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Regulation of Thrombospondin-1 expression in alternatively activated macrophages and adipocytes: role of cellular crosstalk and omega-3 fatty acids

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Abstract

Thrombospondin-1 (TSP-1) expression in human adipose positively correlates with body mass index and may contribute to adipose dysfunction by activating TGF-β and/or inhibiting angiogenesis. Our objective was to determine how TSP-1 is regulated in adipocytes and polarized macrophages using a coculture system and to determine whether fatty acids, including the ω-3 fatty acid DHA, regulate TSP-1 expression. Coculture of M1, M2a, or M2c macrophages with adipocytes induced TSP-1 gene expression in adipocytes (from 2.4 to 4.2-fold, $P<0.05$), and adipocyte coculture induced TSP-1 gene expression in M1 and M2c macrophages (M1:8.6-fold; M2c 26-fold, $P<0.05$). TSP-1 protein levels in the shared media of adipocytes and M2c cells was also strongly induced by coculture (>10 fold, $P<0.05$). DHA treatment during the coculture of adipocytes and M2c macrophages potently inhibited theM2c macrophage TSP-1 mRNA level (97% inhibition, $P<0.05$). Adipocyte coculture induced IL-10 expression in M2c macrophages (10.1-fold, $P<0.05$), and this increase in IL-10 mRNA expression was almost completely blocked with DHA treatment (96% inhibition, $P<0.05$); thus, IL-10 expression closely paralleled TSP-1 expression. Since IL-10 has been shown to regulate TSP-1 in other cell types, we reduced IL-10 expression with siRNA in the M2c cells and found that this caused TSP-1 to be reduced in response to adipocyte coculture by 60% ($P<0.05$), suggesting that IL-10 regulates TSP-1 expression in M2c macrophages. These results suggest that supplementation with dietary ω-3 fatty acids could potentially be beneficial to adipose tissue in obesity by reducing TSP-1 and fibrosis.

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Keywords

docosahexaenoic acid; thrombospondin-1; adipocyte; macrophage; IL-10

1. Introduction

Adipose tissue becomes dysfunctional with obesity, resulting in increased free fatty acids (FFAs) and inflammation, which contribute to insulin resistance. Some of the features of dysfunctional adipose tissue are increased hypoxia, fibrosis, necrotic adipocytes, and macrophages. Macrophages are capable of differentiating into “classically activated” macrophages (M1), which are inflammatory and “alternatively activated” macrophages (M2), which are anti-inflammatory and profibrotic. M2 macrophages can be further characterized as M2a or M2c macrophages, which are activated by IL-4 and IL-10 respectively. Despite the fact that macrophages are implicated in adipose dysfunction, tissue-resident macrophages perform numerous activities to maintain tissue homeostasis, and many tissue macrophages are heterogeneous with respect to their expression of M1 and M2 markers reflecting these diverse functions. In humans, more adipose tissue macrophages are observed with obesity, including macrophages expressing M1 markers, M2 markers, and both types of markers. Thus, macrophages may contribute to both the chronic state of low-grade inflammation and increased fibrosis observed in obese adipose.

Fibrosis is the excess deposition of extracellular matrix (ECM) proteins, and the adverse consequence of increased fibrosis on adipose function has only recently begun to be investigated. In mice, obesity is correlated with increased expression of collagen VI, and collagen VI knockout mice in the ob/ob background display an improved metabolic profile. Another line of evidence suggesting that reducing ECM rigidity improves adipose function is that inhibiting the collagen cross-linking enzyme lysyl oxidase also improves glucose tolerance in mice. One proposed mechanism to explain the adverse effect of fibrosis on adipose function is that a rigid extracellular matrix restricts adipocyte expansion under conditions of increased caloric intake, causing cellular stress. In humans, increased collagen VI gene expression is also positively correlated with BMI and negatively correlated with insulin sensitivity. Adipocytes and macrophages are both capable of producing ECM proteins including collagen VI, and recent studies suggest that coculture of human M2 macrophages with adipocytes induces the expression of fibrotic proteins in both cell types.

In humans, thrombospondin (TSP-1) may contribute to adipose fibrosis in obesity since TSP-1 mRNA expression positively correlates with BMI and negatively correlates with insulin sensitivity; furthermore, the insulin sensitizer pioglitazone reduces TSP-1 expression. TSP-1 induces fibrosis by activating latent TGF-β and by a TGF-β independent mechanism, and levels of both TSP-1 mRNA and protein are induced by coculture in both adipoctyes and macrophages. In mice, TSP-1 protein expression is increased in subcutaneous and gonadal fat pads in a genetic model of obesity (ob/ob mice) and is transiently induced in gonadal fat pads in a diet-induced model of obesity. Furthermore, TSP-1 knockout mice display improved metabolic function when challenged with a high fat diet, which was attributed to the fact that TSP-1 increases macrophage chemotaxis. However, TSP-1 is a multifunctional protein that inhibits angiogenesis and induces fibrosis; these additional TSP-1 functions would also be predicted to contribute to adipose dysfunction.

In this study, we examined the regulation of TSP-1in adipocytes derived from lean subjects and different types of polarized macrophages using an adipocyte-macrophage coculture system. Furthermore, we evaluated the effect of free fatty acids (FFAs) on our adipocyte macrophage cocultures, focusing on the n-3 FFA docosahexaenoic acid (DHA) because it

has anti-fibrotic effects in other cell types including cardiac fibroblasts and mesangial cell. DHA has also been demonstrated to improve metabolic function in rodents. We observed that adipocyte coculture augments the macrophage expression of markers associated with alternative activation and that coculture induces TSP-1 expression in macrophages as well as adipocytes via apparently different mechanisms.

