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DOI:
10.1016/j.imbio.2015.06.019

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PII: S0171-2985(15)30018-8
DOI: http://dx.doi.org/doi:10.1016/j.imbio.2015.06.019
Reference: IMBIO 51359

To appear in:

Received date: 14-11-2014
Revised date: 11-6-2015
Accepted date: 23-6-2015

Please cite this article as: Lenart, Marzena, Rutkowska, Magdalena, Baj-Krzyworzeka, Monika, Szatanek, Rafal, Węglarczyk, Kazimierz, Smallie, Timothy, Ziegler-Heitbrock, L*ddoto*ms, Zembala, Marek, Siedlar, Maciej, Hyaluronan carried by tumor-derived microvesicles induces IL-10 production in classical (CD14++CD16*minus*) monocytes via PI3K/Akt/mTOR-dependent signalling pathway.Immunobiology http://dx.doi.org/10.1016/j.imbio.2015.06.019

Title: **Hyaluronan carried by tumor-derived microvesicles induces IL-10 production in classical ($CD14^{++}CD16^{-}$) monocytes *via* PI3K/Akt/mTOR-dependent signalling pathway**

Short title: **The mechanism of IL-10 induction in monocytes**

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Keywords:

hyaluronan, IL-10, monocytes, monocyte subsets, PI3K/Akt/mTOR pathway, tumor-derived microvesicles,

Abstract

Tumor-derived microvesicles (TMV) can mimic effects of tumor cells leading to an increased anti-inflammatory cytokine production, such as interleukin 10 (IL-10), by tumor-infiltrating monocytes and macrophages. Yet, the mechanism of IL-10 induction by TMV in monocytes remains unclear.

The co-incubation of TMV derived from the human pancreas carcinoma cell line (HPC-4) with human monocytes resulted in a nearly 30-fold increase in IL-10 protein production. This effect operates at the level of transcription since monocytes transduced with an adenovirus containing IL-10-promoter luciferase reporter gene showed a 5-fold induction of luciferase activity after treatment with TMV. Since tumor cells can express hyaluronan (HA), which participates in tumor invasion and metastases, we have tested its effect on IL-10 expression. We showed that HA at the concentration of 100 μg/ml induces IL-10 protein expression and the IL-10 promoter activation in monocytes. Moreover, hyaluronidase treatment of TMV reduced IL-10 protein production by 50% and promoter activity by 40%. Inhibitors of the PI3K/Akt/mTOR pathway reduced both, TMV-induced IL-10 promoter activity and protein production, and the same was observed in monocytes when stimulated by HPC-4 cells or HA. Inhibition of PI3K activity down-regulated phosphorylation of the Akt and (to a lesser extent) mTOR proteins in monocytes following TMV or HA stimulation. When comparing monocyte subsets, TMV induced IL-10 protein and mRNA synthesis only in classical $CD14^{++}CD16^{-}$ but not in CD16-positive monocytes. Our data show that TMV induce IL-10 synthesis in human classical monocytes *via* HA, which, in turn, activates the PI3K/Akt/mTOR pathway.

Introduction

Interleukin 10 (IL-10) is synthesized *in vivo* by a broad variety of immune cells (1-3) and inhibits the release of Th1 cytokines, antigen presentation, expression of co-stimulatory molecules, phagocytosis, but enhances B cell survival and antibody production (3-4). IL-10 production is elevated in various types of cancer, where is produced by both tumor cells and/or tumor-infiltrating monocytes/macrophages (TIM), and is being associated with tumor-mediated immunosuppression. IL-10 production by monocytes/macrophages following interaction with tumor cells and/or TMV results in alteration of their immunophenotype and biological activity (5-7). However, the signalling pathways responsible for IL-10 induction in monocytes/macrophages following their interactions with tumor cells are poorly characterised. IL-10 production by monocytes can be enhanced through the activation of phosphatidylinositol-3 kinase (PI3K) pathway (8). PI3K converts phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-triphosphate, which recruits and activates downstream targets, including Akt, also termed protein kinase B (PKB). The other key cellular signalling pathway, depending on the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, affects broad aspects of cellular functions, such as metabolism, growth, and survival (9). Although, they were initially viewed as two separate pathways, it has been indicated that PI3K and mTOR are connected *via* Akt (10). PI3K/Akt/mTOR pathway has been reported to take part in the regulation of immune cells activity, including monocytes/macrophages (11). In many types of cancer, PI3K pathway is activated by hyaluronan (HA) (12). Moreover, signal transducer and activator of transcription 3 (STAT3) and interferon regulatory factor 1 (IRF-1) have been characterised as important transcription factors inducing IL-10 promoter activation (13-14).

Hyluronan (HA) is a glycosaminoglycan with a molecular weight ranging from 10^5 to 10^7 Da and is a major component of the extracellular matrix (15). HA can be either attached directly to the cell surface by hyaluronan synthases or can bind to cell surface receptors, i.e. CD44, activating intracellular signalling pathways associated with them (15-17). HA accumulates in the cellular division sites (18) and is one of the major extracellular matrix components in human malignancies (19), participating in tumor invasion and metastases (15). TMV, originating from tumor cells, carry some tumor cell surface determinants, growth factors, nucleic acids, and tumor-associated antigens (6, 20-22). It has been previously suggested that tumor-monocyte interactions may involve hyaluronan or other CD44 ligands

carried by TMV (23-24). Therefore, TMV may affect monocytes/macrophages functions, altering their immunophenotype and biological activity. Moreover, TMV differently affect monocyte subsets (7,25). Monocytes can be subdivided into three subsets: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (26), where the latter two may be collectively referred to as $CD16^{+}$ monocytes (27). The $CD16^{+}$ monocytes are characterised by enhanced inflammatory cytokine secretion, including tumor necrosis factor α (TNF), and low secretion of IL-10; increased expression of human leukocyte antigens (HLA) class II and some adhesion molecules (28-30). They are considered to be more mature than classical monocytes (31), and their numbers are elevated in inflammatory diseases (32). In response to a contact with tumor cells, the $CD16^{+}$ monocytes produce more proinflammatory cytokines (TNF, IL-12) and show an increased cytotoxic/cytostatic activity towards tumor cells (30). TMV-activated $CD16^{+}$ monocytes also show an increased release of TNF, IL-12p40 and reactive nitrogen intermediates (RNI), while $CD14^{++}CD16^{-}$ monocytes produce more reactive oxygen species (ROI) and IL-10 (25).

In this study, we have proposed a new possible mechanism responsible for the induction of IL-10 production in monocytes after stimulation with TMV. For the first time, we provide evidence that HA carried by TMV is able to induce IL-10 production in classical $CD14^{++}$ $CD16^{-}$ (but not in $CD16^{+}$) monocytes *via* the PI3K/Akt/mTOR signalling pathway.

