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Syndecan-1 regulates the biological activities of interleukin-34

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ABSTRACT

IL-34 is a challenging cytokine sharing functional similarities with M-CSF through M-CSFR activation. It also plays a singular role that has recently been explained in the brain, through a binding to the receptor protein tyrosine phosphatase RPTPβ/ζ. The aim of this paper was to look for alternative binding of IL-34 on other cell types. Myeloid cells (HL-60, U-937, THP-1) were used as cells intrinsically expressing M-CSFR, and M-CSFR was expressed in TF-1 and HEK293 cells. IL-34 binding was studied by Scatchard and binding inhibition assays, using ^{125}I-radiolabelled cytokines, and surface plasmon resonance. M-CSFR activation was analysed by Western blot after glycosaminoglycans abrasion, syndecan-1 overexpression or repression and addition of a blocking anti-syndecan antibody. M-CSF and IL-34 induced different patterns of M-CSFR phosphorylations, suggesting the existence of alternative binding for IL-34. Binding experiments and chondroitinase treatment confirmed low affinity binding to chondroitin sulfate chains on cells lacking both M-CSFR and RPTPβ/ζ. Amongst the proteoglycans with chondroitin sulphate chains, syndecan-1 was able to modulate the IL-34-induced M-CSFR signalling pathways. Interestingly, IL-34 induced the migration of syndcean-1 expressing cells. Indeed, IL-34 significantly increased the migration of THP-1 and M2a macrophages that was inhibited by addition of a blocking anti-syndecan-1 antibody. This paper provides evidence of alternative binding of IL-34 to chondroitin sulfates and syndecan-1 at the cell surface that modulates M-CSFR activation. In addition, IL-34-induced myeloid cell migration is a synedcan-1 dependent mechanism.

1. INTRODUCTION

The discovery of interleukin-34 (IL-34) in 2008 upset the functional biological concepts that had been admitted for many years between Macrophage Colony Stimulating Factor (M-CSF) and its receptor, the M-CSF receptor (M-CSFR) [1,2]. Like M-CSF, IL-34 promotes the survival and proliferation of monocytes, as well as their differentiation into macrophages [3]. Macrophages induced by IL-34 have phenotypes and functions that are similar to those differentiated by M-CSF, as both cytokines polarize these macrophages into immunosuppressive macrophages M2 [4]. In addition, and in association with the Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), IL-34 can replace M-CSF to induce osteoclast formation by stimulating the proliferation and adhesion of osteoclast precursors [5]. The cytokine also overlaps with the roles played by M-CSF in inflammation in degenerative bone diseases such as rheumatoid arthritis [6] and periodontal inflammation [7]. All these effects are mediated by a cell-surface tyrosine kinase activity receptor, M-CSFR, encoded by the proto-oncogene c-fms, and also called c-fms, CD115 or CSF-1R [8]. The homodimeric cytokines M-CSF or IL-34 bind in a close way to the extracellular domain of M-CSFR, but with a different binding mode [9,10], leading to receptor dimerization and differential auto-phosphorylation on its eight tyrosine residues [11]. M-CSF/M-CSFR and IL-34/M-CSFR crystals have quite a similar shape, except a higher stability for the IL-34/M-CSFR complex [12]. Chihara et al. showed some differences in the kinetics of M-CSFR phosphorylations and in the nature and intensity of phosphorylated tyrosine residues after M-CSF or IL-34 binding, which partly explains their ability to differentially activate the monocyte/macrophage lineage [13].

MCSF-R knockout mice [14] exhibit a similar but more pronounced phenotype than op/op mice [15,16] characterized by a more severe osteopetrosis and reduction of tissue macrophages. This osbservation suggested the existence of another ligand for the M-CSFR,

and IL-34 was a perfect candidat. Although IL-34 expression under the promoter of the M-CSF gene rescues the phenotype of op/op mice, the cytokine also plays singular roles during brain development [17]. Microglia and Langerhans cells effectively decrease dramatically in IL-34 knockout mice, as in M-CSFR deficient mice, while monocytes, lymphoid tissue macrophages and dendritic cells are not affected. Besides, op/op mice show quite a conventional development of these cells [18-20]. These results identify specific IL-34 activity during embryogenesis and tissue homeostasis, and make it possible to better understand the tissue distribution and independent activity of IL-34 [21].

IL-34 is highly expressed in both post-natal and adult brains, whereas M-CSFR expression, which is maximal in early development, dramatically decreases in adult brains [22]. High expression of IL-34 despite an almost undetectable expression of its receptor suggests the existence of other receptors for the cytokine. In spite of overlapping and complementary activities between M-CSF and IL-34 through M-CSFR activation, the "M-CSF twin" may also exert specific functions independently of this receptor. The present study thus investigates a new binding mode for IL-34 that may modulate the bioavailability and/or activities of this cytokine. This paper provides evidence of syndecan-1 as a new molecular actor in the M-CSF/IL-34/M-CSFR triad and proposes one explanation for the differential activation of the M-CSFR observed after M-CSF and IL-34 stimulation.

2. MATERIAL AND METHODS

All experiments described in the manuscript were repeated at least three times in independent experiments and representative experiments are shown.

2.1. Reagents

Recombinant human Macrophage-Colony Stimulating Factor (M-CSF), human interleukin-34 (IL-34), human M-CSF Receptor (M-CSFR), mouse IgG1 (control isotype) and antibody anti-human M-CSFR were obtained from R&D Systems (Abingdon, UK). Diaclone (Besanc□on, France) kindly provided the murine B-B4 anti-syndecan 1 (CD138) IgG1 monoclonal antibody. Antibodies directed against human CD14, human CD200R and human CD86 were purchased from Biolegend (Saint Quentin en Yvelines, France). Antibodies directed against human P-MCSFR, P-Erk1/2, P-mTor, P-Akt and the total form of proteins were purchased from Cell Signalling (Ozyme, Saint Quentin Yvelines, France). Anti-β actin and total phosphorylated tyrosine antibodies were respectively provided by Sigma-Aldrich (Saint Quentin Fallavier, France) and Millipore (Molsheim, France). Anti-Receptor Protein Tyrosine Phosphatase (RPTPβ/ζ, C-19 clone) and HRP-conjugated secondary antibodies were obtained from Santa-Cruz (CliniSciences, Nanterre, France). Chondroitin sulfate salts from shark cartilage, heparin, heparan sulfate salts and chondroitinases A, B, C were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2. Cell cultures

The cell lines used in the present study were purchased from the American Tissue Cell Collection (ATCC, Molsheim, France). HEK293 (HEK), osteosarcoma MNNG/HOS (ATCC® Number: CRL-1547™) (HOS) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Lonza, Levallois-Perret, France) supplemented with 10% foetal bovine

serum (FBS; HyclonePerbio, Bezons, France) and 2 mmol/L of L-glutamine. HL-60, U937, THP-1 and TF-1cell lines were maintained in RPMI (Roswell Park Memorial Institute, Lonza) supplemented with 10% FBS. In addition, TF-1 cells were cultured in the presence of 3 ng/mL of G-MCSF according to ATCC recommendations. The human U251 glioblastoma cell line was kindly provided by Dr Mylène Dorvillius (ATLAB Pharma, Nantes, France) and cultured in DMEM supplemented with 10 % FBS.

2.3. Cloning of the human M-CSFR gene and cell transfection

The human M-CSFR gene (NM_005211.3) was cloned in a pCDNA3.3 TOPO TA vector (Life Technologies, Villebon sur Yvette, France) from the cDNA from a healthy donor $CD14^+$. $CD14^+$ cells were initially isolated from human peripheral blood donors provided by the French blood bank institute (Etablissement Français du Sang, Nantes, France, authorization number: NTS 2000-24), by using MACS microbeads (MiltenyiBiotec, Germany) as previously described [5]. The RT-PCR product was obtained using the following primer sequences: Forward 5'-CACCATGGGCCCAGGAGTTCTGCTGCT-3' and Reverse 5'-AACTCCTCAGCAGAACTGATAGTTGTTGGGCTGCA-3', and fully sequenced to check for the absence of functional mutations based on the sequences already published in the data bases.

Embryonic HEK293 cells and osteosarcoma HOS cells were transfected as described below with the pCDNA3 empty plasmid or the pCDNA3 plasmid containing the M-CSFR gene. To obtain a polyclonal population expressing the membrane M-CSFR, $5x10^6$ cells were then stained with phycoerythrin (PE) conjugated antibodies directed against the M-CSFR and sorted out on a FACSAria III (Beckman Coulter, Villepinte, France). The cells expressing the M-CSFR were named respectively HEK M-CSFR and HOS M-CSFR. The cells transfected with the empty pCDNA3 vector were called HEK Mock and HOS Mock. Erythroblastic TF-1

cells were transfected using an Amaxa® Cell Line Nucleofector® Kit by Lonza. Cell sorting was also carried out to isolate M-CSFR expressing cells (TF-1 M-CSFR).

2.4. RNA isolation and real-time PCR

Total RNA was extracted using NucleoSpin®RNAII (Macherey Nagel, Duren, Germany) with one stage of DNase I treatment (25 units, 15 min) to prevent genomic contamination. 1 µg of total RNA was used for first strand cDNA synthesis using the ThermoScript RT-PCR System (Invitrogen). Real-time PCR was performed on 20 ng of reverse transcribed total RNA (cDNA), 300 nM of primers and 2x SYBR Green Supermix (Biorad, Marnes-la-Coquette, France). The analysis was performed according to the method described by Vandesompele et al. [23] using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the invariant control. Standard curves were obtained using decreasing quantities of cDNA from the U251 cell line to validate the RPTPβ/ζ primers according to the MIQE guidelines [24]. The sense and antisense primers used were: RPTPβ/ζ Forward: 5'-GCAGAGCTGTACTGTTGACTT-3', Reverse: 5'-TGTGCTAGCTTAACCCTGCT-3'; GAPDH Forward: 5'-TGGGTGTGAACCATGAGAAGTATG-3', Reverse: 5'-GGTGCAGGAGGCATTGCT-3'.

2.5. M-CSF and IL-34 binding assays

M-CSF and IL-34 were radio-labelled with iodine-125 (^{125}I) (which has specific radioactivity of around 2,000 cpm/fmol) using the chloramine T method, as described by Tejedor and Ballesta [25] and Godard et al [26]. To determine the ability to bind to the cells of both cytokines, $1x10^6$ cells were incubated with increasing concentrations of iodinated M-CSF or IL-34 (up to 1nM) for 1 h at 4°C. Non-specific binding was determined in the presence of 100-fold excess of the corresponding unlabelled cytokine. Specific binding was subsequently

obtained by subtraction of this non-specific binding from the total binding signal. Regression analysis of the binding data was carried out using a one-site equilibrium binding equation (Graphit; Erithacus Software, Staines, U.K.) and data were plotted in the Scatchard coordinate system. Cross competition binding curves were obtained by adding simultaneously to the cells increasing amounts of a competitor cytokine together with a fixed, non-saturable concentration of a labelled cytokine. The inhibition curves obtained were analysed with an equation for competitive inhibition between two ligands for a common binding site. Curve fittings were performed using the Graphit data analysis programme.

2.6. Surface Plasmon Resonance (SPR) assays

The SPR experiments were carried out on a BIAcore 2000 biosensor (BIAcore, Uppsala, Sweden). Recombinant human IL-34 (5µg/mL, in 5 mM maleate buffer pH=6.2) was covalently linked to the dextran matrix of a CM5 sensor chip (BIAcore, Uppsala, Sweden), previously activated with an ethyl (dimethylaminopropyl) carbodiimide/ N-hydroxysuccinimide (EDC/NHS) mixture. Immobilisation levels in the 1300 RU range were then obtained. Residual active groups were inactivated by injection of ethanolamine 1M pH=8.5 for 10 min. The binding of decreasing concentrations of chondroitin sulfate salts, heparin or heparan sulfate salts was monitored, from 100 µg/mL and with a serial dilution of 1:3. Concentrations up to 500 µg/mL were assessed for the heparin and heparan sulfate. The flow rate was around 30 µL/min, and the chip was regenerated with 20 mM NaOH between each cycle. The resulting sensorgrams were fitted using BiaEval 4.1 software.

Cell binding experiments were carried out in the SPRiplex II. The SPRi biochip-CH (H0550) was activated with an EDC/NHS mixture, and 200 µg/mL IL-34 was spotted on it with a 500µM tip for 30 min. The protein binding capacity of the IL-34 was controlled by injections of 1 and 2 µg/mL of recombinant human M-CSFR on the chip after each

regeneration cycle performed with 10mM glycine pH=1.5. HEK Mock cells ($2x10^5$ cells/mL) were then injected on to the chip at 20 µL/min for 10 min.

2.7. Flow-cytometry experiments

Membrane expression of the M-CSFR, syndecan-1, CD14, CD220R, CD86 were assessed with flow cytometry (Cytomics FC500; Beckman Coulter). Cells were incubated with PE-conjugated anti-human M-CSFR antibody or mouse IgG1 as the control for 30 min. For syndecan-1 expression, the cells were first incubated for 90 min at 4°C with the anti-syndecan-1 B-B4, before incubation with the PE-conjugated secondary antibody for 60 min. Similar method was used to analyse CD14, CD220R and CD86 expression. M-CSFR and syndecan-1 expressions were analysed using the CXP Analysis software 2.2 (Beckman Coulter).

2.8. Western Blot Analysis

The cells were collected in a RIPA buffer (10 mM Tris pH8, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS containing a cocktail of protease and phosphatase inhibitors: 1 mM sodium orthovanadate (Na_2VO_4), 1 mM phenylmethylsulforyl fluoride (PMSF), 10 mM sodium fluoride (NaF), 10 mM N-ethylmaleimide (NEM), 2 µg/ml leupeptin and 1 µg/ml pepstatine). The protein concentration was determined using a BCA (bicinchoninic acid) protein assay (Sigma Aldrich). 40 µg of total protein extracts were prepared in a Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and then separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer, the immobilon-P membranes (Millipore, Molsheim, France) were blotted with the antibodies referenced in the reagents section. The membranes were then probed with secondary antibodies coupled with horseradish peroxidase. Antibody binding was

visualised with a Pierce enhanced chemiluminescence (ECL) kit (ThermoSientific, Illkirch, France). The luminescence detected with a Charge Couple Device (CCD) camera was quantified using the GeneTools programme (Syngene, Cambridge, United Kingdom).

2.9. siRNA gene silencer

The syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA (Ambion, France) and the INTERFERin^{TM} transfection reagent (Polyplus transfection, France). 700 x 10^3 HEK M-CSFR cells per well were seeded in a 6-well plate 1 day before transfection. In each well 50 nm siRNA (Syndecan-1 siRNA : ref#s12634 ; Ambion® Silencer® Negative Control #1 : ref#AM4611) duplexes diluted in serum-free medium were incubated with 2 µl of INTERFERin^{TM} for 30 min at room temperature. The expression of membrane syndecan-1 was measured by flow cytometry. 48 h after the transfection, 50 ng/mL of IL-34 (5 min, 37°C) were added to the cells and M-CSFR activation was determined by Western blot as previously described.

2.10. M2a macrophage differentiation and cell migration assays

$CD14^+$ monocytes were isolated from peripheral blood of 3 healthy donors as previously described in paragraph 2.3. For M2a macrophage differentiation, $CD14^+$ cells were cultured in RPMI (Roswell Park Memorial Institute medium, Lonza) supplemented with 1% Human Serum (Invitrogen), 1% penicillin/streptomycin, 50 ng/mL of IL-4 (R&D System) for 5 days. Half volume of the medium was renewed at day 2. After the differentiation culture period, M2a adherent cells were collected by gentle scraping. Their phenotype was analysed by flow cytometry ($CD14^{low}$, $CD220R^+$, $CD86^+$) before use in migration assays. $2x10^5$ M2a macrophages or $5x10^5$ THP-1 were pre-treated for 30 min at 37°C with a blocking anti syndecan-1 antibody (SDC-1 Ab, B-B4) or the corresponding isotype (IgG1 Ab). Cell

migration was then assessed using a 8µm Boyden chamber (top well) in RPMI 1% FCS, with or without 100 ng/mL of IL-34 at the bottom well. Cells migrating at the bottom well were counted after 6 hours of culture. Each experiment was done in triplicate and repeated three times.

2.11. Statistical analysis

Results were analyzed using a Kruskal-Wallis test was performed followed by a Dunn's post-hoc between all conditions using GraphPad InStat v3.02 software. The results of the migration assays are given as a mean ± SD of three independent experiments done in triplicate. Results were considered significant at p values ≤ 0.05. All Western blots were performed three or four times in independent experiments and a representative blot was changed in each figure.

3. RESULTS

3.1. IL-34 and M-CSF induce differential activation of the M-CSFR

We first analysed the membrane expression of M-CSFR in various human cells with flow cytometry. As expected, the HL-60, U937 and THP-1 myeloid cell lines intrinsically expressed this cytokine receptor, in contrast to the erythroblastic TF-1, epithelial HEK293 and HOS osteosarcoma cells which did not express the M-CSFR (Figure 1A). The lack of M-CSFR expression by the TF-1, HEK and HOS cells was confirmed by quantitative PCR (data not shown). Marked expression of the M-CSFR at the cell membrane was observed in M-CSFR transfected TF-1, HEK and HOS cells (Figure 1A).

Based on these observations, we investigated the functionality of the receptor by studying the cell signalling induced by M-CSF and IL-34, the two identified ligands of the M-CSFR (Figures 1B-E). In HEK M-CSFR cells, both cytokines induced in a dose- (data not shown) and time-dependent manner the tyrosine 546, 699, 708, 723 and 923 phosphorylation of the receptor's intracellular chains with a peak at 5 min (Figure 1B). Similarly, M-CSF and IL-34 induced the phosphorylation of Erk1/2. Compared to M-CSF, IL-34 induced a weaker activation of the signalling pathway mediated by M-CSFR activation, at the same dose and time (Figure 1B). In the HOS M-CSFR cells, high doses of IL-34 (200 ng/mL) appeared more efficient at inducing the phosphorylation of the M-CSFR compared to M-CSF, unlike low doses of the cytokine (50 ng/mL) (Figure 1C). This higher activation of the M-CSFR by IL-34 was also observed with 100 ng/mL of cytokine in the TF-1 M-CSFR cells as demonstrated by fast and huge phosphorylation levels of the M-CSFR (Figure 1D), data in agreement with Chihara et al. [13]. We then investigated M-CSFR activation in the THP-1 cells which intrinsically expressed the receptor chains (Figure 1E). Consistent with the findings obtained with the TF-1 M-CSFR cells, the M-CSFR and MAPK downstream signalling pathways were

more activated by IL-34 than by M-CSF (Figure 1E, Supplementary data 1A). Similar results were obtained in the U937 cells (Supplementary data 1B). Overall, these data revealed that IL-34 and M-CSF differentially activated the M-CSFR signalling pathway according to the cell type studied.

3.2. IL-34 binds to HEK and HOS cells lacking the M-CSFR

In light of the differential signalling pathway induced by IL-34 and M-CSF, binding assays using ^{125}I-ligands were carried out on the different cell lines of interest. A saturation binding curve was obtained, which after Scatchard transformation, made it possible to estimate the affinity of IL-34 for the membrane M-CSFR. ^{125}I-IL34 bound all cell lines studied with an equilibrium dissociation constant around 2 nM, 0.55 nM, 1 nM and 0.75 nM for U937, THP-1, HEK M-CSFR and HOS M-CSFR cells respectively (Figure 2A-D). As expected, ^{125}I-MCSF bound to the HEK M-CSFR and HOS M-CSFR with a similarly high affinity (K_d = 0.35 nM). These data demonstrated the capacity of IL-34 to bind with a high affinity to cell lines expressing the M-CSFR (Figure 2D).

We next assessed the potency of recombinant IL-34 and/or M-CSF to compete with ^{125}I-IL34 binding to cell surface receptors (Figures 3A, B). The results demonstrated that cold IL-34 and M-CSF differentially competed with the ^{125}I-IL34 binding sites expressed in the HEK M-CSFR, as revealed by the slope factor of the inhibition curves (0.8 and 2 for M-CSF and IL-34 respectively) (Figure 3A,B). Similar results were obtained with the HOS M-CSFR cells (data not shown). ^{125}I-IL34 binding assays were then performed in HEK Mock cells transfected with the empty control vector that did not express the M-CSFR (Figure 1A, Figure 3C). Surprisingly, specific ^{125}I-IL34 specific binding sites were detected at the surface of the HEK Mock cells (Figure 3C,D). This binding was characterised by a lower affinity (K_d = 14.6

nM) than those observed in the HEK M-CSFR (Figure 2B). In contrast to ^{125}I-IL34, ^{125}I-M-CSF did not bind to cells lacking the M-CSFR (Supplementary data 2). Complementary SPR experiments reinforced these results and confirmed the ability of the HEK Mock cells to bind to recombinant IL-34 spotted on a sensorchip, in contrast to recombinant M-CSF, as measured by the increasing percentage of reflectivity (Figure 3E). The results clearly demonstrated an additional binding mode for IL-34 independent of the M-CSFR.

