammatory markers. A 2-tailed $p<0.05$ was considered statistically significant.

3. Results

3.1. CD163 and TWEAK are associated in human carotid atherosclerotic plaques.

CD163 and TWEAK expression was analyzed in atherosclerotic plaques from 25 consecutively subjects who underwent carotid endarterectomy. As shown in figure 1, both CD163 and TWEAK are present in human carotid atherosclerotic plaques. As expected, CD163 expression was higher in the shoulder (macrophage-rich area) than in the cap region (10.32±1.28 vs 6.97±0.91; respectively; $p<0.011$). TWEAK expression was not significantly different between shoulder and cap region (8.95±1.49 vs 8.14±1.37; respectively; N.S.). Double immunostaining revealed that TWEAK and CD163 are colocalized by some of the same cells (Fig. 1). When we analyzed the association between CD163 and TWEAK levels in human atherosclerotic plaques we observed an inverse correlation between the expression of both proteins ($r=-0.36$; $p=0.082$) (Fig. 1B). This inverse association was higher when CD163 and TWEAK expression was only considered in the shoulder region of atherosclerotic plaques ($r=-0.51$; $p=0.008$) (Fig. 1C).

3.2. CD163-expressing macrophages have less endogenous TWEAK.

To assess the relationship between CD163 expression and TWEAK levels, THP-1 cells were treated with PMA (10^{-7} M) for 48 hours and then with dexamethasone (DXM) (2.5×10^{-7} M) for 24 hours to induce maximal CD163 expression (20). Cells incubated with PMA or PMA+DXM expressed Fn14 (Fig. 2A). As expected, CD163 was highly expressed in cells in the presence of DXM. Interestingly, TWEAK protein was expressed in macrophages but its expression diminished in the presence of DXM in a time-dependent manner showing an opposite trend to CD163 expression (Fig. 2A). We hypothesize that CD163 could mediate TWEAK uptake and subsequent degradation by macrophages. For this purpose, we used an anti-CD163 blocking antibody to avoid the uptake of endogenous TWEAK. CD163 expressing-macrophages that were preincubated with an anti-CD163 blocking antibody (100 ng/ml) showed similar TWEAK levels to non CD163-expressing cells. No effects were observed when cells were incubated in the presence of non-specific IgG (data nor shown). Furthermore, when

CD163-expressing macrophages were incubated in excess of soluble form of r-HuTWEAK (1 ng/mL), endogenous TWEAK levels were restablished (Fig. 2B).

To analyze whether TWEAK diminution is a consequence of TWEAK mRNA downregulation, THP-1 cells were treated with PMA for 48 hours and then with dexamethasone for 24 hours to induce maximal CD163 expression. At this time (0 hours), macrophages exposed to DXM express both CD163 and TWEAK mRNA (Fig. 2C). In addition, CD163 mRNA expression was downregulated in a time-dependent manner (Fig. 2C) although protein expression remained unaltered (Fig. 2A). In contrast, TWEAK mRNA expression remained without changes for up to 24 hours, indicating that the diminution in TWEAK protein expression observed in CD163-expressing macrophages was not due to a reduction in mRNA expression.

3.3. CD163-expressing macrophages recognize and internalize sTWEAK in vitro.

To analyze whether CD163-expressing macrophages can take up sTWEAK from supernatants, cells were exposed to 1 ng/mL of soluble r-HuTWEAK. As shown in figure 3A, quantification of sTWEAK in macrophage supernatants shows a higher consumption by cells incubated with PMA+DXM compared with those with PMA, although non statistical significance was observed (Fig. 3A). Since Fn14 is present in macrophages independently of CD163 expression, cells were preincubated with an anti-Fn14 blocking antibody for 2 hours and then incubated with r-HuTWEAK. In these conditions, sTWEAK levels were decreased only in the supernatants from CD163-expressing macrophages, indicating that CD163 binds and allows uptake of r-HuTWEAK in macrophages (Fig. 3A). Furthermore, when cells were preincubated with both anti-Fn14 and anti-CD163 blocking antibodies, we observed that CD163-expressing macrophages can not take up r-HuTWEAK from supernatants, indicating a direct interaction between CD163 and r-HuTWEAK. No effects were observed when cells were incubated in the presence of non-specific IgG.

To further examine the cellular interaction between sTWEAK with CD163 and/or Fn14, we labelled r-HuTWEAK with Cy5 fluorescent dye. CD163-expressing macrophages exhibited

a binding to TWEAK-Cy5 in both membrane and cytoplasmatic compartments (Fig 3B-A). To confirm whether CD163 or Fn14 are responsible of TWEAK-Cy5 binding, cells were preincubated with either anti-CD163 or anti-Fn14 blocking antibodies. When cells were exposed to both blocking antibodies, no fluorescence was observed in macrophages (Fig. 3B-B).In addition, when cells were incubated with anti-CD163, TWEAK-Cy5 showed a membrane-staining pattern, indicating its potential interaction with Fn14 (Fig 3B-C). Moreover, when cells were preincubated with anti-Fn14, TWEAK-Cy5 labelling was present in both, membrane and cytoplasmic compartments, indicating that CD163 can bind and internalize TWEAK protein (Fig. 3B-D). No changes were observed in TWEAK-Cy5 distribution when non-specific IgG were used as a control (not shown).