Materials and Methods

Isolation of monocytes and their subsets

Monocytes were isolated by counter-flow centrifugal elutriation from peripheral blood mononuclear cells (PBMC) obtained from 10 healthy blood donors. Briefly, PBMC were isolated from EDTA-treated whole peripheral blood by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were then separated from PBMC with the JE-5.0 elutriation system, equipped with the Sanderson separation chamber (Beckman, Palo Alto, CA, USA), as described previously (33). Purity of isolation was above 95% as tested by staining with anti-CD14 mAb (BD Biosciences Pharmingen, San Diego, CA) and flow cytometry analysis (FACSCanto flow cytometer, Becton Dickinson, San Jose, CA, USA).

The following monoclonal antibodies (mAbs) were used to stain $CD14^{++}CD16^{-}$ (further called: $CD14^{+}$) and $CD16^{+}$ monocytes: anti-CD14-APC (clone MφP9, BD Bioscience) and anti-CD16-PE-Cy7 (clone 3G8, BD Bioscience), in 1:25 dilution v/v. The stained monocytes were then incubated for 30 min at 4°C after which they were sorted using the FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA) into foetal bovine serum (FBS)-coated polypropylene tubes (BD Biosciences), at 12.000 cells/s, in order to gain pure (usually 97-98%) $CD14^{+}$ and $CD16^{+}$ subsets. The $CD16^{+}$ monocytes were in the range 5-10%.

Cell culture

The HPC-4 cell line (34) was cultured by biweekly passages in RPMI 1640 supplemented with 5% FBS (Sigma, St. Louis, MO). FBS used in all experiments was earlier microvesicles-depleted by appropriate centrifugation, as described previously (6). Cell lines were regularly tested for *Mycoplasma sp*. contamination by polymerase chain reaction (PCR) ELISA test (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol.

Monocytes were cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine (Invitrogen Life Technologies, Gaithersburg, MD), and 25 μg/ml gentamycin (Invitrogen Life Technologies), 1–2× nonessential amino acids (Invitrogen Life Technologies), and OPI

supplement (contains oxalacetic acid, sodium pyruvate, and insulin; Sigma-Aldrich, Munich, Germany).

Monocytes were co-cultured with HPC-4 (4 x 10^4/well) cells (2,5:1 ratio) in supplemented medium overnight at 37°C, 5% CO_2 in humidified atmosphere. Then, supernatants were collected and kept frozen in -20°C.

TMV isolation

TMV were obtained from the HPC-4 cell line (TMV_{HPC}) as previously described (7). Briefly, HPC-4 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) with 5% FBS and gentamycin (25 μg/ml). Supernatants from well grown cell cultures were collected and centrifuged at 300×g for 20 min to remove cell debris. Then, supernatants were again centrifuged at 50,000×g for 1 h at 4°C. Pellets were washed several times in PBS and resuspended in serum-free RPMI 1640 medium. Protein concentration of TMV_{HPC} suspension was estimated by the Bradford method (BioRad, Hercules, CA). TMV_{HPC} were tested for endotoxin contamination by the Limulus test (Charles River Laboratories, Inc., Wilmington, MA), stored at −20°C, and used for experiments only when TMV suspension was LPS-free. TMV were characterised in detail by flow cytometry and electron microscopy previously (6).

Hyaluronan, hyaluronidase treatment, kinase inhibitors

A low-molecular-weight potassium salt of hyaluronic acid (HA) from human umbilical cord (Calbiochem, Darmstadt, Germany), was added to cell cultures at the final concentration of 100 μg/ml (35,36).

TMV_{HPC} were added to cell cultures at the final protein concentration of 3 μg/ml. In particular experiments, TMV_{HPC} were treated with hyaluronidase (HAase), at the final concentration of 100 U/ml, for 3 h at 37°C, with gentle shaking (5) and then centrifuged at 50,000×*g* for 15 min at 4°C. Pellets were resuspended in serum-free RPMI 1640 medium. HAase-treated TMV_{HPC} were added to cell cultures at the final protein concentration of 3 μg/ml.

The presence of HA in TMV_{HPC} suspension and supernatants from HPC-4 cell culture was determined using Hyaluronan Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

The following inhibitors of the PI3K/Akt/mTOR pathway were used: PI3K (10 μM; LY-294002; Sigma, St Louis, MO), Akt (10 μM; Akt inhibitor) and mTOR (10 ng/ml; rapamycin), both from Calbiochem (Darmstadt, Germany).

Viability assay

Elutriated monocyte and sorted monocyte subsets were cultured for 6 h in the presence of PI3K, Akt or mTOR inhibitor, or left untreated. Then, cells were labelled with Annexin V-FITC Apoptosis Detection Kit II (BD Pharmingen), in order to detect apoptotic cells according the manufacturer's protocol. Immediately before flow cytometry analysis (FACSCanto flow cytometer, Becton Dickinson), propidium iodide was added.

Monocyte transduction with IL-10-promoter-containing adenoviral (AdV) vectors

Monocytes were transduced with the IL-10 (-237) wt lux AdV vectors containing the IL-10 gene promoter with STAT3 and IRF-1 motifs, reporter luciferase gene, and green fluorescent protein gene with -237 bp upstream of the IL-10 transcription start (37). The $1x10^6$ monocytes or dummy sorted monocytes and monocyte subsets ($CD14^+$ and $CD16^+$) were incubated with AdV vectors for 2 h in serum-free medium in 24-well ultra-low attachment plates (Costar, Corning, Lowell, MA) at the 100 MOI. Then, FBS was added to cultures at the final concentration of 10%, and the cells were cultured overnight at 37°C, 5% CO_2 in humidified atmosphere. Cells were then washed and resuspended in fresh medium with 10% FBS. A percentage of transduced cells was determined by the analysis of GFP-positive cells on FACSCanto flow cytometer. The average percentage of infected cells was 37 ± 12%.

Reporter gene analysis

Transduced cells were cultured in ultra-low attachment 96-well plates, at the concentration of 2×10^5 cells/well in 200 μl of monocyte medium. Monocytes were preincubated with PI3K, Akt or mTOR inhibitors for 30 min at 37°C and then stimulated with TMV_{HPC}, HA or TMV_{HPC} treated with HAase, for 6 h at 37°C, or left intact. Cells were then harvested, lysed in

Reporter Lysis Buffer (Promega, Madison, WI) and frozen at -20°C. Luciferase activity in cell lysates was determined using Luciferase Assay System (Promega) and Wallac Victor 2 plate-reader (Perkin-Elmer, Turku, Finland). Protein concentration in each sample was determined by the Bradford method. Luciferase activity was normalised by protein concentration in each sample (cpm per 1 µg of protein) and the results were presented as a fold difference between normalised luciferase level in each sample, in comparison to the control.