2. Materials and methods

2.1. Cell culture and coculture

THP-1 cells were maintained and induced to differentiate into polarized macrophages as described. Briefly, Corning® CellBind® 6-well tissue culture plates (Corning, NY) were coated with 1 mL per well of phenyl-ethyl-enimine(PEI) solution (1mL/L PEI in 38.8 mM boric acid, 10 mM sodium borate) for 3-hours at room temperature, washed twice with 2 mL dH_2O, dried overnight, and stored at −20°C until use. The THP-1 cells (1×10^6 cells per well) were then differentiated as described on the PEI coated plates. Briefly, the treatments were as follows. For M1 macrophages, the THP-1 cells were washed with macrophage serum free medium (MSFM, Life Sciences, Grand Island, NY) and incubated overnight in MSFM containing 20 ng/ml LPS from Escherichia coli 0111:B4 (Sigma, St. Louis, MO) and 20 ng/ml IFN-γ (R&D Systems, Minneapolis, MN). For M2a macrophages, the THP-1 cells were incubated in MSFM containing 25 nM phorbalmyristate acetate (PMA) for 5-minuteswhile centrifuging at 100 × g. The medium was then replaced with MSFM containing 20 ng/mL IL-4 (R&D Systems) for overnight incubation. For M2c macrophages, the treatment was the same as for M2a macrophages except that 20 ng/mL IL-10 (R&D Systems) was used instead of IL-4. After the overnight differentiation, the macrophages were cocultured with differentiated ADHASC (adult-derived human adipose stem cells) adipocytes grown on coculture inserts, which were placed in the 6-well plates containing the macrophages. The coculture media was α-MEM with 1X penicillin-streptomycin and 2% fetal bovine serum. The ADHASC cells were plated in inserts and induced to differentiate for 10–14 days as described ; the ADHASC cells used in this study were greater than 90% differentiated at the time of coculture. The ADHASC cells used in this study were isolated from women with a BMI less than 25 in the laboratories of Dr. Kern and Dr. McGehee.

2.2. Analysis of gene expression

Total RNA was prepared using an Ambion RNA queous kit (Life Technologies)and was analyzed for RNA integrity and quantity with an Agilent Bioanalyzer (Palo Alto, CA, USA). Total RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and then subjected to real time polymerase chain reaction analysis with SYBR Green PCR Master Mix (Applied Biosystems) as described. A standard curve was prepared from a pool of the cDNA to determine relative changes in gene expression, and the data fell within the curve. Gene expression was normalized to 18S expression.

2.3.Treatment with TGF-β neutralizing antibody and TGF-β receptor inhibitor

M2cmacrophages and adipocytes were differentiated and cocultured as described above in the presence of 1D11 TGF-β neutralizing antibody or SB505124 TGF-β receptor inhibitor. In the TGF-β neutralizing antibody experiment, macrophages and adipocytes were cococultured in the presence of 10μg/ml 1D11 TGF-β neutralizing antibody(R&D Systems) or a control monoclonal antibody(R&D Systems) for 24hrs. In the TGF-β receptor inhibitor experiment, macrophages and adipocytes were cococultured in the presence of DMSO vehicle control or 1μM SB505124(Sigma) for 24hrs.

2.4 Analysis of TSP-1 protein levels

The level of TSP-1 protein in the media was analyzed with a Thrombospondin-1 ELISA (R&D Systems, Minneapolis MN) according to the manufacturer's instructions.

2.5. Recombinant IL-10 and TGF-β treatment

M2c macrophages were prepared as described above. They were then subjected to serum starvation in macrophage serum-free medium for 12-hours and then were treated with either20 ng/ml IL-10 or 5ng/mL TGF-β (R&D Systems) for 6-hours. The cells were then harvested and analyzed for RNA expression.

2.6. siRNA-mediated knockdown of IL-10

M2c macrophages were prepared described above. The macrophages were then treated for 24 hours with control or IL-10siRNA (25 nMol) and 10 μL transit TKO transfection reagent (Mirus Bio LLC, Madison, WI) as indicated. The macrophages were then cultured alone or cocultured with ADHASC cells as indicated for 24 hours and harvested for analysis of RNA expression.

2.7. Free fatty acid treatment

DHA (Cayman Chemical, Ann Arbor, MI) and all other FFAs (Sigma, St. Louis, MO) were dissolved in ethanol and then were conjugated to fatty acid free BSA (Sigma) as described. M2c macrophages were prepared in 6-well plates and cultured alone or cocultured with ADHASC cells for 24 hours in the presence of 100 μM free fatty acid or ethanol (vehicle control) as indicated. IL-10 was added at a concentration of 20 ng/mL in the presence of DHA as indicated to reverse DHA inhibition.

2.8. Conditioned medium preparation and treatments

Conditioned medium was prepared from ADHASC cells by incubating differentiated ADHASC cells in 6-well plates with 2 mL alpha MEM with 2% FBS (coculture medium). The medium was then applied to M2c macrophages (prepared as described above) with the indicated free fatty acid for 24 hours. During the same experiment, M2c macrophages were cocultured with ADHASC cells and the indicated free fatty acid as described above.

2.9. Statistical Analysis

SAS v9.3 was used for all statistical tests and *P*-values of less than 0.05 were considered statistically significant. The data were treated as continuous variables and were described with means and standard errors by cell expression type and coculture type. Two-way ANOVAs were used to investigate the main effects of expression type, FFA treatment (as appropriate),coculture type, and the interaction of expression type, FFA treatment (as appropriate), and coculture type. In order to make further comparisons between means, least squared means post-hoc t-tests were used. When the data suggested potential interactions ($P \leq 0.2$), comparisons were made investigating the interaction between expression type and coculture type. One-way ANOVA was used to investigate the effects of recombinant IL-10 or TGF-β treatment or free fatty acid treatment on IL-10 and TSP-1 expression. In order to make further comparisons between means, least squared means post-hoc t-tests were used.

3. Results

3.1. Adipocyte coculture augments the expression of markers associated with alternative macrophage activation

THP-1 monocytes were specifically treated to differentiate into three different subtypes of macrophages: classically activated M1 macrophages and alternatively activated M2a and

M2c macrophages, and these were then cultured alone or cocultured for 24 hours with primary human adipocytes as described previously. We then analyzed the media for TSP-1 protein expression and the RNA from the macrophages and adipocytes isolated separately for gene expression. First, we determined whether adipocyte coculture altered the phenotype of the macrophages since we had previously observed that adipocyte coculture enhances the alternatively activated phenotype. The mannose receptor is a marker of alternatively activated macrophages, and we found that the MR was expressed at higher levels in M2a and M2c alternatively activated macrophages than M1 macrophages (Fig. 1A, macrophages alone, $P<0.05$). Adipocyte coculture further induced mannose receptor mRNA expression in M2a cells (Fig. 1A,3.7-fold increase, $P<0.05$). IL-10 is an anti-inflammatory cytokine, and adipocyte coculture induced IL-10 mRNA expression in M2c macrophages (Fig. 1B, 10.1-fold increase, $P<0.05$), consistent with previous results; however, we also observed that adipocyte coculture decreased IL-10 in M2a cells (Fig. 1B).CD163 is a marker of M2c macrophages that is induced by IL-10.CD163 mRNA was expressed in M2c macrophages and was induced by adipocyte coculture (Fig. 1C, 2.9-fold increase, $P<0.05$). Thus, adipocyte coculture enhances the mRNA expression of markers of alternative activation in M2a and M2c macrophages. However, it should be noted that adipocyte coculture slightly increased IL-1 mRNA levels in M2a and M2c macrophages, consistent with our previous observations (data not shown). It is important to note that we do not know the extent to which the initial cytokine treatment polarizes the cells; therefore, it is possible that the adipocytes may provide a secondary stimulus for differentiation if significantly less than 100% of the cells are initially of the M2c phenotype.

