Biology of Reproduction, 2020, 102(1), 185–198
doi:10.1093/biolre/ioz124
Research Article
Advance Access Publication Date: 18 July 2019

Research Article

Endogenous retrovirus-encoded Syncytin-2 contributes to exosome-mediated immunosuppression of T cells†

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†**Grant Support:** This work was supported by the NSERC (#298527-2013), by the CIHR (MOP130561), and by the March of Dimes USA (#6-FY13-155).

Conference Presentation: Presented in part at the 17th World Congress of Gynaecological Endocrinology 2016, Firenze, Italy, at the Society for Reproductive Investigation 63rd Annual Scientific Meeting 2017, Montreal, Canada and at the 33rd Annual Meeting of the European Society of Human Reproduction and Embryology 2017, Geneva, Switzerland.

Received 15 August 2018; Revised 21 December 2018; Accepted 11 July 2019

Abstract

Modulation of the activation status of immune cell populations during pregnancy depends on placental villous cytotrophoblast (VCT) cells and the syncytiotrophoblast (STB). Failure in the establishment of this immunoregulatory function leads to pregnancy complications. Our laboratory has been studying Syncytin-2 (Syn-2), an endogenous retroviral protein expressed in placenta and on the surface of placental exosomes. This protein plays an important role not only in STB formation through its fusogenic properties, but also through its immunosuppressive domain (ISD). Considering that Syn-2 expression is importantly reduced in preeclamptic placentas, we were interested in addressing its possible immunoregulatory effects on T cells. Activated Jurkat T cells and peripheral blood mononuclear cells (PBMCs) were treated with monomeric or dimerized version of a control or a Syn-2 ISD peptide. Change in phosphorylation levels of ERK1/2 MAP kinases was selectively noted in Jurkat cells treated with the dimerized ISD peptide. Upon incubation with the dimerized Syn-2 ISD peptide, significant reduction in Th1 cytokine production was further demonstrated by ELISA and Human Th1/Th2 Panel Multi-Analyte Flow Assay. To determine if exosome-associated Syn-2 could also be immunosuppressive placental exosomes were incubated with activated Jurkat and PBMCs. Quantification of Th1 cytokines in the supernatants revealed severe reduction in T cell activation. Interestingly, exosomes from

Syn-2-silenced VCT incubated with PBMCs were less suppressive when compared with exosome derived from VCT transfected with control small interfering RNA (siRNA). Our results suggest that Syn-2 is an important immune regulator both locally and systemically, via its association with placental exosomes.

Summary sentence

Human endogenous retroviral Syncytin-2 negatively downmodulates Th1 response via its immunosuppressive domain and acts through its association to exosomes.

Key words: Syncytin-2, Th1 cytokines, syncytiotrophoblast, placenta, exosomes, immunosuppression.

Introduction

During normal pregnancy, the maternal immune system has been known to be modulated in order for the immunologically foreign fetus to be protected from rejection [1]. Recent studies have however highlighted that placenta-mediated modulation of the activity of the immune response is more complex and involves pro-inflammatory and anti-inflammatory phases, in which placental trophoblasts play an essential role [2]. Several immune cell populations are targeted by trophoblasts at different stages of pregnancy and include monocytes/macrophages, Treg, NKs, and dendritic cells, which are recruited and modulated by various secreted chemokines and cytokines [3]. CD4+ and CD8+ T cells also importantly participate in the immune response through their presence in circulating maternal blood [4].

Placenta development involves the differentiation of trophoblasts into extravillous and villous cytotrophoblasts (VCTs) [5]. Unlike extravillous cytotrophoblasts, VCTs are noninvasive but fuse to form and maintain the structure of the peripheral syncytiotrophoblast (STB) layer through a well-regulated process. A number of studies have demonstrated that envelope (Env) genes from human endogenous retrovirus (HERV), remnant of former ancestral retroviral infectious events, are implicated in this fusion through their fusogenic domain [6, 7]. Convincing data have clearly suggested that Env Syncytin-1 and Syncytin-2 indeed participate in the formation of the STB layer by mediating fusion with underlying cytotrophoblast cells through interaction with their specific receptors [8].