Western blot analysis.

Elutriated monocytes were plated ($3x10^6$/well) on 12-well plates in RPMI 1640 (Sigma, St. Louis, MO) with 5% FBS and gentamycin (25 µg/ml). After 2 h of incubation at 37°C in humidified atmosphere containing 5 % CO_2, PI3K inhibitor was added for 1 h to the appropriate wells. Next, monocytes were stimulated with TMV_{HPC} (final concentration -3 µg/ml) and HA (100 µg/ml) for 30 min. Monocytes were than harvested and lysed in M-PER lysing buffer (Pierce, Rockford, IL, USA), containing proteases inhibitors cocktail (Roche, Mannheim, Germany). The protein concentration was measured on micro-volume spectrophotometer Q5000 (QUAWELL, San Jose, CA, USA) using Bradford Dye Reagent (Bio-Rad, Hercules, CA, USA) and Bovine Gamma Globulin (Bio-Rad), used as a protein concentration standard. The 20 µg of isolated protein of each sample was mingled with NuPAGE LDS Sample Buffer (4X) (Life Technologies, Carlsbad, CA, USA) and NuPAGE Sample Reducing Agent (10X) (Life Technologies). Samples were heated (70°C, 10 minutes) and electrophoresed in 12% polyacrylamide gel containing SDS. Next, electrophoresed proteins were transferred to the polyvinylidene fluoride membrane (Bio-Rad). Then, after blocking for 1 h at room temperature in Tris buffered saline (TBS) with 0,1% Tween-20 (Sigma, St. Louis, MO, USA) and 1% bovine serum albumin (BSA, Sigma), the membranes were incubated overnight at 4°C with monoclonal antibodies (dilution 1:1000): rabbit anti-Akt, rabbit anti-phospho-Akt (Ser473), rabbit anti-mTOR, rabbit anti-phospho-mTOR (Ser2448) (all Cell Signalling, Beverly, MA, USA). As a loading control, rabbit anti-GAPDH antibodies (Cell Signalling) were used. After overnight incubation, membranes were washed in TBS supplemented with BSA and Tween-20 and incubated (1 h) in RT with secondary goat anti-rabbit antibodies (dilution 1:4000), conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were visualized with the SuperSignal West Pico Chemiluminescence Substrate kit, as recommended by the

manufacturer (Pierce, Rockford, IL, USA), and analysed with KODAK GEL LOGIC 1500 Digital Imaging System (KODAK, Rochester, NY, USA). Densitometry analysis was done on the Western Blot results using KODAK MI SE 4.5 software to determine the abundance of studied protein. Saturation of GAPDH protein bands was used as control. Data are presented as a ratio between individual protein and GAPDH band saturation.

Real-time PCR

Dummy sorted monocytes and monocyte subsets ($CD14^+$ and $CD16^+$) were cultured in ultra-low attachment 96-well plates in serum free medium, with TMV_{HPC} or HA for 3 h at 37°C, or left untreated. Then, the total RNA was extracted from cells using RNeasy Protect Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. cDNA was obtained from the RNA samples with Maloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Sigma) and oligo-dT primer (Sigma), as specified by the manufacturer's protocol. The quantitative polymerase chain reactions (PCR) for IL-10 and β-actin was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany), as previously described (7), with the following primer pairs: IL-10 sense, 5′-GGA-CTT-TAA-GGG-TTA-CCT-GG and antisense, 5′-GAA-CTC-CTG-ACC-TCA-AGT-GA; β-actin sense, 5′-GGA-TGC-AGA-AGG-AGA-TCA-CTG; and antisense, 5′-CGA-TCC-ACA-CGG-AGT-ACT-TG. Each LightCycler PCR run consisted of 40 cycles with initial denaturation time of 10 min at 95°C. The cycling profile for IL-10 was set at: 95°C for 10 sec, 62°C for 10 sec, 72°C for 40 sec; and for β-actin: 95°C for 10 sec, 60°C for 60 sec. The fluorescent signals generated during the informative log-linear phase were used to calculate the relative amount of mRNA. The specificity of the amplified products was verified by the melting curve analysis. The mRNA expression was indicated as a fold difference from untreated dummy sorted monocytes, normalised to the β-actin expression level ($\Delta\Delta Ct$ method).

Determination of IL-10 secretion

$2x10^5$ monocytes or dummy sorted monocytes and monocyte subsets ($CD14^+$ and $CD16^+$) were cultured in 96-well plates (BD Falcon, Franklin Lakes, NJ). The control cells were left untreated or preincubated for 30 min with PI3K, Akt or mTOR inhibitors and then TMV_{HPC},

HA, or whole HPC-4 cells were added. The cells were cultured for 18 h at 37°C, 5% CO_2 in humidified atmosphere. The cell culture supernatants were collected and IL-10 level was determined using Cytokine Bead Array (CBA) and Human IL-10 Flex Set (BD Biosciences, San Jose, CA, USA), according to manufacturer's protocol (lower limit of detection – 0,13 pg/ml).

Statistical analysis

Statistical analysis was performed using Mann-Whitney, when applicable, or two-tailed Student *t* test using GraphPad InStat Software (GraphPad Soft Inc., La Jolla, CA). Differences were considered significant at $p<0,05$. Data represent the mean values ± SD of five independent experiments, each performed on cells of different donor.

Results

HA is involved in TMV-mediated IL-10 induction in monocytes

Initially, we have determined the effect of TMV_{HPC} on IL-10 induction in the whole population of human monocytes. TMV_{HPC} significantly upregulated IL-10 protein production by monocytes from 46 ± 95 to 1344 ± 1251 pg/ml. Moreover, co-culture of monocytes with the HPC-4 cells resulted in even higher levels of IL-10 production (2463 ± 1014 pg/ml) (Fig 1A). In order to test whether TMV_{HPC} act *via* induction of the IL-10 gene promoter, we have transduced the monocytes with adenoviral vectors containing a luciferase reporter gene controlled by the IL-10 promoter containing intact wild-type binding sites for STAT3 and IRF-1 (37). In monocytes transduced with AdV vectors and stimulated with TMV_{HPC}, the luciferase activity increased by an average of 5.2-folds when compared to unstimulated cells (Fig. 1B).