3.2. Adipocyte coculture augments the expression of TSP-1mRNA in M1 and M2c macrophages

TSP-1 expression is increased in PMA-activated macrophages by adipocyte coculture, but its regulation in cytokine-polarized macrophages is unknown. Adipocyte coculture strongly induced the mRNA expression of TSP-1 inM1 (Fig. 2A, 8.6-fold increase, $P<0.05$) and M2c (Fig. 2A, 26-fold increase, $P<0.05$) macrophages, but TSP-1 was not induced in M2a macrophages, indicating that this induction is specific to the polarization state of the macrophages. Since TSP-1 could potentially activate TGF-β, we examined the mRNA expression of connective tissue growth factor (CTGF) and collagen I, downstream targets of TGF-β signaling, and found that CTGF mRNA expression is induced in macrophages by coculture with adipocytes (Fig. 2B, effect of adipocyte coculture: $P<0.05$).Collagen I mRNA was also induced by adipocyte coculture in M1 (Fig. 2C,2.7-fold increase, $P<0.05$) and M2c macrophages (Fig. 2C, 2.4-fold increase, $P<0.05$). Thus, the mRNA expression patterns of CTGF and collagen I corresponded with macrophage TSP-1 expression.

Adipocytes cultured alone expressed TSP-1 mRNA, and coculture with each of the three types of polarized macrophages induced TSP-1 mRNA in adipocytes (Fig. 2D, 2.4-fold by M1, 3.6-fold by M2a, and 4.2-fold by M2c macrophages, $P<0.05$). CTGF mRNA was expressed in adipocytes cultured alone and was also induced in adipocytes by coculture with M1 (Fig. 2E, 1.6-fold increase, $P<0.05$) and M2c (Fig. 2E, 2.4-fold increase, $P<0.05$) macrophages. The expression pattern of CTGF in adipocytes did not exactly match the adipocyte TSP-1 expression pattern as it did in macrophages (Fig. 1A and Fig. 1B); however, the cells share the same media, which may explain the increase of CTGF in adipocytes cocultured with M1 and M2c macrophages (see next section). Collagen I was not induced in adipocytes by macrophage coculture, suggesting that it may be regulated differently in macrophages and adipocytes (Fig. 2F)

The results of these studies suggest that TGF-β signaling is increased by the increased expression of TSP-1 during M2c macrophage coculture with adipocytes. To test this, we included control or TGF-β neutralizing monoclonal antibody 1D11 in aM2c macrophage-

adipocyte coculture experiment; we also performed this experiment with vehicle control (DMSO) or the TGF-β receptor inhibitor SB505124. As shown in Fig. 2G, 1D11 inhibited CTGF expression by 48% ($P<0.05$), and SB505124 inhibited CTGF expression by 40% ($P=0.056$), suggesting that coculture induces TGF-β signaling, which then induces CTGF mRNA expression.

3.3. Effect of coculture on TSP-1 protein expression

Since TSP-1 was induced by coculture in both adipocytes and macrophages, we evaluated TSP-1 protein expression in the media shared by both of these cells in the coculture experiment. The TSP-1 protein level was low(128 ng/mL)in the media from adipocytes cultured alone and barely detectable in the media from macrophages cultured alone (Fig. 3).Coculture induced TSP-1 protein levels in the media from M1 (Fig. 3, $P<0.05$) and M2c (Fig. 3, $P<0.05$) macrophages cocultured with adipocytes. An interesting observation from this experiment is that the pattern of TSP-1protein expression in the media (Fig. 3) was more similar to the expression of TSP-1 mRNA in the macrophages (Fig. 2A) than the adipocytes (Fig. 2D), suggesting that more TSP-1 is secreted into the media when M1 or M2c macrophages are cocultured with adipocytes than when M2a macrophages are cocultured with adipocytes. This may explain the increased expression of the TGF-β regulated gene CTGF in adipocytes cocultured with M1 and M2c macrophages.

3.4. Mechanism of TSP-1induction in M2c macrophages by adipocyte coculture

Next, we wanted to begin to understand the mechanism of TSP-1induction by coculture. We did these experiments in M2c cells since there was such a strong correlation in the mRNA expression patterns between IL-10 and TSP-1 in M2c macrophages in response to coculture(Fig. 1B) and IL-10 has previously been shown to up regulate TSP-1 in dendritic cells. Therefore, it is possible that the strong induction of TSP-1 expression in M2c macrophages by adipocyte coculture(Fig. 1B and Fig. 2A) may be due to IL-10.To begin to assess this, we treated M2c macrophages with either recombinant IL-10 or TGF-β, which also induces TSP-1in dendritic cells as a control. As expected, IL-10 gene expression was induced by IL-10 treatment (Fig. 4A, $P<0.05$) but not by TGF-β. TSP-1 gene expression was induced by IL-10 in M2c macrophages (Fig. 4B, $P<0.05$) and tended to be induced by TGF-β(Fig. 4B, $P=.07$). We then determined whether IL-10 induction of TSP-1 by adipocyte coculture was physiologically relevant by treating M2c macrophages with control or IL-10 siRNA and then coculturing these cells with adipocytes to induce IL-10 and TSP-1 gene expression. M2c macrophages transfected with IL-10 siRNA expressed less IL-10 mRNA than macrophages transfected with a control siRNA in response to adipocyte coculture as expected (Fig. 5A, $P<0.05$). IL-10 siRNA transfected M2c macrophages also expressed less TSP-1 mRNA than control cells (Fig. 5B, $P<0.05$) in response to adipocyte coculture, suggesting that IL-10 induction is necessary for TSP-1 induction in M2c macrophages in response to adipocyte coculture. Of note, IL-10 was not induced in adipocytes by M2c macrophage coculture (data not shown).