Interestingly, HERV Envproteins have been reported to contain an immunosuppressive domain (ISD) in their sequence, which shared similarity to a previously described immunoregulatory region of envelope proteins from exogenous retroviruses [9–11]. We and others have further provided evidence that these HERV Env ISD were functional [12–16]. Importantly, the potential immunomodulatory role of HERV Env proteins in pregnant women has been suggested to be altered in pregnancy disorders, such as preeclampsia [17–21]. In all these studies, including those which were focussed in the ISD region of exogenous retroviruses, the dimerized form of a 17 amino acid-long peptide was shown to be sufficient to mediate immunosuppression [16, 22]. Although the exact molecular mechanisms and interacting partners involved in the modulation of the immune response are not known, the ISD domain-induced cell signaling has been shown to result in the activation of ERK1/2(Extracellular signal-regulated kinase) MAP (mitogen activated protein) kinases and other cellular effectors [22]. Studies on the Friend murine leukemia virus have further indicated that the ISD affects both the innate and adaptive immunity [11].

A number of studies have suggested that the HERV Env-specific immunosuppressive activity might be attributed to their incorporation in extracellular microvesicles, such as exosomes. These various vesicles are released from the STB and have been shown to functionally alter several immune cell types [23, 24]. We and others have clearly demonstrated that Syncytin-1 is present on the surface of these extracellular vesicles and that, in this context, Syncytin-1 was suggested to be immunosuppressive [16, 25].

A recent study from our team has demonstrated that like Syncytin-1, Syncytin-2 is also incorporated in the surface of VCT-derived extracellular vesicles [26, 30]. As limited studies have been aimed at the immunosuppressive activity of Syncytin-2, we were interested in studying the ISD activity of Syncytin-2 in the form of a dimerized peptides and in association to exosomes.

Materials and methods

Reagents, antibodies, and peptides

The following activating agents were purchased from Millipore Sigma (Oakville, Canada): phorbol 12-myristate 13-acetate (PMA) (P1585) and ionomycin (I3909). In this study, the following antibodies were used (see also Supplementary Table 1): mouse anti-CD28 (#302902, Biolegend, San Diego CA), mouse anti-CD3 (#317318, Biolegend), rabbit anti-phospho-ERK1/2 (#9101, Cell Signaling, Danvers MA), rabbit anti-ERK1/2 (#4695, Cell Signaling), mouse anti-TSG101 (ab83, Abcam, Cambridge MA), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit/anti-mouse antibodies (#7074 and #7076; Cell Signaling). Antibodies against Syncytin-2 have been described previously and target the extracellular surface (SU) subunit [26]. Peptides were synthesized by solid phase peptide synthesis and were characterized by LC/MS-TOF (liquid chromatography/mass spectrometry-time-of flight). Dimerization was achieved by dimethyl sulfoxide oxidation. Amino acid sequence of the peptides were as follow: LQNRRGLDMLTAAQGGI (Sync-2 ISD) and IGGQAATLMDLGRRNQL (Rev Sync-2-ISD), the latter containing the same amino acid in reverse sequence (negative control).

Cell line

The human Jurkat cell line was obtained from American Type Culture Collection (Manassas VA) and was maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (350–000-CL; Wisent, Montreal, Canada) and 10% exosome-free FBS (fetal bovine serum) (ultracentrifuged overnight at 100 000*g* at 4 °C; 12483-020; Life technologies, Burlington, Canada).

Isolation of primary cytotrophoblast cells and peripheral blood mononuclear cells

This study was approved by the Ethic committees of both Université du Québec à Montréal and the Ste Justine Hospital and conducted in accordance with the specific guidelines and standards of the Society for the Study of Reproduction. Venous blood was collected from