Then, we asked whether HA, which is produced in abundance by tumor cells, may be responsible for IL-10 induction by TMV. First, we tested the TMV_{HPC} for the presence of HA using ELISA and found out that the average HA concentration in TMV_{HPC} suspension was 0,51 ± 0,16 ng/µg per 1 µg of TMV (n=5). Moreover, HA presence was also detected in supernatants from HPC-4 cell culture (99 ± 14 ng/ml), as previously suggested (23). We then asked whether HA is able to induce the human IL-10 promoter and lead to production of IL-10 protein in monocytes. Indeed, HA stimulated both IL-10 secretion (to 2240 ± 1327 pg/ml) (Fig. 2A) and IL-10 promoter-driven luciferase activity (2,3-fold increase) (Fig. 2B) in human monocytes, and both were statistically significant. The amount of IL-10 protein produced by monocytes in response to HA was somewhat higher when compared to TMV_{HPC}, albeit not significant, while IL-10 promoter induction after HA stimulation was about 2-folds lower.

Then we analysed whether TMV stimulation of IL-10 is mediated by HA. The induction of IL-10 protein production was halved when monocytes were stimulated with TMV that were pretreated with HAase for 3h, and the difference was statistically significant (Fig. 2A). Similar results were obtained in the case of IL-10 promoter activation in monocytes also stimulated with HAase-treated TMV_{HPC}, as HAase treatment of TMV_{HPC} resulted in significant reduction of IL-10 promoter activation (Fig. 2B). Although, the reduction of IL-10 expression caused by HAase treatment of TMV_{HPC} was no absolute, longer TMV_{HPC}

treatment with HAase (beyond 3h) did not reduce IL-10 expression any further (data not shown).

TMV-driven induction of IL-10 in monocytes is mediated via PI3K/Akt/mTOR pathway

Next, we asked whether the expression of IL-10 induced by TMV_{HPC} and HA is mediated by the PI3K/Akt/mTOR signalling pathway. To address this, IL-10 protein production and its promoter induction were determined in the whole population of monocytes preincubated with specific inhibitors of this signalling pathway. First, a viability assay was performed to evaluate the toxicity of the inhibitors. The results revealed less than 10% of dead cells following the incubation of monocytes with the inhibitors, when compared to untreated cells. Therefore, the effects of the inhibitors on the IL-10 production, observed in our experiments, were specific and did not result from the cytotoxicity of the inhibitors.

The co-culture of monocytes preincubated with the inhibitors of PI3K, Akt or mTOR, with HPC-4 cells, resulted in significant down-regulation of IL-10 protein production (1,4-, 1,4- and 2-fold decrease, respectively, ($p<0,001$)) (Fig. 3). Similar results were obtained when monocytes were stimulated with HA (Fig. 4A, B). IL-10 secretion by monocytes stimulated with HA decreased 3,3-, 3,3- and 2,5-folds (Fig. 4A, $p<0.001$), while IL-10 promoter activation decreased 1,8-, 2,1- and 1,8-folds (Fig. 4B, $p<0.05$), when cells were preincubated with PI3K, Akt or mTOR inhibitors, respectively. Inhibition of the PI3K signalling cascade in monocytes stimulated with TMV_{HPC} resulted in a considerable decrease in IL-10 expression. IL-10 protein production was reduced 3,3-, 2,5- and 1,7-folds when using PI3K, Akt or mTOR inhibitors, respectively (Fig. 4C, $p<0.05$). IL-10 promoter activation in monocytes stimulated with TMV_{HPC} also decreased when cells were treated with the inhibitors (1,5-, 2- and 1,6-fold decrease in the case of PI3K, Akt or mTOR inhibitor, respectively), yet only in the case of Akt inhibitor, the difference was statistically significant (Fig. 4D).

The activation of PI3K/Akt/mTOR signalling pathway in monocytes was confirmed in Western-blot analysis (Fig. 5). Unphosphorylated and phosphorylated Akt and mTOR proteins were analysed in monocytes stimulated with TMV_{HPC} or HA after PI3K inhibition. The expression of phospho-Akt and phospho-mTOR was down-regulated when monocytes were pre-treated with PI3K inhibitor. **However, although the effect of PI3K inhibitor on**

TMV and HA-induced phospho-Akt activation is obvious, this effect on phospho-mTOR inhibition was rather moderate. That is why, we have measured the intensity of mTOR and phospho-mTOR bands using densitometry analysis and the results were normalized according to GAPDH intensity (see Supplementary Fig. 1). As a result, we have noticed significant reduction of phospho-mTOR bands intensity in the case of MO cultured alone and MO stimulated with TMV_{HPC} (decrease of 38% and 36%, respectively). Nonetheless, in the case of HA stimulation, the PI3K inhibitor had only slight effect on phospho-mTOR band intensity (decrease of 3%; Supplementary Fig. 1B). In contrast, the level of unphosphorylated mTOR was not reduced when monocytes where preincubated with PI3K inhibitor (Supplementary Fig. 1A).

TMV and HA-mediated IL-10 induction in monocyte subsets

In order to determine, which monocyte subset ($CD14^+$ classical monocytes or $CD16^+$ intermediate/non-classical monocytes) is responsible for IL-10 production in response to TMV_{HPC} or HA stimulation, we have analysed IL-10 promoter activity, mRNA expression and protein production in the cells of both sorted subpopulations.

After TMV_{HPC} and HA stimulation we observed increased IL-10 promoter activity in classical monocytes with a 4,4- and 2-fold increase, respectively. In the CD16-positive monocytes there was only a 2- and 1,1-fold increase after stimulation with TMV_{HPC} and HA, respectively (basal levels of luciferase activity was 46 ± 18 and 4 ± 1 RLU/μg of protein, for $CD14^+$ and $CD16^+$ monocytes, respectively), however, due to a considerable variation between experiments, the induction of the promoter activity was not significant (Fig. 6A). A clearer pattern emerged at the IL-10 mRNA level. Here, IL-10 expression increased 2,2-folds in HA-stimulated and 1,9- folds in TMV_{HPC}-stimulated $CD14^+$ monocytes (Fig 6B, $p<0,001$ and $p<0,01$, respectively), while in $CD16^+$ monocytes there was no induction. The IL-10 mRNA expression of untreated $CD14^+$ and $CD16^+$ monocytes was similar (ΔCt value: 11,3 ± 1,9 for $CD14^+$ monocytes and 10,21 ± 1,8 for $CD16^+$ monocytes), indicating that basal IL-10 expression in these subsets is comparable, yet only $CD14^+$ monocytes responded to TMV_{HPC} and HA stimulation.

The average level of IL-10 protein produced by $CD14^+$ monocytes was 71 ± 20 pg/ml in unstimulated cells. HA treatment resulted in an increase of IL-10 production to 605 ± 418

pg/ml, while in monocytes treated with TMV_{HPC} an increase to 619 ± 904 pg/ml was observed. By contrast, the $CD16^{+}$ monocytes show negligible amounts of IL-10 protein production when untreated (about 2 pg/ml) and there was no increase after stimulation by either HA or TMV_{HPC} (Figure 6C). These data demonstrate that in response to HA or TMV_{HPC} stimulation, the classical ($CD14^{++}CD16^{-}$) monocytes are the main source of IL-10.

