

MicroRNA-4443 regulates mast cell activation by T cell-derived microvesicles

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Background: The mechanism by which mast cells (MCs) are activated in T cell-mediated inflammatory processes remains elusive. Recently, we have shown that microvesicles derived from activated T cells (mvT*s) can stimulate MCs to degranulate and release several cytokines.

Objective: The aim of this study was to characterize the contribution of microRNAs (miRs) delivered by microvesicles to MC activation.

Methods: miR profiling was performed with NanoString technology and validated by using real-time PCR. The biological role of mvT* miR was verified by overexpression of miRs in MCs using mimic or inhibitory molecules and analyzing the effect on their predicted targets.

Results: mvT*s were found to downregulate the expression of the tyrosine phosphatase protein tyrosine phosphatase receptor type J (PTPRJ), a known extracellular signal-regulated kinase inhibitor. Bioinformatics analysis predicted that miR-4443 regulates the *PTPRJ* gene expression. Indeed, miR-4443, which was present in mvT*s, was also found to be overexpressed in human MCs stimulated with these MVs. α -Amanitin insensitivity confirmed that overexpression of miR-4443 was not due to transcriptional activation. The luciferase reporter assay indicated that the 3' untranslated region of *PTPRJ* was targeted by this miR. Transfection of MCs with mimic or inhibitor of miR-4443 resulted in decreased or enhanced *PTPRJ* expression, respectively. Furthermore, miR-4443 regulated extracellular signal-regulated kinase phosphorylation and IL-8 release in MCs activated by mvT*s.

Conclusion: These results support a scenario by which T cell-derived microvesicles act as intercellular carriers of functional miR-4443, which might exert heterotypic regulation of *PTPRJ* gene expression in MCs, leading to their activation in the context of T cell-mediated inflammatory processes. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: MicroRNA, mast cells, microvesicles, T cells

Mast cells (MCs) are key effector cells in the elicitation of IgE-mediated allergic responses. Close physical proximity between T cells and MCs and activation of the latter have been demonstrated in several T cell-mediated inflammatory processes and have led investigators to propose a bidirectional interaction between these 2 cell populations.¹⁻⁴ However, the molecular mechanism by which MCs are activated in these conditions remains elusive. Previously, we have reported that direct physical contact between MCs and activated T lymphocytes or their membranes is associated with Ras activation and sustained extracellular signal-regulated kinase (ERK) phosphorylation that results in MC activation and mediator release.⁵⁻⁸ Furthermore, MCs could also be stimulated by microvesicles released from activated T cells, thereby promoting a response in the absence of direct physical contact with T cells at the site of inflammation.⁹ These T cell-derived microvesicles are actively internalized into MCs, a process that can be detected as early as 1 hour after initiation and requires 24 hours for completion, culminating in both degranulation and cytokine release.¹⁰ In the present study we aimed to further uncover the mechanism by which T cell-derived microvesicles affect MC activation.

In addition to sharing surface markers of their parental cells, microvesicles carry a broad variety of cytoplasmic components, including proteins, DNA, and RNA.¹¹ These microvesicles might act as intercellular carriers and might deliver bioactive proteins and RNA to recipient cells, including mRNAs¹² and microRNAs (miRs),^{13,14} which can affect their properties. miRs are a large class of small noncoding regulatory molecules (19-24 nucleotides) that regulate genes expression by binding directly to the 3'-untranslated region (UTR) of specific mRNA target, leading to reduction of protein synthesis by means of repression of translation or by inducing mRNA degradation.¹⁵

The ability of microvesicles to contain and transfer miRs to recipient cells prompted us to investigate whether and which miRs might be involved in activation of microvesicles derived from activated T cells (mvT*)-induced MCs.

Here we demonstrate that stimulation of MCs with mvT*s leads to overexpression of miR-4443, which has not been recognized thus far in MCs. MiR-4443 served as a negative regulator of the protein tyrosine phosphatase receptor type J (*PTPRJ*) gene, which is known to modulate the Ras-signaling pathway through dephosphorylation of ERK.¹⁶ Furthermore, overexpression of miR-4443 was found to regulate ERK phosphorylation and IL-8 release in MCs activated by mvT*s. Thus, by delivering miR-4443, T cell-derived microvesicles can play an important role in MC activation within T cell-mediated inflammatory processes in which MCs were found to be involved.

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Abbreviations used

CBMC: Human cord blood mast cell
 ERK: Extracellular signal-regulated kinase
 GUSB: β -Glucuronidase
 MC: Mast cell
 miR: MicroRNA
 mvT: Microvesicle derived from resting T cells
 mvT*: Microvesicle derived from activated T cells
 PTPRJ: Protein tyrosine phosphatase receptor type J
 qRT-PCR: Quantitative real-time PCR
 SCF: Stem cell factor
 UTR: Untranslated region

METHODS**Antibodies and reagents**

The following antibodies were used for this study: anti-PTPRJ (R&D Systems, Minneapolis, Minn), anti-tubulin (Sigma-Aldrich, St Louis, Mo), anti-phospho-ERK (Cell Signaling Technology, Danvers, Mass), and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa). The RNA polymerase inhibitor α -amanitin was purchased from Sigma-Aldrich.

Cell culture

Reagents for cell culture were purchased from Biological Industries (Beit Haemek, Israel). Jurkat T-cell leukemia cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 12.5 U/mL nystatin. The human LAD2 MCs were maintained in StemPro-34 SFM (GIBCO Invitrogen Corporation, Grand Island, NY) supplemented with 2 mmol/L L-glutamine, 50 μ g/mL streptomycin, 100 IU/mL penicillin, and 100 ng/mL recombinant human stem cell factor (SCF; PeproTech, Rocky Hill, NJ), as previously described.¹⁷

Cell activation

LAD2 MCs were activated by means of incubation with 50 μ g/mL purified microvesicles derived from resting T cells (mvTs) or activated T cells (mvT*s), as previously described.⁹ For IgE-mediated activation, cells were sensitized overnight with 500 ng/mL human myeloma IgE (Calbiochem, Merck KGaA, Darmstadt, Germany) and then stimulated with 10 μ g/mL anti-human IgE (Dako, Glostrup, Denmark). Primary cultured human cord blood mast cells (CBMCs) were derived from human cord blood mononuclear cells in the presence of SCF, IL-6, and prostaglandin E₂ and used as previously described.⁸ Jurkat T cells were activated with 75 ng/mL phorbol 12-myristate 13-acetate (PMA) (Calbiochem) for 1 hour, followed by extensive washes, as previously described.⁸ Human peripheral blood-derived T lymphocytes were activated by anti-CD3/anti-CD28 antibodies, as previously described.⁹

RNA isolation

The experimental protocol is detailed in the Method section in this article's Online Repository.

NanoString technology

miRNA (miR) expression profiling of LAD2 cells activated with mvTs or mvT*s or left untreated was performed with the human V1 miRNA assay kit (NanoString Technologies, Seattle, Wash), as previously reported.¹⁸

Real-time PCR for miRs

miR-4443 was quantified by using the qScript miRNA cDNA synthesis kit, followed by real-time PCR analysis with PerfeCta miRNA assays and

PerfeCta SYBR Green master mix (Quanta Biosciences, Gaithersburg, Md). The small nuclear RNA RNU44 was used as reference for gene quantitation.