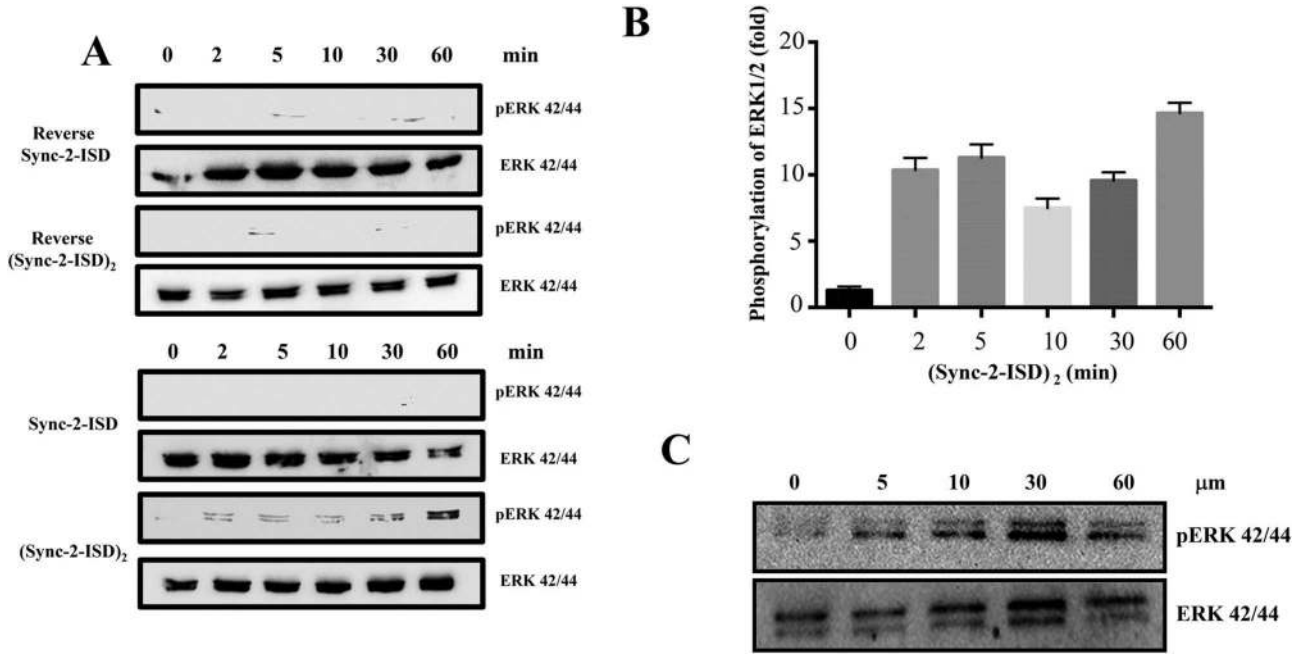

Figure 1. The dimerized Syncytin-2 ISD peptide induces ERK1/2 phosphorylation in Jurkat cells. (A and B) Jurkat cells were either left untreated or treated with monomeric Sync-2-ISD, dimeric (Sync-2-ISD)$_2$, monomeric Reverse Sync-2-ISD, or dimeric Reverse (Sync-2-ISD)$_2$ (60 µM) from 2 to 60 min. Cell lysates were prepared and the phosphorylation of MAP kinase was determined by Western blot using antibodies against phosphorylated ERK1 and ERK2 (pERK 42/44) (upper panels) or total ERK1/2 (ERK 42/44) (lower panels) (A). The phosphorylation of MAP kinase in this time course experiment was quantified by scanning densitometry for dimeric (Sync-2-ISD)$_2$-treated cells (B). Data represent fold increase of pERK1/2 (normalized with signals for total ERK1/2) compared with non-stimulated control (set at a value of 1). (C) Jurkat cells were either left untreated or treated with different concentrations (5, 10, 30, and 60 µM) of dimerized ISD (Sync-2-ISD)$_2$ for 1 h. Western blot analysis was performed as indicated in A and B. Results are representative of three independent experiments.