To summarise, our findings suggest that IL-10 production in $CD14^{+}$ monocytes is induced by TMV and is mediated by HA via the PI3K/Akt/mTOR pathway (Fig.6).

Discussion

Previous findings show that both, tumor cells and TMV released by these cells, are able to induce IL-10 expression in monocytes at both the mRNA and protein level (5-7, 38). IL-10 promotes the differentiation of monocytes to mature macrophages and blocks their differentiation into dendritic cells (39). High frequency of TIMs has been usually associated with poor prognosis in cancer patients (40). Moreover, macrophages exposed to IL-10 polarise into M2 cells that reveal poor anti-tumor response (41). Therefore, the present study investigated the molecular mechanism of IL-10 induction in primary human monocytes, and in particular: i) which component of TMV_{HPC} shedded by HPC-4 tumor cells is responsible for IL-10 induction in monocytes, and ii) which signalling pathways are involved in this process. Our findings suggest that IL-10 stimulation observed in monocytes exposed to TMV_{HPC} is mediated by low molecular weight HA carried by these microvesicles. The presence of HA in TMV_{HPC} suspension has been confirmed. Moreover, IL-10 promoter activation, IL-10 gene expression and cytokine production was significantly induced by TMV_{HPC} and HA, as well as by HPC-4 tumor cells. The level of IL-10 induction may differ between TMV_{HPC} and HPC cells stimulation, since TMV only mimic tumor cells presence. Pre-treatment of TMV_{HPC} with HAase reduced IL-10 expression by half, when compared to untreated TMV_{HPC}. Thus, our data strongly suggest that HA carried by TMV_{HPC} may be involved in interactions of TMV with monocytes and thus corroborate previous reports (7). Besides, we found HA in TMV originated not only from HPC-4 cells but also from another cancer cell lines, e.g. colon cancer cell lines (data not shown). It has been shown that CD44, a major receptor for HA, is expressed on monocytes and takes part in interactions of monocytes with cancer cells, mediating TMV-induced release of TNF, IL-1 and probably ROI (7, 23, 42). Yet, HA involvement in IL-10 induction has never been investigated.

The observed decrease of IL-10 induction after addition of TMV_{HPC} pre-treated with HAase did not reach the level observed in control samples. It may suggest that either HA stimulation was not fully efficient, or, apart from HA, another, yet still unknown factor/s carried by TMV_{HPC} may be also associated with IL-10 induction.

In our studies, HA more efficiently induced IL-10 secretion, while IL-10 gene promoter was stimulated more effectively by TMV_{HPC}. This observation may support the suggestion that HA is not the only component of TMV_{HPC} that is responsible for IL-10 gene induction. It is plausible that the presence of only STAT3 and IRF1 binding sites, contained

by IL-10 promoter in AdV vectors used in these studies, is insufficient for excessive HA induction of such a construct. Apart from STAT3 and IRF1, other transcription factors binding sites have been identified in IL-10 promoter, such as specific protein 1 (SP1), SP3, and CCAAT/enhancer binding protein-β (C/EBPβ) (43-45), which may suggest that HA is able to activate one of these factors. On the other hand, the additional, still unknown component of TMV_{HPC} that can also induce IL-10 gene expression, may act mainly through STAT3 and/or IRF1 transcription factors.

Moreover, it has been shown that IL-10 expression is also regulated post-transcriptionally, through IL-10 mRNA stabilization dependant on regulation of its 3′-UTR. In unstimulated cells, the constitutively expressed IL-10 mRNA is kept at low levels through mRNA-destabilizing signals, while upon activation, IL-10 transcription is up-regulated through activation of IL-10 promoter, and then IL-10 mRNA levels are further increased through RNA stabilization controlled by regulatory regions located in the 3′-UTR (46). Thus, it cannot be excluded, that the differences in IL-10 secretion levels and promoter activation, mediated by TMV_{HPC} and HA, observed in our studies, might result from differences in IL-10 mRNA de- or stabilizing signals induced by stimulation with each of the factors.

The IL-10 gene expression and/or protein production after TMV_{HPC}, HA and HPC-4 tumor cells stimulation was significantly down-regulated when monocytes were preincubated with the inhibitors of PI3K, Akt or mTOR. What is more, Western-blot analysis with PI3K inhibition demonstrated the specificity of PI3K/Akt/mTOR signalling cascade activated in monocytes, mostly by TMV_{HPC} and to a lesser extent by HA. These data strongly suggest that IL-10 induction in monocytes by TMV_{HPC} carrying HA is PI3K/Akt/mTOR pathway-dependent which is consistent with previous data where PI3K and Akt contribution to IL-10 production has been demonstrated in monocytes and macrophages (47, 48), while inhibition of mTOR down-regulates expression of IL-10 in human monocytes (9). Also, TLR activation results in IL-10 synthesis by the engagement of the Akt/mTOR pathway and the MAP kinase p38 (49). However, it has not been shown before that HA is able to activate the PI3K/Akt/mTOR pathway in monocytes. Wu Y. *et al.* indicated that HA fragments released by tumor cells activate neutrophils through the TLR4/PI3K/Akt signalling pathway (50). In PBMC and dendritic cells, the stimulatory effect of HA was mediated also by TLR4 and included phosphorylation of p38 and p42/p44 MAPK followed by translocation of NF-κB to the nucleus (17, 51). In the case of TMV-mediated IL-10 production by monocytes, Akt phosphorylation has been already reported (6).

Furthermore, our results indirectly suggest that IL-10 expression, mediated by the PI3K/Akt/mTOR signalling pathway, is mediated - among others - by STAT3 and IRF-1 transcription factors. The luciferase reporter vectors, used in our studies, contain IL-10 promoter composed of only STAT3 and IRF-1-binding motifs, so that activation of the promoter by TMV_{HPC} or HA stimulation must result from one or both of the transcription factors activation. Our previous observations could confirm STAT3 involvement in IL-10 induction in monocytes, since we have observed even before STAT3 phosphorylation in monocytes stimulated by TMV (7).

Based on the previous findings reporting TNF induction in monocytes by TMV (7), we have also determined whether the observed mechanism of activation is also involved in TNF production. In fact, both, TMV_{HPC} and HA, stimulated TNF protein production in monocytes and this was independent of the PI3K/Akt/mTOR signalling (data not shown). Therefore, we suggest that the PI3K/Akt/mTOR dependent induction of IL-10 production differs substantially from that observed in the case of TNF induction.