3.3. IL-34 binding to chondroitin sulfate chains modulates M-CSFR activation

As IL-34 bound with a relatively low affinity to cells that did not express the M-CSFR (HEK Mock and HOS Mock), cell signalling was further investigated in these cells after stimulation with a high dose of IL-34 (200ng/mL). As shown in Supplementary data 3, the profile of the phosphorylated tyrosines was not modified after IL-34 stimulation (Supplementary data 3A) and no modulation of the different signalling pathways such as mTor, Akt, (Supplementary data 3B), β-catenin, p38 and Erk1/2 (data not shown) was observed in the presence of IL-34. Consistent with the ability of IL-34 to bind to glycosaminoglycans [27], we assessed the IL-34 binding to chondroitin sulfate and heparin/heparan sulfate chains by SPR. The SPR sensorgrams revealed a low affinity binding of the various types of chondroitin sulfate to IL-34, with a K_d of 132 nM, 775 nM and 16.1 nM for chondroitin sulfates A (Figure 4A), B (Figure 4B) and C (Figure 4C) respectively. On the contrary, heparin (Figure 4D) and heparan sulfate (data not shown) were not able to bind IL-34, even with high doses of up to 500 µg/mL.

In light of these data, the chondroitin sulfate chains may be behind the IL-34 binding to the HEK Mock and HOS Mock cells, as well as the differential signalling pathways induced by M-CSF or IL-34 in the HEK M-CSFR. To explore this hypothesis, HEK M-CSFR

cells were then treated with chondroitinases A, B, C solution (chABC) to abrogate the chondroitin sulfates (CS) present at the cell surface prior to study of the M-CSF- or IL-34-induced signalling pathways. Consistent with the results presented in Figure 1B, the M-CSFR was less activated after addition of 50 ng/mL of IL-34 compared to stimulation with the same dose of M-CSF, as illustrated by the phosphorylation levels of tyrosines 546, 708 and 723 (Figure 5A). Interestingly, the chABC treatment markedly increased the levels of M-CSFR phosphorylations induced by IL-34 as demonstrated by the phosphorylation levels of M-CSFR, whereas the response induced by M-CSF remained unchanged (Figure 5A). Similar results were obtained for ERK1/2. Both cytokines were also pre-incubated with 1, 3 or 10 µg/mL CS salts for 1h at 37°C prior to their addition to the HEK M-CSFR cells. The addition of CS did not modulate the M-CSF-induced signalling pathway (Figure 5B). On the contrary, pre-incubation of CS with IL-34 led to increased phosphorylation of the receptor tyrosines 708 and 723 (Figure 5B). Overall, these data clearly demonstrated that CS strongly regulates the biological activities of IL-34.

3.4. IL-34 binding to chondroitin sulfate is independent of the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ)

RPTPβ/ζ, a proteoglycan mainly expressed in the central nervous system, was recently identified on the glioblastoma cell line U251 as another receptor for IL-34 through its CS chains, explaining the role of the cytokine in brain development [27]. We then analysed RPTPβ/ζ expression in THP-1, TF-1, HOS Mock and HEK Mock cells. The RPTPβ/ζ was not expressed at either the transcriptional or the protein levels in any of these cells in contrast to the U251 cells used as a positive control (Figure 6). IL-34 binding to the HEK Mock and HOS Mock cells was consequently independent of RPTPβ/ζ.

3.5. The IL-34-induced migration of myeloid cells is syndecan-1 dependent

Within the proteoglycan families, the syndecans control a lot of biological functions, such as angiogenesis, cytokine bioavailability, cell adhesion/interactions, etc. To investigate the functional involvement of syndecans in IL-34 biology, syndecans 1, 2 and 4 were individually overexpressed in the HEK M-CSFR cells and the phosphorylation levels of the M-CSFR were then analysed in the presence of IL-34. The, overexpression of syndecan-1 in the HEK M-CSFR cells exacerbated the cell response to IL-34 (Figure 7A,B). Indeed, this overexpression induced stronger activation of the M-CSFR when IL-34 was added compared to the effects in non-transfected cells (NT) or cells transfected with an empty plasmid (Mock), as shown by the increased phosphorylations of 708, 723 tyrosines (Figure 7B). In contrast, the overexpression of syndecan-2 or -4 did not have any impact on M-CSFR activation by IL-34 (Supplementary data 4). The addition of a blocking syndecan-1 antibody reduced the IL-34-induced M-CSFR activation as measured by the levels of 708, 723 tyrosine phosphorylations in cells overexpressing syndecan-1 (Figure 7C). Anti-syndecan-1 antibody did not modulate by itself the M-CSFR phosphorylation while it upmodulated IL-34 effects on M-CSFR activation in non transfected cells (Figure 7D). To confirm the involvement of syndecan-1 in the IL-34 induced signalization, the syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA. As expected, syndecan-1 siRNA significantly down regulated the expression of the membrane protein as observed by flow cytometry (Figure 8A). Similarly to the addition of a blocking syndecan-1 antibody, the knocking down of syndecan-1 upregulated the M-CSFR phosphorylations (Figure 8B). Similar results were obtained for ERK1/2. We then compared the IL-34 dose response on the M-CSFR phosphorylations in HEK M-CSFR subpopulations expressing differentially syndecan-1 (Supplementary data 5). The data showed that IL-34-induced M-CSFR phosphorylations reached a plateau in the

presence of 25 ng/mL for pTyr-708 whereas no plateau was observed in cells expressing higher syndecan-1 expression even in the presence of 100 ng/mL of IL-34 (Supplementary data 5). These data demonstrated that syndecan-1 was able to modulate the biological activities of IL-34 through the M-CSFR.

To elucidate the functional implication of the syndecan-1/IL-34 interactions, we first analysed the expression of syndecan-1 in various cell types (Figure 9A). Syndecan-1 appears highly expressed by several cell types with hematopoietic (THP-1), mesenchymal (HOS Mock) and epithelial (HEK Mock) origin in contrast to U937 and TF-1 cells. IL-34 significantly increased the migration of THP-1 cells compared to the control (Figure 9B, $p < 0.001$). This IL-34-induced THP-1 cell migration was abolished by the addition of a blocking anti-syndecan-1 antibody. We then investigated the expression of syndecan-1 by human monocytes/macrophages (Figure 9C). $CD14^+$ cells expressed a very low level of syndecan-1 and interestingly, when $CD14^+$ cells were differentiated toward M2a macrophages ($CD14^{low}$, $CD200R^+$, $CD86^+$), differentiated cells highly expressed the syndecan-1 (Figure 9C). We then investigated the effects IL-34 on syndecan-1 expressing M2a macrophages. IL-34 significantly stimulated the migration of M2a macrophages (Figure 9C, $p < 0.001$). Similarly to the THP-1 cells, the addition of a blocking anti-syndecan-1 antibody totally inhibited the IL-34-induced M2a macrophage migration. Anti-syndecan-1 antibody had no effect on cell proliferation or on IL-34-associated monocyte survival (data not shown). Overall, these investigations demonstrated that IL-34 induced the migration of myeloid cells in a syndecan-1 dependent manner.

4. DISCUSSION

IL-34 and M-CSF are considered as “twin” cytokines sharing common M-CSFR chains. However, while both cytokines have some functional overlaps, they also have specific activities, especially in the differentiation and activation of the monocyte/macrophage lineage, effects in favour of the existence of an additional, unknown receptor for IL-34 [13, 18-20]. Recently, Nandi et al. [27] highlighted an alternative receptor for IL-34, the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), known to increase tyrosine phosphorylations of downstream proteins such as β-catenin, Src family kinases, focal adhesion kinase or Erk1/2. The present manuscript characterized a new binding mode for IL-34 to cells that express neither the M-CSFR, nor the chondroitin sulfate proteoglycan RPTPβ/ζ. This new IL-34 binding is dependent on chondroitin sulfate chains, and syndecan-1 was identified as regulating the IL-34 activation levels of the M-CSFR.

RPTPβ/ζ interacts with IL-34 through its chondroitin sulfate chains [27]. These authors demonstrated with enzymatic treatment and CS addition that chondroitin sulfate competed with IL-34 for binding to the extracellular domain of RPTPβ/ζ, revealing binding dependence on RPTPβ/ζ chondroitin sulfate chains. Like RPTPβ/ζ, chondroitin sulfate moieties seem to be a prerequisite for IL-34 binding to syndecan-1. RPTPβ/ζ and syndecan-1 are transmembrane proteoglycans with common features. Both proteins have glycosaminoglycans on their ectodomain, and their intracellular C-terminal moiety interacts with proteins containing the PDZ domain [28,29]. The polymorphism of glycosaminoglycans attached to the protein core of syndecan-1 is due to different post-translational modifications and is directly linked to tissue-specific functions [30]. Thus, the degree of glycosylation of the extracellular domain explains the versatility of the syndecan family. Syndecan-1 belongs to a proteoglycan family including four members: syndecan-1 mainly expressed in epithelial cells

[31], syndecan-2 mainly present in mesenchymal cells [32], syndecan-3 mostly found in neuronal and cartilage tissues [33] and syndecan-4 which is ubiquitously expressed [34]. All members of this family have heparan sulphate chains but only syndecan-1 and syndecan-3 display chondroitin sulfate chains on their protein core. Syndecan-4 has been also described, to a lesser extent, in a chondroitin sulphate-associated form [35]. Based on these data, IL-34 binding to the various syndecans must be considered according to tissue context and, for instance, IL-34 binding to syndecan-3 must not be excluded in the brain, especially regarding the importance of both IL-34 and syndecan-3 in the neuronal environment.

Proteoglycans are commonly devoted to numerous biological functions [36] and it is accepted that they control the activities of a lot of cytokines/growth factors with or without glycosaminoglycan binding domains such as the heparin binding domain. Thus, the various proteoglycans serve as a reservoir for cytokines/growth factors and also modulate their induced signalling pathways by acting directly on the cytokine/growth factors-receptor interaction or by the regulation of cytokine activation and/or turnover [37]. In light of these pleiotropic activities, glycosaminoglycans may be involved in the sequestration of IL-34 at the cell membrane as revealed by the chondroitinase treatment that increased the phosphorylation levels of the M-CSFR induced by IL-34 (Figure 5A). Abrogation of membrane chondroitin sulphate chains may then reduce the trapping of IL-34 in these chains and may facilitate IL-34 binding to the M-CSFR (Figure 10A, B). Similarly, the pre-incubation of chondroitin sulphate chains with IL-34 upmodulated the signal transduction through the M-CSFR (Figure 5B). In this context, soluble chondroitin sulphate chains may act as competitors to their homologous membrane forms and may limit IL-34 sequestration at the cell surface, facilitating its interaction with the M-CSFR (Figure 10C). Interestingly, soluble chondroitin sulfates compete with IL-34 for binding to the extracellular domain of RPTPβ/ζ [27], in contrast to the extracellular domain of M-CSFR (present data).

The co-receptor functions of proteoglycans are well illustrated by their interaction with the fibroblast growth factor and its receptor (FGF/FGFR). The FGF was the first growth factor identified as being dependent on heparin/heparan sulphate chains for its binding to the FGFR. The ternary complex FGF/FGFR/proteoglycans formed directly modulates the signalling pathways induced by the growth factor [38]. The co-receptor role of glycosaminoglycans has then also been demonstrated for epidermal growth factor (EGF) [39], hepatocyte growth factor (HGF) [40], vascular endothelial growth factor (VEGF) [41], Wnt factors [42] or members of the transforming growth factors (TGFs) [43]. In these cases, syndecan-1, via its heparan sulphate chains, acts as a co-receptor for these growth factors. Our present results suggest that syndecan-1 may also act as a modulator for the tyrosine kinase M-CSFR, but through its chondroitin sulfate chains. Syndecan-1 exhibits a stimulatory or inhibitory role, probably depending on its expression level. Thus, a low/moderate level of syndecan-1 may sequestrate IL-34 at the cell surface through its chondroitin sulfate chains, limiting the interaction between IL-34 and the M-CSFR (Figure 8D). The overexpression of syndecan-1 induced significant phosphorylation of the M-CSFR, reinforced by the presence of IL-34. Interestingly, the overexpression of syndecan-1 modulate the pTyr723 of the M-CSFR that was already associated with the activation of specific signaling pathways (PI3K, Rho GTPases, paxilline, SFKs/Pyk2, etc) involved in cell migration [2, 44]. In contrast, a blocking anti-syndecan-1 antibody significantly reduced the effects of IL-34. The enhanced activation by syndecan-1 may be explained by the membrane proximity between the M-CSFR and syndecan-1, the biodistribution of syndecan-1 at the cell membrane being directly related to its expression level. IL-34 stimulation did not induce detectable signalling pathways (Supplementary data 2) in MOCK cells expressing syndecan-1 but not the M-CSFR and the RPTPβ/ζ. These findings strengthen the hypothesis of a regulator for syndecan-1 on IL-34 signalling through the M-CSFR. Previous works have already identified specialised docking

sites for other receptors in the syndecan extracellular domains, such as integrins and tyrosine kinase receptors. Thus, αvβ3 integrin and insulin-like growth factor 1 receptor (IGF1R) are captured by syndecan-1 to form a ternary receptor mainly expressed on tumour cells and endothelial cells and involved in the angiogenesis process [45]. Further investigations will be necessary to investigate the potential role of syndecan-1 in the IL-34 trapping.

Syndecan-1 is implicated in the control of cellular chemoattraction. Indeed, membrane proteoglycans, including the syndecan family, are responsible of a chemotactif gradient on endothelial cells linked to the adherence and migration of numerous circulating cells such as neutrophils [46]. Syndecan-1 mediated the osteoprotegerin-induced chemotaxis in human peripheral blood monocytes and may control by this mechanism, the inflammatory process and the bone remodelling [47, 48]. Proteoglycans like syndecan-1 contributes to the control of the pathogenesis of osteolysis [49, 50]. Thus, tumor-derived syndecan-1 is associated a crosstalk with bone and an increase of osteoclastogenesis [49, 50]. By interacting with syndecan-1, IL-34 may have a double involvement in osteoclastogenesis, by inducing the migration of myeloid osteoclast precursors expressing syndecan-1 and by substituting for M-CSF in RANKL-induced osteoclastogenesis [5]. In addition, IL-34 was already shown to drives in vitro the polarization of macrophages towards an M2 immunosuppressive phenotype and function [4]. Several reports have described the accumulation of TAMs in tissue during the tumour development and TAMs are considered to facilitate the metastatic process [51]. We showed that syndecan-1 controls the IL-34-induced M2 macrophage migration (Figure 9), which can be related to the role of IL-34 in the biology of M2 macrophages [4]. The increased expression of syndecan-1 by macrophages was already observed during the myeloid differentiation and during the inflammatory process [52, 53]. These authors found a link between macrophage motility and the expression level of syndecan-1.

Syndecan-1 can now be added to the list of key regulators of IL-34 activities in addition to M-CSFR and RPTPβ/ζ, but not of M-CSF. By controlling IL-34 bioavailability, by modulating the recruitment of M-CSFR chains or the affinity of IL-34 to the receptor chains, and/or by acting as a regulator signalling of the M-CSFR, syndecan-1 may play a part in the role of IL-34 in development [22], bone [5] and inflammatory diseases [6]. Consequently, new therapies targeting the IL-34/M-CSFR axis must now take into account the existence of other receptors for the cytokine.

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5. REFERENCES

[1] R.P. Bourette, J.A.J Rohrschneider, Early events in M-CSF receptor signaling, Growth Factors Chur. Switz. 17 (2000) 155–166.

[2] F.J., E.R. Stanley, CSF-1 regulation of the wandering macrophage: complexity in action, Trends Cell Biol. 14 (2004) 628–638.

[3] H. Lin, E. Lee, K. Hestir, C. Leo, M. Huang, E. Bosch, R. Halenbeck, G. Wu, A. Zhou, D. Behrens, D. Hollenbaugh, T. Linnemann, M. Qin, J. Wong, K. Chu, S.K. Doberstein, L.T. Williams, Discovery of a Cytokine and Its Receptor by Functional Screening of the Extracellular Proteome, Science 320 (2008) 807–811.

[4] E.D. Foucher, S. Blanchard, L. Preisser, E. Garo, N. Ifrah, P. Guardiola, Y. Delneste, P. Jeannin, IL-34 Induces the Differentiation of Human Monocytes into Immunosuppressive Macrophages, Antagonistic Effects of GM-CSF and IFNγ, PloS One 8 (2013) e56045.

[5] M. Baud'Huin, R. Renault, C. Charrier, A. Riet, A. Moreau, R. Brion, F. Gouin, L. Duplomb, D. Heymann, Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis, J. Pathol. 221 (2010) 77–86.

[6] M. Chemel, B. Le Goff, R. Brion, C. Cozic, M. Berreur, J. Amiaud, G. Bougras, S. Touchais, F. Blanchard, M.F. Heymann, J.M. Berthelot, F. Verrecchia, D. Heymann, Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients, Ann. Rheum. Dis. 71 (2012) 50–154.

[7] E.A. Boström, P. Lundberg, The Newly Discovered Cytokine IL-34 Is Expressed in Gingival Fibroblasts, Shows Enhanced Expression by Pro-Inflammatory Cytokines, and Stimulates Osteoclast Differentiation, PLoS ONE 8 (2013) e81665.

[8] P. Roth, E.R. Stanley, The biology of CSF-1 and its receptor, Curr. Top. Microbiol. Immunol. 181 (1992) 141–167.

[9] J. Felix, J. Elegheert, I. Gutsche, A.V. Shkumatov, Y. Wen, N. Bracke, E. Pannecoucke, I. Vandenberghe, B. Devreese, D.I. Svergun, E. Pauwels, B. Vergauwen, S.N. Savvides, Human IL-34 and CSF-1 establish structurally similar extracellular assemblies with their common hematopoietic receptor, Structure 21 (2013) 528–539.

[10] X. Ma, W.Y. Lin, Y. Chen, S. Stawicki, K. Mukhyala, Y. Wu, F. Martin, J.F. Bazan, M.A. Starovasnik, Structural Basis for the Dual Recognition of Helical Cytokines IL-34 and CSF-1 by CSF-1R, Structure 20 (2012) 676–687.

[11] C.J. Sherr, Colony-stimulating factor-1 receptor, Blood 75 (1990) 1–12.

[12] H. Liu, C. Leo, X. Chen, B.R. Wong, L.T. Williams, H. Lin, X. He, The mechanism of shared but distinct CSF-1R signaling by the non-homologous cytokines IL-34 and CSF-1, Biochim. Biophys. Acta 1824 (2012) 938–945.

[13] T. Chihara, S. Suzu, R. Hassan, N. Chutiwitoonchai, M. Hiyoshi, K. Motoyoshi, F. Kimura, S. Okada;, IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation, Cell Death Differ. 17 (2010)1917–1927.

[14] X.M. Dai, G.R. Ryan, A.J. Hapel, M.G. Dominguez, R.G. Russell, S. Kapp, V. Sylvestre, E.R. Stanley, Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects, Blood 99 (2002) 111–120.

[15] H. Yoshida, S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo, L.D. Shultz, S. Nishikawa, The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene, Nature 345 (1990) 442–444.

[16] W. Wiktor-Jedrzejczak, A. Bartocci, A.W. Jr. Ferrante, A. Ahmed-Ansari, K.W. Sell , J.W. Pollard, E.R. Stanley, Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4828–4832.

[17] S. Wei, S. Nandi, V. Chitu, Y.G. Yeung, W. Yu, M. Huang, L.T. Williams, H. Lin, E.R. Stanley, Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells, J. Leukoc. Biol. 88 (2010) 495 – 505.

[18] F. Ginhoux, M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M.F. Mehler, S.J. Conway, L.G. Ng, E.R. Stanley, I.M. Samokhvalov IM, M. Merad M, Fate mapping analysis reveals that adult microglia derive from primitive macrophages, Science 330 (2010) 841–845.

[19] M. Greter, I. Lelios, P. Pelczar, G. Hoeffel, J. Price, M. Leboeuf, T.M. Kündig, K. Frei, F. Ginhoux, M. Merad, B. Becher, Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia, Immunity 37 (2012) 1050–1060.