3.5. Effect of FFAs on the induction of TSP-1 and IL-10 in M2c macrophages by adipocyte coculture

We next tested the effect of different FFAs on the adipocyte-macrophage cocultures. IL-10 and TSP-1 gene expression was induced in M2c macrophages by adipocyte coculture as expected (Fig. 6A: TSP-1, $P<0.05$; Fig 6B: IL-10, $P<.0001$). Interestingly, in contrast to linoleic, oleic, and palmitic acid, the omega-3 fatty acids, DHA and EPA significantly inhibited the coculture-induced increases in TSP-1 and IL-10 mRNA. DHA potently inhibited both TSP-1 (Fig. 6A, 97 % inhibition, $P<0.05$) and IL-10 (Fig. 6B, 96 % inhibition, $P<0.05$) mRNA induction by adipocyte coculture in M2c macrophages. EPA was also effective but not as potent as DHA(Fig. 6A and B, $P<0.05$),suggesting that the

inhibition is specific to omega-3 fatty acids. When we performed the coculture experiment described in Fig. 3, we also treated an identical set of cells with 100 μM DHA. As shown in Fig. 6C, DHA inhibited the protein level of TSP-1 in the shared coculture media of M2c macrophages and adipocytes ($P<0.05$), consistent with its inhibition of TSP-1 mRNA in M2c macrophages as described above (Fig 6A). DHA treatment of adipocytes alone did not alter the level of TSP-1 protein secreted into the media (Fig. 6C).TSP-1 and IL-10 displayed remarkably similar patterns of expression in response to the different fatty acids tested (compare Fig. 6A and B), which would be consistent with IL-10 regulating TSP-1 expression (see above). We further evaluated this by adding exogenous IL-10 to the coculture experiement in the presence of DHA to determine whether this would reverse the inhibition of DHA on TSP-1 and IL-10. As shown in Fig. 6D, the addition of IL-10 completely reversed the inhibition of DHA on both IL-10 and TSP-1 ($P<0.05$), consistent with IL-10 regulating TSP-1 expression in M2c macrophages (Figs. 4 and 5).

These data suggest that adipocytes secrete a factor that can up-regulate TSP-1 expression in M2c macrophages and that this up-regulation is potently inhibited by DHA. In order to determine whether DHA acts on adipocytes, or on M2c macrophages, or on both, we compared adipocyte conditioned media to adipocyte coculture for their ability to induce TSP-1 in M2c macrophages in the presence of different FFAs. Coculture of adipocytes induced TSP-1 gene expression approximately 4-fold higher than adipocyte conditioned media (Fig. 7, compare controls from conditioned media and coculture, $P<0.05$), suggesting that the factor that induces TSP-1 is labile. Of more interest, DHA only inhibited TSP-1 expression in the coculture conditions (Fig. 7, $P<0.05$), suggesting that DHA might act on the adipocyte to regulate the factor that induces TSP-1 in M2c cells. Alternatively, the effect of DHA may be mediated by one of its many known biologically active metabolites. To test this, medium from adipocytes conditioned in the presence of DHA was evaluated. This media also failed to inhibit the induction of TSP-1 (data not shown).Thus, the effect of DHA is complex and requires the presence of both cells to be effective at inhibiting the induction of TSP-1 gene expression in M2c macrophages by adipocytes.

4. Discussion

Thrombospondin-1 is increased in the adipose of obese human subjects and, therefore, may contribute to the increased fibrosis and decreased angiogenesis that is also observed. This study demonstrates that TSP-1 is increased in adipocytes by coculture with macrophages and that the magnitude of the increase is dependent on the polarity of the cocultured macrophage. TSP-1 is also induced in M1 and M2c macrophages but not M2a macrophages by adipocyte coculture. The induction of TSP-1 by adipocyte coculture in M2c macrophages likely is due to the induction of IL-10 since siRNA-mediated knockdown of IL-10 inhibits induction of TSP-1in M2c macrophages by adipocytes. Interestingly, this induction is inhibited by n-3 FFAs, especially DHA, but not other FFAs. DHA potently inhibits both TSP-1 induction and IL-10 induction, and the addition of exogenous IL-10 reverses this inhibition, further suggesting that IL-10 regulates TSP-1 expression in M2c macrophages. The factor that is secreted by adipocytes to cause IL-10 and TSP-1 induction is unknown, but is likely labile since adipocyte co-culture induces TSP-1 in M2c macrophages more effectively than conditioned medium. Finally, the mechanism of inhibition by DHA is complex since it requires coculture; adding DHA to adipocyte conditioned medium is not effective at inhibiting TSP-1 induction.

When we characterized the phenotype of the differentiated macrophages used in this study, we found that adipocyte coculture augmented the expression of the mannose receptor in M2c macrophages and augmented the expression of IL-10 and CD163 in M2c cells. This suggests that human adipocytes promote the M2 phenotype, and the expression of M2

markers is a prominent feature of human adipose tissue macrophages under both lean and obese conditions. These M2 markers are important to the anti-inflammatory functions of M2 macrophages since IL-10 is well known to be anti-inflammatory, and engaging the mannose receptor induces IL-10 production. These results suggest that adipocytes may program adipose resident macrophages to be less inflammatory although it should be noted that IL-1 levels increased slightly in response to coculture, which is consistent with previous results. In another study using mouse adipocytes and macrophages, a strong inflammatory response was induced by coculture. It will be interesting to determine whether coculture of human macrophages with adipocytes reduces their inflammatory response to toll like receptor (TLR) agonists such as LPS. It will also be important to identify the adipocyte factors that enhance the expression of M2 markers in macrophages and why this occurs since macrophages that express M2 markers in adipose are associated with obesity in humans. Finally, the adipocytes used in this study were derived from adipose stem cells of lean subjects. It would therefore be of interest to determine whether adipocytes derived from obese subjects display the same properties or are less effective at inducing Il-10 and TSP-1.

One of the important functions of alternatively activated macrophages, especially M2c macrophages, is the resolution of inflammation and wound healing, and TSP-1 is important in both of these processes. However, aberrant TSP-1 expression may lead to increased TGF-β signaling, fibrosis, and reduced angiogenesis, and increased TSP-1 expression is associated with obesity. Since adipocyte coculture enhances the expression of IL-10 in M2c macrophages and coculture increases TSP-1 in phorbalmyristate acetate-activated macrophages, we decided to evaluate TSP-1 expression in this coculture study using macrophages activated to three specific polarizations by cytokine treatment. TSP-1 was induced in both adipocytes and macrophages by coculture, and coculture with M2c macrophages resulted in the highest TSP-1 expression in both adipocytes and M2c macrophages. Furthermore, the highest TSP-1 protein levels were observed in the M2c macrophage-adipocyte cocultures. This may explain the strong association of M2c macrophages with fibrosis in adipose with obesity.