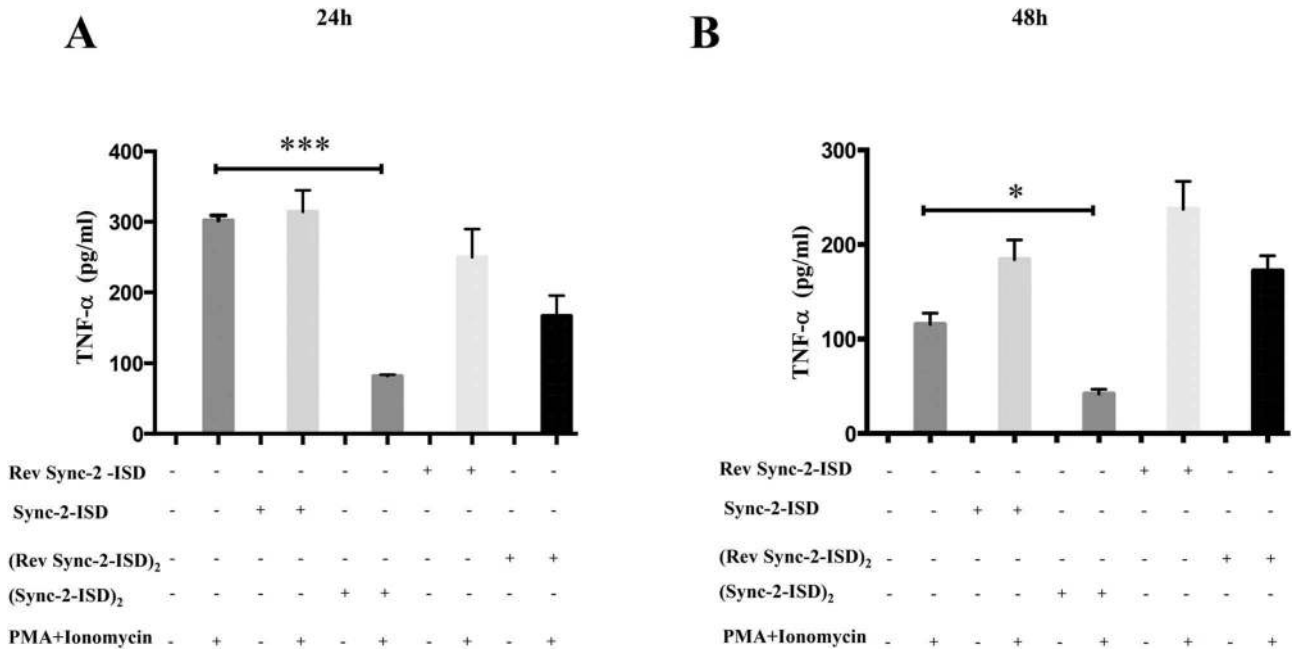

Figure 2. (Sync-2-ISD)$_2$ inhibits TNF-α production in Jurkat T cells. Jurkat cells were incubated with 60 µM of monomeric or dimerized Sync-2-ISD versus control Rev Sync-2-ISD peptides. After 2 h, cells were stimulated with PMA (20 ng/ml)/ionomycin (1 µM) and TNF-α was measured in the supernatant after 24 h (A) and 48 h (B) by ELISA. Means ± SE were calculated from triplicates and results are representative of three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$.