[20] Y. Wang, K.J. Szretter, W. Vermi, S. Gilfillan, C. Rossini, M. Cella, A.D. Barrow, M.S. Diamond, M. Colonna, IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia, Nat. Immunol. 13 (2012) 753–760.

[21] D. Heymann, Interleukin-34: An Enigmatic Cytokine, IBMS BoneKEy 7 (2010) 406–413.

[22] S. Nandi, S. Gokhan, XM Dai, S. Wei, G. Enikolopov, H. Lin, M.F. Mehler, E.R. Stanley, The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation, Dev. Biol. 367 (2012) 100–113.

[23] J. Vandesompele, K.D. Preter, F. Pattyn, B. Poppe, N.V. Roy, A.D. Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034.

[24] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622.

[25] F. Tejedor, J.P. Ballesta, Iodination of biological samples without loss of functional activity, Anal. Biochem. 127 (1982) 143–149.

[26] A. Godard, D. Heymann, S. Raher, I. Anegon, M.A. Peyrat, B. Le Mauff, E. Mouray, M. Gregoire, K. Virdee, J.P. Soulillou, High and low affinity receptors for human interleukin for DA cells/leukemia inhibitory factor on human cells. Molecular characterization and cellular distribution, J. Biol. Chem. 267(1992) 3214–3222.

[27] S. Nandi, M. Cioce, Y.G. Yeung, E. Nieves, L. Tesfa, H. Lin, Hsu A.W., R. Halenbeck, H.Y. Cheng, S. Gokhan, M.F. Mehler, E.R. Stanley, Receptor-type protein tyrosine phosphatase zeta is a functional receptor for interleukin-34, J. Biol. Chem. 288 (2013) 21972-21986

[28] A. Fujikawa, M. Fukada, Y. Makioka, R. Suzuki, J.P.H. Chow, M. Matsumoto, M. Noda Consensus substrate sequence for protein-tyrosine phosphatase receptor type Z, J. Biol. Chem. 286 (2011) 37137–37146.

[29] J.R. Couchman, Transmembrane Signaling Proteoglycans, Annu. Rev. Cell Dev. Biol. 26 (2010) 89–114.

[30] R.D. Sanderson, M. Bernfield, Molecular polymorphism of a cell surface proteoglycan: distinct structures on simple and stratified epithelia, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9562–9566.

[31] S. Saunders, M. Jalkanen, S. O'Farrell, M. Bernfield, Molecular cloning of syndecan, an integral membrane proteoglycan, J. Cell Biol. 108 (1989) 1547–1556.

[32] P. Marynen, J. Zhang, J.J. Cassiman, H. Van den Berghe, G. David, Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. Prediction of an integral membrane domain and evidence for multiple distinct core proteins at the cell surface of human lung fibroblasts, J. Biol. Chem. 264 (1989) 7017–7024.

[33] D.J. Carey, D.M. Evans, R.C. Stahl, V.K. Asundi, K.J. Conner, P. Garbes, G. Cizmeci-Smith, Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan, J. Cell Biol. 117 (1992) 191–201.

[34] G. David, B. van der Schueren, P. Marynen, J.J. Cassiman, H. van den Berghe Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells, J. Cell Biol. 118 (1992) 961–969.

[35] C. Yeaman, A.C. Rapraeger, Membrane-anchored proteoglycans of mouse macrophages: P388D1 cells express a syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form, J. Cell Physiol. 157 (1993) 413–425.

[36] G.A. Karasneh, M. Ali, D. Shukla, An Important Role for Syndecan-1 in Herpes Simplex Virus Type-1 Induced Cell-to-Cell Fusion and Virus Spread, PLoS ONE 6 (2011) e25252.

[37] H. Kresse , E. Schönherr, Proteoglycans of the extracellular matrix and growth control. J. Cell Physiol. 189 (2001) 266–274.

[38] N. Jastrebova, M. Vanwildemeersch, U. Lindahl, D. Spillmann, Heparan sulfate domain organization and sulfation modulate FGF-induced cell signaling, J. Biol. Chem. 285 (2010) 26842–26851.

[39] R. Takazaki, Y. Shishido, R. Iwamoto, E. Mekada, Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like Growth Factor, J. Biol. Chem. 279 (2004) 47335–47343.

[40] L.E. Kemp, B. Mulloy, E. Gherardi, Signalling by HGF/SF and Met: the role of heparan sulphate co-receptors Biochem. Soc. Trans. 34 (2006) 414–417.

[41] J. Dai, A.B.M. Rabie, VEGF: an essential mediator of both angiogenesis and endochondral ossification, J. Dent. Res. 86 (2007) 937–950.

[42] C. Niehrs, The complex world of WNT receptor signalling, Nat. Rev. Mol. Cell. Biol. 13 (2012) 767–779.

[43] C.C. Rider, Heparin/heparan sulphate binding in the TGF-β cytokine superfamily, Biochem. Soc. Trans. 34 (2006) 458.

[44] N.G. Sampaio, W. Yu, D. Cox, J. Wyckoff, J. Condeelis, E.R. Stanley, F.J. Pixley, Phosphorylation of CSF-R Y721 mediates its association with PI3K to regulate macrophage motility and enhancement of tumor invasion, J. Cell Sci. 124 (2011) 2021-2031.

[45] A.C. Rapraeger, Synstatin: a selective inhibitor of the syndecan-1-coupled IGF1R-αvβ3 integrin complex in tumorigenesis and angiogenesis, FEBS J. 280 (2013) 2207–2215.

[46] S. Massena, G. Christoffersson, E. Hjertström, E. Zcharia, I. Vlodavsky, N. Ausmees, C. Rolny, J.P. Li, M. Phillipson, A chemotactif gradient sequestered on endothelial heparan sulfate induces directionnal intraluminal crawling of netutrophils, Blood 116 (2010) 1942-1931.

[47] B.A. Mosheimer, N.C. Kaneider, C. Feistritzer, A.M. Djanani, D. H. Sturn, J.R. Patsch, C.J. Wiedermann, Syndecan-1 is involved in osteoprotegerin-induced chemotaxis in human peripheral blood monocytes, J. Clin.Endocrinol. Metab. 90 (2005) 2964-2971.

[48] Z. Bensliman-Ahmin, F. Poirier, C. Delomenie, A. Lokajczyk, F. Grelac, I. Galy-Fauroux, A. Mohamedi, A.M. Fischer, D. Heymann, D. Lutomski, C. Boisson-Vidal, Mechanistic study of the pro-angiogenic effect of osteoprotegerin, Angiogenesis 16 (2013) 575-593.

[49] T. Kelly, L.J. Suva, K.M. Nicks, V. MacLeod, R.D. Sanderson, Tumor-derived syndecan-1 mediates distal cross-talk with bone that enhances osteoclastogenesis, J. Bone Miner. Res. 25 (2010) 1295-1304.

[50] M. Baud'huin, C. Charrier, G. Bougras, R. Brion, F. Lezot, M. Padrines, D. Heymann, Proteoglycans and osteolysis, Methods Mol. Biol. 836 (2012) 323-337.

[51] J. Cook, T. Hagemann T., Tumour-associated macrophages and cancer. Curr. Opin. Pharmacol. 13 (2013) 595-601.

[52] M. Averbeck, C. Gebbhardt, U. Anderegg, C. Termeer, J.P., Sleeman, J.C. Simon, Swith in syndecan-1 and syndecan-4 expression controls maturation associated dendritic cell motility. Exp. Dermatol. 16 (2007) 580-589

[53] C. Yeaman, A.C. Rapraeger, Post-transcriptional regulation of syndecan-1 expression by cAMP in peritoneal macrophages. J. Cell Biol. 122 (1993) 941-950.

FIGURE LEGENDS

Figure 1: IL-34 and M-CSF differentially activate the M-CSFR. (**A**) M-CSFR expression on the cell membrane of human cells analysed by flow cytometry. TF-1: erythroblastic leukaemia cell line, HEK293: embryonary cell line; HOS: osteosarcoma cell line; HL-60, U-937 and THP-1: myeloid leukaemic cell lines; HOS M-CSFR and HEK M-CSFR: HOS and HEK stably overexpressing M-CSFR; HOS Mock and HEK Mock: HOS and HEK cell lines transfected with the empty control vector. The isotype IgG1 was used as the control (in grey on the graphs). (**B-E**) Western blot investigations of tyrosine phosphorylation patterns of M-CSFR and Erk1/2 phosphorylation after addition of 50, 100 or 200 ng/mL of IL-34 or M-CSF for 1 to 30 minutes, in HEK M-CSFR (**B**), HOS M-CSFR (**C**), TF-1 M-CSFR (**D**) and THP-1 cells (**E**). All experiments were performed three times in independent conditions and a representative set of experiments is shown.

Figure 2: IL-34 binds to M-CSFR with a high affinity. Increasing concentrations of ^{125}I-labelled IL-34 (IL-34*) or ^{125}I-labelled M-CSF (M-CSF*) were incubated for 60 min at 4°C with 10^6 cells and bound and unbound fractions were determined as described in "Experimental Procedures". Curve fitting was performed as described in the "Materials and Methods" section. Typical curves revealing a specific binding fraction of iodinated M-CSF on HEK M-CSFR cells (**A**) and of iodinated IL-34 on HEK M-CSFR **(B**) and THP-1 cells (**C**). Table summarizing the binding mode of M-CSF and IL-34 on the cells of interest (**D**). These experiments are representative of three times in independent conditions and a representative experiment is shown.

Figure 3: A new IL-34 binding independent of the M-CSFR. (**A-B**) Inhibition binding assays were carried out on HEK M-CSFR cells. After incubation of HEK M-CSFR cells with a saturating concentration of ^{125}I-labelled IL-34 (IL-34*), increasing doses of unlabelled IL-34 (**A**) or unlabelled M-CSF (**B**) were added to the cells. (**C**) Binding of ^{125}I-labelled IL-34 was assessed by Scatchard assay on HEK Mock cells which did not express the M-CSFR. (**D**) Competition assay of ^{125}I-labelled IL-34 with unlabelled IL-34 was performed on HEK Mock cells. (**E**) Binding of HEK Mock cells on IL-34 or M-CSF spotted on a chip analysed by Surface Plasmon Resonance. All experiments were repeated three times in independent times.

Figure 4: IL-34 binds to chondroitin sulfate but not to heparin/heparan sulfate chains. Interactions between IL-34 and glycosaminogycans were studied by surface plasmon resonance technology. Chondroitin sulfate A (**A**), chondroitin sulfate B (**B**), chondroitin sulfate C (**C**), or heparin (**D**) were injected on immobilised IL-34 using the parameters described in the "Materials and Methods" section.

Figure 5: Chondroitin sulfate chains regulate the M-CSFR phosphorylations induced by IL-34. (**A**) HEK M-CSFR cells were treated with a mixture of chondroitinases A, B and C for 90 min at 37°C, before addition of 50 ng/mL of M-CSF or IL-34 for 5 min. (**B**) HEK M-CSFR cells were pre-incubated with 1, 3 or 10 µg/mL of chondroitin sulfate salts for 90 min at 37°C before addition of 50 ng/mL of M-CSF or IL-34 for 5 min. The phosphorylation patterns of the M-CSFR and ERK1/2 were assessed by Western blot. Total forms of M-CSFR, ERK1/2 and β-actin were used as a loading control. Bar graphs show relative densitometric values of pTyr-723 normalized to the M-CSFR. Similar densitometric measurments were obtained for pTyr-746 or pTyr-708, normalized to the M-CSFR or to β-actin. These

experiments were performed three times in independent conditions and a representative experiment is shown.

Figure 6: HEK, HOS, THP-1 and TF-1 do not express the receptor protein tyrosine phosphatase RPTPβ/ξ. The expression of the receptor protein tyrosine phosphatase RPTPβ/ξ was assessed in the different cells used in the study both at the transcriptional level by quantitative RT-PCR (**A**), where expression was normalised against GAPDH mRNA levels, and at the protein level by Western Blot, where β-actin was used as a loading control (**B**). These experiments were performed three times in independent conditions and a representative experiment is shown.

Figure 7: Syndecan-1 modulates the M-CSFR phosphorylations induced by IL-34. HEK M-CSFR were transiently transfected with a plasmid-encoding human syndecan-1. (**A**) Syndecan-1 expression analysed by flow cytometry, 48 h after transient transfection. Syndecan-1 expression in Mock M-CSFR transfected with an empty vector of syndecan-1 (empty histogram). Syndecan-1 overexpressing cells were stimulated with or without 50 ng/mL of IL-34 for 5 min (**B**) in the presence or absence of 20 µg/mL of syndecan-1 blocking antibody pre-incubated for 90 min (**C**). Similar experiments were performed on HEK M-CSFR (non transfected for syndecan-1) with or without 100 ng/mL of IL-34 and/or 10 µg/ml of anti-syndecan-1 antibody for 1 and 5 minutes (**D**). M-CSFR- and ERK1/2-phosphorylations were assessed by Western Blot, with the M-CSFR, ERK1/2 total forms of and GAPDH as a loading control. Bar graphs show relative densitometric values of pTyr-723 and pTyr-708 normalized to the M-CSFR. These experiments were performed four times in independent conditions and a representative experiment is shown.

Figure 8: Syndecan-1 siRNA increases the phoshorylation M-CSFR induced by IL-34.

The syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA. (**A**) Syndecan-1 expression analysed by flow cytometry, 48 h after siRNA transfection. (**B**) Syndecan-1 knocked down cells were stimulated with or without 50 ng/mL of IL-34 for 5 min and M-CSFR-, ERK1/2-phosphorylations were assessed by Western Blot, with the total forms of M-CSFR and ERK1/2 as a loading control. Bar graphs show relative densitometric values of pTyr-723 normalized to the M-CSFR. Similar densitometric measurments were obtained for pTyr-708, normalized to the M-CSFR or to GAPDH. A representative experiment out of three independent experiments is shown.

Figure 9: IL-34 increases the migration of myeloid cells through the syndecan-1. (**A**) Syndecan-1 expression by various myeloid, mesenchymal and epithelial cells analysed by flow cytometry. (**B**) THP-1 cells were preincubated with antibody to syndecan-1 (B-B4) or -4 the correspond isotype matched IgG (IgG1) for 15 min. After washing twice, migration toward IL-34 (100 ng/mL) was tested for 6 hours. (**C**) M2a macrophage were obtained from $CD14^+$ cells isolated from peripheral blood of healthy donors, cultured and differentiated in in RPMI supplemented with 1% human serum, 1% penicillin/streptomycin, 50 ng/mL of IL-4 for 5 days. The expression of syndecan-1 and their phenotype ($CD14^{low}$, $CD220R^+$, $CD86^+$) were analysed by flow cytometry. M2a cells were collected by gentle scraping and their migration ability in the presence of IL-34 with or withoutan a blocking anti-syndecan-1 antibody was assessed as described for the THP-1 cells. The histograms show the percentage of the migrated cells compared to the IgG1 control. Graphs represent the mean ±SD of three independent experiments done in triplicate in each case. A Kruskal-Wallis test was performed followed by a Dunn's post-hoc between all the conditions. *** $p < 0.001$.

Figure 10: Schematic diagram hypothesizing the functional involvement of syndecan-1 in the bioavailability and activities of IL-34. Potential interactions of IL-34 with M-CSFR, Syndecan-1 and chondroitin sulfates in control conditions (**A**), after chondroitinase ABC treatment (**B**), in the presence of exogenous chondroitin sulfate chains (**C**), and according to the levels of syndecan-1 expression (**D**).

Syndecan-1 regulates the biological activities of interleukin-34

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ABSTRACT

IL-34 is a challenging cytokine sharing functional similarities with M-CSF through M-CSFR activation. It also plays a singular role that has recently been explained in the brain, through a binding to the receptor protein tyrosine phosphatase RPTPβ/ζ. The aim of this paper was to look for alternative binding of IL-34 on other cell types. Myeloid cells (HL-60, U-937, THP-1) were used as cells intrinsically expressing M-CSFR, and M-CSFR was expressed in TF-1 and HEK293 cells. IL-34 binding was studied by Scatchard and binding inhibition assays, using ^{125}I-radiolabelled cytokines, and surface plasmon resonance. M-CSFR activation was analysed by Western blot after glycosaminoglycans abrasion, syndecan-1 overexpression or repression and addition of a blocking anti-syndecan antibody. M-CSF and IL-34 induced different patterns of M-CSFR phosphorylations, suggesting the existence of alternative binding for IL-34. Binding experiments and chondroitinase treatment confirmed low affinity binding to chondroitin sulfate chains on cells lacking both M-CSFR and RPTPβ/ζ. Amongst the proteoglycans with chondroitin sulphate chains, syndecan-1 was able to modulate the IL-34-induced M-CSFR signalling pathways. Interestingly, IL-34 induced the migration of syndcean-1 expressing cells. Indeed, IL-34 significantly increased the migration of THP-1 and M2a macrophages that was inhibited by addition of a blocking anti-syndecan-1 antibody. This paper provides evidence of alternative binding of IL-34 to chondroitin sulfates and syndecan-1 at the cell surface that modulates M-CSFR activation. In addition, IL-34-induced myeloid cell migration is a synedcan-1 dependent mechanism.

1. INTRODUCTION

The discovery of interleukin-34 (IL-34) in 2008 upset the functional biological concepts that had been admitted for many years between Macrophage Colony Stimulating Factor (M-CSF) and its receptor, the M-CSF receptor (M-CSFR) [1,2]. Like M-CSF, IL-34 promotes the survival and proliferation of monocytes, as well as their differentiation into macrophages [3]. Macrophages induced by IL-34 have phenotypes and functions that are similar to those differentiated by M-CSF, as both cytokines polarize these macrophages into immunosuppressive macrophages M2 [4]. In addition, and in association with the Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), IL-34 can replace M-CSF to induce osteoclast formation by stimulating the proliferation and adhesion of osteoclast precursors [5]. The cytokine also overlaps with the roles played by M-CSF in inflammation in degenerative bone diseases such as rheumatoid arthritis [6] and periodontal inflammation [7]. All these effects are mediated by a cell-surface tyrosine kinase activity receptor, M-CSFR, encoded by the proto-oncogene c-fms, and also called c-fms, CD115 or CSF-1R [8]. The homodimeric cytokines M-CSF or IL-34 bind in a close way to the extracellular domain of M-CSFR, but with a different binding mode [9,10], leading to receptor dimerization and differential auto-phosphorylation on its eight tyrosine residues [11]. M-CSF/M-CSFR and IL-34/M-CSFR crystals have quite a similar shape, except a higher stability for the IL-34/M-CSFR complex [12]. Chihara et al. showed some differences in the kinetics of M-CSFR phosphorylations and in the nature and intensity of phosphorylated tyrosine residues after M-CSF or IL-34 binding, which partly explains their ability to differentially activate the monocyte/macrophage lineage [13].

MCSF-R knockout mice [14] exhibit a similar but more pronounced phenotype than op/op mice [15,16] characterized by a more severe osteopetrosis and reduction of tissue macrophages. This osbservation suggested the existence of another ligand for the M-CSFR,

and IL-34 was a perfect candidat. Although IL-34 expression under the promoter of the M-CSF gene rescues the phenotype of op/op mice, the cytokine also plays singular roles during brain development [17]. Microglia and Langerhans cells effectively decrease dramatically in IL-34 knockout mice, as in M-CSFR deficient mice, while monocytes, lymphoid tissue macrophages and dendritic cells are not affected. Besides, op/op mice show quite a conventional development of these cells [18-20]. These results identify specific IL-34 activity during embryogenesis and tissue homeostasis, and make it possible to better understand the tissue distribution and independent activity of IL-34 [21].

IL-34 is highly expressed in both post-natal and adult brains, whereas M-CSFR expression, which is maximal in early development, dramatically decreases in adult brains [22]. High expression of IL-34 despite an almost undetectable expression of its receptor suggests the existence of other receptors for the cytokine. In spite of overlapping and complementary activities between M-CSF and IL-34 through M-CSFR activation, the "M-CSF twin" may also exert specific functions independently of this receptor. The present study thus investigates a new binding mode for IL-34 that may modulate the bioavailability and/or activities of this cytokine. This paper provides evidence of syndecan-1 as a new molecular actor in the M-CSF/IL-34/M-CSFR triad and proposes one explanation for the differential activation of the M-CSFR observed after M-CSF and IL-34 stimulation.

2. MATERIAL AND METHODS

All experiments described in the manuscript were repeated at least three times in independent experiments and representative experiments are shown.