3.4. CD163/sTWEAK ratio is associated with subclinical atherosclerosis

We have previously reported that sTWEAK plasma levels are diminished and inversely correlated with IMT in asymptomatic subjects (12). To test whether sCD163 is associated with sTWEAK plasma levels, we have analyzed its plasma levels in 90 asymptomatic subjects in whom IMT and sTWEAK were measured. Characteristics of the studied population are summarized in Table 1, demonstrating variable intensity of conventional atherosclerotic risk factors and antiatherosclerotic therapy. The percentage of diabetics free from overt cardiovascular disease was 16%, without differences in the sCD163/sTWEAK ratio as compared with nondiabetic patients (13.2±7.8 vs 11.2±6.3). An univariate analysis shows a negative correlation between sCD163 and sTWEAK ($r=-0.25$; $p=0.016$) (Fig. 4A).

Since sCD163 and sTWEAK show opposite trends, we calculated sCD163/sTWEAK ratio and tested its potential as a marker of subclinical atherosclerosis by evaluating its potential association with IMT. Univariate analysis shows a positive correlation between sCD163 and IMT ($r=0.36$; $p<0.001$) and between sCD163/sTWEAK ratio and IMT ($r=0.51$; $p<0.001$) (Fig. 4B). As shown in table I (online), no evidence of association between sCD163 or sCD163/sTWEAK ratio and the other clinical parameters was observed. Interestingly,

correlation between sCD163/sTWEAK and IMT was superior to that observed between IMT and sCD163 or sTWEAK and other clinical parameters analyzed including C-reactive protein, a marker of systemic inflammation (Table I online).

The association between sCD163 or sCD163/sTWEAK ratio and carotid IMT remained significant after adjusting for traditional risk factors and inflammatory markers, explaining 39% (sCD163) or 48% (sCD163/sTWEAK ratio) of IMT variance (Table II online).

4. Discussion

Our results show that CD163 and TWEAK are expressed in an opposite trend in human carotid atherosclerotic plaques and that CD163-expressing macrophages are able to bind and internalize sTWEAK in vitro. More importantly, sCD163 plasma levels are negatively associated with sTWEAK concentrations and sCD163/sTWEAK ratio is positively correlated with IMT in asymptomatic subjects.

CD163 is a member of the class B scavenger receptors restricted to monocyte/macrophages lineage, which is expressed as a membrane protein and actively shed from the cell surface (21). The principal characteristic of CD163 is to be a scavenger receptor for Hp-Hb complex. Hp proteins represents the first line of defense against the toxic effects of free Hb. Hp binds to free Hb forming a Hp–Hb complex which may be cleared by CD163 receptor (22-23). Clearance of the Hp–Hb complex from the atherosclerotic plaque is mediated exclusively by CD163. In addition to its scavenger function, this receptor is now recognized as an immunomodulator of the atherosclerotic plaque, with anti-inflammatory and antiatherogenic properties (4-5). In agreement with previous reports in which CD163 expression has been shown in coronary and aortic lesions (20;24), we have observed that CD163 is expressed in human carotid atherosclerotic plaques, principally in the shoulder region, an area characterized by a high inflammatory content (18). CD163 has been recently identified as a new receptor for TWEAK (6), a novel member of the TNF superfamily. Through interaction with its receptor Fn14, TWEAK increases the production of different molecules such as proinflammatory cytokines and metalloproteinases, and activates proliferation, migration and angiogenesis (11). In this context, we have previously reported that TWEAK is expressed by both SMCs and macrophages within atherosclerotic plaques (12). In the present study, we showed that TWEAK is present in human carotid atherosclerotic plaques colocalizing with CD163. Moreover, we have observed an inverse association between CD163 and TWEAK expression in the shoulder of atherosclerotic plaques, indicating that co-expression or interaction between CD163 and TWEAK can take place *in vivo*.

It has been demonstrated that TWEAK mediates signal transduction in cells lacking Fn14, suggesting the existence of an alternative TWEAK receptor (25). In this respect, CD163 from CD14-positive monocytes not expressing Fn14 has been related with TWEAK binding (6). However, the functionality of CD163 in cells expressing Fn14 has not been yet analyzed. Since CD163 and TWEAK colocalize within atherosclerotic plaques as we previously reported for TWEAK and Fn14 (10), we evaluated the potential significance of CD163/TWEAK interaction in cells-expressing both receptors, CD163 and Fn14. We have observed that Fn14/CD163-expressing macrophages have less amount of endogenous TWEAK protein compared with those without CD163. Furthermore, TWEAK mRNA expression was not affected and remained constant in Fn14/CD163-expressing macrophages, suggesting that TWEAK protein diminution was not related with its mRNA expression. Interestingly, this diminution was reversed either by an anti-CD163 blocking antibody or in competition studies with soluble r-HuTWEAK in the culture medium, indicating that CD163/TWEAK interaction was responsible of endogenous TWEAK reduction.