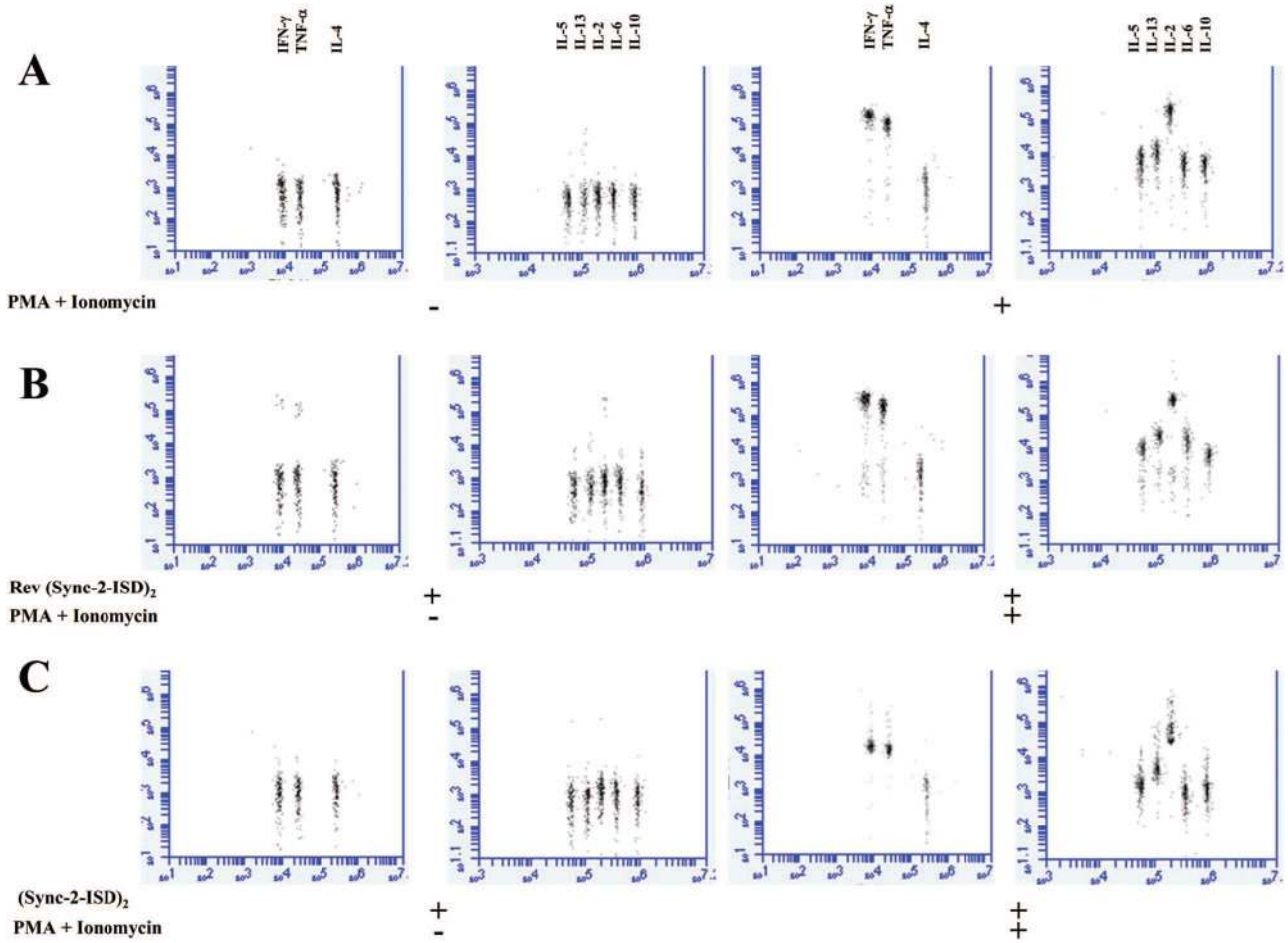

Figure 3. (Sync-2-ISD)$_2$ inhibits Th1 cytokine production in PMA/ionomycin-activated PBMC. Peripheral blood mononuclear cells were left untreated (A) or incubated with 60 μM Rev (Sync-2-ISD)$_2$ (B) or (Sync-2-ISD)$_2$ (C). After 2 h, cells were stimulated with PMA (20 ng/ml)/ionomycin (1 μM) and cytokines were measured in supernatants after 24 h through the LEGENDplex Human Th1/Th2 Panel kit. Data are depicted as the distribution of signals corresponding to each detected bead and are presented in terms of intensity for each measured cytokine.

four healthy male adult donors and PBMCs were prepared from Ficoll gradient, as previously described [27]. Human placentas of uncomplicated term pregnancies from 37 to 41 weeks were obtained after spontaneous vaginal delivery. All participating women signed an informed consent form. Primary human VCTs were isolated through a previously published protocol [28]. Briefly, placental villi were cut, thoroughly washed to remove blood, and digested four times in Hanks' balanced salt solution containing trypsin (from 9.6×10^5 to 1.8×10^6 U) and DNase I (Millipore Sigma; from 15 to 30 mg per digestion) for 30 min at 37 °C in a water bath under continuous shaking. Dispersed cells were layered on top of a discontinuous 5–70% Percoll gradient and centrifuged for 23 min at 507*g*. Intermediate layers, with a density between 1.048 and 1.062, containing cytotrophoblast cells, were collected and washed extensively. Cells were seeded at a density of 1.5×10^6 cells/well in Dulbecco's modified Eagle's medium (Wisent, Montreal, Canada), 2 mM glutamine, 10% exosome-depleted FBS, and penicillin/streptomycin/neomycin (Invitrogen Canada, Inc., Burlington, Canada) and cultured for a maximum of 4 days. The purity of each cytotrophoblast preparation was evaluated by flow cytometry with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-cytokeratin 7 antibody (1:500) (CBL194F, Millipore Sigma), a specific marker of trophoblasts. Only preparations with a minimum of 96% of cytotrophoblast cells were used. All experiments with primary cytotrophoblast cells were conducted in triplicate with different placenta donors.