2.1. Reagents

Recombinant human Macrophage-Colony Stimulating Factor (M-CSF), human interleukin-34 (IL-34), human M-CSF Receptor (M-CSFR), mouse IgG1 (control isotype) and antibody anti-human M-CSFR were obtained from R&D Systems (Abingdon, UK). Diaclone (Besanc□on, France) kindly provided the murine B-B4 anti-syndecan 1 (CD138) IgG1 monoclonal antibody. Antibodies directed against human CD14, human CD200R and human CD86 were purchased from Biolegend (Saint Quentin en Yvelines, France). Antibodies directed against human P-MCSFR, P-Erk1/2, P-mTor, P-Akt and the total form of proteins were purchased from Cell Signalling (Ozyme, Saint Quentin Yvelines, France). Anti-β actin and total phosphorylated tyrosine antibodies were respectively provided by Sigma-Aldrich (Saint Quentin Fallavier, France) and Millipore (Molsheim, France). Anti-Receptor Protein Tyrosine Phosphatase (RPTPβ/ζ, C-19 clone) and HRP-conjugated secondary antibodies were obtained from Santa-Cruz (CliniSciences, Nanterre, France). Chondroitin sulfate salts from shark cartilage, heparin, heparan sulfate salts and chondroitinases A, B, C were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2. Cell cultures

The cell lines used in the present study were purchased from the American Tissue Cell Collection (ATCC, Molsheim, France). HEK293 (HEK), osteosarcoma MNNG/HOS (ATCC® Number: CRL-1547™) (HOS) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Lonza, Levallois-Perret, France) supplemented with 10% foetal bovine

serum (FBS; HyclonePerbio, Bezons, France) and 2 mmol/L of L-glutamine. HL-60, U937, THP-1 and TF-1cell lines were maintained in RPMI (Roswell Park Memorial Institute, Lonza) supplemented with 10% FBS. In addition, TF-1 cells were cultured in the presence of 3 ng/mL of G-MCSF according to ATCC recommendations. The human U251 glioblastoma cell line was kindly provided by Dr Mylène Dorvillius (ATLAB Pharma, Nantes, France) and cultured in DMEM supplemented with 10 % FBS.

2.3. Cloning of the human M-CSFR gene and cell transfection

The human M-CSFR gene (NM_005211.3) was cloned in a pCDNA3.3 TOPO TA vector (Life Technologies, Villebon sur Yvette, France) from the cDNA from a healthy donor $CD14^+$. $CD14^+$ cells were initially isolated from human peripheral blood donors provided by the French blood bank institute (Etablissement Français du Sang, Nantes, France, authorization number: NTS 2000-24), by using MACS microbeads (MiltenyiBiotec, Germany) as previously described [5]. The RT-PCR product was obtained using the following primer sequences: Forward 5'-CACCATGGGCCCAGGAGTTCTGCTGCT-3' and Reverse 5'-AACTCCTCAGCAGAACTGATAGTTGTTGGGCTGCA-3', and fully sequenced to check for the absence of functional mutations based on the sequences already published in the data bases.

Embryonic HEK293 cells and osteosarcoma HOS cells were transfected as described below with the pCDNA3 empty plasmid or the pCDNA3 plasmid containing the M-CSFR gene. To obtain a polyclonal population expressing the membrane M-CSFR, $5x10^6$ cells were then stained with phycoerythrin (PE) conjugated antibodies directed against the M-CSFR and sorted out on a FACSAria III (Beckman Coulter, Villepinte, France). The cells expressing the M-CSFR were named respectively HEK M-CSFR and HOS M-CSFR. The cells transfected with the empty pCDNA3 vector were called HEK Mock and HOS Mock. Erythroblastic TF-1

cells were transfected using an Amaxa® Cell Line Nucleofector® Kit by Lonza. Cell sorting was also carried out to isolate M-CSFR expressing cells (TF-1 M-CSFR).

2.4. RNA isolation and real-time PCR

Total RNA was extracted using NucleoSpin®RNAII (Macherey Nagel, Duren, Germany) with one stage of DNase I treatment (25 units, 15 min) to prevent genomic contamination. 1 µg of total RNA was used for first strand cDNA synthesis using the ThermoScript RT-PCR System (Invitrogen). Real-time PCR was performed on 20 ng of reverse transcribed total RNA (cDNA), 300 nM of primers and 2x SYBR Green Supermix (Biorad, Marnes-la-Coquette, France). The analysis was performed according to the method described by Vandesompele et al. [23] using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the invariant control. Standard curves were obtained using decreasing quantities of cDNA from the U251 cell line to validate the RPTPβ/ζ primers according to the MIQE guidelines [24]. The sense and antisense primers used were: RPTPβ/ζ Forward: 5'-GCAGAGCTGTACTGTTGACTT-3', Reverse: 5'-TGTGCTAGCTTAACCCTGCT-3'; GAPDH Forward: 5'-TGGGTGTGAACCATGAGAAGTATG-3', Reverse: 5'-GGTGCAGGAGGCATTGCT-3'.

2.5. M-CSF and IL-34 binding assays

M-CSF and IL-34 were radio-labelled with iodine-125 (^{125}I) (which has specific radioactivity of around 2,000 cpm/fmol) using the chloramine T method, as described by Tejedor and Ballesta [25] and Godard et al [26]. To determine the ability to bind to the cells of both cytokines, $1x10^6$ cells were incubated with increasing concentrations of iodinated M-CSF or IL-34 (up to 1nM) for 1 h at 4°C. Non-specific binding was determined in the presence of 100-fold excess of the corresponding unlabelled cytokine. Specific binding was subsequently

obtained by subtraction of this non-specific binding from the total binding signal. Regression analysis of the binding data was carried out using a one-site equilibrium binding equation (Graphit; Erithacus Software, Staines, U.K.) and data were plotted in the Scatchard coordinate system. Cross competition binding curves were obtained by adding simultaneously to the cells increasing amounts of a competitor cytokine together with a fixed, non-saturable concentration of a labelled cytokine. The inhibition curves obtained were analysed with an equation for competitive inhibition between two ligands for a common binding site. Curve fittings were performed using the Graphit data analysis programme.

2.6. Surface Plasmon Resonance (SPR) assays

The SPR experiments were carried out on a BIAcore 2000 biosensor (BIAcore, Uppsala, Sweden). Recombinant human IL-34 (5µg/mL, in 5 mM maleate buffer pH=6.2) was covalently linked to the dextran matrix of a CM5 sensor chip (BIAcore, Uppsala, Sweden), previously activated with an ethyl (dimethylaminopropyl) carbodiimide/ N-hydroxysuccinimide (EDC/NHS) mixture. Immobilisation levels in the 1300 RU range were then obtained. Residual active groups were inactivated by injection of ethanolamine 1M pH=8.5 for 10 min. The binding of decreasing concentrations of chondroitin sulfate salts, heparin or heparan sulfate salts was monitored, from 100 µg/mL and with a serial dilution of 1:3. Concentrations up to 500 µg/mL were assessed for the heparin and heparan sulfate. The flow rate was around 30 µL/min, and the chip was regenerated with 20 mM NaOH between each cycle. The resulting sensorgrams were fitted using BiaEval 4.1 software.

Cell binding experiments were carried out in the SPRiplex II. The SPRi biochip-CH (H0550) was activated with an EDC/NHS mixture, and 200 µg/mL IL-34 was spotted on it with a 500µM tip for 30 min. The protein binding capacity of the IL-34 was controlled by injections of 1 and 2 µg/mL of recombinant human M-CSFR on the chip after each

regeneration cycle performed with 10mM glycine pH=1.5. HEK Mock cells ($2x10^5$ cells/mL) were then injected on to the chip at 20 µL/min for 10 min.

2.7. Flow-cytometry experiments

Membrane expression of the M-CSFR, syndecan-1, CD14, CD220R, CD86 were assessed with flow cytometry (Cytomics FC500; Beckman Coulter). Cells were incubated with PE-conjugated anti-human M-CSFR antibody or mouse IgG1 as the control for 30 min. For syndecan-1 expression, the cells were first incubated for 90 min at 4°C with the anti-syndecan-1 B-B4, before incubation with the PE-conjugated secondary antibody for 60 min. Similar method was used to analyse CD14, CD220R and CD86 expression. M-CSFR and syndecan-1 expressions were analysed using the CXP Analysis software 2.2 (Beckman Coulter).

2.8. Western Blot Analysis

The cells were collected in a RIPA buffer (10 mM Tris pH8, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS containing a cocktail of protease and phosphatase inhibitors: 1 mM sodium orthovanadate (Na_2VO_4), 1 mM phenylmethylsulforyl fluoride (PMSF), 10 mM sodium fluoride (NaF), 10 mM N-ethylmaleimide (NEM), 2 µg/ml leupeptin and 1 µg/ml pepstatine). The protein concentration was determined using a BCA (bicinchoninic acid) protein assay (Sigma Aldrich). 40 µg of total protein extracts were prepared in a Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and then separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer, the immobilon-P membranes (Millipore, Molsheim, France) were blotted with the antibodies referenced in the reagents section. The membranes were then probed with secondary antibodies coupled with horseradish peroxidase. Antibody binding was

visualised with a Pierce enhanced chemiluminescence (ECL) kit (ThermoSientific, Illkirch, France). The luminescence detected with a Charge Couple Device (CCD) camera was quantified using the GeneTools programme (Syngene, Cambridge, United Kingdom).

2.9. siRNA gene silencer

The syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA (Ambion, France) and the INTERFERin^{TM} transfection reagent (Polyplus transfection, France). 700 x 10^3 HEK M-CSFR cells per well were seeded in a 6-well plate 1 day before transfection. In each well 50 nm siRNA (Syndecan-1 siRNA : ref#s12634 ; Ambion® Silencer® Negative Control #1 : ref#AM4611) duplexes diluted in serum-free medium were incubated with 2 µl of INTERFERin^{TM} for 30 min at room temperature. The expression of membrane syndecan-1 was measured by flow cytometry. 48 h after the transfection, 50 ng/mL of IL-34 (5 min, 37°C) were added to the cells and M-CSFR activation was determined by Western blot as previously described.

2.10. M2a macrophage differentiation and cell migration assays

$CD14^+$ monocytes were isolated from peripheral blood of 3 healthy donors as previously described in paragraph 2.3. For M2a macrophage differentiation, $CD14^+$ cells were cultured in RPMI (Roswell Park Memorial Institute medium, Lonza) supplemented with 1% Human Serum (Invitrogen), 1% penicillin/streptomycin, 50 ng/mL of IL-4 (R&D System) for 5 days. Half volume of the medium was renewed at day 2. After the differentiation culture period, M2a adherent cells were collected by gentle scraping. Their phenotype was analysed by flow cytometry ($CD14^{low}$, $CD220R^+$, $CD86^+$) before use in migration assays. $2x10^5$ M2a macrophages or $5x10^5$ THP-1 were pre-treated for 30 min at 37°C with a blocking anti syndecan-1 antibody (SDC-1 Ab, B-B4) or the corresponding isotype (IgG1 Ab). Cell

migration was then assessed using a 8µm Boyden chamber (top well) in RPMI 1% FCS, with or without 100 ng/mL of IL-34 at the bottom well. Cells migrating at the bottom well were counted after 6 hours of culture. Each experiment was done in triplicate and repeated three times.

2.11. Statistical analysis

Results were analyzed using a Kruskal-Wallis test was performed followed by a Dunn's post-hoc between all conditions using GraphPad InStat v3.02 software. The results of the migration assays are given as a mean ± SD of three independent experiments done in triplicate. Results were considered significant at p values ≤ 0.05. All Western blots were performed three or four times in independent experiments and a representative blot was changed in each figure.

3. RESULTS

3.1. IL-34 and M-CSF induce differential activation of the M-CSFR

We first analysed the membrane expression of M-CSFR in various human cells with flow cytometry. As expected, the HL-60, U937 and THP-1 myeloid cell lines intrinsically expressed this cytokine receptor, in contrast to the erythroblastic TF-1, epithelial HEK293 and HOS osteosarcoma cells which did not express the M-CSFR (Figure 1A). The lack of M-CSFR expression by the TF-1, HEK and HOS cells was confirmed by quantitative PCR (data not shown). Marked expression of the M-CSFR at the cell membrane was observed in M-CSFR transfected TF-1, HEK and HOS cells (Figure 1A).

Based on these observations, we investigated the functionality of the receptor by studying the cell signalling induced by M-CSF and IL-34, the two identified ligands of the M-CSFR (Figures 1B-E). In HEK M-CSFR cells, both cytokines induced in a dose- (data not shown) and time-dependent manner the tyrosine 546, 699, 708, 723 and 923 phosphorylation of the receptor's intracellular chains with a peak at 5 min (Figure 1B). Similarly, M-CSF and IL-34 induced the phosphorylation of Erk1/2. Compared to M-CSF, IL-34 induced a weaker activation of the signalling pathway mediated by M-CSFR activation, at the same dose and time (Figure 1B). In the HOS M-CSFR cells, high doses of IL-34 (200 ng/mL) appeared more efficient at inducing the phosphorylation of the M-CSFR compared to M-CSF, unlike low doses of the cytokine (50 ng/mL) (Figure 1C). This higher activation of the M-CSFR by IL-34 was also observed with 100 ng/mL of cytokine in the TF-1 M-CSFR cells as demonstrated by fast and huge phosphorylation levels of the M-CSFR (Figure 1D), data in agreement with Chihara et al. [13]. We then investigated M-CSFR activation in the THP-1 cells which intrinsically expressed the receptor chains (Figure 1E). Consistent with the findings obtained with the TF-1 M-CSFR cells, the M-CSFR and MAPK downstream signalling pathways were

more activated by IL-34 than by M-CSF (Figure 1E, Supplementary data 1A). Similar results were obtained in the U937 cells (Supplementary data 1B). Overall, these data revealed that IL-34 and M-CSF differentially activated the M-CSFR signalling pathway according to the cell type studied.

3.2. IL-34 binds to HEK and HOS cells lacking the M-CSFR

In light of the differential signalling pathway induced by IL-34 and M-CSF, binding assays using ^{125}I-ligands were carried out on the different cell lines of interest. A saturation binding curve was obtained, which after Scatchard transformation, made it possible to estimate the affinity of IL-34 for the membrane M-CSFR. ^{125}I-IL34 bound all cell lines studied with an equilibrium dissociation constant around 2 nM, 0.55 nM, 1 nM and 0.75 nM for U937, THP-1, HEK M-CSFR and HOS M-CSFR cells respectively (Figure 2A-D). As expected, ^{125}I-MCSF bound to the HEK M-CSFR and HOS M-CSFR with a similarly high affinity (K_d = 0.35 nM). These data demonstrated the capacity of IL-34 to bind with a high affinity to cell lines expressing the M-CSFR (Figure 2D).

We next assessed the potency of recombinant IL-34 and/or M-CSF to compete with ^{125}I-IL34 binding to cell surface receptors (Figures 3A, B). The results demonstrated that cold IL-34 and M-CSF differentially competed with the ^{125}I-IL34 binding sites expressed in the HEK M-CSFR, as revealed by the slope factor of the inhibition curves (0.8 and 2 for M-CSF and IL-34 respectively) (Figure 3A,B). Similar results were obtained with the HOS M-CSFR cells (data not shown). ^{125}I-IL34 binding assays were then performed in HEK Mock cells transfected with the empty control vector that did not express the M-CSFR (Figure 1A, Figure 3C). Surprisingly, specific ^{125}I-IL34 specific binding sites were detected at the surface of the HEK Mock cells (Figure 3C,D). This binding was characterised by a lower affinity (K_d = 14.6

nM) than those observed in the HEK M-CSFR (Figure 2B). In contrast to ^{125}I-IL34, ^{125}I-M-CSF did not bind to cells lacking the M-CSFR (Supplementary data 2). Complementary SPR experiments reinforced these results and confirmed the ability of the HEK Mock cells to bind to recombinant IL-34 spotted on a sensorchip, in contrast to recombinant M-CSF, as measured by the increasing percentage of reflectivity (Figure 3E). The results clearly demonstrated an additional binding mode for IL-34 independent of the M-CSFR.

3.3. IL-34 binding to chondroitin sulfate chains modulates M-CSFR activation

As IL-34 bound with a relatively low affinity to cells that did not express the M-CSFR (HEK Mock and HOS Mock), cell signalling was further investigated in these cells after stimulation with a high dose of IL-34 (200ng/mL). As shown in Supplementary data 3, the profile of the phosphorylated tyrosines was not modified after IL-34 stimulation (Supplementary data 3A) and no modulation of the different signalling pathways such as mTor, Akt, (Supplementary data 3B), β-catenin, p38 and Erk1/2 (data not shown) was observed in the presence of IL-34. Consistent with the ability of IL-34 to bind to glycosaminoglycans [27], we assessed the IL-34 binding to chondroitin sulfate and heparin/heparan sulfate chains by SPR. The SPR sensorgrams revealed a low affinity binding of the various types of chondroitin sulfate to IL-34, with a K_d of 132 nM, 775 nM and 16.1 nM for chondroitin sulfates A (Figure 4A), B (Figure 4B) and C (Figure 4C) respectively. On the contrary, heparin (Figure 4D) and heparan sulfate (data not shown) were not able to bind IL-34, even with high doses of up to 500 µg/mL.

In light of these data, the chondroitin sulfate chains may be behind the IL-34 binding to the HEK Mock and HOS Mock cells, as well as the differential signalling pathways induced by M-CSF or IL-34 in the HEK M-CSFR. To explore this hypothesis, HEK M-CSFR

cells were then treated with chondroitinases A, B, C solution (chABC) to abrogate the chondroitin sulfates (CS) present at the cell surface prior to study of the M-CSF- or IL-34-induced signalling pathways. Consistent with the results presented in Figure 1B, the M-CSFR was less activated after addition of 50 ng/mL of IL-34 compared to stimulation with the same dose of M-CSF, as illustrated by the phosphorylation levels of tyrosines 546, 708 and 723 (Figure 5A). Interestingly, the chABC treatment markedly increased the levels of M-CSFR phosphorylations induced by IL-34 as demonstrated by the phosphorylation levels of M-CSFR, whereas the response induced by M-CSF remained unchanged (Figure 5A). Similar results were obtained for ERK1/2. Both cytokines were also pre-incubated with 1, 3 or 10 µg/mL CS salts for 1h at 37°C prior to their addition to the HEK M-CSFR cells. The addition of CS did not modulate the M-CSF-induced signalling pathway (Figure 5B). On the contrary, pre-incubation of CS with IL-34 led to increased phosphorylation of the receptor tyrosines 708 and 723 (Figure 5B). Overall, these data clearly demonstrated that CS strongly regulates the biological activities of IL-34.

3.4. IL-34 binding to chondroitin sulfate is independent of the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ)

RPTPβ/ζ, a proteoglycan mainly expressed in the central nervous system, was recently identified on the glioblastoma cell line U251 as another receptor for IL-34 through its CS chains, explaining the role of the cytokine in brain development [27]. We then analysed RPTPβ/ζ expression in THP-1, TF-1, HOS Mock and HEK Mock cells. The RPTPβ/ζ was not expressed at either the transcriptional or the protein levels in any of these cells in contrast to the U251 cells used as a positive control (Figure 6). IL-34 binding to the HEK Mock and HOS Mock cells was consequently independent of RPTPβ/ζ.

3.5. The IL-34-induced migration of myeloid cells is syndecan-1 dependent

Within the proteoglycan families, the syndecans control a lot of biological functions, such as angiogenesis, cytokine bioavailability, cell adhesion/interactions, etc. To investigate the functional involvement of syndecans in IL-34 biology, syndecans 1, 2 and 4 were individually overexpressed in the HEK M-CSFR cells and the phosphorylation levels of the M-CSFR were then analysed in the presence of IL-34. The, overexpression of syndecan-1 in the HEK M-CSFR cells exacerbated the cell response to IL-34 (Figure 7A,B). Indeed, this overexpression induced stronger activation of the M-CSFR when IL-34 was added compared to the effects in non-transfected cells (NT) or cells transfected with an empty plasmid (Mock), as shown by the increased phosphorylations of 708, 723 tyrosines (Figure 7B). In contrast, the overexpression of syndecan-2 or -4 did not have any impact on M-CSFR activation by IL-34 (Supplementary data 4). The addition of a blocking syndecan-1 antibody reduced the IL-34-induced M-CSFR activation as measured by the levels of 708, 723 tyrosine phosphorylations in cells overexpressing syndecan-1 (Figure 7C). Anti-syndecan-1 antibody did not modulate by itself the M-CSFR phosphorylation while it upmodulated IL-34 effects on M-CSFR activation in non transfected cells (Figure 7D). To confirm the involvement of syndecan-1 in the IL-34 induced signalization, the syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA. As expected, syndecan-1 siRNA significantly down regulated the expression of the membrane protein as observed by flow cytometry (Figure 8A). Similarly to the addition of a blocking syndecan-1 antibody, the knocking down of syndecan-1 upregulated the M-CSFR phosphorylations (Figure 8B). Similar results were obtained for ERK1/2. We then compared the IL-34 dose response on the M-CSFR phosphorylations in HEK M-CSFR subpopulations expressing differentially syndecan-1 (Supplementary data 5). The data showed that IL-34-induced M-CSFR phosphorylations reached a plateau in the

presence of 25 ng/mL for pTyr-708 whereas no plateau was observed in cells expressing higher syndecan-1 expression even in the presence of 100 ng/mL of IL-34 (Supplementary data 5). These data demonstrated that syndecan-1 was able to modulate the biological activities of IL-34 through the M-CSFR.