Moreover, we have shown that CD163-expressing macrophages can sequester and internalize sTWEAK from culture medium. Recovery experiments demonstrated that both Fn14 and CD163 can sequester soluble r-HuTWEAK from the culture medium. When cells were preincubated with anti-Fn14 or anti-CD163 blocking antibody, we observed an increment in soluble r-HuTWEAK recovery from the culture medium. Moreover, when both blocking antibodies were present in the culture medium soluble r-HuTWEAK was not sequestered by macrophages. Consistently with these results, staining of a soluble form of r-HuTWEAK labelled with Cy5 showed that macrophages in the presence of an anti-Fn14 antibody could internalize sTWEAK, probably through interaction with CD163. In contrast, macrophages incubated with an anti-CD163 antibody were capable of binding sTWEAK but could not internalize it, indicating that sTWEAK was bound to Fn14. In addition, macrophages incubated with both anti-CD163 and anti-Fn14 antibodies could not bind sTWEAK, indicating that macrophages could only interact with sTWEAK by either CD163 or Fn14 since both receptors are functional in cells co-expressing them. Furthermore, internalization of sTWEAK by CD163

could be followed by its degradation, as observed in CD163-expressing cells without Fn14 expression (6). In this context, it is important to note that TWEAK protein structure mimics the Hp-Hb complex (6), indicating that TWEAK could compete with Hp-Hb for CD163 binding. In this condition, the presence of sTWEAK could have deleterious consequences since it could induce an augmentation of free Hp-Hb levels, increasing the pathological effects of this complex in the arterial wall (4). However, future research efforts regarding TWEAK-CD163 interaction could help to address this hypothesis.

As commented above, CD163 has been shown to be present in a natural soluble form in plasma (2). Because of a constant shedding of the receptor from the membrane, the plasma concentration of the receptor may reflect its general expression level (2). The elevation of sCD163 in diseases associated with macrophage activation has been described. Patients with rheumatoid arthritis (26), Gaucher disease (27), hemophagocytosis (28), sepsis (29), and myelomonocytic leukaemia (29) have increased soluble CD163 levels in plasma relative to healthy controls. Furthermore, sCD163 concentrations have been a predictor of coronary artery disease extent independently of conventional risk factors such as age, hyperlipidemia, hypertension and smoking status (30). Since sTWEAK plasma levels concentrations are diminished in subjects with carotid atherosclerosis, diabetes or chronic kidney disease (12-13) and are negatively associated with IMT, an index of subclinical atherosclerosis (12), we have explored whether CD163/TWEAK interaction could be reflected by sCD163 and sTWEAK plasma concentrations. We have shown that sCD163 plasma levels inversely correlated with sTWEAK concentrations in asymptomatic subjects in whom IMT were measured (12). These data are in agreement with the observation that subjects with diabetes have increased sCD163 plasma levels (20) and decreased sTWEAK concentrations (13) compared with those without diabetes. Moreover, sCD163 plasma levels were associated with IMT in asymptomatic subjects and this association remained significant after adjustment by traditional cardiovascular risk factors and other inflammatory biomarkers. In addition, when considered sCD163/sTWEAK ratio, an even higher association with IMT was observed. Whether sCD163/sTWEAK ratio

could be a potential novel biomarker of atherosclerosis warrants further investigation in prospective studies.

In conclusion, our results shows that TWEAK/CD163 interaction could occur within atherosclerotic plaques and CD163 could be responsible of sTWEAK plasma diminution observed in subjects with cardiovascular disease. Furthermore, sCD163/sTWEAK ratio could be a potential biomarker of atherosclerosis in asymptomatic subjects.

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Figure legends

Figure 1.- TWEAK and CD163 expression in human arteries.

(A) Colocalization studies showing the expression of CD163, macrophages and TWEAK in sections from human carotid atherosclerotic plaques. Magnification ×200 and ×400. (**B**) Correlation between TWEAK and CD163 in the whole or (**C**) in the shoulder region of atherosclerotic plaques.

Figure 2.- TWEAK levels in CD163-expressing macrophages.

A, Western blot showing the expression of TWEAK and their receptors Fn14 and CD163 in macrophages incubated with or without DXM and anti-CD163. Results are expressed as mean±SEM of three independent experiments.

B, Western blot showing TWEAK expression in macrophages in the presence of sTWEAK and incubated with or without DXM and anti-CD163. Results are expressed as mean±SEM of three independent experiments.

