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Human Tribbles Homolog 2 is Expressed in Unstable Regions of Carotid Plaques and Regulates Macrophage IL-10 in Vitro

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Short Title: Human Trb2 in Unstable Carotid Plaque

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Abstract

Mammalian orthologs of the *Drosophila* tribbles protein (Trb1, Trb2 and Trb3) are a recently described family of signalling molecules that regulate gene expression by modulation of protein kinase signalling pathways. In this study, a screen for mRNA species specifically regulated in vulnerable regions of human atherosclerotic plaque demonstrated upregulation of both Trb1 and Trb2, the latter by more than 8-fold. *In vitro* experiments in primary human monocyte-derived macrophages showed that Trb2 expression was upregulated by treatment with oxidised low density lipoprotein (LDL), and that expression of recombinant Trb2 specifically reduced macrophage levels of IL-10 mRNA. Our results thus identify Trb2 as a highly regulated gene in vulnerable atherosclerotic lesions, and demonstrate inhibition of macrophage IL-10 biosynthesis as a potential pro-inflammatory consequence of high Trb2 expression that may contribute to plaque instability.

Introduction

There is increasing evidence that vessel wall inflammation plays a central role in the development of the atherosclerotic plaque, and crucially in the process of plaque destabilisation that results in the local thrombosis underlying the common clinical presentations of atherosclerosis [1]. Recruitment of monocytes into the lesion, and their subsequent differentiation into macrophages and transformation into foam cells via ingestion of modified LDL particles plays a central role in determining the inflammatory environment within the plaque. Several endogenous and microbial molecules are able to activate macrophages within the plaque, leading to production of inflammatory cytokines, proteases and oxygen radicals [2]. The anti-inflammatory cytokine, IL-10, is also produced by macrophages within atherosclerotic plaques [3]; since there are a number of lines of evidence suggesting an atheroprotective role for IL-10 [4-7], control of plaque macrophage IL-10 production may be an important determinant of inflammatory load, and hence of progression towards instability and rupture.

Three mammalian orthologs of the *Drosophila* Tribbles protein (Trb1, Trb2 and Trb3) have recently been identified as intracellular signalling molecules that resemble serine-threonine kinases but lack canonical ATP-binding sites [8]. They are thought to act principally as adaptor proteins, with a scaffold-like regulatory function in a number of signalling pathways [9,10]. Trb3 has been reported to be a negative regulator of PKB signalling in the liver [11]; it also acts as a scaffold to bind the E3 ligase COP1 to acetyl-coenzyme A carboxylase, promoting ubiquitin-dependent degradation of the latter and thus inhibiting fatty acid synthesis [12]. Trb1 and Trb3 have also been shown to modulate activity of mitogen activated protein kinase (MAPK) cascades by binding MAPKKs [9]. In this context, a recent paper has identified Trb1 as a negative regulator of c-jun N-terminal kinase (JNK) activation by MAPKK4 in vascular smooth muscle cells [13]. Less is known about the function of mammalian Trb2; it was originally reported as a labile phosphoprotein upregulated by mitogens in dog thyroid cells [14]. More recently it has been identified as a protein upregulated by inflammatory stimuli in myeloid (THP-1) cells [15] and also as an oncogene that inactivates the transcription factor CEBP/α and causes acute myelogenous leukaemia [16].

Here we show that Trb2 mRNA is expressed at high levels specifically in vulnerable regions of human atherosclerotic plaques, and that expression correlates with that of the macrophage marker CD68. Overexpression of Trb2 in human monocyte-derived macrophages *in vitro* caused specific suppression of IL-10 biosynthesis. These findings suggest a possible role for Trb2 as a mediator of atherosclerotic plaque destabilisation via negative regulation of macrophage IL-10 synthesis.

Methods

Human carotid endarterectomy samples

Human atherosclerotic plaque samples were collected (with informed consent) from patients undergoing carotid endarterectomy. Classification of plaque segments as stable or unstable, and RNA isolation from these segments, was as previously described [17].

Peripheral blood mononuclear cells (PBMC) preparation

Fresh blood (50–100 ml, taken into EDTA) was obtained from healthy volunteers (with informed consent) and stored on ice for a maximum of 60 min. PBMCs were isolated under sterile conditions as described [18]. Briefly, whole blood was layered on endotoxin-free Histopaque-1077 medium, and PBMCs present at the Histopaque interface, following centrifugation at 400 g for 30 min at 25 °C, were collected and subjected to brief treatment with cell lysis solution (Promega, Madison, WI 53711-5399). After washing in 50 ml PBS, PBMC pellets were gently resuspended in 5 ml PBS. CD14⁺ cells were quantified by flow cytometry with fluorescein-conjugated anti-CD14 (Beckman Coulter Inc. Fullerton CA 92835).