In order to determine, which monocyte subset is responsible for IL-10 production after HA or TMV_{HPC} stimulation, we have examined IL-10 production by $CD14^+$ and $CD16^+$ subpopulations of monocytes. Previous reports indicated that classical ($CD14^{++}CD16^-$) monocytes, in response to LPS, produced more IL-10 in comparison to non-classical ($CD14^+CD16^{++}$) monocytes (52, 53). It has been shown that $CD16^+$ monocytes stimulated with HPC-4 tumor cells show lower IL-10 release, in comparison to $CD14^+$ cells (30). When monocyte subsets are treated with TMV_{HPC}, secretion of IL-10 by $CD16^+$ cells is also significantly lower than that observed in the whole monocyte population and $CD14^+$ cells (25). To extend these reports, we have determined IL-10 production in monocyte subsets at each level of protein expression (promoter activation, mRNA expression and protein secretion). As a result, IL-10 gene expression, IL-10 mRNA production and protein production after TMV_{HPC} or HA stimulation was significantly higher in $CD14^+$ subset. The differences in IL-10 expression in monocyte subsets seem not to result from differential expression of CD44, since the expression level of CD44 on $CD14^+$ and $CD16^+$ monocytes is similar (data not shown). These data clearly corroborate previous findings that $CD14^+$ monocytes are the main producers of IL-10 among the whole monocyte population.

Our observations may have also some clinical relevance. They may, at least partially, explain the observations that increased serum level of IL-10 is an independent unfavourable

prognostic factor in patients with advanced neoplastic disease, i.e. gastric cancer (54), which may be a result of monocytes overstimulation for IL-10 production by TMV known to be elevated in circulation of advanced tumor-bearing hosts (55, 56). Indeed, we have also noticed that TMV released by gastric cancer cell lines, e.g. cell lines 1401 and 1415, derived in our laboratory, induce IL-10 promoter activation, mRNA expression and protein production in monocytes at a similar level to TMV_{HPC} (data not shown).

In conclusion, our findings demonstrate that TMV-induced IL-10 production in monocytes, mainly $CD14^+$ cells, is mediated by HA via the PI3K/Akt/mTOR pathway.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgements

We would like to thank Mariola Ożóg, Irena Ruggiero and Barbara Hajto for skillful technical assistance.

Footnotes

This study was supported by the Jagiellonian University Medical College (grant no. K/ZDS/001477 and K/ZDS/002906) and the National Committee for Scientific Research grant no. UMO-2012/07/B/NZ6/03499 and NN 401 290236.

Abbreviations used in this article:

DCs	dendritic cells
HA	hyaluronan
IRF	interferon regulatory factor
MOI	multiplicity of infection

PKB	protein kinase B
RNI	reactive nitrogen intermediates
ROI	reactive oxygen species
mTOR	mammalian target of rapamycin
TIM	tumor-infiltrating monocytes/macrophages
TMV	tumor microvesicles

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Figure legends

Fig. 1. Effect of TMV_{HPC} or HPC-4 cells on IL-10 production and promoter activation in monocytes. Monocytes were cultured with TMV_{HPC} or HPC-4 cells for 18h. Release of IL-10 was significantly elevated after TMV_{HPC} or HPC-4 cell stimulation, in comparison to untreated monocytes (A). Monocytes were transduced with AdV vector containing IL-10 promoter, with STAT3 and IRF1 motifs, and luciferase gene. The results are presented as a relative level, represent a fold difference between normalized luciferase level in samples and the control. The IL-10 promoter induction was significantly elevated after TMV_{HPC}

stimulation, in comparison to untreated TMV_{HPC} (B). Statistical analysis was performed using Mann-Whitney (A) or two-tailed Student *t* test (B).

Fig. 2. Effect of HA stimulation on IL-10 production and promoter activation in monocytes. Monocytes were stimulated with HA, TMV_{HPC} or HAase-treated TMV_{HPC} for 18h. Release of IL-10 was significantly elevated after HA and TMV_{HPC} stimulation, in comparison to untreated monocytes, while HAase treatment of TMV_{HPC} resulted in lesser IL-10 production, when compared to untreated TMV_{HPC} (A). Monocytes were transduced with AdV vector containing IL-10 promoter, with STAT3 and IRF1 motifs, and luciferase gene. The results are presented as a relative level, represent a fold difference between normalized luciferase level in samples and the control. IL-10 promoter activation was significantly elevated after HA and TMV_{HPC} stimulation, in comparison to untreated monocytes, while HAase treatment of TMV_{HPC} resulted in lesser IL-10 production and promoter activation, when compared to untreated TMV_{HPC} (B). Statistical analysis was performed using Mann-Whitney (A) or two-tailed Student *t* test (B).

Fig. 3. Effect of inhibition of PI3K, Akt or mTOR on HPC-4 cell-stimulated IL-10 secretion. IL-10 level was significantly decreased when monocytes were pre-incubated with selected protein kinase inhibitors. The results are presented as a relative level, represent a fold difference between IL-10 secretion level in each samples and the control. Statistical analysis was performed using two-tailed Student *t* test.

Fig. 4. The effect of inhibition of PI3K, Akt or mTOR on HA or TMV_{HPC}-stimulated IL-10 production – IL-10 protein production (A,C) and IL-10 promoter activation (B,D). Monocytes were preincubated with inhibitors of PI3K, Akt or mTOR and then HA (A,B) or TMV_{HPC} (C,D) was added. IL-10 protein production following stimulation with HA (A) or TMV_{HPC} (C) was significantly decreased when monocytes were pre-incubated with PI3K, Akt or mTOR inhibitors. IL-10 promoter induction was significantly decreased when HA-stimulated monocytes were pre-incubated with appropriate inhibitors (B). In the case of stimulation of monocytes with TMV_{HPC}, the promoter induction was also down-regulated after inhibition of analysed kinases, yet the difference was only statistically significant after use of Akt inhibitor (D). The results are presented as a relative level, represent a fold difference between IL-10 secretion level in each samples and the control (A,C) or as a relative level representing a fold difference between normalized luciferase level in each samples and the control (B,D). Statistical analysis was performed using two-tailed Student *t* test.

Fig. 5. Western-blot analysis of unphosphorylated and phosphorylated Akt and mTOR proteins expression in monocytes. Monocytes were pre-treated with PI3K inhibitor and then stimulated with TMV_{HPC} or HA. The PI3K inhibition results in Akt and mTOR down-regulation following TMV_{HPC}/HA stimulation. One representative experiment out of four performed is presented.