To elucidate the functional implication of the syndecan-1/IL-34 interactions, we first analysed the expression of syndecan-1 in various cell types (Figure 9A). Syndecan-1 appears highly expressed by several cell types with hematopoietic (THP-1), mesenchymal (HOS Mock) and epithelial (HEK Mock) origin in contrast to U937 and TF-1 cells. IL-34 significantly increased the migration of THP-1 cells compared to the control (Figure 9B, $p < 0.001$). This IL-34-induced THP-1 cell migration was abolished by the addition of a blocking anti-syndecan-1 antibody. We then investigated the expression of syndecan-1 by human monocytes/macrophages (Figure 9C). $CD14^+$ cells expressed a very low level of syndecan-1 and interestingly, when $CD14^+$ cells were differentiated toward M2a macrophages ($CD14^{low}$, $CD200R^+$, $CD86^+$), differentiated cells highly expressed the syndecan-1 (Figure 9C). We then investigated the effects IL-34 on syndecan-1 expressing M2a macrophages. IL-34 significantly stimulated the migration of M2a macrophages (Figure 9C, $p < 0.001$). Similarly to the THP-1 cells, the addition of a blocking anti-syndecan-1 antibody totally inhibited the IL-34-induced M2a macrophage migration. Anti-syndecan-1 antibody had no effect on cell proliferation or on IL-34-associated monocyte survival (data not shown). Overall, these investigations demonstrated that IL-34 induced the migration of myeloid cells in a syndecan-1 dependent manner.

4. DISCUSSION

IL-34 and M-CSF are considered as “twin” cytokines sharing common M-CSFR chains. However, while both cytokines have some functional overlaps, they also have specific activities, especially in the differentiation and activation of the monocyte/macrophage lineage, effects in favour of the existence of an additional, unknown receptor for IL-34 [13, 18-20]. Recently, Nandi et al. [27] highlighted an alternative receptor for IL-34, the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), known to increase tyrosine phosphorylations of downstream proteins such as β-catenin, Src family kinases, focal adhesion kinase or Erk1/2. The present manuscript characterized a new binding mode for IL-34 to cells that express neither the M-CSFR, nor the chondroitin sulfate proteoglycan RPTPβ/ζ. This new IL-34 binding is dependent on chondroitin sulfate chains, and syndecan-1 was identified as regulating the IL-34 activation levels of the M-CSFR.

RPTPβ/ζ interacts with IL-34 through its chondroitin sulfate chains [27]. These authors demonstrated with enzymatic treatment and CS addition that chondroitin sulfate competed with IL-34 for binding to the extracellular domain of RPTPβ/ζ, revealing binding dependence on RPTPβ/ζ chondroitin sulfate chains. Like RPTPβ/ζ, chondroitin sulfate moieties seem to be a prerequisite for IL-34 binding to syndecan-1. RPTPβ/ζ and syndecan-1 are transmembrane proteoglycans with common features. Both proteins have glycosaminoglycans on their ectodomain, and their intracellular C-terminal moiety interacts with proteins containing the PDZ domain [28,29]. The polymorphism of glycosaminoglycans attached to the protein core of syndecan-1 is due to different post-translational modifications and is directly linked to tissue-specific functions [30]. Thus, the degree of glycosylation of the extracellular domain explains the versatility of the syndecan family. Syndecan-1 belongs to a proteoglycan family including four members: syndecan-1 mainly expressed in epithelial cells

[31], syndecan-2 mainly present in mesenchymal cells [32], syndecan-3 mostly found in neuronal and cartilage tissues [33] and syndecan-4 which is ubiquitously expressed [34]. All members of this family have heparan sulphate chains but only syndecan-1 and syndecan-3 display chondroitin sulfate chains on their protein core. Syndecan-4 has been also described, to a lesser extent, in a chondroitin sulphate-associated form [35]. Based on these data, IL-34 binding to the various syndecans must be considered according to tissue context and, for instance, IL-34 binding to syndecan-3 must not be excluded in the brain, especially regarding the importance of both IL-34 and syndecan-3 in the neuronal environment.

Proteoglycans are commonly devoted to numerous biological functions [36] and it is accepted that they control the activities of a lot of cytokines/growth factors with or without glycosaminoglycan binding domains such as the heparin binding domain. Thus, the various proteoglycans serve as a reservoir for cytokines/growth factors and also modulate their induced signalling pathways by acting directly on the cytokine/growth factors-receptor interaction or by the regulation of cytokine activation and/or turnover [37]. In light of these pleiotropic activities, glycosaminoglycans may be involved in the sequestration of IL-34 at the cell membrane as revealed by the chondroitinase treatment that increased the phosphorylation levels of the M-CSFR induced by IL-34 (Figure 5A). Abrogation of membrane chondroitin sulphate chains may then reduce the trapping of IL-34 in these chains and may facilitate IL-34 binding to the M-CSFR (Figure 10A, B). Similarly, the pre-incubation of chondroitin sulphate chains with IL-34 upmodulated the signal transduction through the M-CSFR (Figure 5B). In this context, soluble chondroitin sulphate chains may act as competitors to their homologous membrane forms and may limit IL-34 sequestration at the cell surface, facilitating its interaction with the M-CSFR (Figure 10C). Interestingly, soluble chondroitin sulfates compete with IL-34 for binding to the extracellular domain of RPTPβ/ζ [27], in contrast to the extracellular domain of M-CSFR (present data).

The co-receptor functions of proteoglycans are well illustrated by their interaction with the fibroblast growth factor and its receptor (FGF/FGFR). The FGF was the first growth factor identified as being dependent on heparin/heparan sulphate chains for its binding to the FGFR. The ternary complex FGF/FGFR/proteoglycans formed directly modulates the signalling pathways induced by the growth factor [38]. The co-receptor role of glycosaminoglycans has then also been demonstrated for epidermal growth factor (EGF) [39], hepatocyte growth factor (HGF) [40], vascular endothelial growth factor (VEGF) [41], Wnt factors [42] or members of the transforming growth factors (TGFs) [43]. In these cases, syndecan-1, via its heparan sulphate chains, acts as a co-receptor for these growth factors. Our present results suggest that syndecan-1 may also act as a modulator for the tyrosine kinase M-CSFR, but through its chondroitin sulfate chains. Syndecan-1 exhibits a stimulatory or inhibitory role, probably depending on its expression level. Thus, a low/moderate level of syndecan-1 may sequestrate IL-34 at the cell surface through its chondroitin sulfate chains, limiting the interaction between IL-34 and the M-CSFR (Figure 8D). The overexpression of syndecan-1 induced significant phosphorylation of the M-CSFR, reinforced by the presence of IL-34. Interestingly, the overexpression of syndecan-1 modulate the pTyr723 of the M-CSFR that was already associated with the activation of specific signaling pathways (PI3K, Rho GTPases, paxilline, SFKs/Pyk2, etc) involved in cell migration [2, 44]. In contrast, a blocking anti-syndecan-1 antibody significantly reduced the effects of IL-34. The enhanced activation by syndecan-1 may be explained by the membrane proximity between the M-CSFR and syndecan-1, the biodistribution of syndecan-1 at the cell membrane being directly related to its expression level. IL-34 stimulation did not induce detectable signalling pathways (Supplementary data 2) in MOCK cells expressing syndecan-1 but not the M-CSFR and the RPTPβ/ζ. These findings strengthen the hypothesis of a regulator for syndecan-1 on IL-34 signalling through the M-CSFR. Previous works have already identified specialised docking

sites for other receptors in the syndecan extracellular domains, such as integrins and tyrosine kinase receptors. Thus, αvβ3 integrin and insulin-like growth factor 1 receptor (IGF1R) are captured by syndecan-1 to form a ternary receptor mainly expressed on tumour cells and endothelial cells and involved in the angiogenesis process [45]. Further investigations will be necessary to investigate the potential role of syndecan-1 in the IL-34 trapping.

Syndecan-1 is implicated in the control of cellular chemoattraction. Indeed, membrane proteoglycans, including the syndecan family, are responsible of a chemotactif gradient on endothelial cells linked to the adherence and migration of numerous circulating cells such as neutrophils [46]. Syndecan-1 mediated the osteoprotegerin-induced chemotaxis in human peripheral blood monocytes and may control by this mechanism, the inflammatory process and the bone remodelling [47, 48]. Proteoglycans like syndecan-1 contributes to the control of the pathogenesis of osteolysis [49, 50]. Thus, tumor-derived syndecan-1 is associated a crosstalk with bone and an increase of osteoclastogenesis [49, 50]. By interacting with syndecan-1, IL-34 may have a double involvement in osteoclastogenesis, by inducing the migration of myeloid osteoclast precursors expressing syndecan-1 and by substituting for M-CSF in RANKL-induced osteoclastogenesis [5]. In addition, IL-34 was already shown to drives in vitro the polarization of macrophages towards an M2 immunosuppressive phenotype and function [4]. Several reports have described the accumulation of TAMs in tissue during the tumour development and TAMs are considered to facilitate the metastatic process [51]. We showed that syndecan-1 controls the IL-34-induced M2 macrophage migration (Figure 9), which can be related to the role of IL-34 in the biology of M2 macrophages [4]. The increased expression of syndecan-1 by macrophages was already observed during the myeloid differentiation and during the inflammatory process [52, 53]. These authors found a link between macrophage motility and the expression level of syndecan-1.

Syndecan-1 can now be added to the list of key regulators of IL-34 activities in addition to M-CSFR and RPTPβ/ζ, but not of M-CSF. By controlling IL-34 bioavailability, by modulating the recruitment of M-CSFR chains or the affinity of IL-34 to the receptor chains, and/or by acting as a regulator signalling of the M-CSFR, syndecan-1 may play a part in the role of IL-34 in development [22], bone [5] and inflammatory diseases [6]. Consequently, new therapies targeting the IL-34/M-CSFR axis must now take into account the existence of other receptors for the cytokine.

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5. REFERENCES

[1] R.P. Bourette, J.A.J Rohrschneider, Early events in M-CSF receptor signaling, Growth Factors Chur. Switz. 17 (2000) 155–166.

[2] F.J., E.R. Stanley, CSF-1 regulation of the wandering macrophage: complexity in action, Trends Cell Biol. 14 (2004) 628–638.

[3] H. Lin, E. Lee, K. Hestir, C. Leo, M. Huang, E. Bosch, R. Halenbeck, G. Wu, A. Zhou, D. Behrens, D. Hollenbaugh, T. Linnemann, M. Qin, J. Wong, K. Chu, S.K. Doberstein, L.T. Williams, Discovery of a Cytokine and Its Receptor by Functional Screening of the Extracellular Proteome, Science 320 (2008) 807–811.

[4] E.D. Foucher, S. Blanchard, L. Preisser, E. Garo, N. Ifrah, P. Guardiola, Y. Delneste, P. Jeannin, IL-34 Induces the Differentiation of Human Monocytes into Immunosuppressive Macrophages, Antagonistic Effects of GM-CSF and IFNγ, PloS One 8 (2013) e56045.

[5] M. Baud'Huin, R. Renault, C. Charrier, A. Riet, A. Moreau, R. Brion, F. Gouin, L. Duplomb, D. Heymann, Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis, J. Pathol. 221 (2010) 77–86.

[6] M. Chemel, B. Le Goff, R. Brion, C. Cozic, M. Berreur, J. Amiaud, G. Bougras, S. Touchais, F. Blanchard, M.F. Heymann, J.M. Berthelot, F. Verrecchia, D. Heymann, Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients, Ann. Rheum. Dis. 71 (2012) 50–154.

[7] E.A. Boström, P. Lundberg, The Newly Discovered Cytokine IL-34 Is Expressed in Gingival Fibroblasts, Shows Enhanced Expression by Pro-Inflammatory Cytokines, and Stimulates Osteoclast Differentiation, PLoS ONE 8 (2013) e81665.

[8] P. Roth, E.R. Stanley, The biology of CSF-1 and its receptor, Curr. Top. Microbiol. Immunol. 181 (1992) 141–167.

[9] J. Felix, J. Elegheert, I. Gutsche, A.V. Shkumatov, Y. Wen, N. Bracke, E. Pannecoucke, I. Vandenberghe, B. Devreese, D.I. Svergun, E. Pauwels, B. Vergauwen, S.N. Savvides, Human IL-34 and CSF-1 establish structurally similar extracellular assemblies with their common hematopoietic receptor, Structure 21 (2013) 528–539.

[10] X. Ma, W.Y. Lin, Y. Chen, S. Stawicki, K. Mukhyala, Y. Wu, F. Martin, J.F. Bazan, M.A. Starovasnik, Structural Basis for the Dual Recognition of Helical Cytokines IL-34 and CSF-1 by CSF-1R, Structure 20 (2012) 676–687.

[11] C.J. Sherr, Colony-stimulating factor-1 receptor, Blood 75 (1990) 1–12.

[12] H. Liu, C. Leo, X. Chen, B.R. Wong, L.T. Williams, H. Lin, X. He, The mechanism of shared but distinct CSF-1R signaling by the non-homologous cytokines IL-34 and CSF-1, Biochim. Biophys. Acta 1824 (2012) 938–945.

[13] T. Chihara, S. Suzu, R. Hassan, N. Chutiwitoonchai, M. Hiyoshi, K. Motoyoshi, F. Kimura, S. Okada;, IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation, Cell Death Differ. 17 (2010)1917–1927.

[14] X.M. Dai, G.R. Ryan, A.J. Hapel, M.G. Dominguez, R.G. Russell, S. Kapp, V. Sylvestre, E.R. Stanley, Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects, Blood 99 (2002) 111–120.

[15] H. Yoshida, S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo, L.D. Shultz, S. Nishikawa, The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene, Nature 345 (1990) 442–444.

[16] W. Wiktor-Jedrzejczak, A. Bartocci, A.W. Jr. Ferrante, A. Ahmed-Ansari, K.W. Sell , J.W. Pollard, E.R. Stanley, Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4828–4832.

[17] S. Wei, S. Nandi, V. Chitu, Y.G. Yeung, W. Yu, M. Huang, L.T. Williams, H. Lin, E.R. Stanley, Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells, J. Leukoc. Biol. 88 (2010) 495 – 505.

[18] F. Ginhoux, M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M.F. Mehler, S.J. Conway, L.G. Ng, E.R. Stanley, I.M. Samokhvalov IM, M. Merad M, Fate mapping analysis reveals that adult microglia derive from primitive macrophages, Science 330 (2010) 841–845.

[19] M. Greter, I. Lelios, P. Pelczar, G. Hoeffel, J. Price, M. Leboeuf, T.M. Kündig, K. Frei, F. Ginhoux, M. Merad, B. Becher, Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia, Immunity 37 (2012) 1050–1060.

[20] Y. Wang, K.J. Szretter, W. Vermi, S. Gilfillan, C. Rossini, M. Cella, A.D. Barrow, M.S. Diamond, M. Colonna, IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia, Nat. Immunol. 13 (2012) 753–760.

[21] D. Heymann, Interleukin-34: An Enigmatic Cytokine, IBMS BoneKEy 7 (2010) 406–413.

[22] S. Nandi, S. Gokhan, XM Dai, S. Wei, G. Enikolopov, H. Lin, M.F. Mehler, E.R. Stanley, The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation, Dev. Biol. 367 (2012) 100–113.

[23] J. Vandesompele, K.D. Preter, F. Pattyn, B. Poppe, N.V. Roy, A.D. Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034.

[24] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622.

[25] F. Tejedor, J.P. Ballesta, Iodination of biological samples without loss of functional activity, Anal. Biochem. 127 (1982) 143–149.

[26] A. Godard, D. Heymann, S. Raher, I. Anegon, M.A. Peyrat, B. Le Mauff, E. Mouray, M. Gregoire, K. Virdee, J.P. Soulillou, High and low affinity receptors for human interleukin for DA cells/leukemia inhibitory factor on human cells. Molecular characterization and cellular distribution, J. Biol. Chem. 267(1992) 3214–3222.

[27] S. Nandi, M. Cioce, Y.G. Yeung, E. Nieves, L. Tesfa, H. Lin, Hsu A.W., R. Halenbeck, H.Y. Cheng, S. Gokhan, M.F. Mehler, E.R. Stanley, Receptor-type protein tyrosine phosphatase zeta is a functional receptor for interleukin-34, J. Biol. Chem. 288 (2013) 21972-21986

[28] A. Fujikawa, M. Fukada, Y. Makioka, R. Suzuki, J.P.H. Chow, M. Matsumoto, M. Noda Consensus substrate sequence for protein-tyrosine phosphatase receptor type Z, J. Biol. Chem. 286 (2011) 37137–37146.

[29] J.R. Couchman, Transmembrane Signaling Proteoglycans, Annu. Rev. Cell Dev. Biol. 26 (2010) 89–114.

[30] R.D. Sanderson, M. Bernfield, Molecular polymorphism of a cell surface proteoglycan: distinct structures on simple and stratified epithelia, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9562–9566.

[31] S. Saunders, M. Jalkanen, S. O'Farrell, M. Bernfield, Molecular cloning of syndecan, an integral membrane proteoglycan, J. Cell Biol. 108 (1989) 1547–1556.

[32] P. Marynen, J. Zhang, J.J. Cassiman, H. Van den Berghe, G. David, Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. Prediction of an integral membrane domain and evidence for multiple distinct core proteins at the cell surface of human lung fibroblasts, J. Biol. Chem. 264 (1989) 7017–7024.

[33] D.J. Carey, D.M. Evans, R.C. Stahl, V.K. Asundi, K.J. Conner, P. Garbes, G. Cizmeci-Smith, Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan, J. Cell Biol. 117 (1992) 191–201.

[34] G. David, B. van der Schueren, P. Marynen, J.J. Cassiman, H. van den Berghe Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells, J. Cell Biol. 118 (1992) 961–969.

[35] C. Yeaman, A.C. Rapraeger, Membrane-anchored proteoglycans of mouse macrophages: P388D1 cells express a syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form, J. Cell Physiol. 157 (1993) 413–425.

[36] G.A. Karasneh, M. Ali, D. Shukla, An Important Role for Syndecan-1 in Herpes Simplex Virus Type-1 Induced Cell-to-Cell Fusion and Virus Spread, PLoS ONE 6 (2011) e25252.

[37] H. Kresse , E. Schönherr, Proteoglycans of the extracellular matrix and growth control. J. Cell Physiol. 189 (2001) 266–274.

[38] N. Jastrebova, M. Vanwildemeersch, U. Lindahl, D. Spillmann, Heparan sulfate domain organization and sulfation modulate FGF-induced cell signaling, J. Biol. Chem. 285 (2010) 26842–26851.

[39] R. Takazaki, Y. Shishido, R. Iwamoto, E. Mekada, Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like Growth Factor, J. Biol. Chem. 279 (2004) 47335–47343.

[40] L.E. Kemp, B. Mulloy, E. Gherardi, Signalling by HGF/SF and Met: the role of heparan sulphate co-receptors Biochem. Soc. Trans. 34 (2006) 414–417.

[41] J. Dai, A.B.M. Rabie, VEGF: an essential mediator of both angiogenesis and endochondral ossification, J. Dent. Res. 86 (2007) 937–950.

[42] C. Niehrs, The complex world of WNT receptor signalling, Nat. Rev. Mol. Cell. Biol. 13 (2012) 767–779.

[43] C.C. Rider, Heparin/heparan sulphate binding in the TGF-β cytokine superfamily, Biochem. Soc. Trans. 34 (2006) 458.

[44] N.G. Sampaio, W. Yu, D. Cox, J. Wyckoff, J. Condeelis, E.R. Stanley, F.J. Pixley, Phosphorylation of CSF-R Y721 mediates its association with PI3K to regulate macrophage motility and enhancement of tumor invasion, J. Cell Sci. 124 (2011) 2021-2031.

[45] A.C. Rapraeger, Synstatin: a selective inhibitor of the syndecan-1-coupled IGF1R-αvβ3 integrin complex in tumorigenesis and angiogenesis, FEBS J. 280 (2013) 2207–2215.