C, Real-time PCR showing the effect of PMA (white) or PMA+DXM (black) on CD163 (upper panel) or TWEAK (lower panel) mRNA expression in macrophages. *$p<0.05$ versus PMA. Results are expressed as mean±SEM of four independent experiments.

Figure 3.- sTWEAK is sequestered and internalized by CD163-expressing macrophages.

A, Recovery of soluble r-HuTWEAK from supernatants of CD163-expressing macrophages. Soluble r-HuTWEAK (1 ng/mL) was incubated with CD163-expressing macrophages in absence or presence of both anti-CD163 and anti-Fn14 blocking antibodies during 6 hours. After that, sTWEAK levels were measured by ELISA in supernatants.

B, In situ detection of TWEAK binding to CD163-expressing macrophages. rTWEAK-Cy5 staining in macrophages incubated PMA and DXM (a) in presence of anti-CD163 (c), anti-Fn14 (d) or both antibodies (b).

Figure 4.- A, Correlation between sCD163 and sTWEAK in asymptomatic individuals. sCD163 negatively correlates with sTWEAK concentration (r=-0.25; p=0.016).

B, IMT and sCD163/sTWEAK ratio in asymptomatic individuals. The IMT of common carotid arteries correlates with sCD163 concentration (r=0.36; P<0.001) and sCD163/sTWEAK ratio (r=0.51;p<0.001).

Table 1. Baseline clinical characteristics of the studied populations.

Total Population (n=90)	
Age, years	56.6±12.3
Sex , male/female	69/21
Smokers, yes/no	27/63
BMI, kg/m^2	28.1±3.6
SBP, mm Hg	132.6±24.4
DBP, mm Hg	81.6 ±10.7
Arterial hypertension , yes/no	49/41
Diabetes mellitus, yes/no	15/75
Glucose, mg/dL	108.9±36.4
Total cholesterol, mg/dL	216.0±39.6
HDL-cholesterol, mg/dL	49.8±13.6
LDL-cholesterol mg/dL	143.3±34.4
Triglycerides, mg/mL	113.9±58.8
CRP, mg/L, #	4.8±0.8
Fibrinogen, mg/dL	319.8±91.0
vWF, %	124.2±75.9
sTWEAK (pg/mL)	200.1±43.3
sCD163	2145.2±1017.0
sCD163/sTWEAK ratio, #	11.5±6.6 (0.7)
Mean carotid IMT, mm	0.74±0.2

Values are expressed as mean±SD, number of subjects and mean±SEM (#).

BMI indicates body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; IMT, intima-media thickness; SBP, systolic blood pressure; vWF, von Willebrand factor.