Fig. 6. Effect of TMV_{HPC} or HA stimulation on IL-10 production by $CD14^+$ or $CD16^+$ monocyte subsets. The induction of IL-10 promoter was observed in each subset after TMV_{HPC} or HA stimulation, yet lower in case of $CD16^+$ subset. The results are presented as a relative level, which represents a fold difference between normalized luciferase level in each

samples and the control (A). The expression of IL-10 mRNA after stimulation with TMV_{HPC} and HA was significantly higher in $CD14^+$ monocyte subset than observed in $CD16^+$ cells. The figure presents relative level, showing the fold difference between normalized IL-10 mRNA level in each sample and the control (B). In response to TMV_{HPC} or HA stimulation, IL-10 secretion was upregulated in $CD14^+$ monocytes, while $CD16^+$ cells did not respond to the stimulation. The amount of secreted IL-10 by $CD14^+$ subset was also notably higher than that secreted by $CD16^+$ subset (C). Statistical analysis was performed using two-tailed Student *t* test.

Fig. 7. Scheme of proposed mechanism of IL-10 production activation in monocytes. Tumor cells shed TMV that contain low weight HA. HA molecules stimulate IL-10 production by activation of PI3K/Akt/mTOR signaling pathway that may act through STAT3 and IRF-1 transcription factors. The latter, activate IL-10 gene promoter, what leads to IL-10 transcription activation and, in the end, IL-10 protein production and secretion.

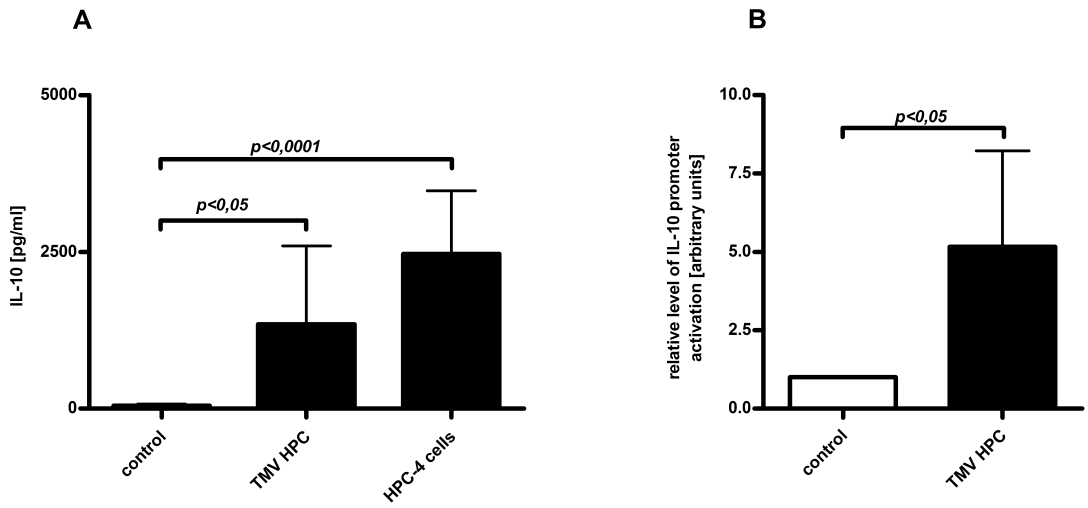


fig 1 .

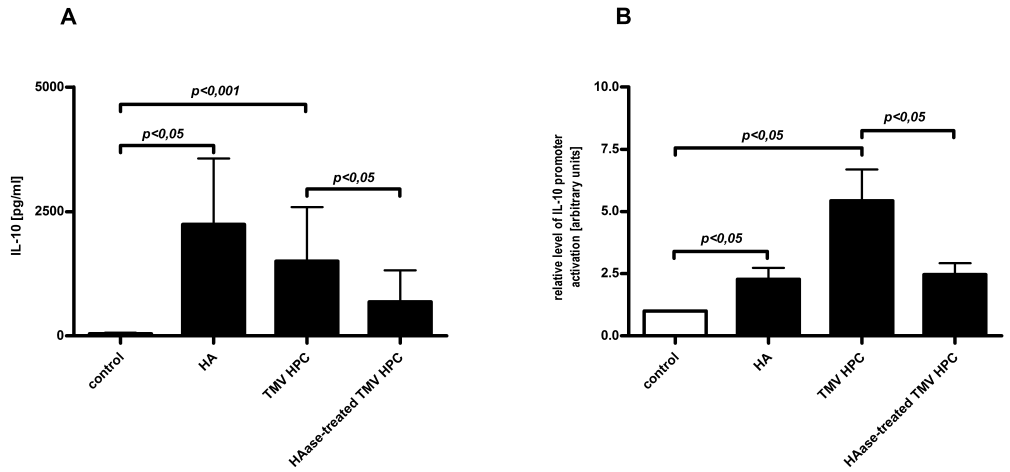


fig 2 .

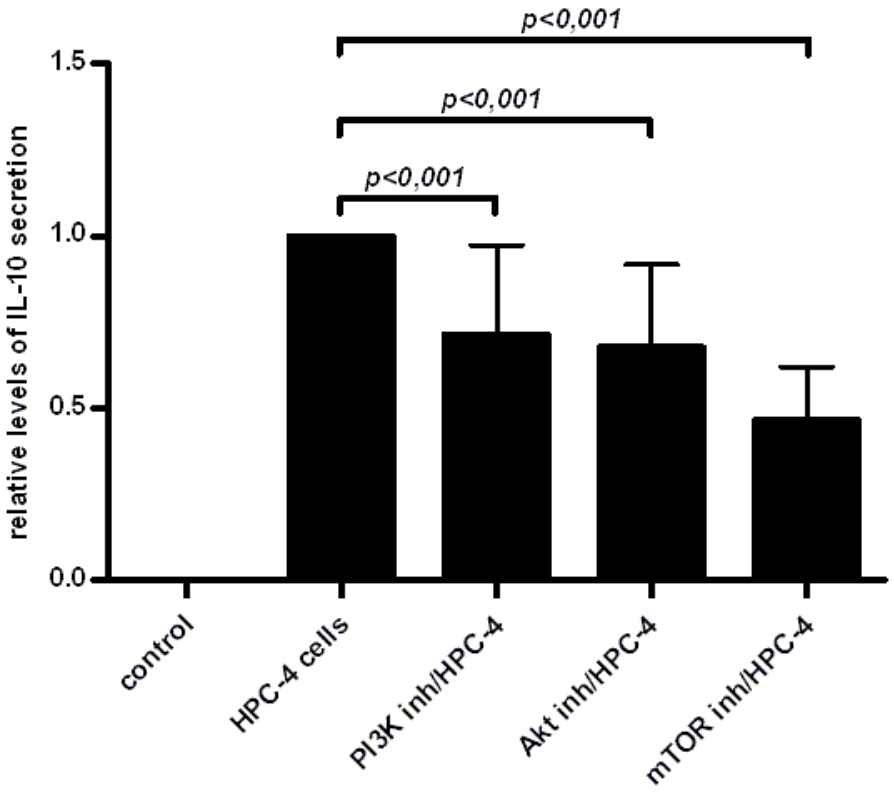


fig 3 .