[46] S. Massena, G. Christoffersson, E. Hjertström, E. Zcharia, I. Vlodavsky, N. Ausmees, C. Rolny, J.P. Li, M. Phillipson, A chemotactif gradient sequestered on endothelial heparan sulfate induces directionnal intraluminal crawling of netutrophils, Blood 116 (2010) 1942-1931.

[47] B.A. Mosheimer, N.C. Kaneider, C. Feistritzer, A.M. Djanani, D. H. Sturn, J.R. Patsch, C.J. Wiedermann, Syndecan-1 is involved in osteoprotegerin-induced chemotaxis in human peripheral blood monocytes, J. Clin.Endocrinol. Metab. 90 (2005) 2964-2971.

[48] Z. Bensliman-Ahmin, F. Poirier, C. Delomenie, A. Lokajczyk, F. Grelac, I. Galy-Fauroux, A. Mohamedi, A.M. Fischer, D. Heymann, D. Lutomski, C. Boisson-Vidal, Mechanistic study of the pro-angiogenic effect of osteoprotegerin, Angiogenesis 16 (2013) 575-593.

[49] T. Kelly, L.J. Suva, K.M. Nicks, V. MacLeod, R.D. Sanderson, Tumor-derived syndecan-1 mediates distal cross-talk with bone that enhances osteoclastogenesis, J. Bone Miner. Res. 25 (2010) 1295-1304.

[50] M. Baud'huin, C. Charrier, G. Bougras, R. Brion, F. Lezot, M. Padrines, D. Heymann, Proteoglycans and osteolysis, Methods Mol. Biol. 836 (2012) 323-337.

[51] J. Cook, T. Hagemann T., Tumour-associated macrophages and cancer. Curr. Opin. Pharmacol. 13 (2013) 595-601.

[52] M. Averbeck, C. Gebbhardt, U. Anderegg, C. Termeer, J.P., Sleeman, J.C. Simon, Swith in syndecan-1 and syndecan-4 expression controls maturation associated dendritic cell motility. Exp. Dermatol. 16 (2007) 580-589

[53] C. Yeaman, A.C. Rapraeger, Post-transcriptional regulation of syndecan-1 expression by cAMP in peritoneal macrophages. J. Cell Biol. 122 (1993) 941-950.

FIGURE LEGENDS

Figure 1: IL-34 and M-CSF differentially activate the M-CSFR. (**A**) M-CSFR expression on the cell membrane of human cells analysed by flow cytometry. TF-1: erythroblastic leukaemia cell line, HEK293: embryonary cell line; HOS: osteosarcoma cell line; HL-60, U-937 and THP-1: myeloid leukaemic cell lines; HOS M-CSFR and HEK M-CSFR: HOS and HEK stably overexpressing M-CSFR; HOS Mock and HEK Mock: HOS and HEK cell lines transfected with the empty control vector. The isotype IgG1 was used as the control (in grey on the graphs). (**B-E**) Western blot investigations of tyrosine phosphorylation patterns of M-CSFR and Erk1/2 phosphorylation after addition of 50, 100 or 200 ng/mL of IL-34 or M-CSF for 1 to 30 minutes, in HEK M-CSFR (**B**), HOS M-CSFR (**C**), TF-1 M-CSFR (**D**) and THP-1 cells (**E**). All experiments were performed three times in independent conditions and a representative set of experiments is shown.

Figure 2: IL-34 binds to M-CSFR with a high affinity. Increasing concentrations of ^{125}I-labelled IL-34 (IL-34*) or ^{125}I-labelled M-CSF (M-CSF*) were incubated for 60 min at 4°C with 10^6 cells and bound and unbound fractions were determined as described in "Experimental Procedures". Curve fitting was performed as described in the "Materials and Methods" section. Typical curves revealing a specific binding fraction of iodinated M-CSF on HEK M-CSFR cells (**A**) and of iodinated IL-34 on HEK M-CSFR **(B**) and THP-1 cells (**C**). Table summarizing the binding mode of M-CSF and IL-34 on the cells of interest (**D**). These experiments are representative of three times in independent conditions and a representative experiment is shown.

Figure 3: A new IL-34 binding independent of the M-CSFR. (**A-B**) Inhibition binding assays were carried out on HEK M-CSFR cells. After incubation of HEK M-CSFR cells with a saturating concentration of ^{125}I-labelled IL-34 (IL-34*), increasing doses of unlabelled IL-34 (**A**) or unlabelled M-CSF (**B**) were added to the cells. (**C**) Binding of ^{125}I-labelled IL-34 was assessed by Scatchard assay on HEK Mock cells which did not express the M-CSFR. (**D**) Competition assay of ^{125}I-labelled IL-34 with unlabelled IL-34 was performed on HEK Mock cells. (**E**) Binding of HEK Mock cells on IL-34 or M-CSF spotted on a chip analysed by Surface Plasmon Resonance. All experiments were repeated three times in independent times.

Figure 4: IL-34 binds to chondroitin sulfate but not to heparin/heparan sulfate chains. Interactions between IL-34 and glycosaminogycans were studied by surface plasmon resonance technology. Chondroitin sulfate A (**A**), chondroitin sulfate B (**B**), chondroitin sulfate C (**C**), or heparin (**D**) were injected on immobilised IL-34 using the parameters described in the "Materials and Methods" section.

Figure 5: Chondroitin sulfate chains regulate the M-CSFR phosphorylations induced by IL-34. (**A**) HEK M-CSFR cells were treated with a mixture of chondroitinases A, B and C for 90 min at 37°C, before addition of 50 ng/mL of M-CSF or IL-34 for 5 min. (**B**) HEK M-CSFR cells were pre-incubated with 1, 3 or 10 µg/mL of chondroitin sulfate salts for 90 min at 37°C before addition of 50 ng/mL of M-CSF or IL-34 for 5 min. The phosphorylation patterns of the M-CSFR and ERK1/2 were assessed by Western blot. Total forms of M-CSFR, ERK1/2 and β-actin were used as a loading control. Bar graphs show relative densitometric values of pTyr-723 normalized to the M-CSFR. Similar densitometric measurments were obtained for pTyr-746 or pTyr-708, normalized to the M-CSFR or to β-actin. These

experiments were performed three times in independent conditions and a representative experiment is shown.

Figure 6: HEK, HOS, THP-1 and TF-1 do not express the receptor protein tyrosine phosphatase RPTPβ/ξ. The expression of the receptor protein tyrosine phosphatase RPTPβ/ξ was assessed in the different cells used in the study both at the transcriptional level by quantitative RT-PCR (**A**), where expression was normalised against GAPDH mRNA levels, and at the protein level by Western Blot, where β-actin was used as a loading control (**B**). These experiments were performed three times in independent conditions and a representative experiment is shown.

Figure 7: Syndecan-1 modulates the M-CSFR phosphorylations induced by IL-34. HEK M-CSFR were transiently transfected with a plasmid-encoding human syndecan-1. (**A**) Syndecan-1 expression analysed by flow cytometry, 48 h after transient transfection. Syndecan-1 expression in Mock M-CSFR transfected with an empty vector of syndecan-1 (empty histogram). Syndecan-1 overexpressing cells were stimulated with or without 50 ng/mL of IL-34 for 5 min (**B**) in the presence or absence of 20 µg/mL of syndecan-1 blocking antibody pre-incubated for 90 min (**C**). Similar experiments were performed on HEK M-CSFR (non transfected for syndecan-1) with or without 100 ng/mL of IL-34 and/or 10 µg/ml of anti-syndecan-1 antibody for 1 and 5 minutes (**D**). M-CSFR- and ERK1/2-phosphorylations were assessed by Western Blot, with the M-CSFR, ERK1/2 total forms of and GAPDH as a loading control. Bar graphs show relative densitometric values of pTyr-723 and pTyr-708 normalized to the M-CSFR. These experiments were performed four times in independent conditions and a representative experiment is shown.

Figure 8: Syndecan-1 siRNA increases the phoshorylation M-CSFR induced by IL-34. The syndecan-1 gene expression was knocked down using specific human syndecan-1 siRNA. (**A**) Syndecan-1 expression analysed by flow cytometry, 48 h after siRNA transfection. (**B**) Syndecan-1 knocked down cells were stimulated with or without 50 ng/mL of IL-34 for 5 min and M-CSFR-, ERK1/2-phosphorylations were assessed by Western Blot, with the total forms of M-CSFR and ERK1/2 as a loading control. Bar graphs show relative densitometric values of pTyr-723 normalized to the M-CSFR. Similar densitometric measurments were obtained for pTyr-708, normalized to the M-CSFR or to GAPDH. A representative experiment out of three independent experiments is shown.

Figure 9: IL-34 increases the migration of myeloid cells through the syndecan-1. (A) Syndecan-1 expression by various myeloid, mesenchymal and epithelial cells analysed by flow cytometry. (**B**) THP-1 cells were preincubated with antibody to syndecan-1 (B-B4) or -4 the correspond isotype matched IgG (IgG1) for 15 min. After washing twice, migration toward IL-34 (100 ng/mL) was tested for 6 hours. (**C**) M2a macrophage were obtained from $CD14^+$ cells isolated from peripheral blood of healthy donors, cultured and differentiated in in RPMI supplemented with 1% human serum, 1% penicillin/streptomycin, 50 ng/mL of IL-4 for 5 days. The expression of syndecan-1 and their phenotype ($CD14^{low}$, $CD220R^+$, $CD86^+$) were analysed by flow cytometry. M2a cells were collected by gentle scraping and their migration ability in the presence of IL-34 with or withoutan a blocking anti-syndecan-1 antibody was assessed as described for the THP-1 cells. The histograms show the percentage of the migrated cells compared to the IgG1 control. Graphs represent the mean ±SD of three independent experiments done in triplicate in each case. A Kruskal-Wallis test was performed followed by a Dunn's post-hoc between all the conditions. *** $p < 0.001$.

Figure 10: Schematic diagram hypothesizing the functional involvement of syndecan-1 in the bioavailability and activities of IL-34. Potential interactions of IL-34 with M-CSFR, Syndecan-1 and chondroitin sulfates in control conditions (**A**), after chondroitinase ABC treatment (**B**), in the presence of exogenous chondroitin sulfate chains (**C**), and according to the levels of syndecan-1 expression (**D**).

Figure 1

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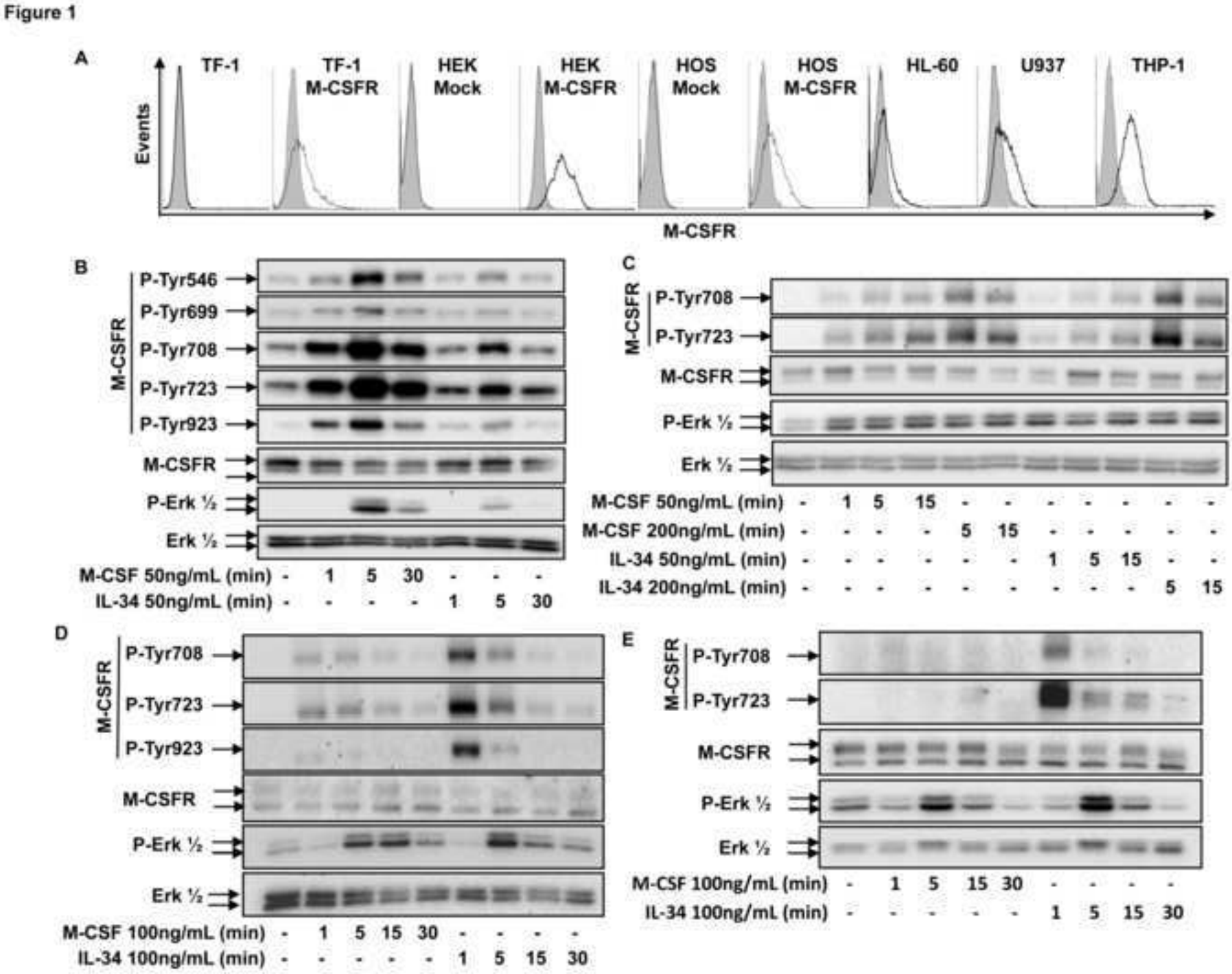

Figure 2
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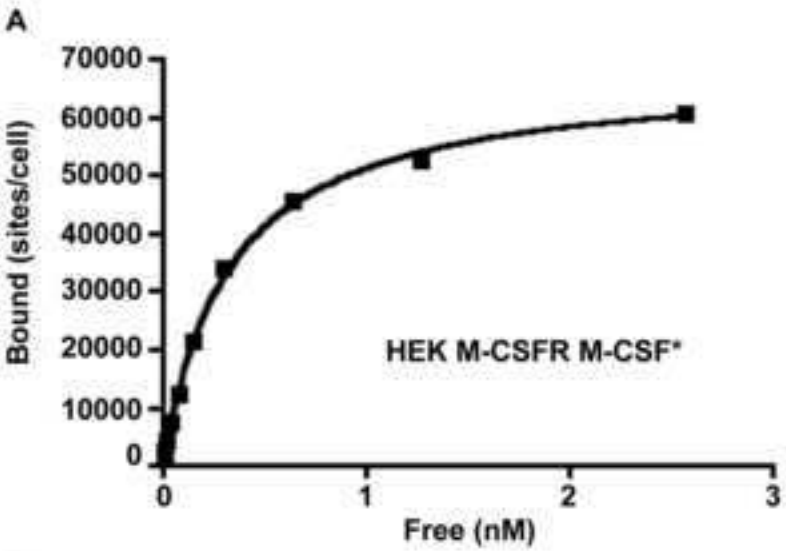
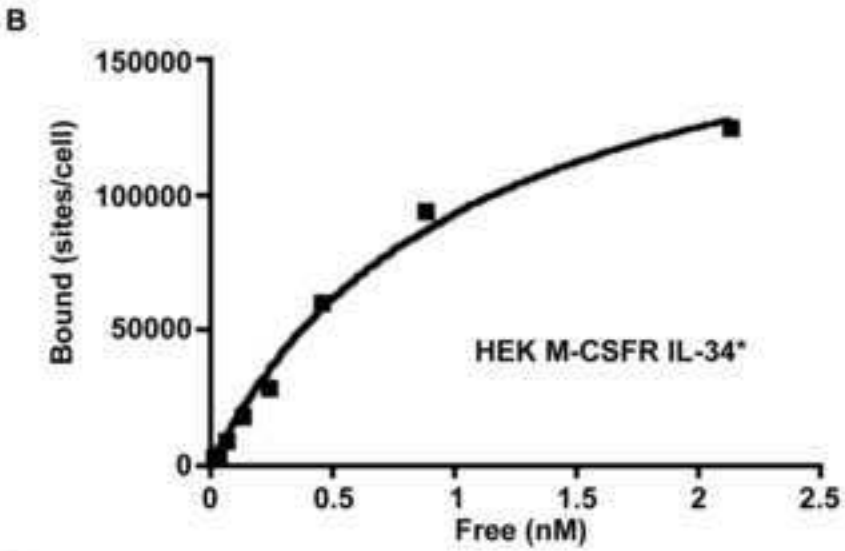
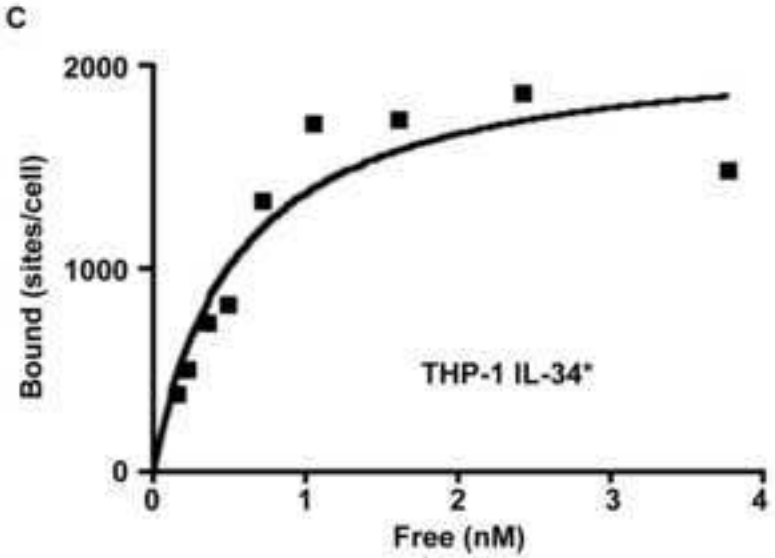
Figure 2







D

Radiolabeled cytokines	Cell lines	K_D (nM)	$Bound_{max}$ (sites/cell)
IL-34	U937	2	9,400
	THP-1	0.55	7,000
	HEK M-CSFR	1	278,700
	HOS M-CSFR	0.75	119,315
M-CSF	HEK M-CSFR	0.32	73,700
	HOS M-CSFR	0.35	9,500

Figure 3

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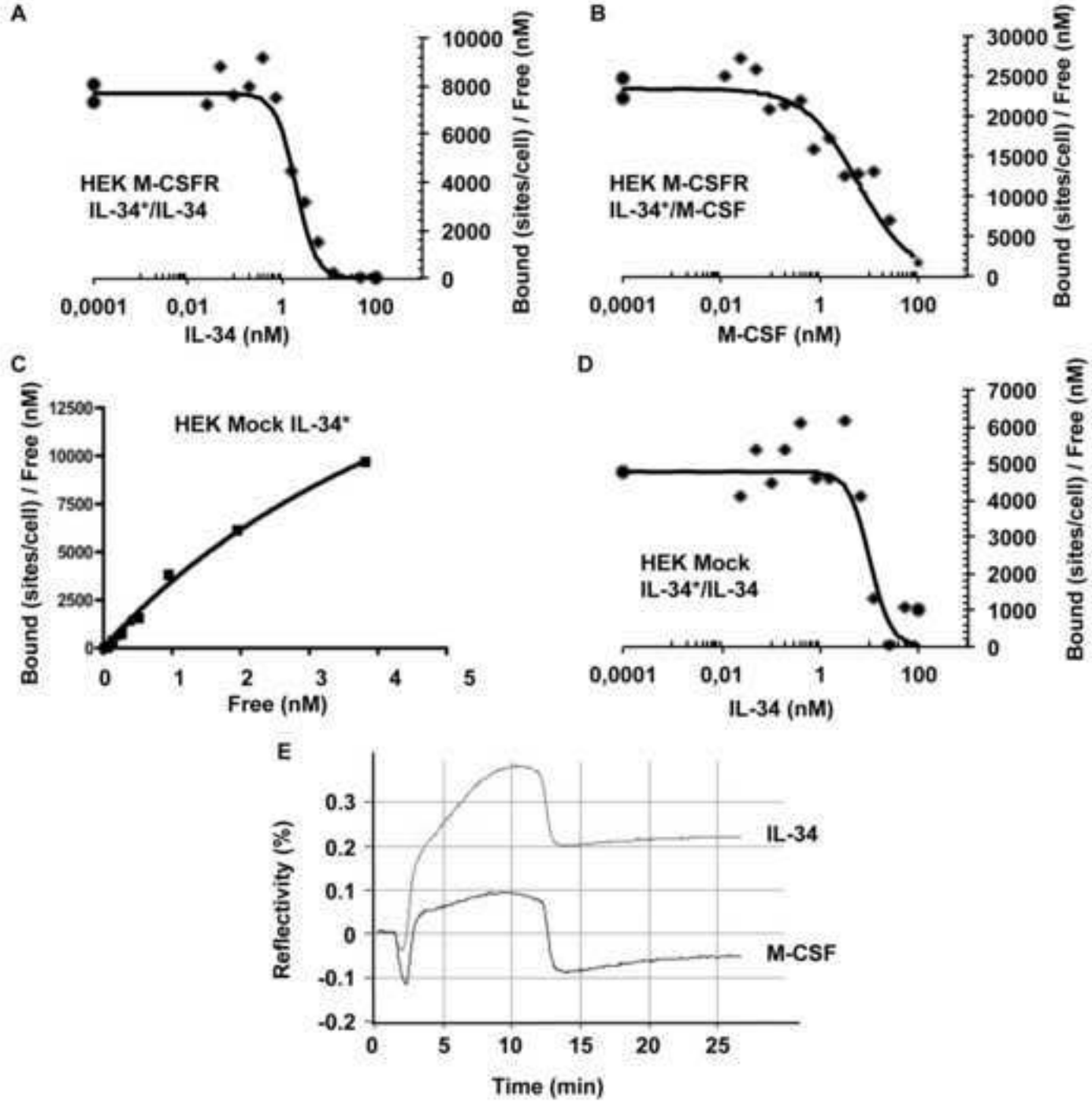