Figure 1

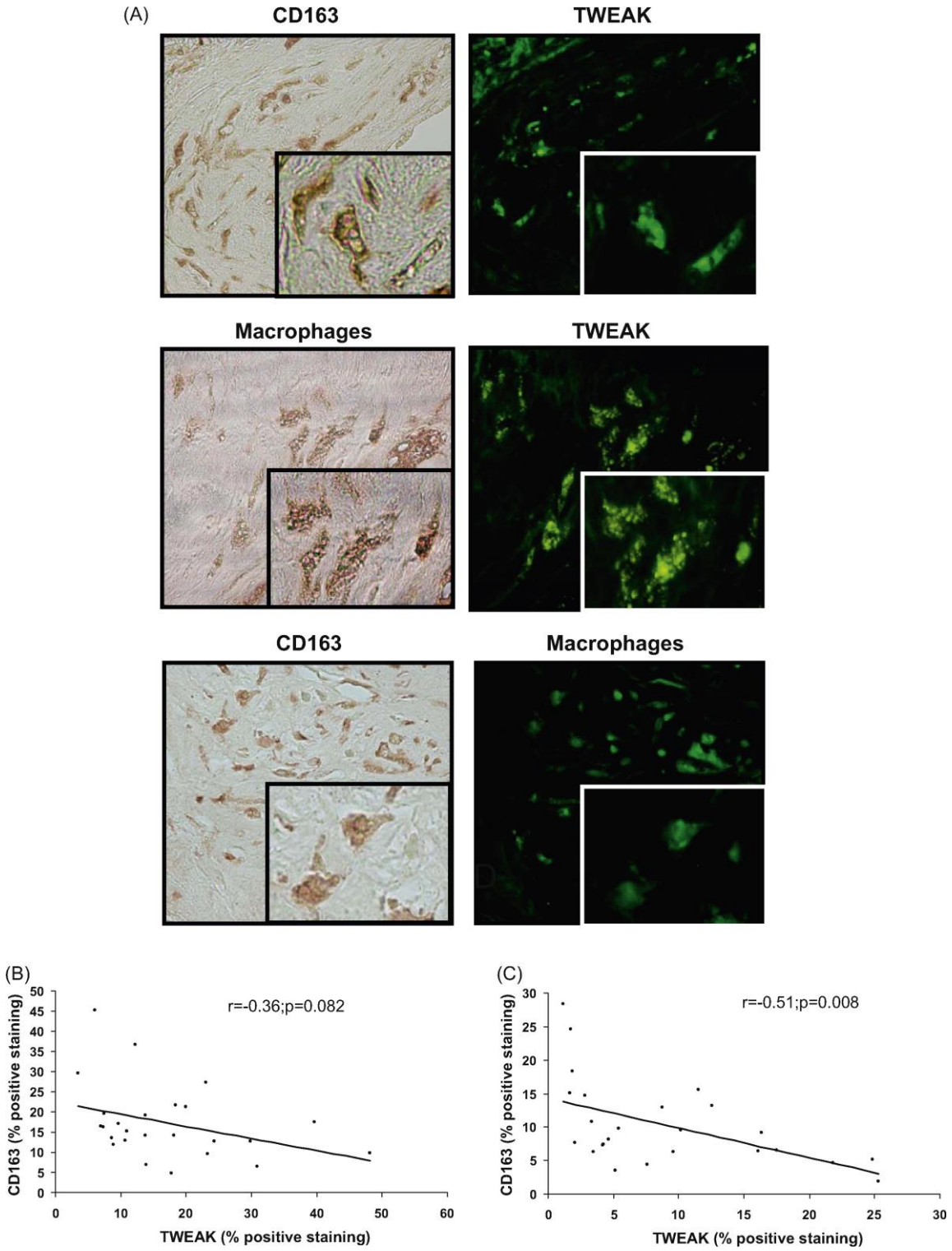

(A)
CD163
TWEAK
Macrophages
TWEAK
CD163
Macrophages
(B)
r=-0.36;p=0.082
CD163 (% positive staining)
TWEAK (% positive staining)
(C)
r=-0.51;p=0.008
CD163 (% positive staining)
TWEAK (% positive staining)