Establishment of macrophage cultures and LDL treatment

PBMCs were resuspended in RPMI 1640/5 % human AB serum (Sigma) and plated in 12-well/6-well plates at 3×10^5 monocytes/ml. After incubation at 37 °C overnight (in 5 % CO₂ in a water-saturated atmosphere), non-adherent cells were removed by repeated PBS wash; adhered monocytes were allowed to differentiate into macrophages by culture in RPMI 1640/5 % human AB serum for 2-4 weeks. For LDL treatment macrophages were exposed to different concentrations of oxidised or native human LDL (Intracel, Frederick MD 21701) in serum-free medium for 4-48 hours.

Quantitative real-time PCR

Single-stranded cDNA was synthesized with random hexamer primers using the Superscript First-Strand Synthesis System, according to the manufacturer's instructions (Invitrogen). mRNA expression was quantified using a TaqMan 7900HT Detection System, with either pre-synthesized primers (Applied Biosystems) for Trb1 (Hs00179769_m1), Trb2 (Hs00222224_m1), Trb3 (Hs00221754_m1) and IL-10 (Hs00174086_m1), or as described for CD3 and CD68 [17]. Reactions (performed in a MicroAmp Optical 96-well reaction plate) contained: 1x Master Mix, 200 μM each primer, and 100 μM probe in a volume of 25 μl. PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 min/60 °C for 1 min. Gene expression data for macrophages were analyzed by normalization against 18S rRNA; those for plaque samples were analyzed by normalization against the geometric mean of the expression of GAPDH, β-actin and β-microglobulin as described [19].

cDNA cloning and production of recombinant Trb2 and Trb3

Human Trb2 and Trb3 open reading frames (ORF) were amplified by PCR using the primer pairs : Trb2: 5'-GAA GTT ATC AGT CGA CAT GAA CAT ACA CAG GTC TAC-3' and 5'-ATG GTC TAG AAA GCT TGC TCA GTT AAA GAA AGG GTC -3'; Trb3: 5'- GAA GTT ATC AGT CGA CAT GCG AGC CAC CCC TCT GGC T-3' and 5'-ATG GTC TAG AAA GCT TTC CTA GCC ATA CAG AAC CAC-3' respectively. The forward primer had a unique SalI site; the reverse primer was designed with a unique HindIII site. PCR was carried out for 30 cycles of 15 s at 92°C/30 s at 55°C, followed by 1 min at 72°C, with *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Ca.). Resulting Trb cDNAs were ligated into the unique SalI and HindIII sites of linearized pDNR-Dual donor vector with the BD In-Fusion Dry-Down PCR cloning kit (BD Biosciences) to make pDNR-Dual/Trb2/3, and sequenced to verify fidelity of the PCR. Macrophages were transiently transfected with Trb2- and Trb3-expressing plasmid (or vector) with lipofectamine (Invitrogen), according to the manufacturer's instructions, 48 hours prior to cell lysis.

Adenovirus constructs were prepared by using BD Adeno-X Expression System 2 (BD Biosciences) according to the manufacturer's protocol. The TRB2/3 genes were transferred from pDNR-

Dual/TRB2/3 constructs (donor vector) to pLP-Adeno-X Viral DNA (acceptor vector). After digestion with PacI, the recombinant adenoviral plasmid was used to transfect HEK293 cells. These were maintained in EMEM medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Paisley, UK) at 37 °C in a tissue culture incubator. Cells were seeded at a density of $1-2 \times 10^5$ cells/ml, and transfected with adenovirus-Trb2 or -Trb3 constructs or acceptor vector alone (AD0) when they had reached 50 – 70 % confluence. After 3-4 days, when approximately 90 % of the cells were detached from the plate, both cells and culture supernatant were collected for virus purification. Adenovirus were isolated by using the freeze-thaw method and purified with an Adeno-X virus purification kit (BD Biosciences); virus titer was determined by using the BD Adeno-X Rapid Titer kit (BD Biosciences).

IgG-OVA complex was prepared by combining IgG fraction of rabbit antiserum to chicken egg albumin (Cappel/MP Biomedicals) and OVA (Worthington Biochemical) at final concentrations of 1.5 mg/ml IgG, and 45µg/ml OVA. Where appropriate, macrophages were infected (in serum-free medium, exchanged for fresh serum-containing medium 4 hours after infection) with purified adenovirus-TRB 2/3 (or AD0) 48 hours before stimulation with IgG-OVA complex (40µl/ml) and *E. coli* lipopolysaccharide (LPS: Sigma, 80ng/ml, added 10 minutes after the IgG-OVA complex), which was for 24 hours (in serum-containing medium). Both cells (for Taqman RT-PCR analysis and Western blot) and culture supernatants (for IL-10 ELISA, measured using an R&D Systems kit according to the manufacturer's instructions) were collected following a further 24 hours incubation.