Real-time PCR

cDNA was synthesized by using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Carlsbad, Calif). Gene expression was determined by means of Fast Real-Time PCR with an ABI 7500 Thermal Cycler (Applied Biosystems). Expression of the gene *PTPRJ* was measured by using specific TaqMan probes (human-PTPRJ NM_002843, Applied Biosystems). β -Glucuronidase gene (*GUSB*) was used as a housekeeping gene for analysis of changes in cycle threshold values.

Transfection of LAD2 cells

miR-4443 mimic, mimic negative control, miR-4443 inhibitor, or inhibitor negative control (50 nmol/L; miRvana, Applied Biosystems) were transfected into LAD2 cells by using the TransIT-X2 Dynamic Delivery System (Mirus, Madison, Wis), according to the manufacturer's instructions, in LAD2 medium (StemPro-34) without addition of StemPro nutrient supplement and antibiotics for 4 hours at 37°C. At the end of the incubation period, StemPro-34 medium with nutrient supplement, 100 ng/mL SCF, and antibiotics was added, and the cells were incubated at 37°C for an additional 24 to 48 hours.

cDNA expression plasmids (5 μ g) were transfected into LAD2 cells with DMRIE-C reagent (Invitrogen, Grand Island, NY), according to the manufacturer's instructions.

Luciferase assay

LAD2 cells were cotransfected with 0.5 μ g of pLightSwitch PTPRJ-3'-UTR plasmid or pLightSwitch reporter gene plasmid (Active Motive, Carlsbad, Calif) and 50 nmol/L miR-4443 mimic or mimic negative control in a 96-well plate by using DMRIE-C Reagent (Invitrogen), according to the manufacturer's instructions. After 24 hours, luciferase activity was measured with the pLightSwitch Luciferase Assay kit (Switchgear; Genomics, Carlsbad, Calif), according to the manufacturer's protocol.

SDS-PAGE and immunoblotting

Cellular extracts were separated by using SDS-PAGE with 7.5% to 10% polyacrylamide gels, transferred to polyvinylidene difluoride filters, and processed for immunoblotting, as previously described.⁷ Immunoreactive bands were visualized with the LAS-3000 Imaging System (Fujifilm, Tokyo, Japan).

Statistical analysis

Results are presented as means $\pm$ SEMs. Statistical analysis was performed by using the unpaired Student *t* test. A *P* value of less than .05 was considered statistically significant.

The experimental protocols for cDNA constructs, isolation of microvesicles, human cytokine assay, and microarray experiment and data analysis are detailed in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS***PTPRJ* levels decrease in mvT*-activated MCs**

As previously reported, microvesicles derived from activated (mvT*s) but not from resting (mvTs) T cells are internalized into MCs, leading to degranulation and cytokine release.^{9,10} These events result in Ras activation and sustained ERK phosphorylation.^{6,7}

To further decipher the effect of mvT*s on MC function, we examined the global gene expression profiles induced by

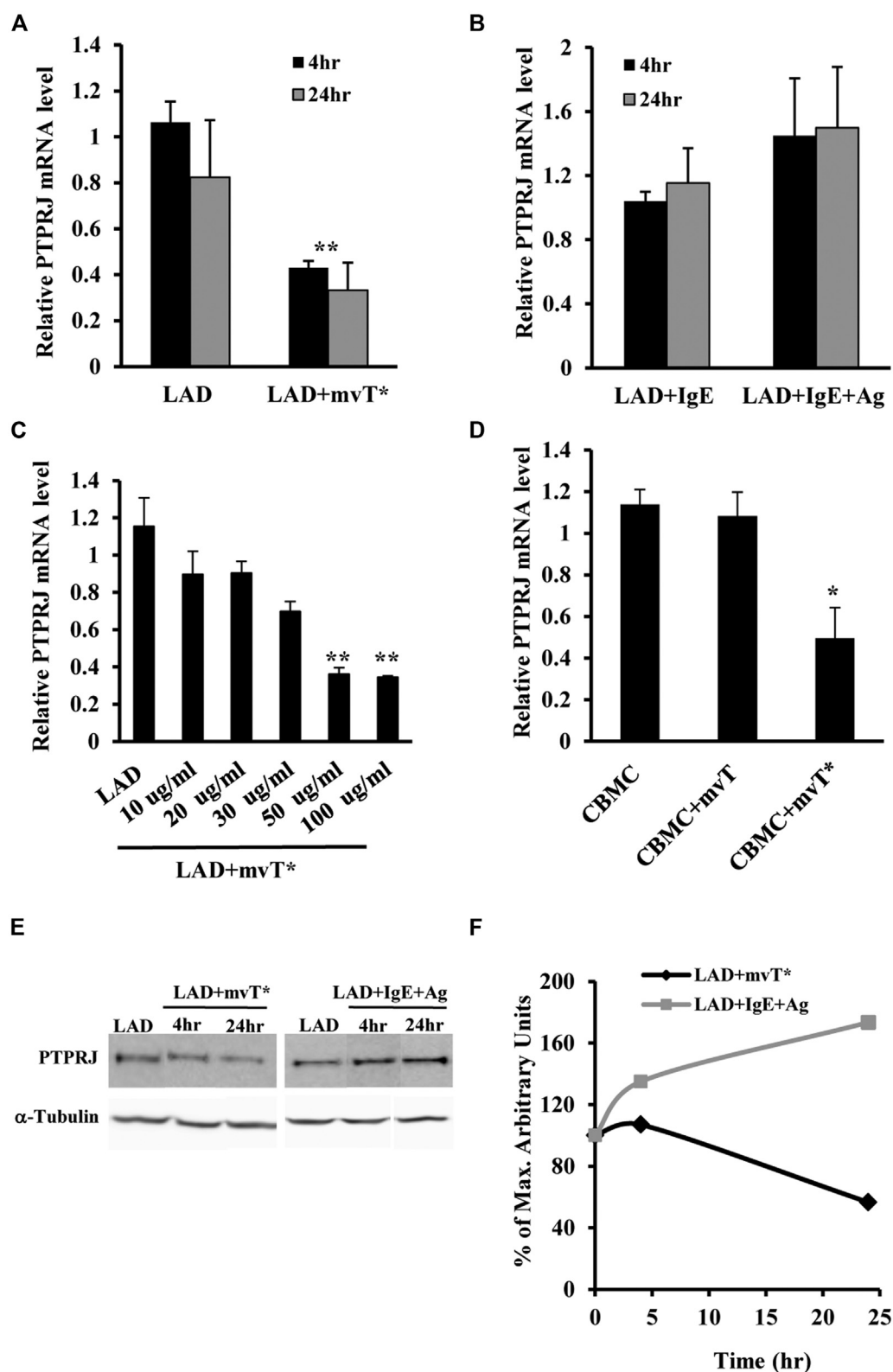


FIG 1. mvT*s downregulate PTPRJ expression in MCs. **A-D**, LAD2 cells (Fig 1, A-C) or CBMCs (Fig 1, D) were incubated with mvTs or mvT*s (Fig 1, A and C) or activated by Fc ϵ RI cross-linking (Fig 1, B). *PTPRJ* mRNA levels were analyzed by using qRT-PCR and normalized to the levels of *GUSB*. Data are relative to untreated cells. **E** and **F**, PTPRJ protein levels were assessed by using Western blot analysis (Fig 1, E) and quantitated by using densitometric analysis (Fig 1, F). Results are shown from 3 independent experiments (means $\pm$ SEMs). * $P < .05$ and ** $P < .01$.