Figure 2

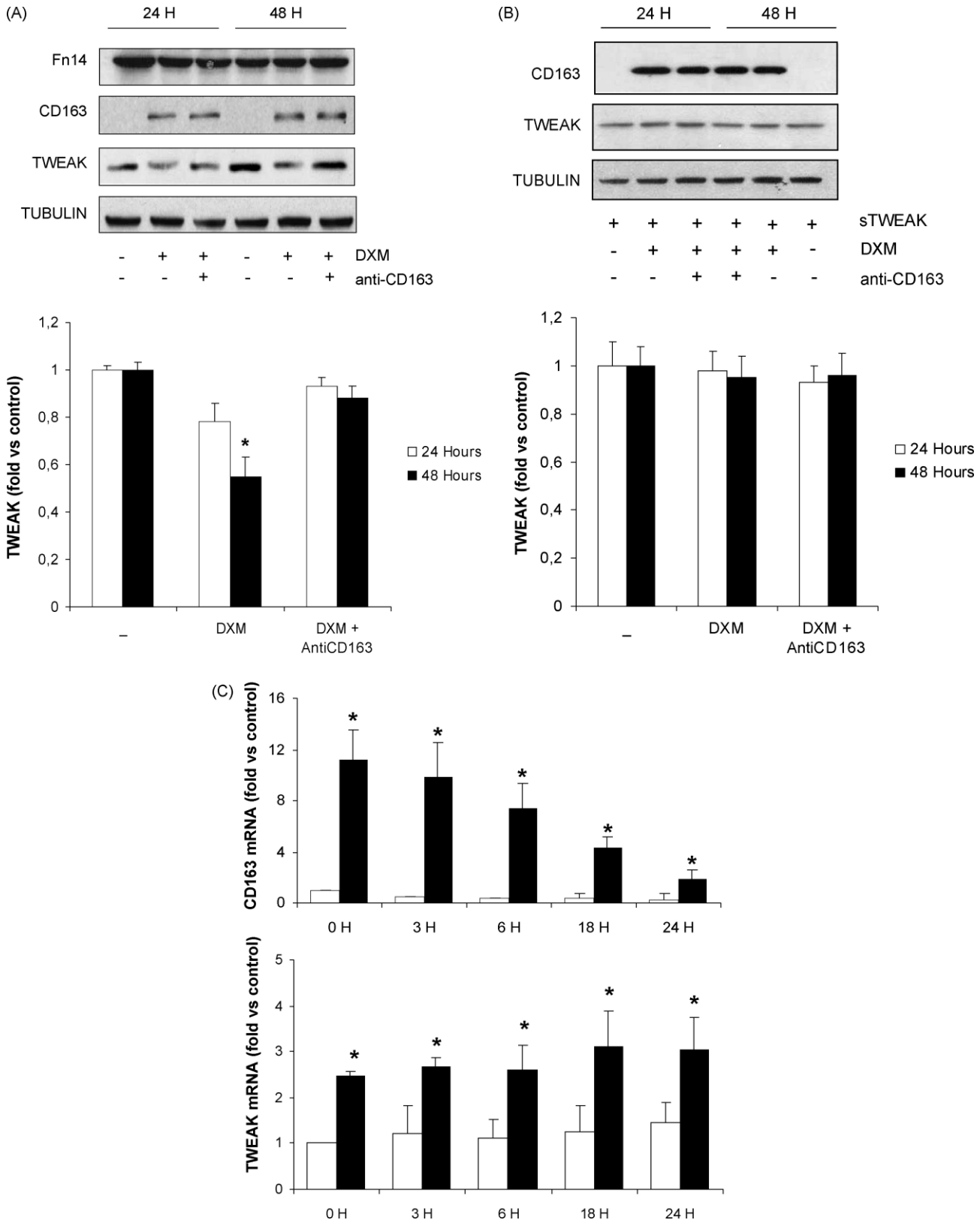
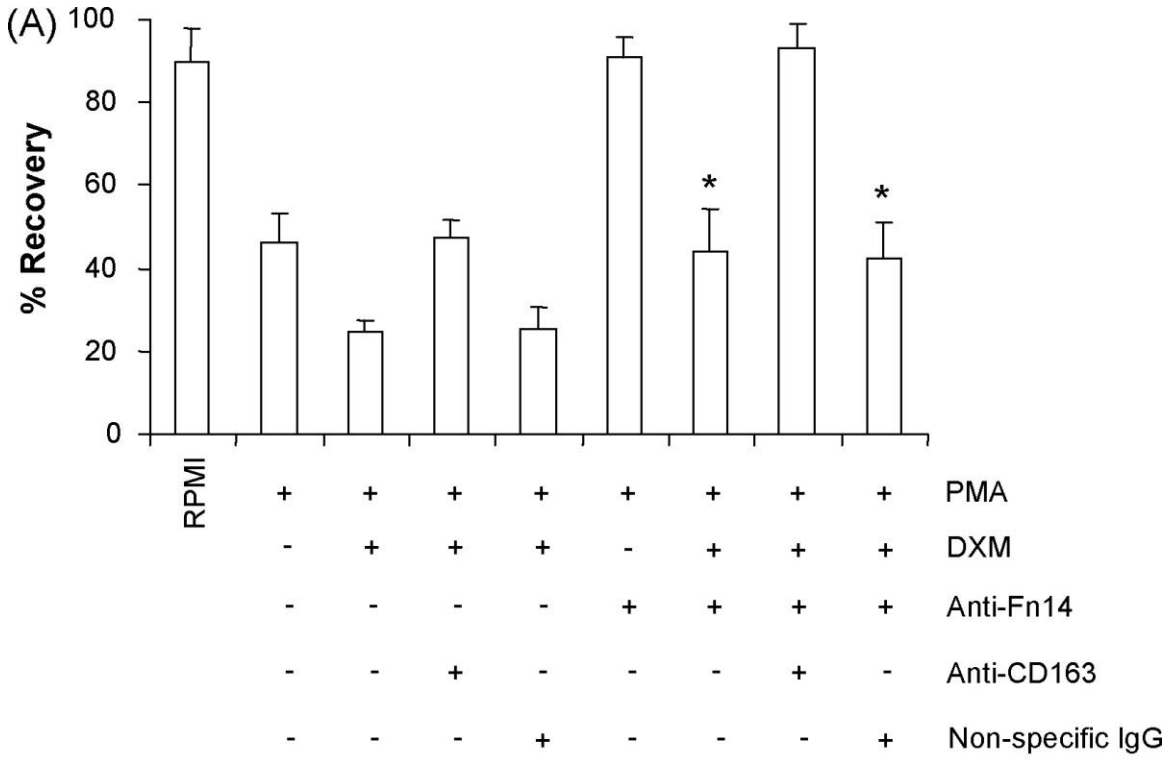

(A)
24 H
48 H
Fn14
CD163
TWEAK
TUBULIN
- + + - + + DXM
- - + - - + anti-CD163
TWEAK (fold vs control)
1,2
1
0,8
0,6
0,4
0,2
0
*
24 Hours
48 Hours
–
DXM
DXM + AntiCD163
(B)
24 H
48 H
CD163
TWEAK
TUBULIN
+ + + + + + sTWEAK
- + + + + - DXM
- - + + - - anti-CD163
TWEAK (fold vs control)
1,2
1
0,8
0,6
0,4
0,2
0
24 Hours
48 Hours
–
DXM
DXM + AntiCD163
(C)
CD163 mRNA (fold vs control)
16
12
8
4
0
*
0 H
3 H
6 H
18 H
24 H
TWEAK mRNA (fold vs control)
5
4
3
2
1
0
*
0 H
3 H
6 H
18 H
24 H