Anti-TRB2/3 antibody generation

Polyclonal antibodies were developed by immunizing rabbits with synthetic peptides (KLH-coupled) corresponding to amino acids 11 - 27 for TRB2 and 314 - 333 for TRB3 (Cambridge Research Biochemicals, Billingham, Cleveland, UK). Antibodies were purified using a Protein A immuno-affinity column (Cambridge Research Biochemicals).

Western Blotting

30 µg protein/well of macrophage lysates were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen). Gels were blotted by using the XCell II blot module (Invitrogen) onto nitrocellulose, and membranes were probed (following blocking for 30 minutes in 5 % non-fat milk) with anti-human-TRB2 or Trb3 antibody (final concentration 0.7 µg/ml) and human-GAPDH antibody (Abcam, Cambridge, UK; 1:2500 dilution) overnight. HRP conjugated secondary antibodies (Abcam) at a dilution of 1:10,000 were used to detect bound primary antibodies; blots were developed with Supersignal West Dura Extended Duration Substrate (Perbio, Cheshire, UK) and Images were captured using a Chemigenius imaging system (Syngene, Cambridge, UK).

Statistics

Data were compared for statistically significant differences by the Wilcoxon Matched-Pairs Signed-Ranks Test. In situations where more than one control was included in the experimental design, the test condition result was compared directly to that for each of the controls in a pairwise manner (since possible differences between controls groups were not of interest). To assess correlations, Rank Correlation coefficients were calculated with associated p-values.

Results and Discussion

Expression of Trb mRNAs in vulnerable regions of human carotid plaques

As part of a wider screen for markers of atherosclerotic plaque vulnerability [17] levels of mRNA for Trb1, Trb2 and Trb3 isolated from regions of human carotid plaques classified as unstable by macroscopic examination [17] were compared to those from regions classified as stable (using an intra-plaque comparison where each measurement in an unstable region was paired with one in a stable region of the same plaque explant). The results are shown in Figure 1; Table 1 gives correlation coefficients (and associated statistical significance) between mRNA levels of the three

Trbs and those for macrophage and T cell markers for the screen. Correlations between mRNA for CD68 and those for some known macrophage proteins that were regulated in vulnerable plaque regions were observed in the wider screen [17], suggesting that this may be a useful way of assigning cell type-specific expression to regulated mRNA species. We found a marked increase in Trb2 expression in unstable compared to stable plaque regions (more than 8-fold regulation, $p < 0.05$) and a significant increase in Trb1 expression (1.7 fold regulation, $p < 0.0001$) but no significant difference in Trb3 mRNA levels. A possible role for Trb1 in atherosclerosis has been suggested by recent observations that Trb1 mRNA is upregulated in coronary arteries from ischaemic heart disease patients, and that enhanced Trb1 expression in vascular smooth muscle cells inhibits proliferation via specific inhibition of the JNK signalling pathway [13]. Our data showing that Trb1 mRNA levels are raised in vulnerable regions of human carotid plaques are also suggestive of a specific role in atherosclerosis, although the positive correlations between (mRNA) levels of Trb1 and both CD3 and CD68 in the plaque segments tested (Table 1) suggest that Trb1 may be mainly expressed by T cells and macrophages within the plaque. Whilst the significant regulation of Trb1 mRNA in vulnerable plaque regions supports a proposed role for the protein in atherosclerosis, the dramatic up-regulation of Trb2 mRNA observed raises the possibility of a hitherto unexplored regulatory function for Trb2 in plaque progression and destabilisation. Further, there is a strong, highly statistically significant correlation observed between expression of Trb2 and CD68 in carotid plaque segments (Table 1). Whilst it is possible that the association is indirect (elevated Trb2 levels in another cell type promoting recruitment of macrophages to the vulnerable plaque region), the finding suggests that macrophages are the cell type primarily responsible for the enhanced Trb2 mRNA levels in unstable plaque regions. The level of Trb2 regulation observed is substantially higher than would be predicted on the basis of higher macrophage content in unstable regions alone [17], suggesting some specific Trb2 regulation in unstable plaque regions.