Figure 4

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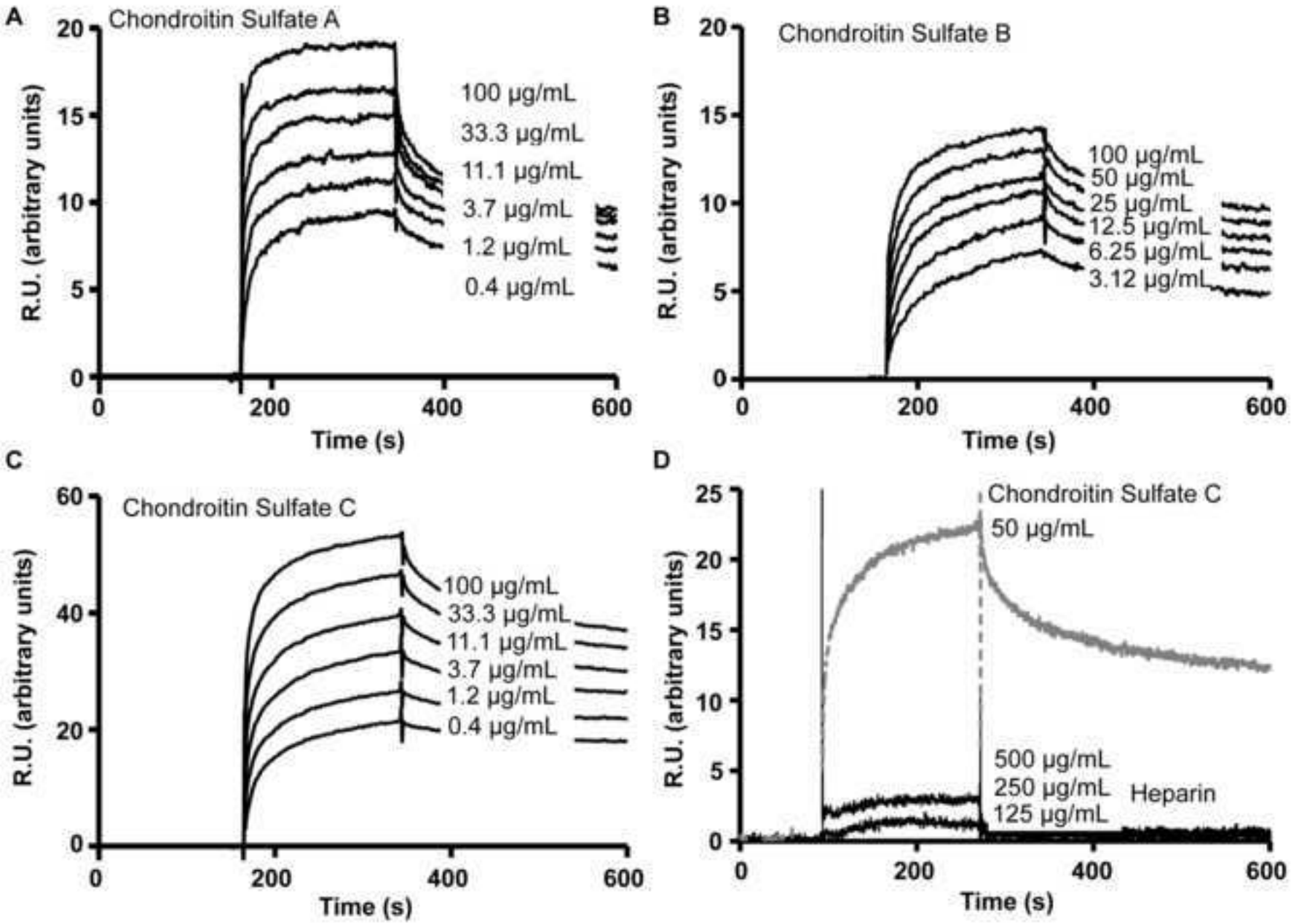

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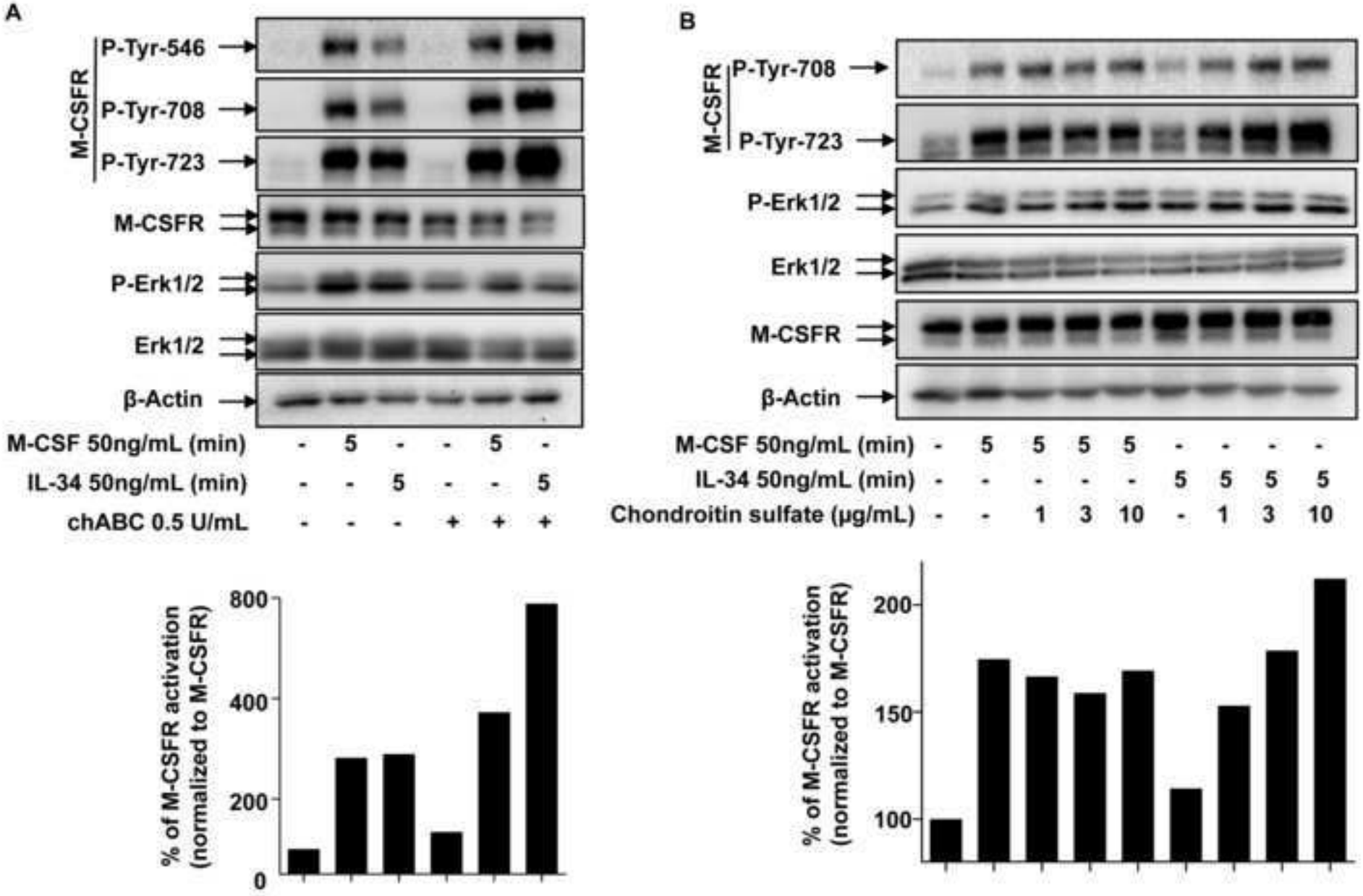

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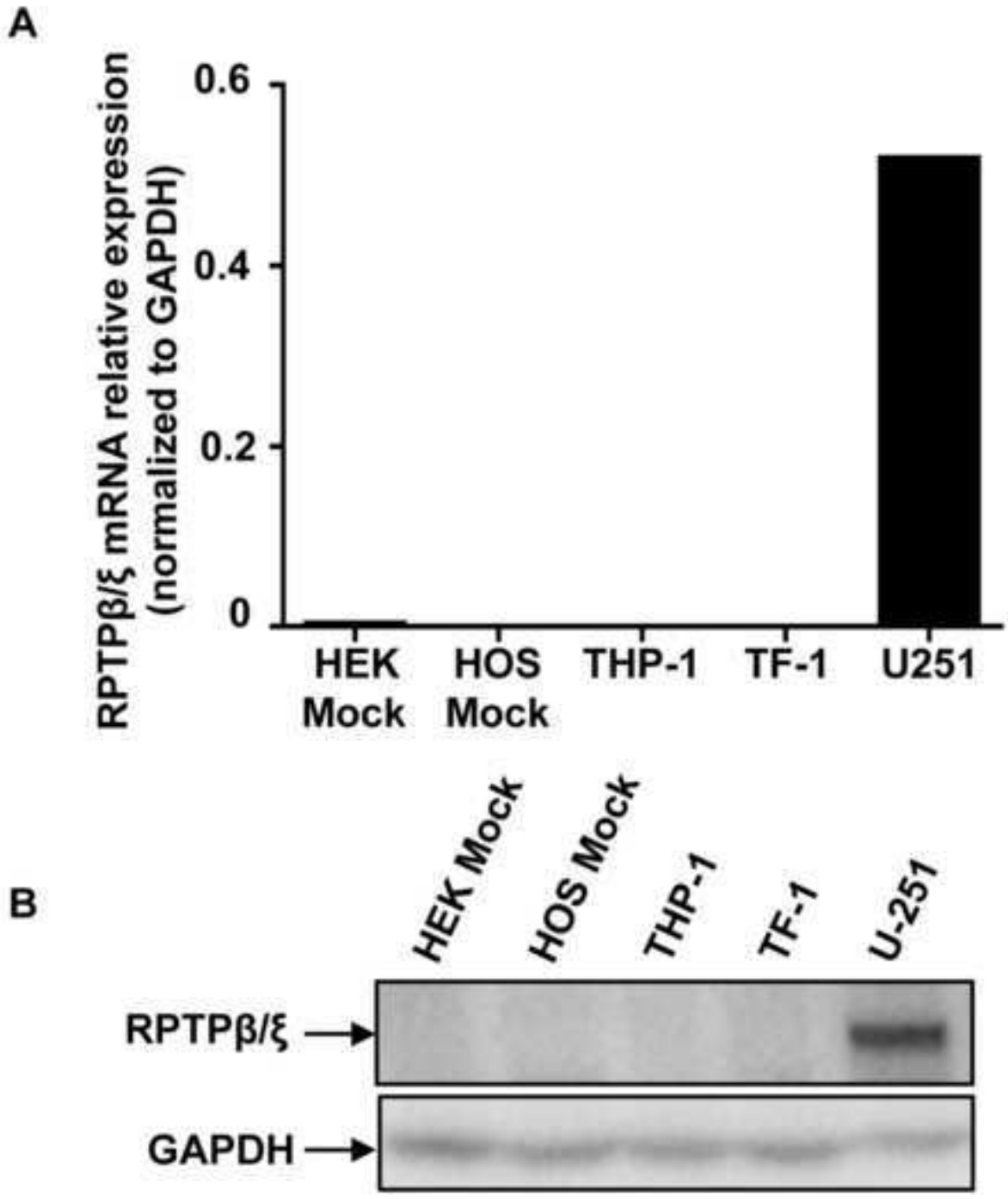

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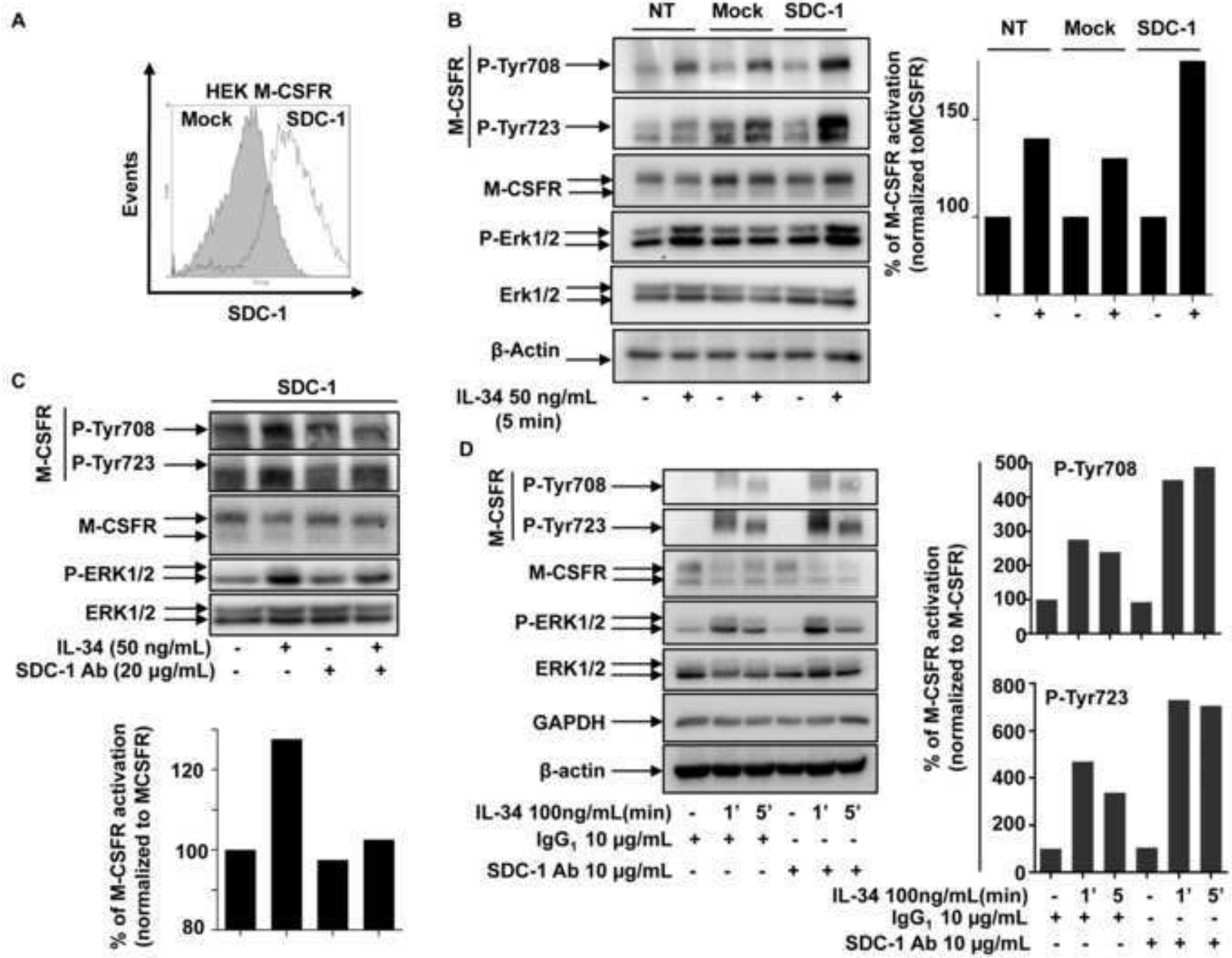

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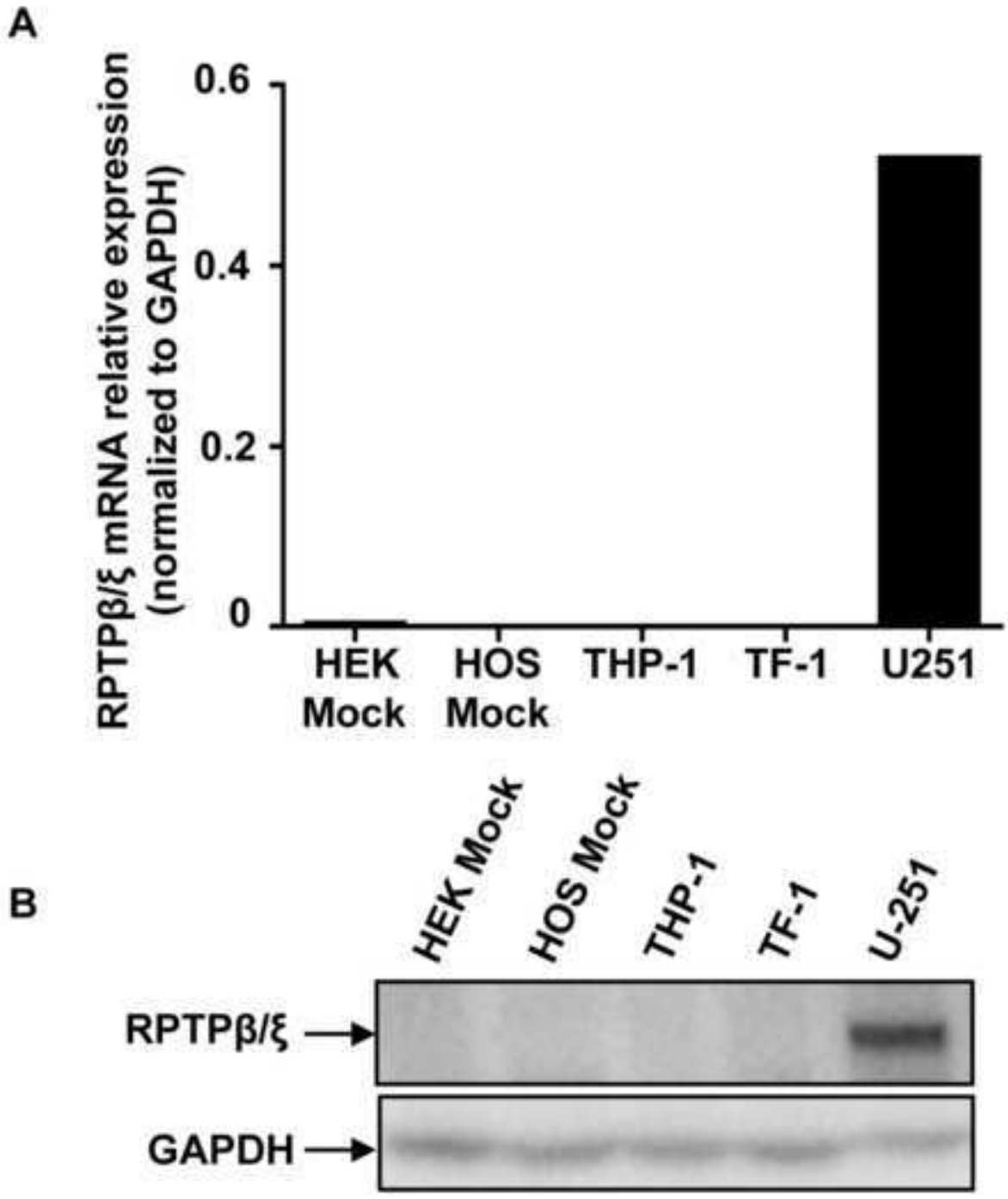