Figure 3


(A)
100
80
60
40
20
0
% Recovery
*
*
RPMI
PMA
DXM
Anti-Fn14
Anti-CD163
Non-specific IgG

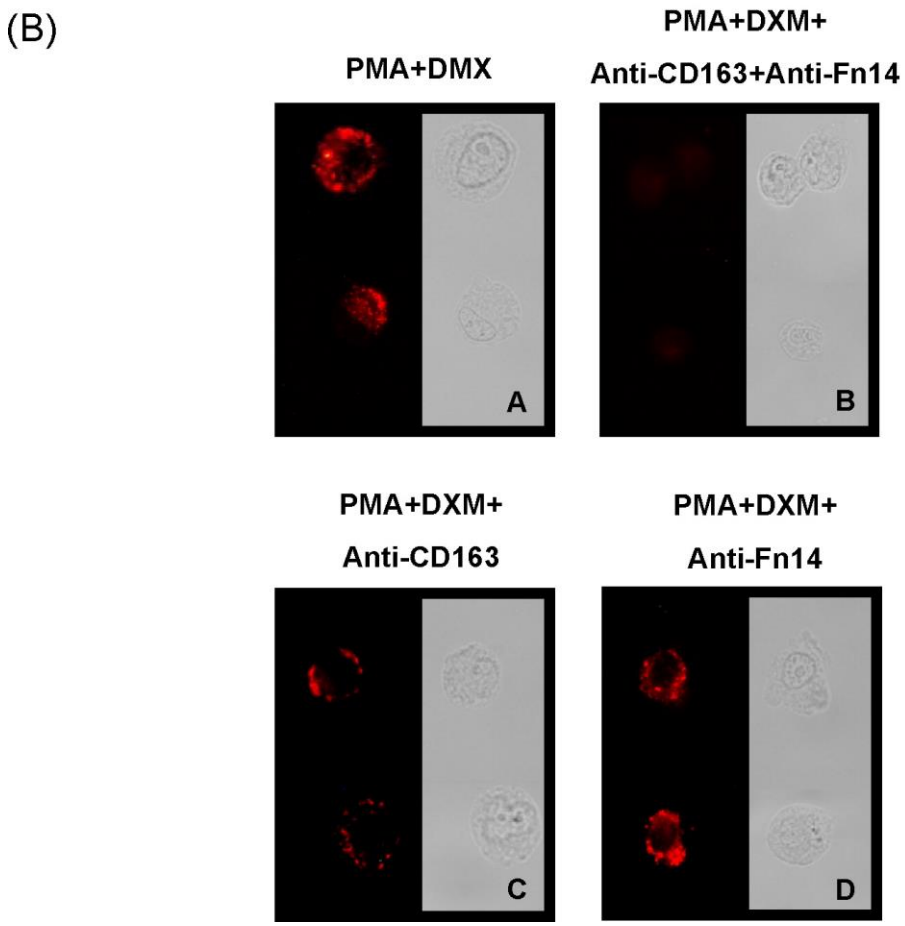

(B)
PMA+DMX
PMA+DXM+
Anti-CD163+Anti-Fn14
A
B
PMA+DXM+
Anti-CD163
PMA+DXM+
Anti-Fn14
C
D