Regulation of monocyte-derived macrophage Trb mRNA expression in response to administration of oxidised LDL

In order to test the possible relevance of macrophage Trb expression in atherosclerosis we examined the regulation of Trb mRNA expression in human monocyte-derived macrophages by oxidised LDL. LDL oxidation is considered a key event in atherogenesis; uptake of oxidized LDL (oxLDL) by macrophages results in foam cell formation and ultimately development of the lipid rich, necrotic core of the lesion [20], whilst lipid products of LDL oxidation act as inflammatory mediators within the plaque that may contribute to instability [22]. Regulation of macrophage Trb2 mRNA by oxLDL would therefore provide evidence of specific expression in foam cells, and of a possible pro-inflammatory role in plaque destabilisation. We found that expression of all 3 Trb mRNAs was altered by 24 hour incubation with oxLDL in a concentration-dependent manner (Figure 2A). Macrophage treatment with 50 μg (total protein)/ml of oxLDL in serum free conditions significantly reduced Trb1 mRNA levels (by over 70 %, $p < 0.01$), whilst significantly enhancing levels of Trb2 (over 7.5-fold; $p < 0.01$) and Trb3 (over 4.5-fold, $p < 0.01$). The time-dependency of Trb mRNA regulation by this concentration of oxLDL is shown in Figure 2 (B, C and D). With all 3 Trb mRNAs the effect of oxLDL treatment was the reverse of that observed with serum withdrawal alone ('control' bars in Figure 2), and in all cases the effect of treatment with non-oxidised LDL (nLDL) was intermediate (although much closer to the control value in the cases of Trb2 and Trb3). As with the carotid plaque analysis, Trb2 mRNA was more variable and more highly regulated than Trb1 or Trb3. These results therefore identify Trb2 as a strongly regulated macrophage protein, and suggest that it may be expressed at high levels by foam cells within atherosclerotic lesions. In contrast, the small negative regulation by oxLDL suggests that macrophage Trb1 expression is less highly regulated; enhanced levels of Trb1 mRNA (Figure 1) may therefore reflect the higher leukocyte content in unstable plaque regions (rather than specific regulation).

Strong up-regulation of Trb2 mRNA in macrophages, specifically in vulnerable regions of carotid plaque tissue *in vivo*, and in response to oxLDL treatment *in vitro*, raises the possibility that Trb2 regulation of macrophage signalling pathways may play a role in plaque progression and destabilisation. Whilst comparatively little is known about regulation of mammalian Trb2 expression,

a single report shows that mRNA levels are up-regulated in response to IL-1 treatment in a myeloid cell line, THP-1 [15] suggesting that Trb2 may be involved in myeloid cell regulation of inflammatory gene expression. Since inflammation within the plaque is a key determinant of vulnerability, and ultimately rupture [2,22,23], we sought to determine directly the effects of Trb2 expression on synthesis and secretion of macrophage inflammatory mediators to provide clues to possible actions of the protein within plaques.

Regulation of macrophage IL-10 mRNA levels by Trb2 overexpression

The mammalian Trb proteins have been shown to play important roles in transcriptional regulation of gene expression in a number of cellular models with possible relevance to human pathologies [9,10,12,16,24,25]. The specific up-regulation of macrophage Trb2 in both unstable carotid plaque regions and cells treated with oxidised LDL *in vitro* suggested that expression of Trb2 in atherosclerotic foam cells may contribute to plaque inflammation and destabilisation, in turn suggesting that it may be involved in (transcriptional) regulation of inflammatory mediators. To test this idea we cloned cDNA for human Trb2 (and Trb3 as a control) into a mammalian expression vector and measured the effect of transfection on secretion of cytokines from monocyte-derived macrophages. We were unable to demonstrate any effect (different from transfection of vector alone) on secretion of the pro-inflammatory cytokines IL-1 β , IL-6 or IL-8 (data not shown). However, TaqMan RT-PCR measurements in transfected macrophages demonstrated that Trb2 expression caused a specific reduction in macrophage IL-10 mRNA levels measured after 24 hours relative to cells expressing Trb3 ($p < 0.05$) or those transfected with vector alone ($p < 0.05$) (Figure 3A). Trb2 overexpression caused no enhancement of macrophage apoptosis over this time period (data not shown). It is interesting to note that, concomitant with enhanced levels of Trb2 mRNA in macrophages (Figure 2D), IL-10 mRNA levels are also reduced by oxLDL treatment *in vitro* (Figure 3B).

We were unable to detect enhancement of macrophage Trb2 and Trb3 expression at the protein level (by Western blot: not shown) using the pDNR-Dual expression system, and we were also unable to demonstrate regulation of IL-10 at the protein level, since the amounts secreted by macrophages in these experiments were below levels of detection for the ELISA kit used (data not shown). In order to overcome these difficulties we used an adenoviral system to express high levels of Trb2 and Trb3 in macrophages, and measured the effects of overexpression on IL-10 secretion stimulated by treating macrophages with aggregated IgG and lipopolysaccharide (IgG/LPS).