Cell transfection

Jurkat cells (1×10^7 cells) were transfected by the diethylaminoethyl (DEAE)-Dextran protocol, as previously described [29]. Plasmids pNFAT-Luc and pNF-κB-Luc have been previously described and, respectively, contains Nuclear factor of activated T cells (NFAT)- and Nuclear factor kappa B (NF-κB)-binding sites positioned upstream of a minimal promoter and the luciferase reporter gene [27]. Small interfering RNAs (siRNAs) were transfected in human primary cytotrophoblast cells using the Microporator MP-100 apparatus (Digital Bio). All siRNAs were synthesized by Invitrogen Canada (Syncytin-2, s53886 and the negative control, AM4642). Freshly isolated primary cytotrophoblast cells (1.5×10^6) were transfected with 300 ng siRNA using the MicroPorator device with 1 pulse at 1300 V (30 ms).

Isolation of exosomes

Supernatants of cultured human primary cytotrophoblast cells (day1 and day 2) were first cleared by successive centrifugations

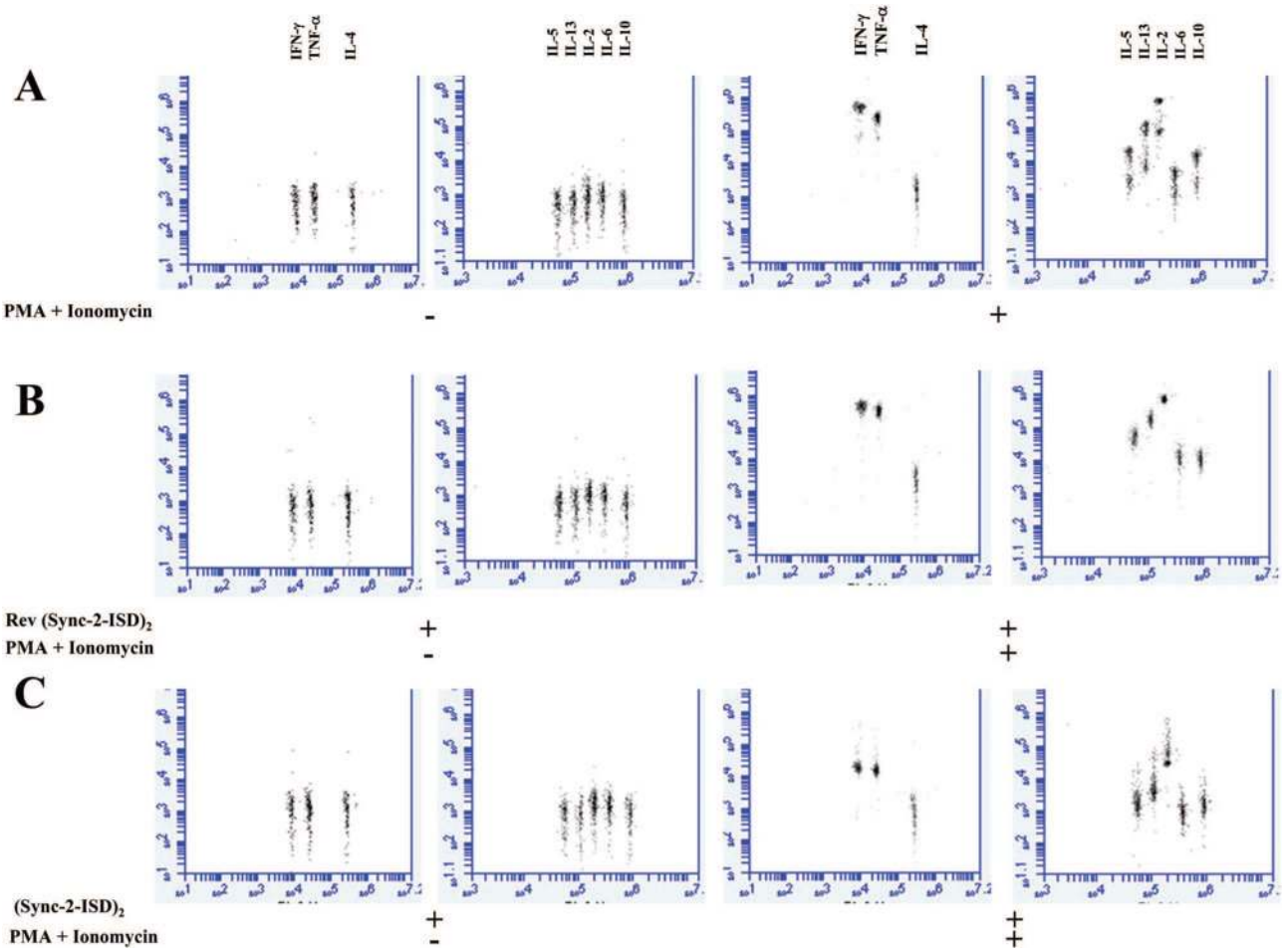

Figure 4. (Sync-2-ISD)$_2$ inhibits Th1 cytokine production in PMA/ionomycin-activated PBMC. Peripheral blood mononuclear cells were left untreated (A) or incubated with 60 μM Rev (Sync-2-ISD)$_2$ (B) or (Sync-2-ISD)$_2$ (C). After 2 h, cells were stimulated with PMA (20 ng/ml)/ionomycin (1 μM) and cytokines were measured in supernatants after 48 h through the LEGENDplex Human Th1/Th2 Panel kit.

at 2000*g* for 15 min and at 10000*g* (4 °C) for 30 min. Following two subsequent 100000*g* centrifugations (4 °C), supernatants were discarded and pellets containing exosomes were resuspended in PBS (phosphate-buffered saline) (100 μL). Exosome preparations were stored at −80 °C in 50 μL aliquots and analyzed by Western blot.

Incubation of Jurkat and peripheral blood mononuclear cells with peptides and exosomes