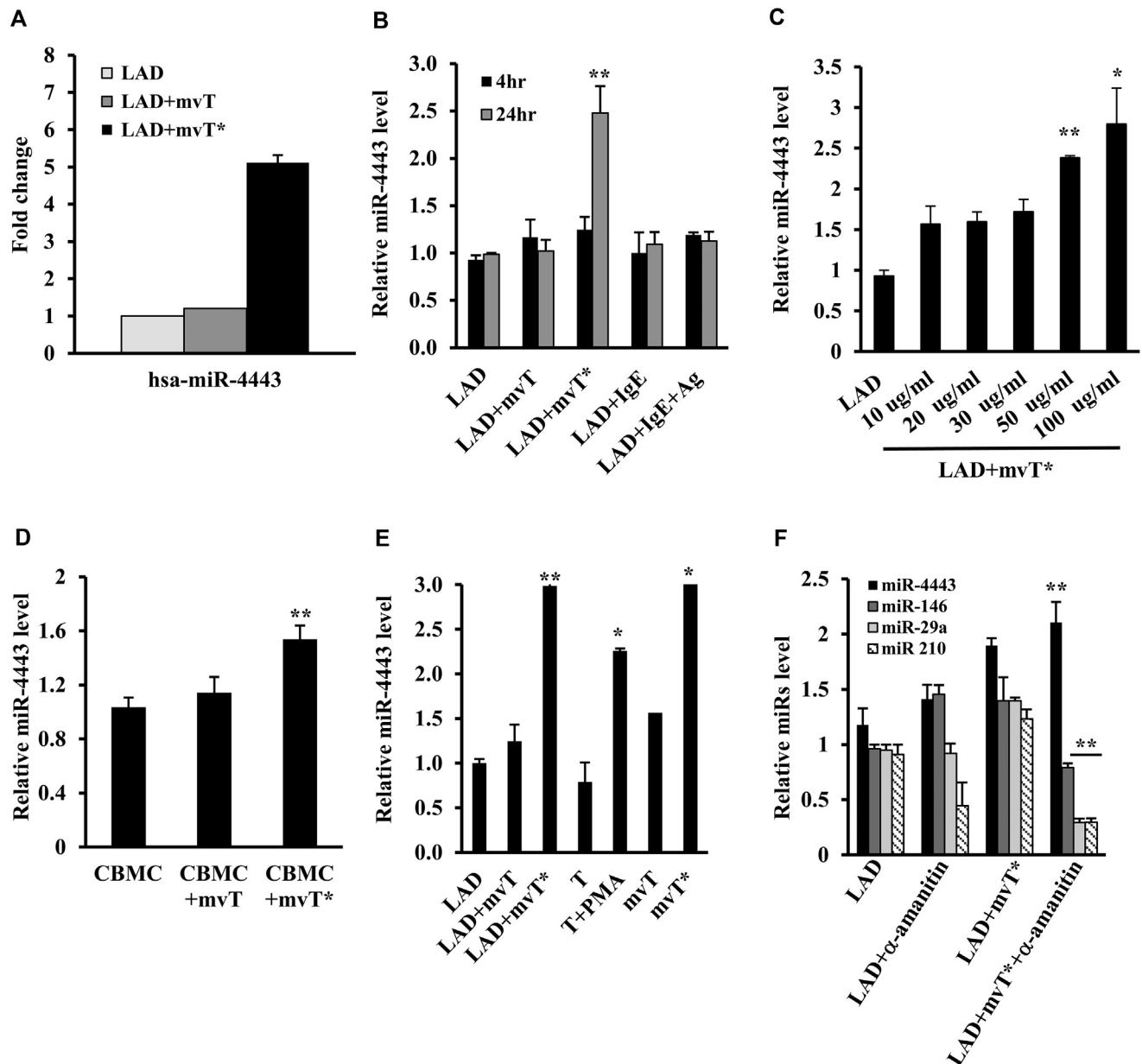


FIG 2. Expression of miR-4443 in MCs stimulated by mvT*s. **A**, NanoString technology of RNA isolated from LAD2 cells activated by mvTs or mvT*s for 24 hours. **B-D**, LAD2 cells (Fig 2, B and C) or CBMCs (Fig 2, D) were incubated as indicated, and miR-4443 levels were measured by using qRT-PCR and normalized to RNU44 levels. Data are relative to untreated cells. **E**, Resting or phorbol 12-myristate 13-acetate (PMA)-activated Jurkat T cells or their microvesicles were analyzed for their miR-4443 content by using qRT-PCR. **F**, LAD2 cells were incubated with mvT*s in the presence of 50 μ g/mL α -amanitin, and the selected miRs were evaluated by using qRT-PCR. Results are shown from 3 independent experiments (means $\pm$ SEMs). * P < .05 and ** P < .01.

incubating MCs with either mvTs or mvT*s. Microarray analysis disclosed a significant downregulation (-3.129 -fold change, P < .05) of the transmembrane receptor protein tyrosine phosphatase *PTPRJ* gene (also known as CD148 or DEP-1) after stimulation of MCs with mvT*s (data not shown). Importantly, *PTPRJ* has previously been shown to act as an inhibitor of the Ras pathway by directly dephosphorylating phosphorylated ERK.¹⁶

These results were validated by using quantitative real-time PCR (qRT-PCR; Fig 1, A-D) and Western blot analysis (Fig 1,

E and F). Indeed, downregulation of *PTPRJ* mRNA levels could be demonstrated after incubation of MCs with mvT*s for 4 and 24 hours (Fig 1, A). Moreover, dose-response analysis with increased concentrations of mvT*s revealed maximal decrease of *PTPRJ* mRNA levels with 50 μ g/mL mvT*s (Fig 1, C). In contrast, IgE-mediated activation (Fc ϵ RI cross-linking) of LAD2 MCs had no effect on *PTPRJ* mRNA expression (Fig 1, B). Decreased expression of *PTPRJ* mRNA was also detected after addition of mvT*s for 24 hours to mature (9 to 10 weeks old) human cord blood-derived MC cultures (CBMCs; Fig 1, D), thus

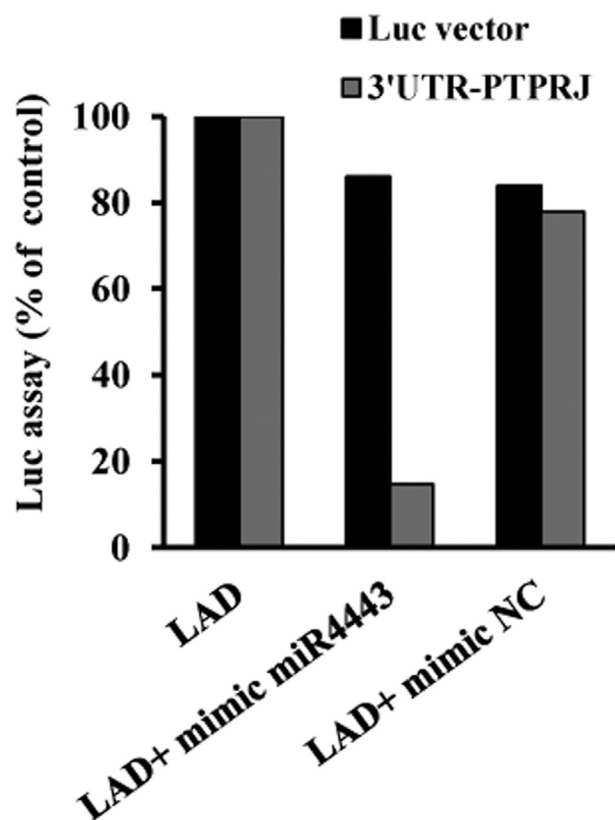


FIG 3. miR-4443 regulates *PTPRJ* gene expression. Luciferase assay of LAD2 cells cotransfected with *PTPRJ*-3'-UTR reporter plasmid (Light-Switch) or empty vector and mimic miR-4443 or mimic. NC, Negative control. Results are representative of 3 different experiments.