Titration of virus multiplicity of infection (MOI) used to infect macrophage cultures showed that both anti-Trb2 and anti-Trb3 antibodies detected recombinant protein at MOI = 200 (by Western blot, Figure 4C), and also that the expression of the two mRNAs in macrophages infected at MOI = 200 was similar (Figure 4D). The relative expression data derived from the threshold cycle values shown in Figure 4D (which are approximately proportional to negative log₂ of specific mRNA concentration) indicate that in non-infected cells Trb3 mRNA was approximately 5.7 times more abundant than Trb2 mRNA, and that in infected cells expression of Trb2 and Trb3 mRNA was approximately equivalent (representing 2600-fold enhancement and 460-fold enhancement over uninfected cells for Trb2 and Trb3 respectively).

Figure 4 (A and B) show that, both at the protein and mRNA level, overexpression of Trb2 in adenovirus-infected macrophages specifically inhibited IgG/LPS-stimulated IL-10 biosynthesis, whilst overexpression of Trb3 did not have this effect. These data thus confirm and extend the observations presented in Figure 3.

Thus, our data indicate that specific suppression of IL-10 is a potentially pro-inflammatory consequence of Trb2 expression in macrophages and, together with the observation of enhanced Trb2 expression associated with macrophages in vulnerable regions of human carotid plaques, suggest a potentially pro-atherogenic role for the protein *in vivo*. An atheroprotective effect of IL-10 has been demonstrated in animal models [5,6], and potentially anti-atherogenic actions have been shown *in vitro* [26-28]. A recent study has also demonstrated genetic variation in the human IL-10 promoter

region to be associated with vascular events [29]. These findings support the view that suppression of macrophage IL-10 biosynthesis within the plaque could contribute to plaque destabilisation, and that this may be a specific consequence of high macrophage Trb2 expression within the plaque. The precise mechanism whereby Trb2 may act to suppress IL-10 synthesis remains to be determined. One possible pathway is suggested by the recent finding that expression of Trb2 in a myeloid cell line can inactivate the transcription factor C/EBP α [16]; previous studies have shown that THP-1 cell IL-10 transcription is (at least in part) controlled by C/EBP α binding to CCAAT motifs in the promoter region [30].

In summary, our results demonstrate strongly upregulated Trb2 expression (that correlates with expression of the macrophage marker, CD68) in regions of human atherosclerotic plaque classified as unstable by macroscopic examination (following carotid endarterectomy), and specific suppression of macrophage IL-10 expression as a consequence of Trb2 expression *in vitro*. These data identify macrophage Trb2 expression as a potentially important determinant in progression of atherosclerosis, adding another possible role in human pathophysiology to the growing list ascribed to members of this recently identified family of signalling molecules.

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Figure Legends

Figure 1. Relative expression of Trb mRNAs in stable and unstable regions of human carotid plaques. Results show mRNA levels measured in unstable segment, relative to those in stable segment of the same plaque explant. Mean + SEM $n = 16$ for Trb1 and Trb3, $n = 10$ for Trb2; $p < 0.05$ (*) or $p < 0.0001$ (**) for unstable vs stable plaque segments.

Figure 2. Regulation of macrophage Trb mRNA levels by treatment with oxidised LDL in serum-free medium. **A:** dose dependency of Trb1, Trb2 and Trb3 mRNA regulation by oxLDL (expressed relative to untreated cells) after 24 hours treatment. Mean + SEM ($n = 5$ for 5 μg and 10 μg treatment, $n = 6$ for 25 μg and 50 μg treatment); $p < 0.05$ (*) or $p < 0.01$ (**) for Trb1, Trb2 or Trb3 expression in oxLDL-treated vs untreated cells; **B, C, D:** time course of regulation by serum withdrawal alone ('control'), or by treatment with 50 μg (total LDL protein)/ml nLDL or oxLDL (expressed relative to cells at time = 0 hours) for Trb1, Trb2 and Trb3 respectively. Mean + SEM ($n = 7$) in all cases; $p < 0.05$ for oxLDL treatment vs control (*) or native LDL (\dagger).

Figure 3. A: Regulation of macrophage IL-10 mRNA levels by expression of recombinant Trb2 and Trb3: mRNA levels expressed relative to untransfected cells from same experiment, mean + SEM ($n = 6$) in all cases; $p < 0.05$ for Trb2 transfected cells vs vector (*) or Trb3 (\dagger) transfected cells. **B:** regulation of macrophage IL-10 mRNA levels by treatment with oxidised LDL: mRNA levels expressed relative to cells at time = 0 hours. Mean + SEM ($n = 6$) in all cases; $p < 0.05$ for oxLDL treatment vs control (*) or native LDL (\dagger).