We found that IL-10 and TSP-1 displayed similar patterns of expression in M2c macrophages and that IL-10 could regulate TSP-1 expression. IL-10 and TSP-1 have been demonstrated to have similar expression patterns in other cell types as well. This would make sense given that both of these proteins are involved in the resolution of wound healing. Interestingly, TSP-1 was also induced inM1 macrophages, which did not express IL-10, suggesting that another mechanism regulates TSP-1induction in M1 macrophages. Also, TSP-1 was induced in adipocytes by all three macrophage subtypes, again suggesting IL-10-independent mechanisms of induction in adipocytes by M1 and M2a macrophage coculture. Finally, the profile of TSP-1 protein expression resembled the macrophage mRNA expression profile more than the adipocyte mRNA expression profile, suggesting that TSP-1 secretion is strongly influenced by the macrophage subtype.

Free fatty acids are an important component of the adipose environment, and n-3 FFAs may be a beneficial nutritional supplement. DHA potently inhibited both IL-10 and TSP-1 induction in M2c macrophages by adipocyte coculture. DHA is considered to be anti-inflammatory so the inhibition of IL-10 was somewhat surprising; however, it is consistent with previous reports. Inhibition of IL-10 would be predicted to have detrimental effects on adipose by increasing inflammation; whereas, inhibition of TSP-1would be predicted to have beneficial effects by reducing fibrosis and increasing angiogenesis. Indeed, TSP-1 knockout mice have improved glucose tolerance in response to high fat feeding. Finally, the mechanism of inhibition of TSP-1 expression by DHA is complex since it requires coculture. This suggests that DHA interferes with macrophage–adipocyte cross talk during coculture to reduce the factor that induces IL-10 and TSP-1. This cross talk may involve

TLR signaling, which is induced in mouse adipocyte macrophage coculture systems and is inhibited by DHA at multiple levels. TLR receptor signaling initiates IL-10 induction in macrophages, and in addition to their well-known role in innate immunity, TLRs can recognize endogenous ligands including saturated fatty acids and proteins, which could be generated by adipocytes. Alternatively, the inhibition of IL-10 by DHA may be by one of its proresolving metabolites.

Previous studies in humans have demonstrated a variety of anti-inflammatory effects of fish oil supplementation ; however, fish oil supplementation does not reduce plasma inflammatory markers or improve insulin sensitivity in humans. This study suggests that fish oil supplementation may have complex effects and may depend on the state of activation of resident macrophages.

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Abbreviations

TSP-1	thrombospondin-1
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FFA	free fatty acid
ECM	extracellular matrix
ADHASC	adult-derived human adipose stem cells
CTGF	connective tissue growth factor
PMA	phorbalmyristate acetate
TLR	Toll like receptor

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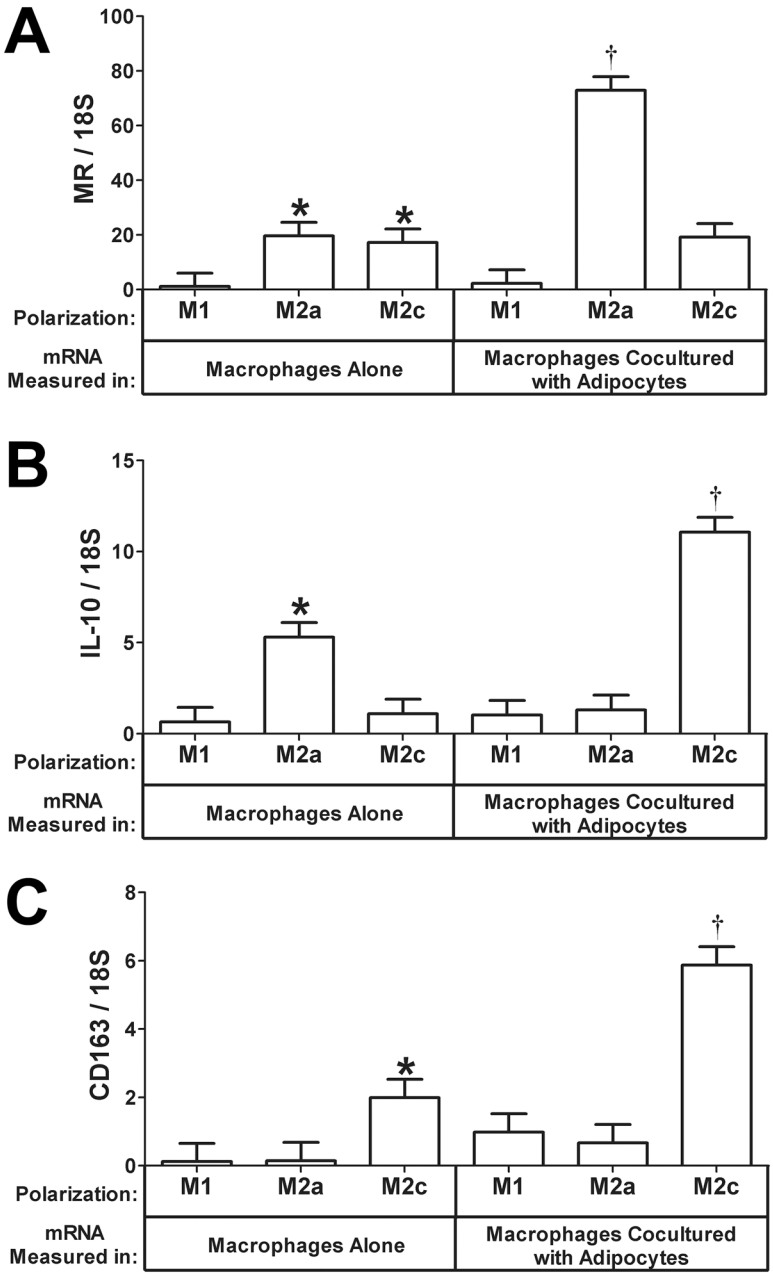

Fig. 1. Effect of adipocyte coculture on the expression of differentiation markers in polarized macrophages

Polarized macrophages (M1, M2a, or M2c) and differentiated adipocytes were prepared as described under “Methods” and cultured alone or with adipocytes as indicated. Total RNA was prepared from the macrophages and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3). A) Mannose receptor (MR) expression in macrophages. *: compare to M1macrophages cultured alone (P<0.05). †: compare M2a macrophages cocultured with adipocytes to M2a macrophages cultured alone (P<0.05). B) IL-10 expression in macrophages. *: compare to M1or M2c macrophages cultured alone (P<0.05). †: compare M2c macrophages cocultured with adipocytes to M2c macrophages cultured alone (P<0.05). C) CD163 expression in macrophages. *: compare to

M1or M2a macrophages cultured alone ($P<0.05$). †: compare M2c macrophages cocultured with adipocytes to M2c macrophages cultured alone ($P<0.05$).