supporting the validity of LAD2 as a human MC model. Furthermore, *PTPRJ* protein levels were also decreased after incubation of MCs for 4 and 24 hours with mvT*s but not with IgE plus antigen (Fig 1, E and F). These results indicate that both mRNA and protein levels of *PTPRJ* are downregulated on specific activation of MCs by mvT*s.

miR expression in mvT*-induced MC activation

We hypothesized that the decrease in *PTPRJ* expression might be attributed to miRs carried by microvesicles. To this end, we examined the global miR gene expression profiles induced by incubation of LAD2 cells with either mvTs or mvT*s by using NanoString technology¹⁸ or qRT-PCR. After stimulation of MCs with mvT*s but not mvTs, microarray analysis disclosed a significant upregulation of 6 miR genes (fold change cutoff, 2.0; see Fig E1 in this article's Online Repository at www.jacionline.org). Among the transcripts, the miR-4443 gene was of particular interest because of strong induction in LAD2 human MCs (fold change, 5; Fig 2, A). Furthermore, the expression of this miR has not been reported in human MCs. qRT-PCR has validated these findings. As shown in Fig 2, B, the miR-4443 level was indeed increased in MCs activated with mvT*s but not with mvTs or IgE plus antigen. The increase in the expression of miR-4443 was first noticed after 6 hours and peaked after activation of the cells for 24 hours (see Fig E2 in this article's Online Repository at www.jacionline.org), consisting of our previous results demonstrating that mvT*s are

actively internalized into the cells after a 24-hour incubation period.¹⁰ Dose-response analysis revealed maximal miR-4443 expression with 100 μ g/mL mvT*s (Fig 2, C). Experiments with mature human CBMCs demonstrated a marked increase in miR-4443 levels in response to activation of primary MC cultures with mvT*s but not mvTs (Fig 2, D). Of note, microvesicles isolated from peripheral blood T cells and activated by anti-CD3/anti-CD28 antibody-coated beads were also found to induce high levels of miR-4443 in LAD2 cells (see Fig E3 in this article's Online Repository at www.jacionline.org).

Microvesicles are known to contain and transfer miRs to other cells.¹¹⁻¹⁴ Therefore qRT-PCR was used to determine the presence of miR-4443 in either T cells or microvesicles derived from activated or resting Jurkat T cells. As shown in Fig 2, E, a high level of miR-4443 was found in both activated Jurkat T cells and their mvT*s but not in resting Jurkat T cells or their mvTs.

LAD2 MCs were treated with α -amanitin, a specific inhibitor of RNA polymerase II, before microvesicle treatment to ensure that the target cells did not upregulate the expression of miR-4443 on microvesicle treatment.¹⁹ As shown in Fig 2, F, similar miR-4443 expression was detected in the microvesicle-treated cells, regardless of the presence of α -amanitin, whereas other miRs, which were found to be upregulated by mvT*s, were significantly decreased. Of note, α -amanitin did not change the cycle threshold values of RNU44 that served as reference for gene quantitation. Thus miR-4443 is indeed present in mvT*s that in turn deliver it into MCs.

miR-4443 regulates *PTPRJ* gene expression and function

Next, we searched for evidence that miR-4443 might actually regulate *PTPRJ* expression. This hypothesis was supported by a recent report that an miR accounts for enhanced IL-4 production by MCs through targeting a specific phosphatase.²⁰

For this purpose, we used microRNA target filter databases, such as TargetScan (www.targetscan.org), which are able to predict target genes of specific miRs based on 3'-UTR complementary sequences. This bioinformatics analysis revealed that the *PTPRJ* gene has a critically conserved binding site for miR-4443, indicating that miR-4443 might interact directly with the 3'-UTR of this gene. Therefore we conducted a luciferase reporter assay to monitor miR-4443 binding to the 3'-UTR of the *PTPRJ* gene. LAD2 cells were cotransfected with reporter plasmid containing the full length of the *PTPRJ*-3'-UTR downstream of the firefly luciferase coding sequence together with miR-4443 mimic. The results showed that the luciferase activity of cells transfected with the miR-4443 overexpression vector was significantly decreased (80% decrease) compared with that in cells transfected with the blank vector control (mimic NC group), demonstrating that *PTPRJ* was inhibited by binding of miR-4443 to its 3'-UTR (Fig 3).

To further verify that *PTPRJ* is a functional target gene of miR-4443, LAD2 cells were transfected with either miR-4443 mimic or its negative control, followed by activation of the cells with mvT*s. Overexpression of miR-4443 in resting cells, but not its negative control, led to a decrease in *PTPRJ* mRNA expression (Fig 4, A). The very same effect was noticed in cells that were exposed to microvesicles, with an approximately 50% decrease