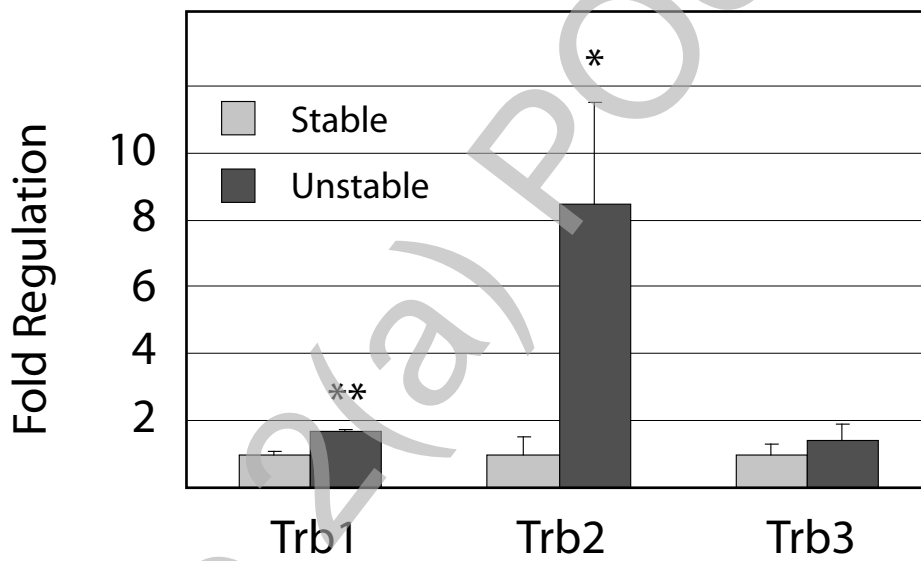
Figure 4. Suppression of IL-10 secretion (**A**) and mRNA levels (**B**) by adenovirus-mediated Trb2 overexpression in aggregated IgG/LPS-treated macrophages. Mean + SEM ($n = 9$ for protein measurements, $n = 7$ for mRNA): $p < 0.05$ (*), $p < 0.005$ (***) for Trb2 adenovirus-infected cells ('Trb2') vs non-infected cells ('IgG/LPS'); $p < 0.05$ (\dagger), $p < 0.01$ ($\dagger\dagger$) for Trb2 adenovirus-infected cells vs control adenovirus-infected cells ('AD0'); $p < 0.05$ ($\hat{\wedge}$), $p < 0.01$ ($\hat{\wedge}\hat{\wedge}$) for Trb2 adenovirus-infected cells vs Trb3 adenovirus-infected cells ('Trb3'). **C:** Western blots of anti-Trb2 (left) and anti-Trb3 (right) detection of proteins in adenovirus-infected macrophages. 'AD0': control adenovirus-infected macrophages (MOI = 200); '100', '200': MOI used to infect macrophages with Trb2- (left) or Trb3- (right) expressing adenovirus. Approximate positions of molecular weight markers (centre) and Trb2, Trb3 and GAPDH indicated. **D:** Relative expression of Trb2 and Trb3 mRNA in non-infected ('control cells') and adenovirus-infected ('Trb2/3-expressing') macrophages (MOI = 200) as detected by TaqMan RT-PCR measurements (threshold cycle is proportional to negative log₂ mRNA concentration; see text for details). Mean + SEM ($n = 7$).

Table 1. Rank Correlation Coefficients (and Associated Statistical Significance) Between Levels of Trb mRNAs and those of Leukocyte Markers in Human Carotid Plaque Tissue

	Trb1	Trb2	Trb3
CD3	0.5213 (P < 0.005)	0.3808 (NS)	0.0323 (NS)
CD68	0.5244 (P < 0.005)	0.8146 (P < 0.0001)	0.3195 (NS)