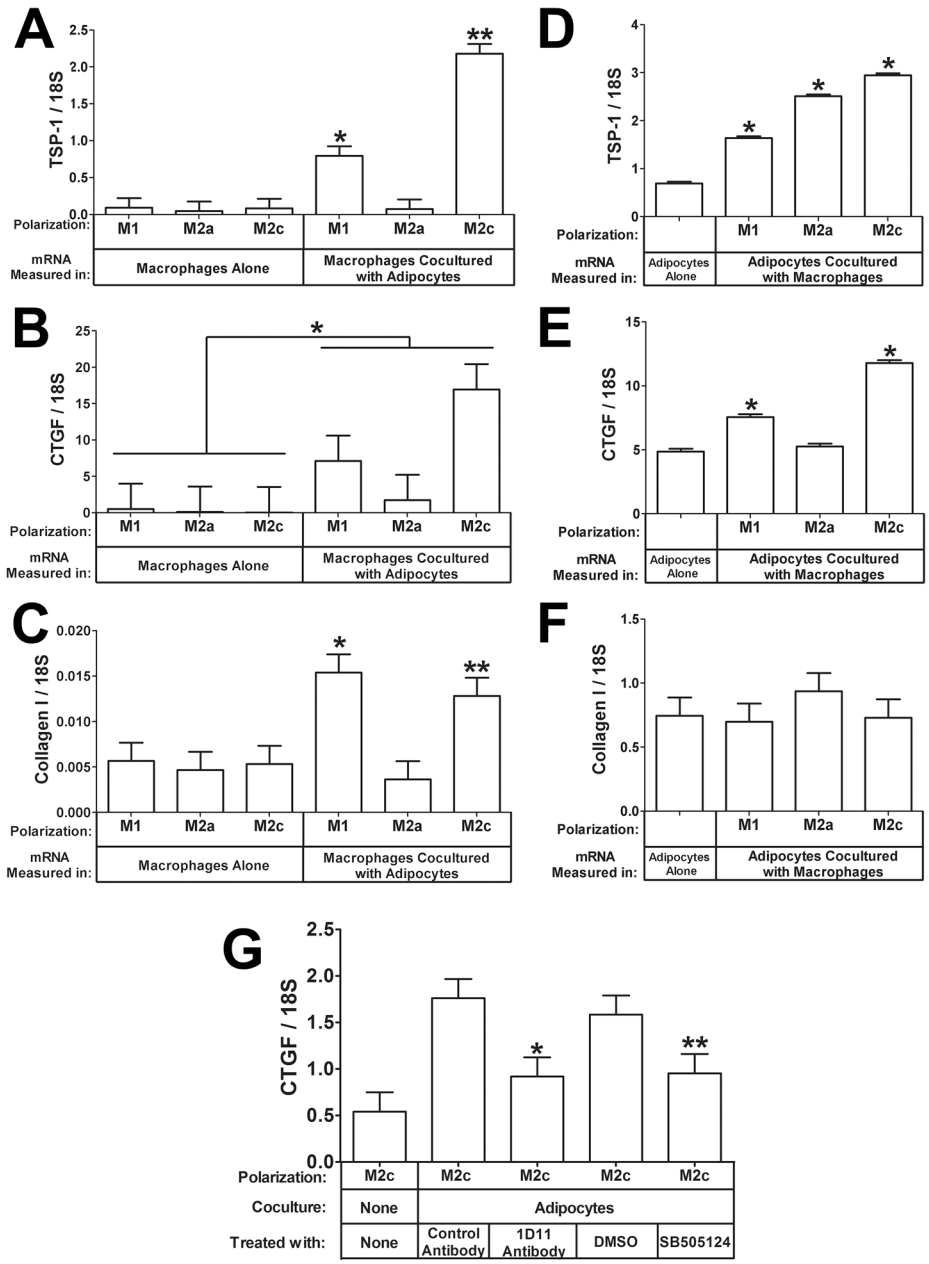

Fig. 2. Effect of adipocyte coculture on the expression of TSP-1, CTGF, and Collagen I in macrophages

Total RNA was prepared from the macrophages from the experiment in Fig. 1 and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3).A) TSP-1 mRNA expression in macrophages. *: compare M1 macrophages cocultured with adipocytes toM1 macrophages cultured alone (P<0.05). **: compare M2c macrophages cocultured with adipocytes to M2c macrophages cultured alone (P<0.05). B) CTGF expression. *: the effect of adipocyte coculture on macrophage TSP-1 expression (P<0.05). C) Collagen I mRNA expression in macrophages. *: compare M1 macrophages cocultured with adipocytes to M1 macrophages cultured alone (P<0.05). **: compare M2c macrophages cocultured with adipocytes to M2c macrophages cultured alone (P<0.05). D) TSP-1 mRNA expression in adipocytes.*: compare adipocytes cocultured with M1, or M2a,

or M2c macrophages to adipocytes cultured alone (P<0.05).E) CTGF mRNA expression in adipocytes. *: compare adipocytes cocultured with M1or M2c macrophages to adipocytes cultured alone (P<0.05). F) Collagen I mRNA expression in adipocytes. G) Inhibition of CTGF induction by inhibiting TGF-β. *: compare 1D11 to control antibody (P< 0.05). **: compare SB505124 to DMSO control (P=0.056).

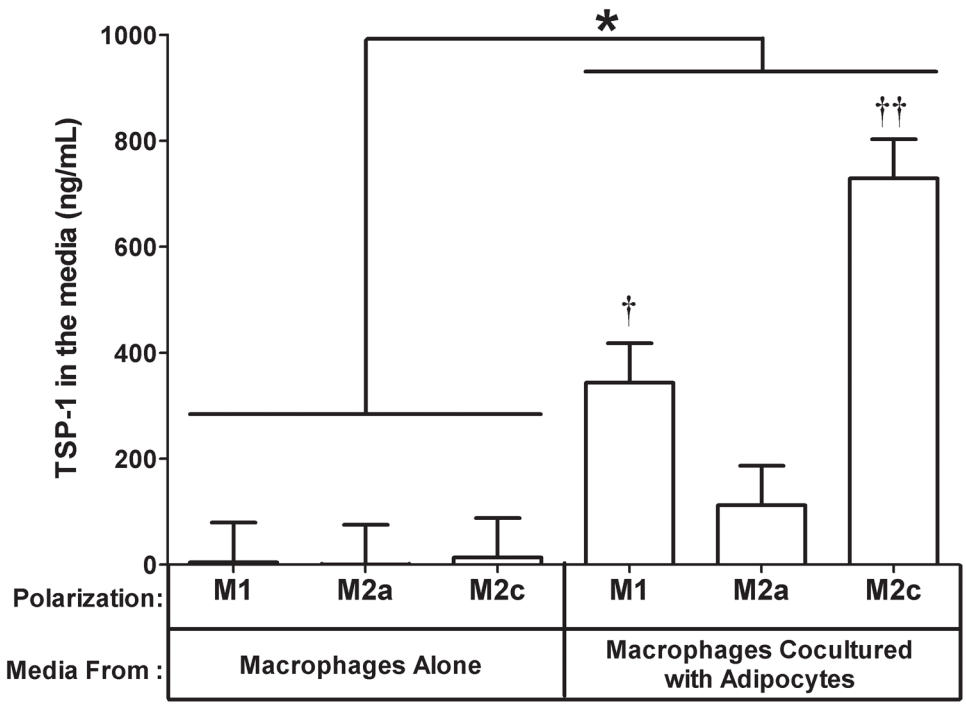

Fig. 3. Effect of adipocyte coculture on the expression of TSP-1 protein in the media