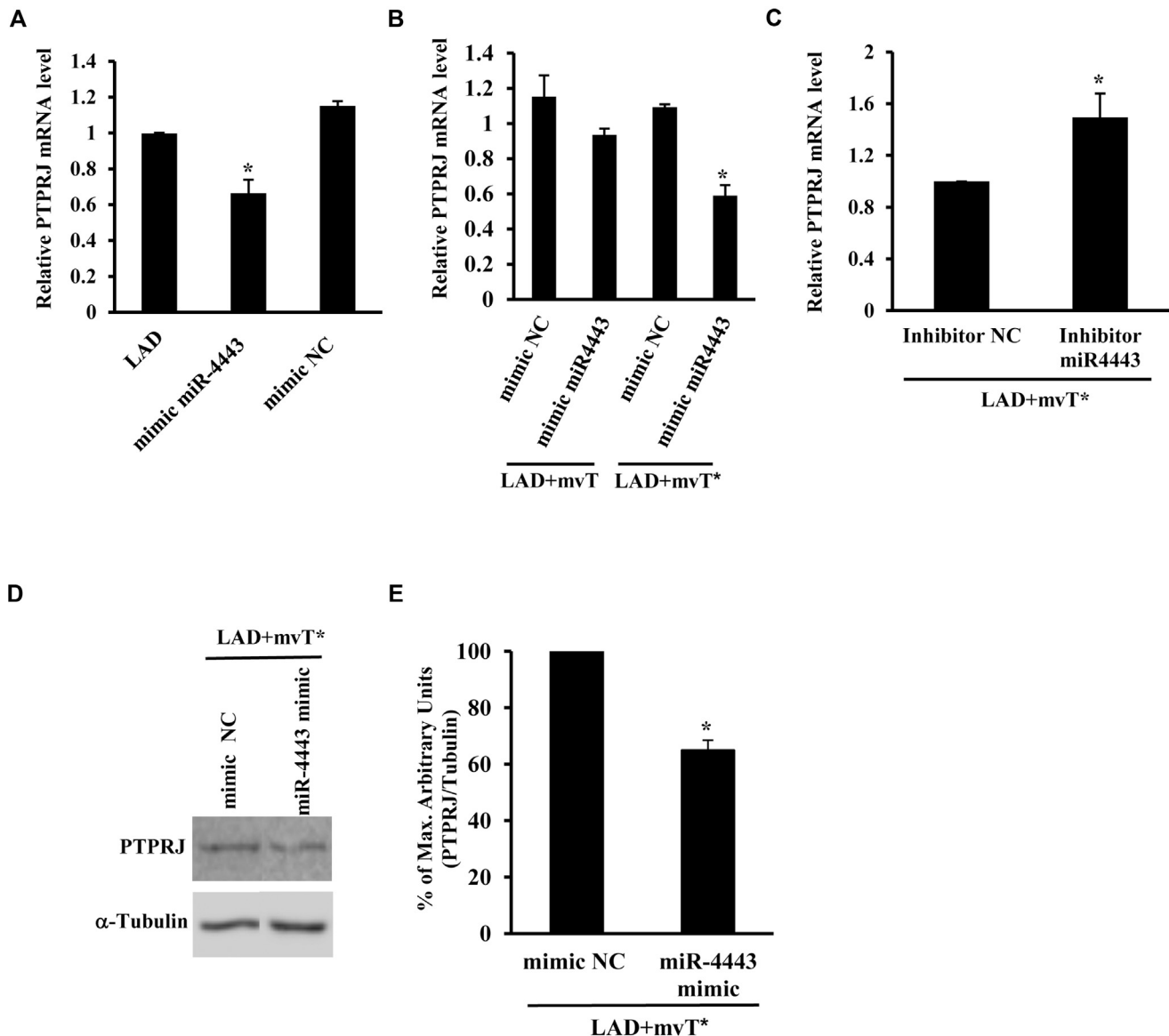


FIG 4. miR-4443 mimic or inhibitor regulates PTPRJ expression. **A-C**, LAD2 cells were transfected with 30 nmol/L miR-4443 mimic, its negative control (NC; Fig 4, **A** and **B**), or 30 nmol/L miR-4443 inhibitor or NC inhibitor (Fig 4, **C**) for 24 hours. Then the transfected cells were activated with mvTs or mvT*s for 24 hours (Fig 4, **B** and **C**). *PTPRJ* mRNA levels were measured by using qRT-PCR and normalized to levels of *GUSB*. Data are compared with mimic/inhibitor-NC-transfected cells. **D**, The PTPRJ protein level was measured by using Western blot analysis and was normalized to α -tubulin. This is a representative experiment of 3 different experiments. **E**, Densitometry presented as a percentage of mimic NC response. * $P < .05$ and ** $P < .01$.

in PTPRJ expression in those activated by mvT*s (Fig 4, **B**). On the other hand, overexpression of miR-146a, which was also found to be upregulated in response to activation of the cells with mvT*s, did not affect PTPRJ expression (data not shown). In contrast, transfection with miR-4443 inhibitor increased *PTPRJ* mRNA expression by approximately 40% (Fig 4, **C**). Overexpression of miR-4443 mimic also led to a decrease in PTPRJ protein levels after activation of LAD2 cells with mvT*s (Fig 4, **D** and **E**). These results indicate that miR-4443 functionally regulates PTPRJ at both the mRNA and protein levels.

miR-4443 regulates ERK phosphorylation

Recently, we reported that stimulation of MCs by mvT*s is associated with Ras activation and sustained ERK phosphorylation.^{6,7} As reported previously, the phosphatase PTPRJ modulates Ras downstream effectors by directly dephosphorylating Tyr-204 of ERK.¹⁶ Thus we hypothesized that the miR-4443-induced decrease in PTPRJ expression might affect ERK phosphorylation and function. LAD2 cells were transfected with miR-4443 mimic or inhibitor, followed by stimulation of the cells with mvT*s, to investigate the effect of miR-4443 on ERK, and the level of phosphorylated ERK was