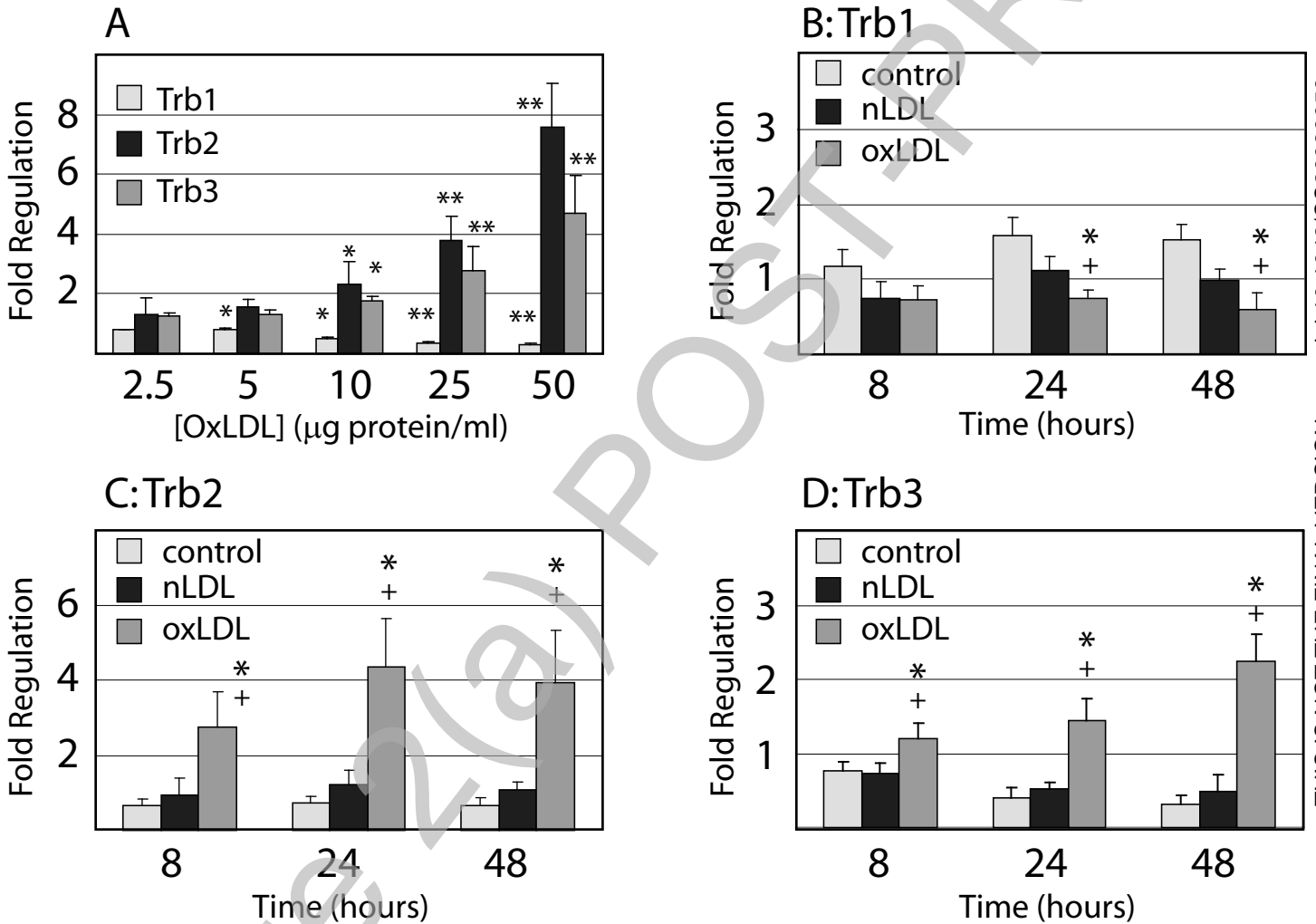
NS: not significant

Figure 1



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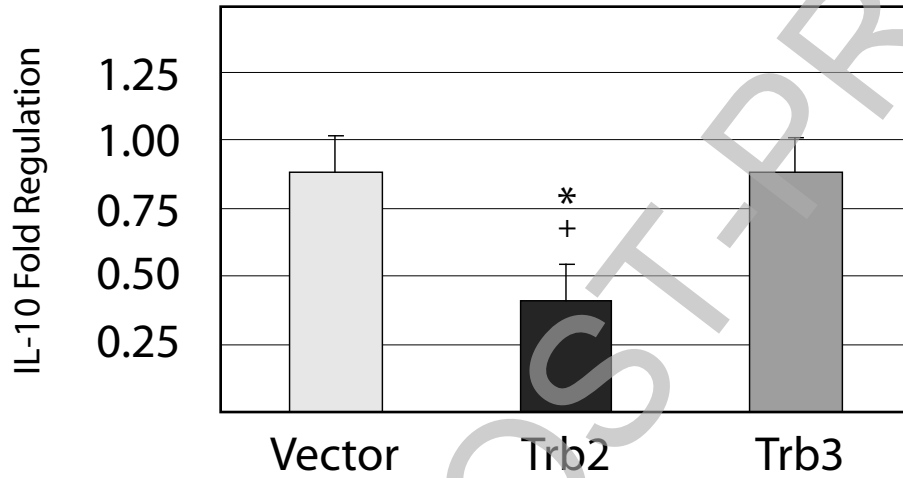
Figure 2