Figure 4

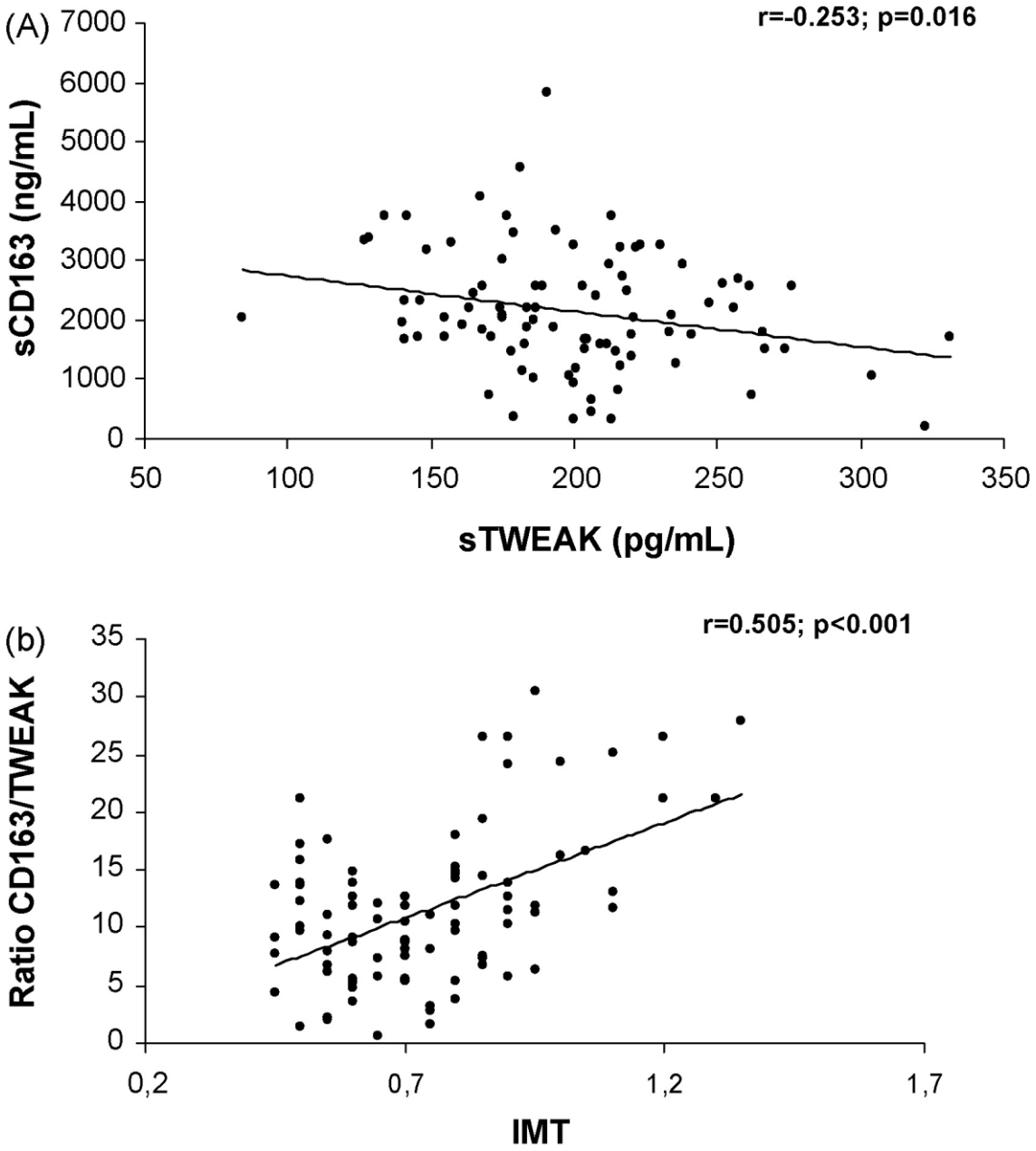

(A)
r=-0.253; p=0.016
sCD163 (ng/mL)
7000
6000
5000
4000
3000
2000
1000
0
50
100
150
200
250
300
350
sTWEAK (pg/mL)
(b)
r=0.505; p<0.001
Ratio CD163/TWEAK
35
30
25
20
15
10
5
0
0,2
0,7
1,2
1,7
IMT

Table I (online). Correlation coefficients of mean IMT, sTWEAK and sCD163 with clinical and laboratory parameters in the studied population

	IMT		sTWEAK		sCD163	
	r	p	r	p	r	p
Age	0.41	<0.001	0.001	0.986	0.05	0.644
BMI	0.21	0.044	-0.05	0.604	0.04	0.665
SBP	0.42	<0.001	-0.14	0.170	0.07	0.467
DBP	0.12	0.245	-0.01	0.859	0.12	0.244
Glucose (mg/dL)	0.28	0.006	-0.19	0.060	0.06	0.548
Cholesterol (mg/dL)	-0.007	0.942	0.21	0.050	-0.04	0.680
Triglycerides (mg/dL)	0.06	0.557	0.01	0.998	-0.03	0.759
LDL-cholesterol (mg/dL)	-0.004	0.969	0.17	0.109	-0.04	0.711
HDL-cholesterol (mg/dL)	-0.15	0.155	0.09	0.381	-0.07	0.465
LogCRP	0.23	0.025	-0.15	0.161	0.01	0.925
Fibrinogen (mg/dL)	0.32	0.001	-0.07	0.470	0.05	0.617
vWF (%)	0.22	0.040	0.09	0.395	0.01	0.860
sTWEAK (pg/mL)	-0.403	<0.001	---	---	-0.25	0.016
sCD163 (ng/mL)	0.36	<0.001	-0.25	0.016	---	---
sTWEAK/CD163 ratio	0.505	<0.001	-0.58	<0.001	0.90	<0.001

Correlations and p values from Pearson correlation coefficient.

Table II (online): Stepwise multiple regression analysis for the association between IMT (mm), cardiovascular risk factors, markers of inflammation and sCD163 or sCD163/sTWEAK ratio in the studied population.

	β	SE (β)	p	Partial R
sCD163	0,000	0,000	0,001	0,359
Age	0,005	0,002	0,003	0,328
SBP	0,002	0,001	0,024	0,253
Adjusted R^2= 0.39				
sCD163/sTWEAK	0,013	0,002	0,000	0,509
Age	0,005	0,001	0,002	0,349
SBP	0,002	0,001	0,023	0,254
Adjusted R^2= 0.48				

Variables excluded were gender, BMI, glucose, cholesterol, fibrinogen, von Willebrand and CRP.