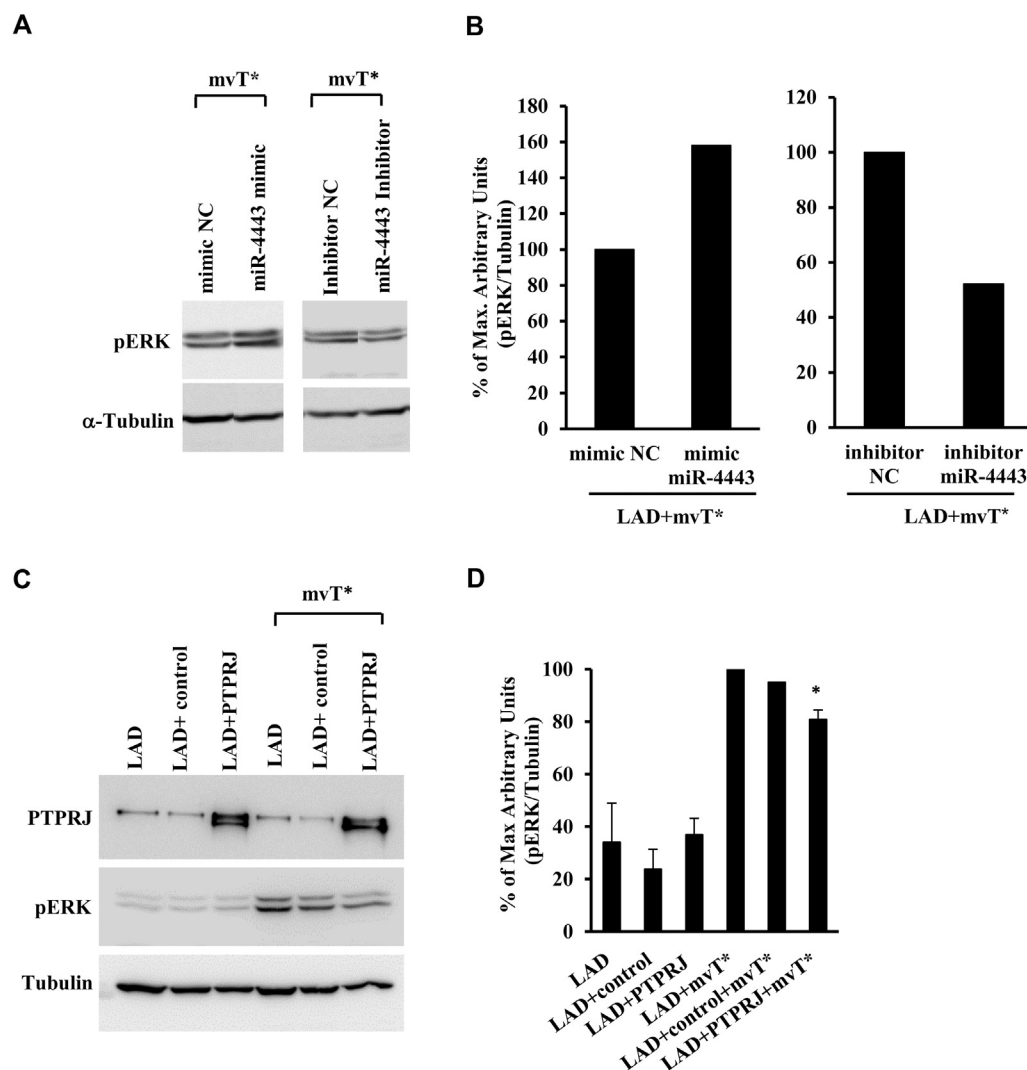


FIG 5. miR-4443 regulates ERK phosphorylation in mvT*-activated MCs. LAD2 cells were transfected with 30 nmol/L miR-4443 mimic, mimic negative control (NC), miR-4443 inhibitor, or NC inhibitor for 24 hours. Thereafter, cells were stimulated with mvT*s for 24 hours. **A**, Levels of phosphorylated ERK (pERK) and α -tubulin were analyzed by using Western blotting. **B**, Densitometry from 1 representative experiment of relative bands of phospho-ERK presented as a percentage of mimic or inhibitor negative control (NC) response. **C**, LAD2 cells were transfected with 5 μ g of PTPRJ expression plasmid or with the empty vector phosphorylated enhanced green fluorescent protein as a control for 48 hours, followed by activation with mvT*s for 24 hours. Levels of phosphorylated ERK, PTPRJ, and α -tubulin were analyzed by means of Western blotting. This is a representative experiment of 2 different experiments. **D**, Densitometry presented as a percentage of LAD2 cells with mvT*s. Results are shown from 2 independent experiments (means $\pm$ SEMs). * P < .05 and ** P < .01.

recorded by using Western blotting. As shown in Fig 5, A and B, enhanced ERK phosphorylation was observed in miR-4443-overexpressing cells, and transfection with the miR-4443 inhibitor reduced the phosphorylated ERK levels.

In contrast, overexpression of the *PTPRJ* gene by using PTPRJ expression plasmid followed by stimulation with mvT*s for 24 hours resulted in a modest but significant decrease (approximately 20%) in ERK phosphorylation (Fig 5, C and D), indicating that miR-4443 retains MC activity in the presence of "normal" PTPRJ levels.

miR-4443 regulates IL-8 release

Previously, it has been shown that sustained ERK phosphorylation in MCs activated by T cells leads to IL-8 release.⁷ Therefore the effect of miR-4443 on cytokine release by mvT*-activated MCs was investigated. miR-4443 mimic or inhibitor was overexpressed in MCs, followed by stimulation with mvT*s. IL-8 release was then detected by using ELISA. As shown in Fig 6, A, when LAD2 cells were transfected with the miR-4443 mimic, a significant increase of approximately 50% in IL-8 release was detected, whereas overexpression of

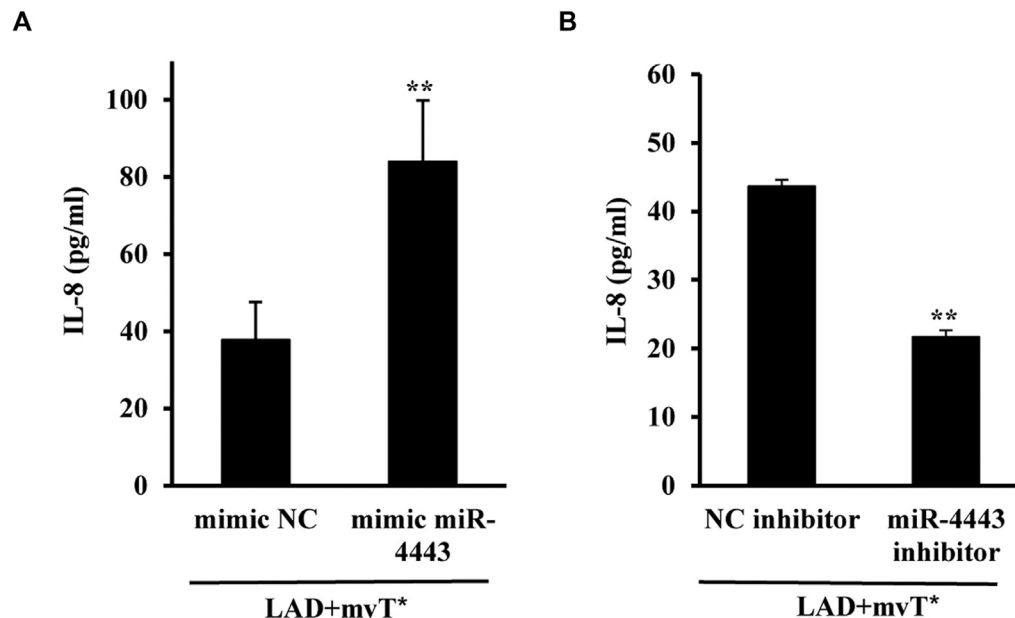


FIG 6. miR-4443 regulates IL-8 release in mvT*-activated MCs. LAD2 cells were transfected with 30 nmol/L miR-4443 mimic, mimic negative control (NC) (**A**), miR-4443 inhibitor, or NC inhibitor (**B**) for 24 hours. Thereafter, cells were stimulated with mvT*s for 24 hours. IL-8 release was assayed by means of ELISA. Results are shown from 3 independent experiments (means $\pm$ SEMs). * $P < .05$ and ** $P < .01$.

the miR-4443 inhibitor resulted in decreased IL-8 release (Fig 6, B). These results indicate that miR-4443 is involved in IL-8 release from mvT*-treated MCs, probably by downregulating PTPRJ.

In summary, these results are consistent with the scenario by which activated T cells might deliver, through microvesicle release, miR-4443 to MCs, which regulates *PTPRJ* gene expression. Downregulation of *PTPRJ* leads to increased ERK phosphorylation and augmented release of cytokines, such as IL-8, from mvT*-treated MCs (Fig 7).

DISCUSSION

Recent studies have shown that MC degranulation and cytokine production can be induced by heterotypic adhesion to activated, but not resting, T cells.⁵⁻⁸ Previously, we postulated that activation of MCs after contact with T cells or their microvesicles might induce an expression profile that would be different from stimulation by “classical” IgE receptor cross-linking.^{3,5,8,10} This unique activation pathway leads to sustained ERK activation⁷ and release of specific cytokines, such as IL-24, IL-8, and oncostatin M.⁸⁻¹⁰ However, the mechanism underlying the stimulation of MCs by microvesicles remains unknown.