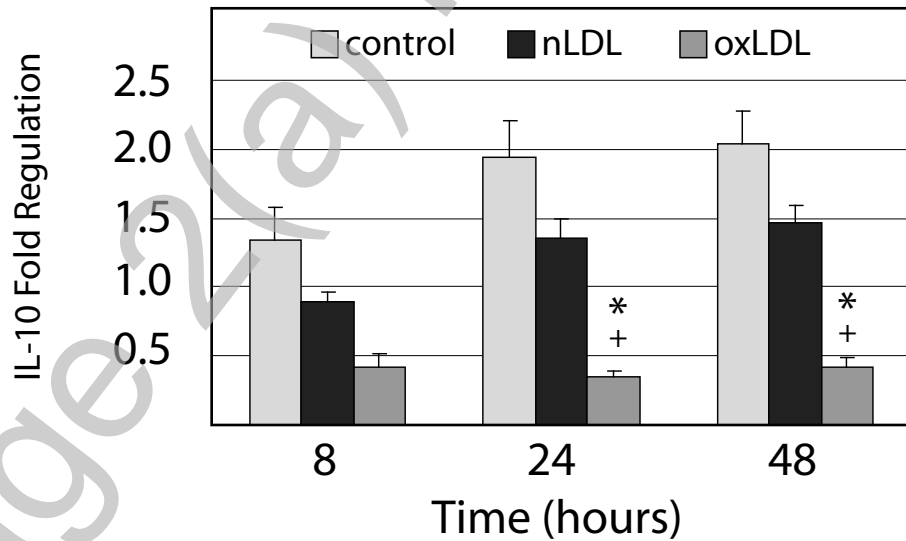
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Figure 3

A: IL-10 regulation by Trb2 and Trb3



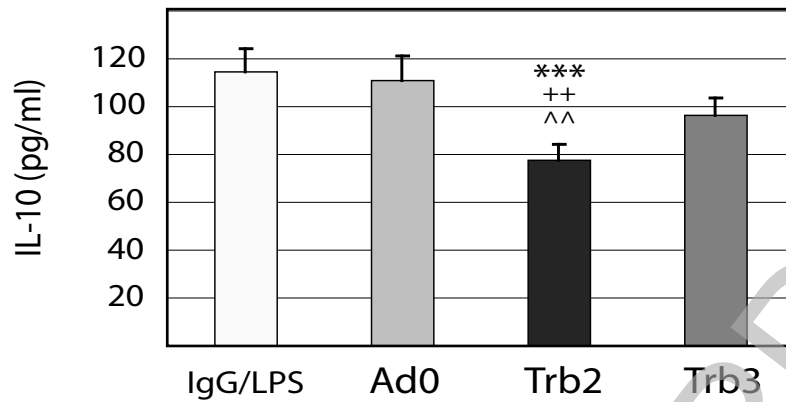
B: IL-10 regulation by oxLDL



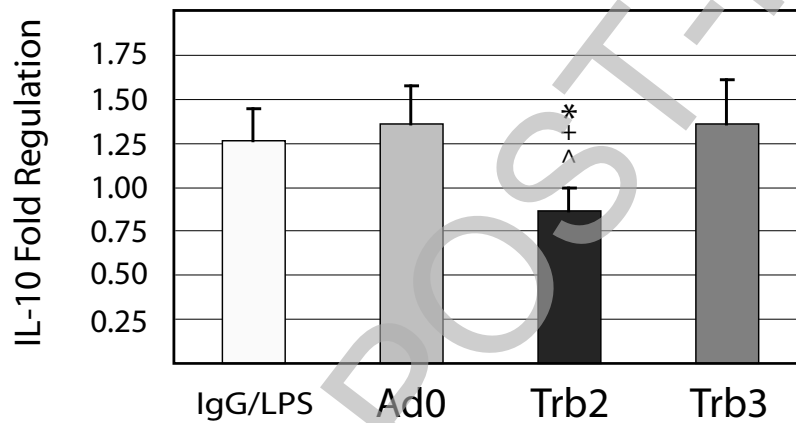
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Figure 4

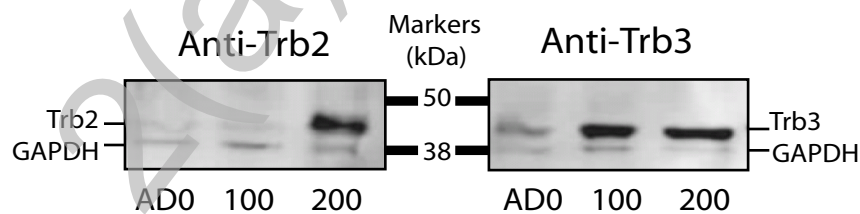
A: IL-10 Secreted from Macrophages



B: Macrophage IL-10 mRNA



C: Trb Protein in Infected Cells



D: Trb mRNA Levels in Infected Cells