The media from the coculture experiment in Fig. 1 was collected and analyzed for TSP-1protein expression by ELISA. The data are represented as means ± SE (n=3).†: compare M1 macrophages cocultured with adipocytes to M1 macrophages cultured alone (P<0.05). ††: compare M2c macrophages cocultured with adipocytes to M2c macrophages cultured alone (P<0.05). *: effect of coculture (P<0.05).

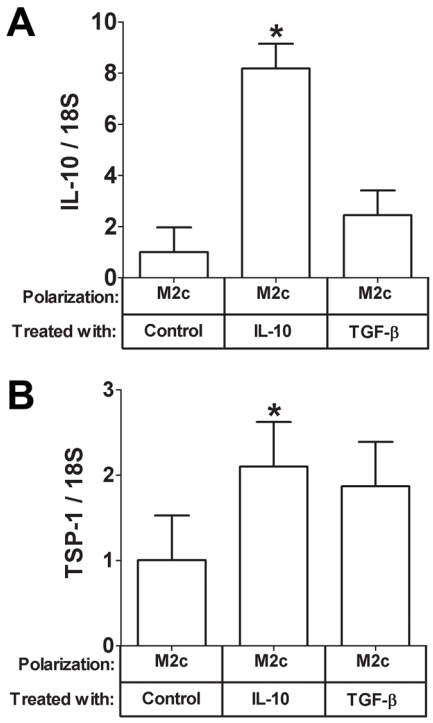

Fig. 4. Effect of treatment with recombinant IL-10 or TGF-β on IL-10 and TSP-1 in M2c macrophages

M2c macrophages were serum starved for 12 hours and then were treated for 6 hours with vehicle control, or IL-10 (20 ng/mL), or TGF-β(5 ng/mL) as indicated. Total RNA was prepared and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3) A) IL-10 expression. *: compare IL-10 expression in cells treated with recombinant IL-10 to control treated M2c macrophages. B) TSP-1expression. *: compare TSP-1 expression in cells treated with recombinant IL-10 to control treated M2c macrophages ($P<0.05$).

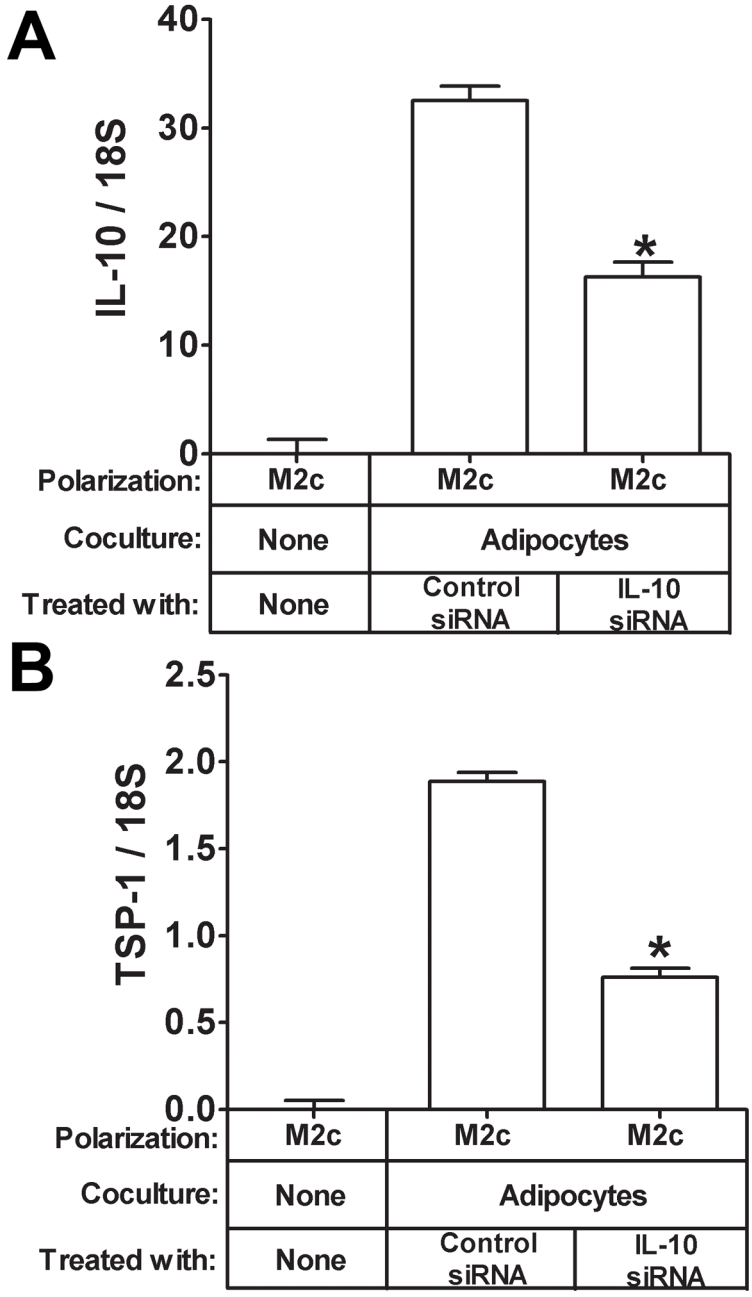

Fig. 5. Effect of siRNA-mediated reduction of IL-10 on IL-10 and TSP-1 in M2c macrophages cocultured with adipocytes

M2c macrophages were treated with the indicated siRNA and then cocultured with adipocytes as indicated. Total RNA was prepared and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3) A) IL-10 expression. *: compare IL-10 expression in M2c macrophages cocultured with adipocytes and treated with IL-10 siRNA to control siRNA treated M2c macrophages (P<0.05). B) TSP-1 expression. *: compare TSP-1 expression in M2c macrophages cocultured with adipocytes and treated with IL-10 siRNA to control siRNA treated M2c macrophages (P<0.05).