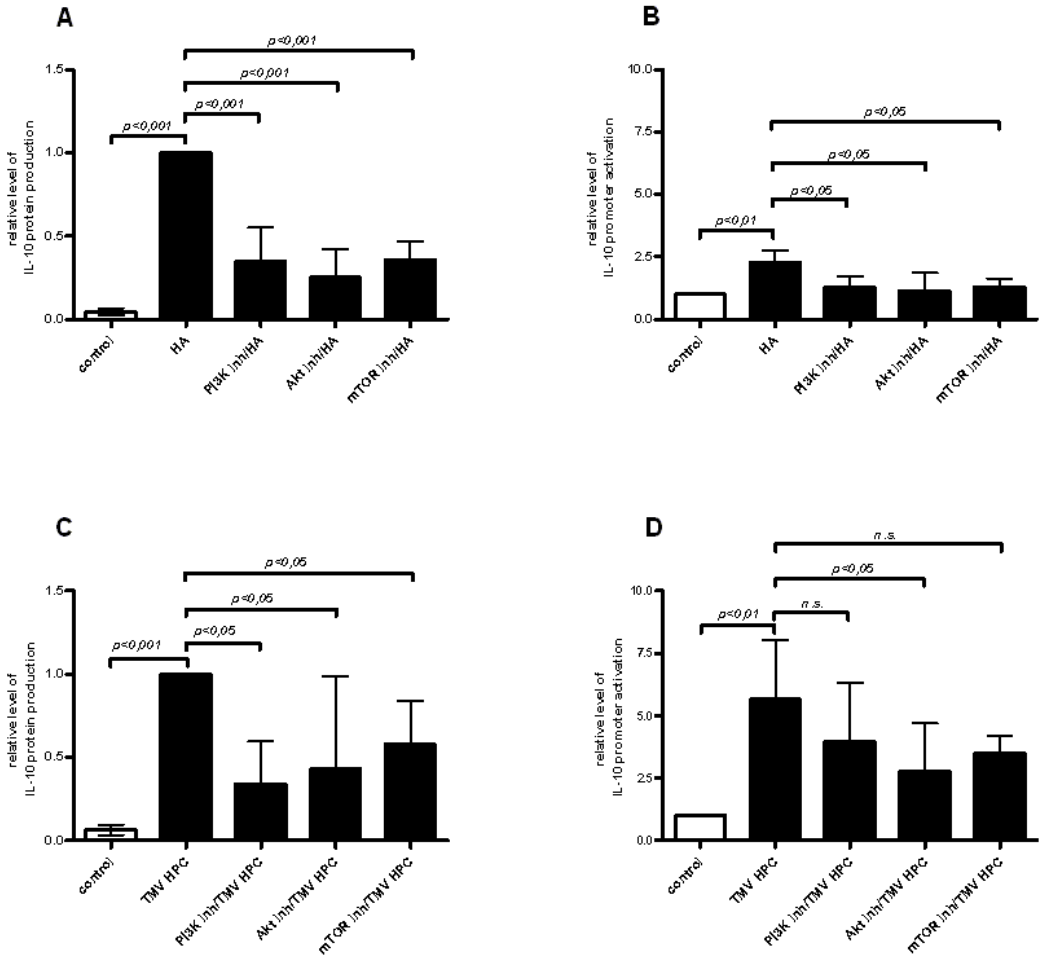


fig 4 .

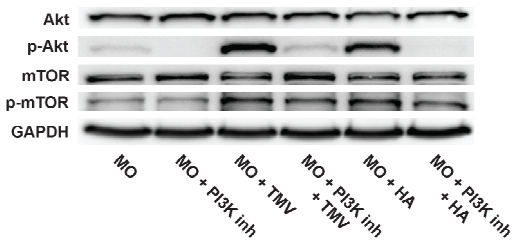
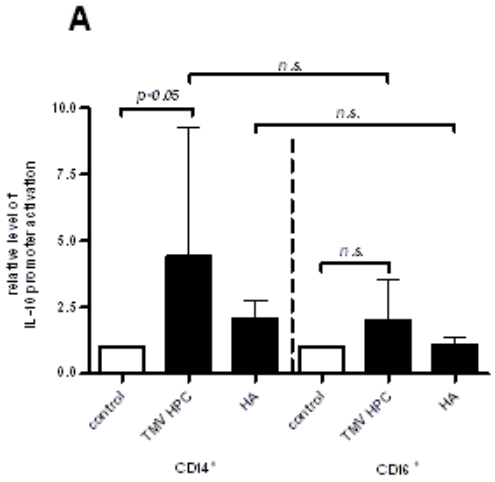
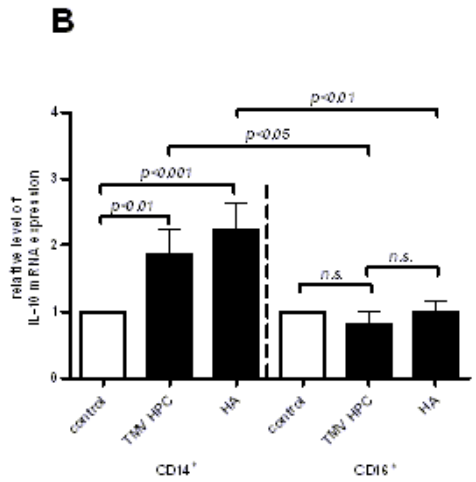


fig 5 .





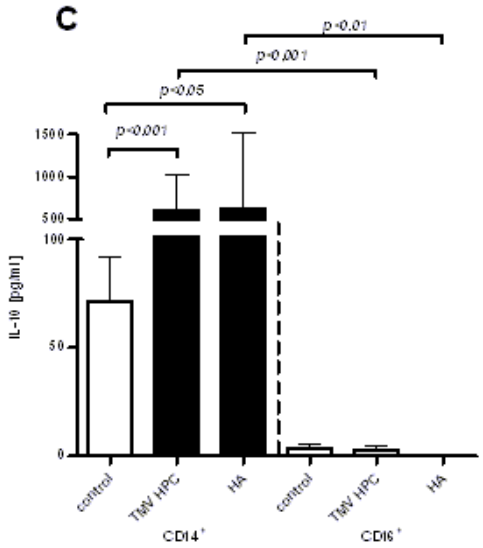


fig 6 .