In the present study we sought to discover factors that mediate the effect of mvT*s on MC function. We now show that activation of MCs with mvT*s, but not by FcεRI cross-linking, leads to downregulation of *PTPRJ*, a ubiquitous receptor-type protein tyrosine phosphatase that modulates the Ras pathway through dephosphorylation of ERK.¹⁶ *PTPRJ* is a widely expressed transmembrane protein that plays a role in multiple signaling pathways.²¹ Substrates of *PTPRJ* include proteins that belong to a family of kinase receptors, such as epidermal growth factor receptor, platelet-derived growth factor, and vascular endothelial growth factor receptor.²²⁻²⁴ Several studies have addressed the role of *PTPRJ* in regulating cellular pathways, underlying the

role of *PTPRJ* as a negative regulator of cell proliferation and function.^{21,22}

A previous finding of ERK activation and downregulation of *PTPRJ* in response to activation of MCs with mvT*s (Fig 1) prompted us to hypothesize that the decrease in *PTPRJ* expression can be attributed to miRs carried by microvesicles. Significant evidence has been accumulated showing that microvesicles released from various cell types can transfer miRs to neighboring or distant cells in which these exogenous miRs function similarly to endogenous miRs in regulating target gene expression and recipient cell function.^{25,26} For instance, platelets activated by thrombin deliver miR-223 through microvesicles to human umbilical vein endothelial cells, leading to accumulation of platelet-derived miR-223 in the latter.¹³ Also, monocyte-derived microvesicles are able to transfer miR-150 to endothelial cells and promote angiogenesis.²⁷

In the present study we performed global screening of miR expression in MCs activated with mvT*s by using NanoString technology. One of the miRs that was specifically expressed at significant levels was miR-4443 (Fig 2). miR-4443 was of particular interest for several reasons: it showed a very strong induction in LAD2 human MCs, its expression was not documented heretofore in human MCs, and it was found to be present in glioma microvesicles.²⁸ miR-4443 levels were increased after a relatively long period of 24 hours of MC activation, although less so after a shorter period of 4 hours. Furthermore, activated T cells or their microvesicles were also found to express this miR, whereas pretreatment with α -amanitin ruled out the possibility of transcriptional activation and *de novo* expression of miR-4443 by LAD2 cells on microvesicle treatment (Fig 2). These findings and the time sequence that paralleled the kinetics found with internalization of microvesicles to human MCs¹⁰ indicate that miR-4443 was transferred to MCs through mvT*s. An earlier decrease in *PTPRJ* expression might be attributed to other mechanisms yet to be determined.

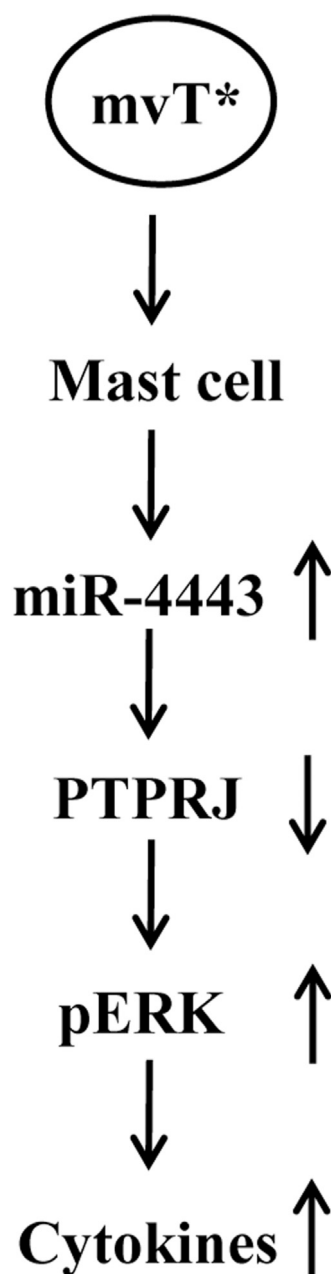


FIG 7. Proposed model of the effect of miR-4443 on mvT*-activated MCs. Activated T cells release microvesicles carrying miRNAs. These microvesicles are internalized into MCs, resulting in increased miR-4443 levels. High levels of miR-4443 downregulate *PTPRJ* gene expression, leading to increased ERK phosphorylation and augmented cytokine release.

miRs function by directly binding to the 3'-UTR of specific target mRNA, leading to repression of protein expression and promotion of target mRNA degradation.²⁹ Because of very short complementarity to the target mRNA, a particular miR can have hundreds of target genes, and therefore collectively, miRs have been suggested to influence the expression of approximately 30% of genes. Moreover, a given target might be regulated by multiple miRs.³⁰ For instance, another miR, namely miR-328, was found to downregulate *PTPRJ* expression in HeLa and SKBr3 cells by targeting directly the 3'-UTR of this gene,

resulting in its decreased expression.³¹ In the present study we show, by using the 3'-UTR luciferase reporter assay, that miR-4443 interacts directly with the 3'-UTR of *PTPRJ* gene as well (Fig 3) and therefore can serve as a target for this miR. Indeed, overexpression of miR-4443 by mimic molecule resulted in reduction of *PTPRJ* mRNA and protein levels, whereas miR-4443 inhibitor enhanced them (Fig 4).

FcεRI-mediated ERK activity is important for cytokine production by MCs.³² Recently, we have reported that stimulation of MCs by mvT*s is associated with Ras activation, ERK phosphorylation, and preferential IL-8 release, whereas activation of MCs by FcεRI cross-linking was followed by transient ERK activation and preferential secretion of TNF-α.^{6,7,9} Because the phosphatase *PTPRJ* was found to modulate the Ras pathway by directly dephosphorylating ERK,¹⁶ and in accordance with our previous findings, we investigated the effect of miR-4443 on ERK activation and IL-8 release. Indeed, overexpression of miR-4443 in MCs activated with mvT*s resulted in increased ERK phosphorylation and IL-8 release, whereas overexpression of the miR inhibitor decreased both ERK phosphorylation and IL-8 release (Figs 5 and 6). Furthermore, overexpression of *PTPRJ* rescued the effect of miR-4443 on ERK phosphorylation (Fig 5), indicating the interrelationship between the miR-*PTPRJ*-phosphorylated ERK. The ability of miRs to regulate ERK activation and cytokine production was also demonstrated in bone marrow-derived MCs activated by cross-linking of FcεRI. Overexpression of miR-126 in these cells suppressed *Spred1* (Sprouty-related Ena/VASP homology-1 domain-containing protein) expression and enhanced ERK activity that was associated with increased FcεRI-mediated cytokine production.³³ Also, IL-10-mediated enhanced MC function has been reported recently to be mediated through miR-155, which targeted suppressor of cytokine signaling 1.³⁴ Relevant to our observation, regulation of phosphatase by miRs has been documented in murine MCs stimulated with LPS. Overexpression of miR-221 was found to decrease expression of the phosphatase and tensin homolog, resulting in enhanced IL-4 secretion.²⁰

In summary, the observations reported herein and summarized in the model presented in Fig 7 suggest that stimulation of MCs by mvT*s results in overexpression of miR-4443, which is carried and transferred to the cells by these mvT*s. miR-4443, which serves as a *PTPRJ*-negative regulator, downregulates *PTPRJ* gene expression. This results in increased/prolonged ERK phosphorylation and IL-8 release. Thus, by delivering miR-4443, T cell-derived microvesicles might play an important role in MC activation within T cell-mediated inflammatory processes in which MCs are found to be involved.

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Key messages

- Microvesicles derived from activated T cells deliver miR-4443 to MCs.
- miR-4443 downregulates *PTPRJ* expression.
- Downregulation of *PTPRJ* results in increased ERK phosphorylation and cytokine release.

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METHODS

Human cytokine assay

Supernatants obtained from the different culture conditions described above were examined for released IL-8 by using a commercial ELISA kit, according to the manufacturer's instructions (Development kit; PeproTech Asia, Rehovot, Israel).