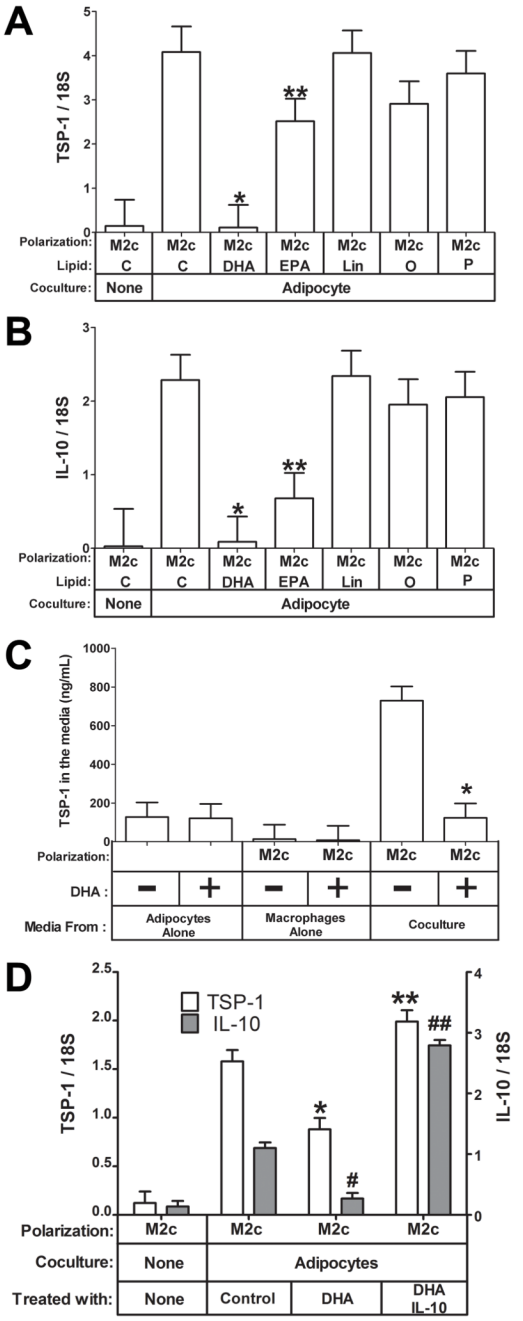

Fig. 6. Effect of Free Fatty Acids on IL-10 and TSP-1 in M2c macrophages cocultured with adipocytes

M2c macrophages were cultured alone or cocultured with adipocytes in the presence of the indicated free fatty acids (C, Control; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; Lin, Linoleic acid; O, Oleic acid; P, palmitic acid) at a concentration of 100 μM. Total RNA was prepared and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3) A) TSP-1 expression. *, **: compare TSP-1 expression in cells treated with the indicated free fatty acid to control treated M2c macrophages (P<0.05). B) *, **: compare IL-10 expression in cells treated with the indicated free fatty acid to control treated M2c macrophages (P<0.05). C)The media from

the coculture experiment done in the absence and presence of 100 μM DHA was collected and analyzed for TSP-1 protein expression by ELISA. The data are represented as means ± SE (n=3). *: effect of DHA treatment on TSP-1 in adipocyte-M2c macrophage cocultures (P<0.05). D) The coculture experiment was repeated in the presence of 20 ng/mL IL-10 as indicated; open columns indicate TSP-1 expression; shaded columns indicate IL-10 expression. The data are represented as means ± SE (n=3).*: compare TSP-1 expression to control cells; **: compare TSP-1 expression to DHA-treated cells (P< 0.05). $^{\#}$: compare IL-10 expression to control cells; $^{\#\#}$: compare IL-10 expression to DHA-treated cells (P< 0.05).

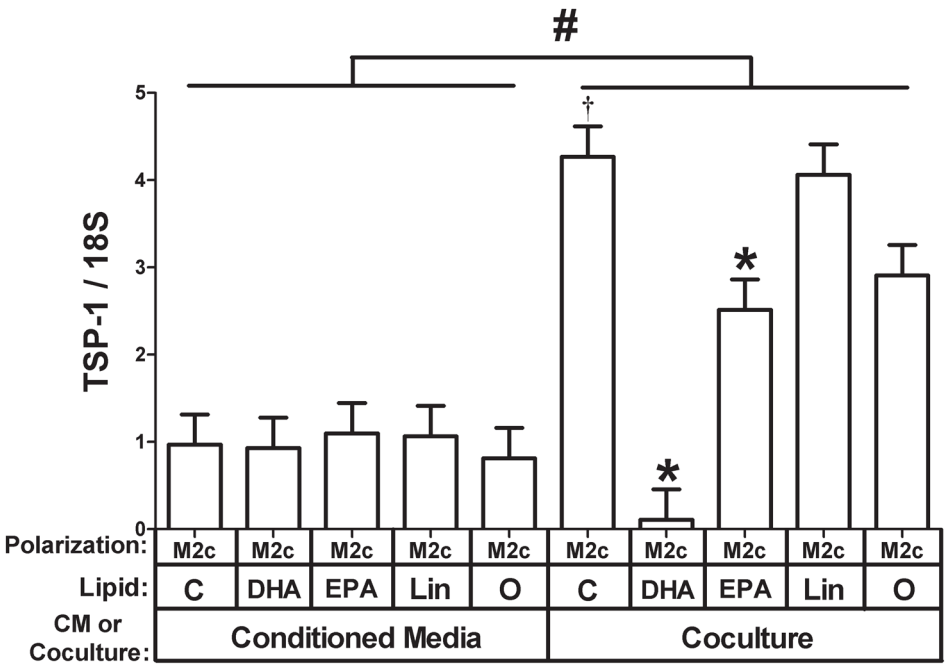

Fig. 7. Effect of using adipocyte conditioned medium versus coculture on the ability of FFAs to inhibit TSP-1 expression

M2c macrophages were treated with adipocyte conditioned media in the presence of the indicated FFA or cocultured with adipocytes in the presence of the indicated FFA as indicated (C, Control; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; Lin, Linoleic acid; O, Oleic acid). Total RNA was prepared and subjected to gene expression analysis by real-time RT PCR. The data are represented as means ± SE (n=3) TSP-1 expression.#: compare TSP-1 expression in cells treated with conditioned media or cocultured with adipocytes ($P<0.05$). *: compare TSP-1 expression in M2c macrophages treated with the indicated free fatty acid to control treated M2c macrophages ($P<0.05$).