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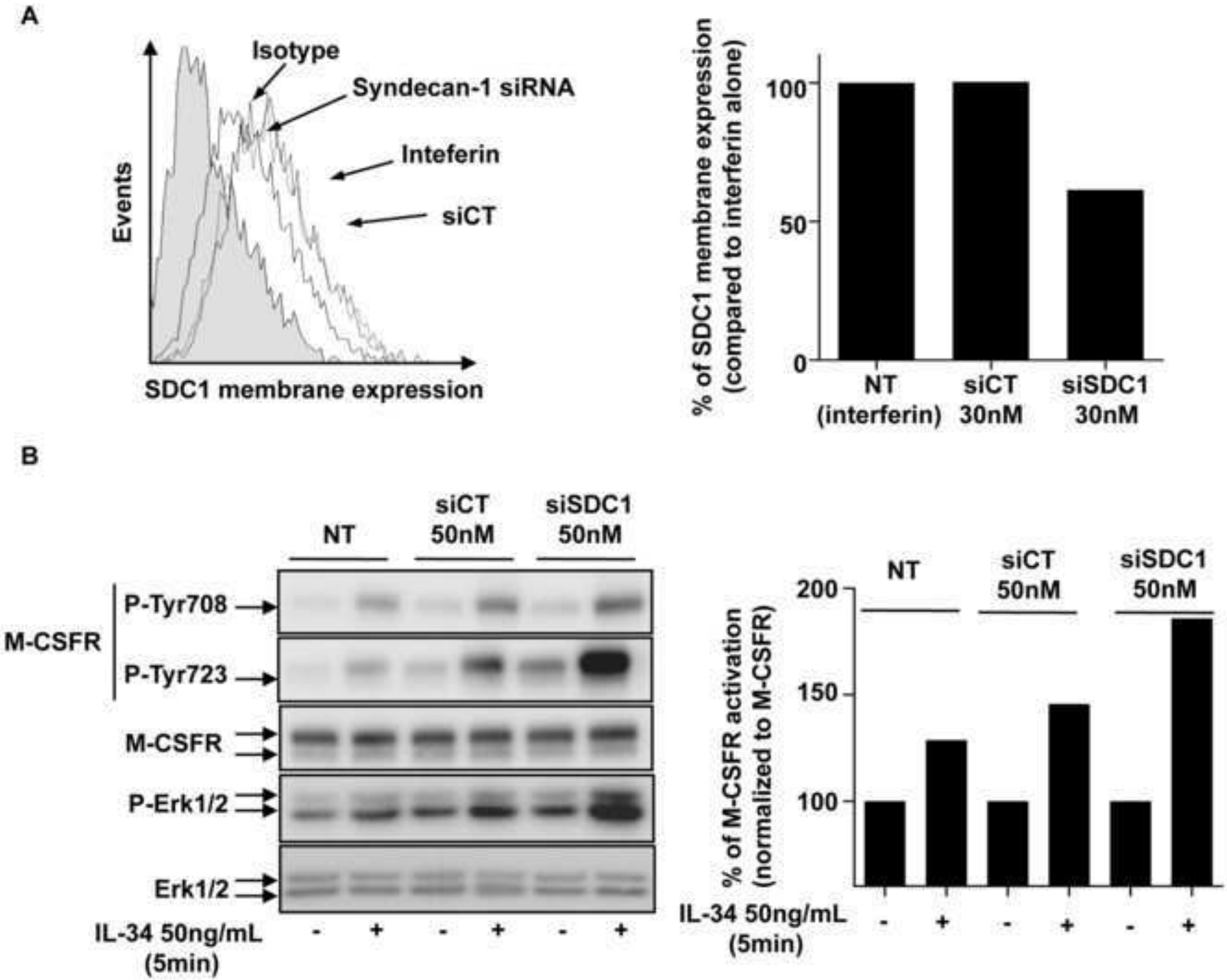

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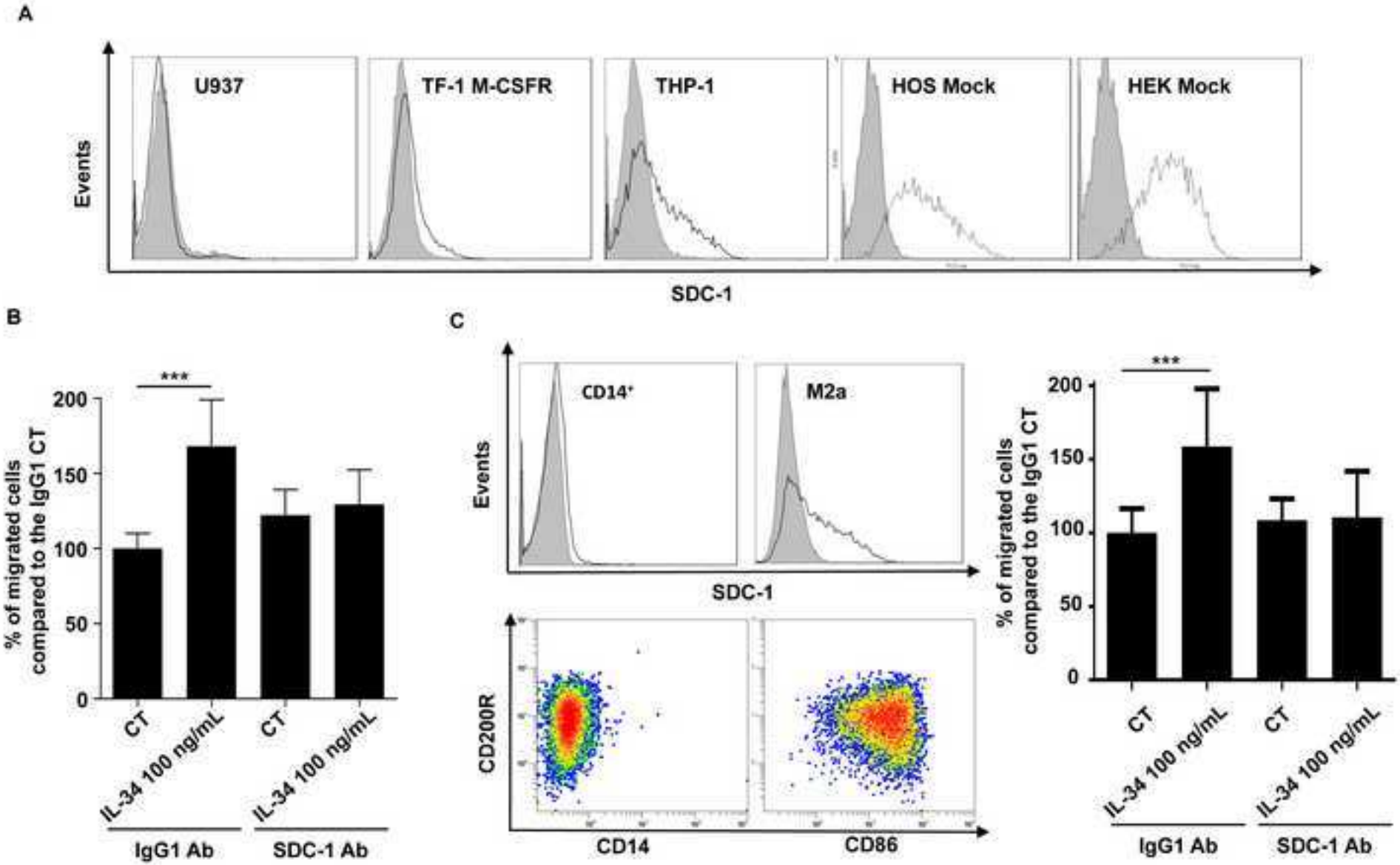

Figure 10

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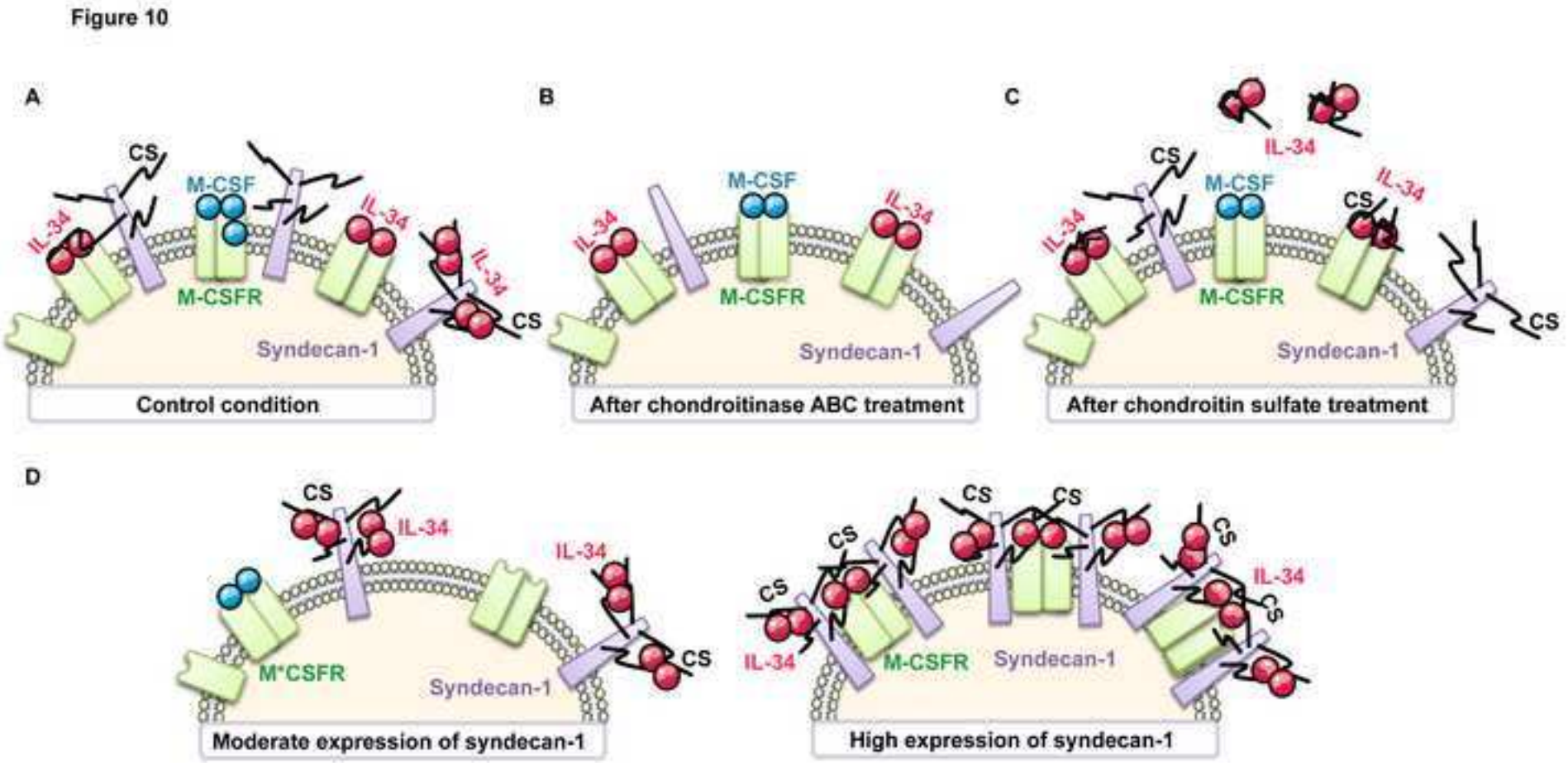
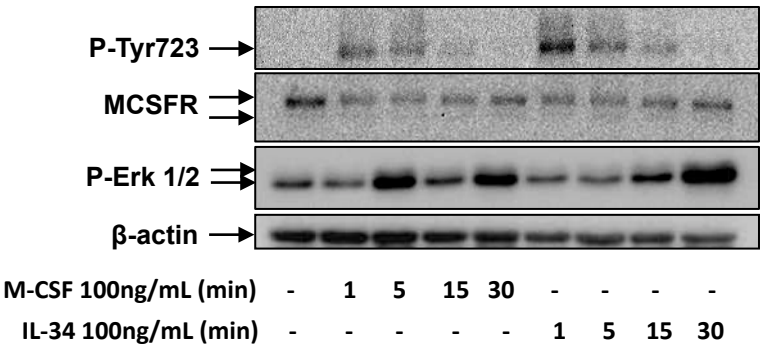
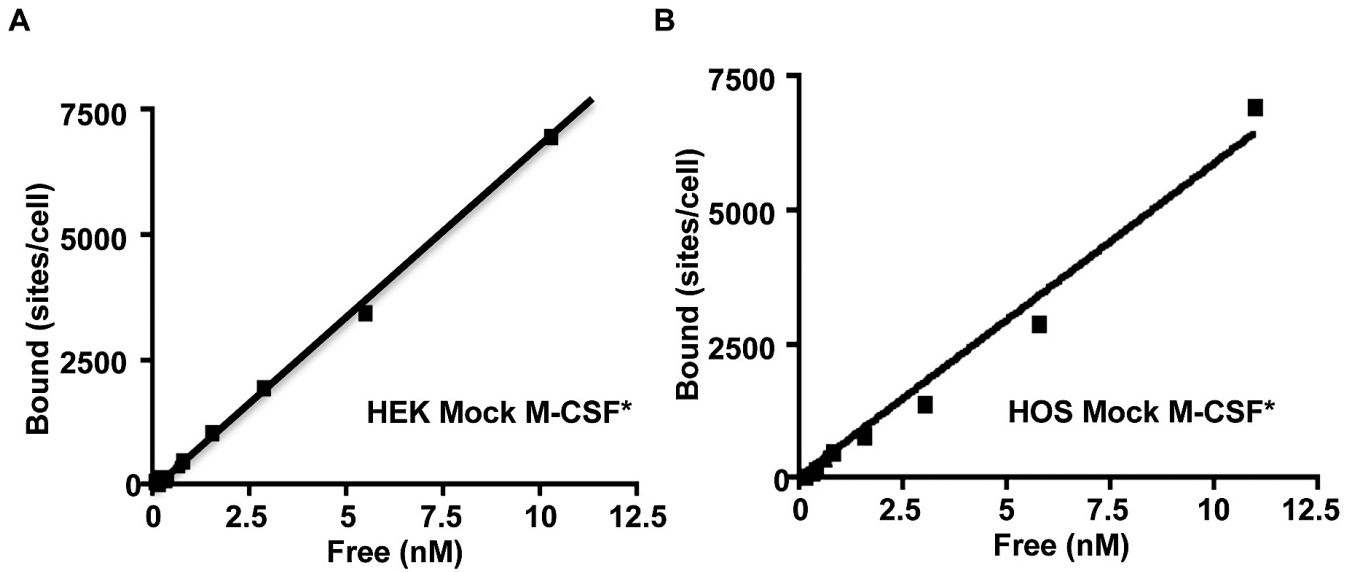
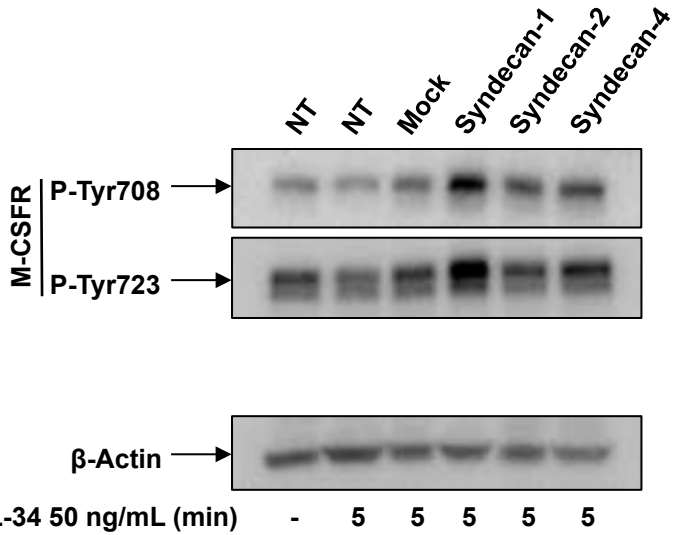


β-actin


P-Tyr723
MCSFR
P-Erk 1/2
β-actin
M-CSF 100ng/mL (min) - 1 5 15 30 - - - -
IL-34 100ng/mL (min) - - - - - 1 5 15 30



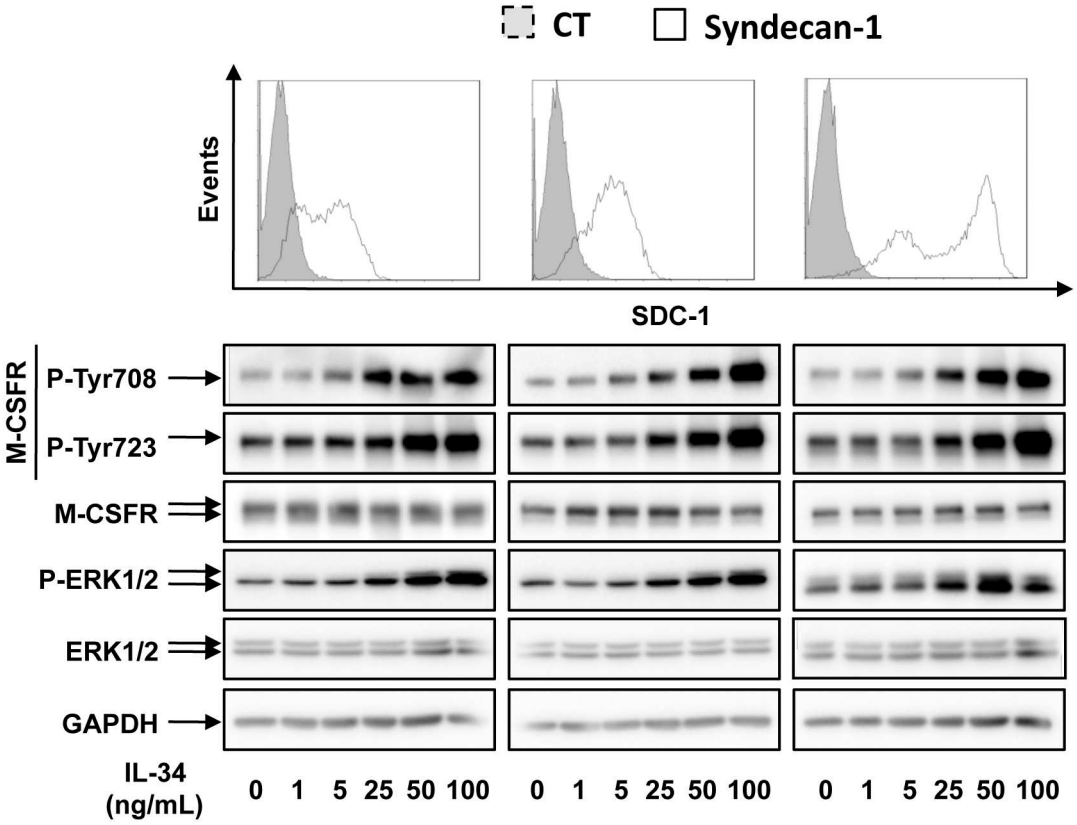
Supplementary data 2: M-CSF did not bind HEK- and HOS-Mock cells licking the M-CSFR. Increasing concentrations of ^{125}I-labelled M-CSF (M-CSF*) were incubated for 60 min at 4°C with 106 cells and bound and unbound fractions were determined as described in « Experimental Procedures ». Specific binding fraction of iodinated cytokines. Curve fitting was performed as described in the « Materials and Methods » section.



Supplementary data 4: Syndecan-2 and -4 have no effect on IL-34 induced signaling pathways in contrast to Syndecan-1. HEK M-CSFR were transfected with the different forms of syndecan (1, 2 and 4). Fourty eight hours after transfection, cells were stimulated with 50 ng/mL of IL-34 for 5 min, and M-CSFR tyrosine phosphorylations were assessed by Western blot. β-actin was used as a loading control. NT: Non-transfected cells; Mock: cells transfected with an empty plasmid; Syndecan-1, -2, -4: cells transfected with a plasmid coding for syndecan-1, -2 or -4.

Supplementary Material (for online publication)

Click here to download Supplementary Material (for online publication): Supplementary data 5.pdf



Supplementary data 5: Syndecan-1 expression regulates the levels IL-34-induced M-CSFR phosphorylation. HEK M-CSF expressing differentially the syndecan-1 expression were isolated by cell sorting flow cytometry. Western blot analysis of tyrosine phosphorylation patterns of M-CSFR and Erk1/2 phosphorylation were carried out in the presence of increasing concentrations of IL-34 for 5 minutes.

***Conflict of Interest**

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