Microarray experiments and data analysis

Affymetrix Human Gene 1.0 ST arrays were used for gene expression analysis according to the instruction manual, as described on the Affymetrix Web site (Affymetrix, Santa Clara, Calif; <http://www.affymetrix.com>). A total of 3 arrays were performed in duplicates. Microarray analysis was performed on CEL files by using the Partek Genomics Suite, version 6.5 (Partek, St Louis, Mo; <http://www.partek.com>). Data were normalized and summarized with the robust multiaverage method, followed by 1-way ANOVA. Genes of interest that were differentially expressed ($P < .05$ and fold-difference cutoff of 3.0) were retrieved.

Isolation of microvesicles

Microvesicles were isolated from supernatants of resting or activated T cells and identified as such, as described previously.^{E1} Briefly, supernatants were obtained after cell removal by means of centrifugation at 800g for

5 minutes and then centrifuged at 4,500g for 5 minutes to discard large debris. After additional centrifugation at 20,000g for 60 minutes at 4°C, microvesicles were washed with PBS by means of additional centrifugation at 20,000g for 60 minutes at 4°C and resuspended in PBS. Microvesicle protein concentrations were measured at 280 nm with a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 1000). Protein quantity of microvesicles was the same in all samples analyzed.

cDNA constructs

The PTPRJ–bio-His cDNA construct was a kind gift from Gavin Wright (plasmid #51816; Addgene, Cambridge, Mass). For control, we used the empty vector pEGFP-N1 (Takara Bio, Mountain View, Calif).

RNA isolation

Total RNA was extracted from LAD2 MCs or CBMCs activated with mvTs or mvT*s from phorbol 12-myristate 13-acetate–treated Jurkat cells or from mvTs or mvT*s by using TRIzol reagent (Sigma-Aldrich), according to the manufacturer's protocol.

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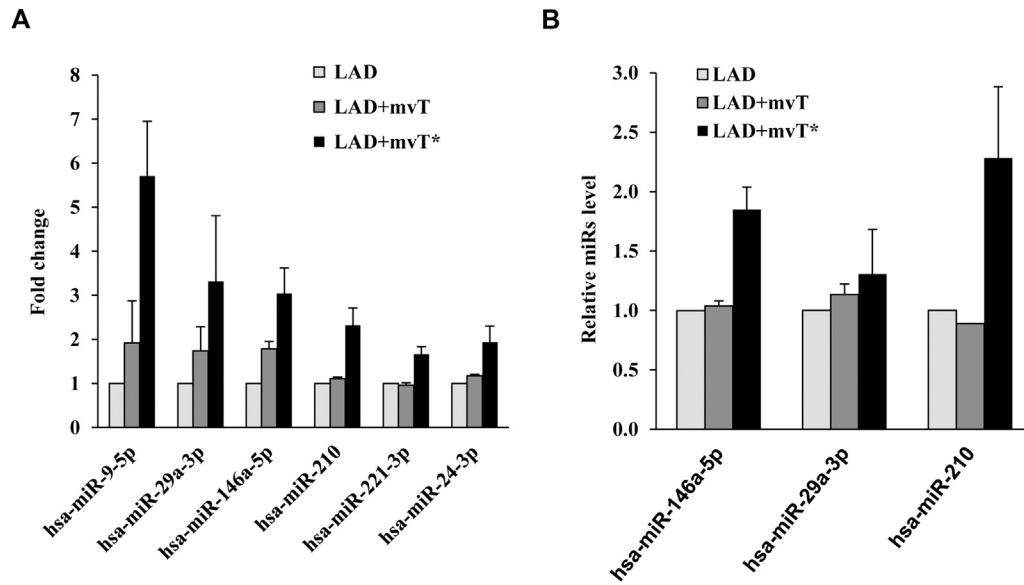


FIG E1. miR expression in MCs treated by mvT*s. RNA isolated from LAD2 cells activated by mvTs or mvT*s was analyzed for miR expression by using NanoString technology (A) or by using qRT-PCR and normalized to RNU44 levels (B).

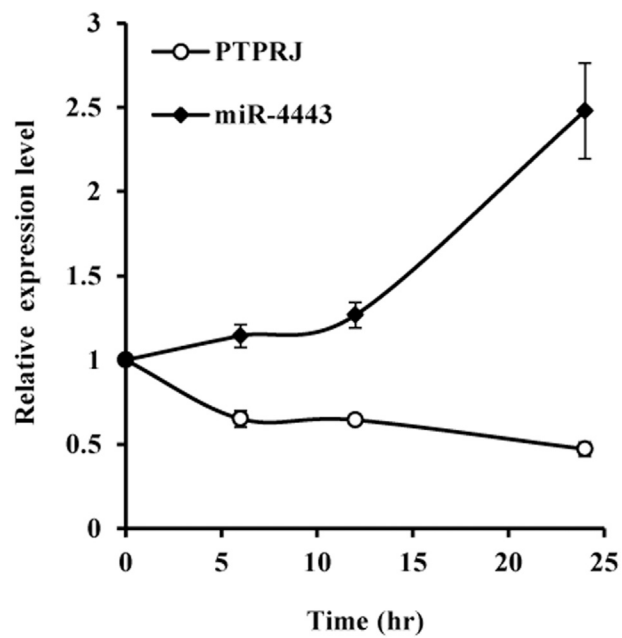


FIG E2. Time course of miR-4443 and PTPRJ expression in MCs treated by mvT*s. LAD2 cells were activated with 50 μ g/mL mvT*s at different time points, and expression of miR-4443 and PTPRJ was measured by using qRT-PCR and normalized to the levels of RNU44 or GUSB, respectively. Data are relative to untreated cells. Results are shown from 3 independent experiments (means $\pm$ SEMs).

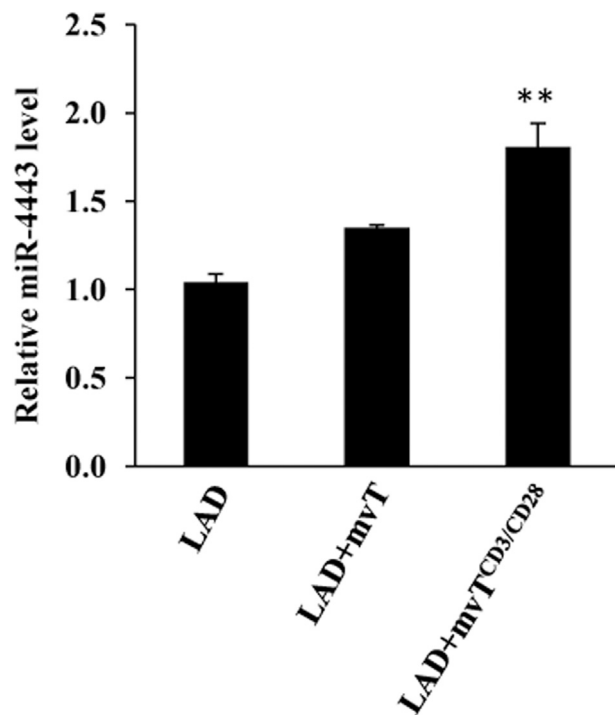


FIG E3. Expression of miR-4443 in MCs treated with anti-CD3/CD28-activated human peripheral blood T lymphocytes ($mvT^{CD3/CD28}$). LAD2 cells were activated by microvesicles derived from resting cells or $mvT^{CD3/CD28}$. miR-4443 levels were measured by using RT-PCR and normalized to RNU44 levels. Data are relative untreated cells. Results are shown from 3 independent experiments (means $\pm$ SEMs). ** $P < .01$.