In a first series of experiments, unstimulated Jurkat cells were incubated in serum-free medium for 2 h at 37 °C and treated with 0–60 μM of monomeric or dimeric Sync-2-ISD or Rev Sync-2-ISD peptides between 2 and 60 min at 37 °C. In subsequent experiments, PBMCs or Jurkat (5×10^5 cells) were cultured in sodium bicarbonate-free supplemented RPMI 1640 medium (20 mM Hepes, 2 mM L-glutamine, 40 μg/mL gentamicin, and 10% FBS). Cells were pre-treated for 2 h with Sync-2-ISD or Rev Sync-2-ISD peptides before activation with PMA (20 ng/ml)/ionomycin (1 μM) or anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) antibodies and cultured for 18–48 h before harvesting the supernatant. For exosomes, PBMC or transfected Jurkat cells (5×10^5 cells) cultured in sodium bicarbonate-free supplemented RPMI 1640 medium (20 mM Hepes, 2 mM L-glutamine, 40 μg/ml gentamicin, and 10% exosomes free FBS) were either left untreated or pre-treated for 24 h with different concentration of exosomes before activation with PMA/ionomycin or anti-CD3/anti-CD28 antibodies. Treated cells were cultured at 37 °C prior to analyses (luciferase activity, RT-PCR (reverse transcriptase- polymerase chain reaction), and harvesting of supernatant). Following incubation, cell viability was determined by lactate dehydrogenase activity assay (MAK066, Sigma-Aldrich, St-Louis MO), according to provided instructions.

Western blot analysis

Cells were resuspended in 2× SDS (sodium dodecyl sulfate) sample buffer containing 10% 2-mercaptoethanol, and a mixture of proteinase (Roche Diagnostics, Laval, Canada) and phosphatase inhibitors (Millipore Sigma) and incubated on ice for 15 min. Protein concentrations were evaluated using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, Rochester, New York). After boiling for 5 min, cell lysates were run on a 10–12% SDS-PAGE and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked in 5% powdered milk in Tris saline, pH 7.5, 0.15% Tween 20 (Millipore Sigma) for 1 h at room temperature. The following primary antibodies were then added: anti-phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti-Syncytin-2 (1:5000), and anti-TSG101

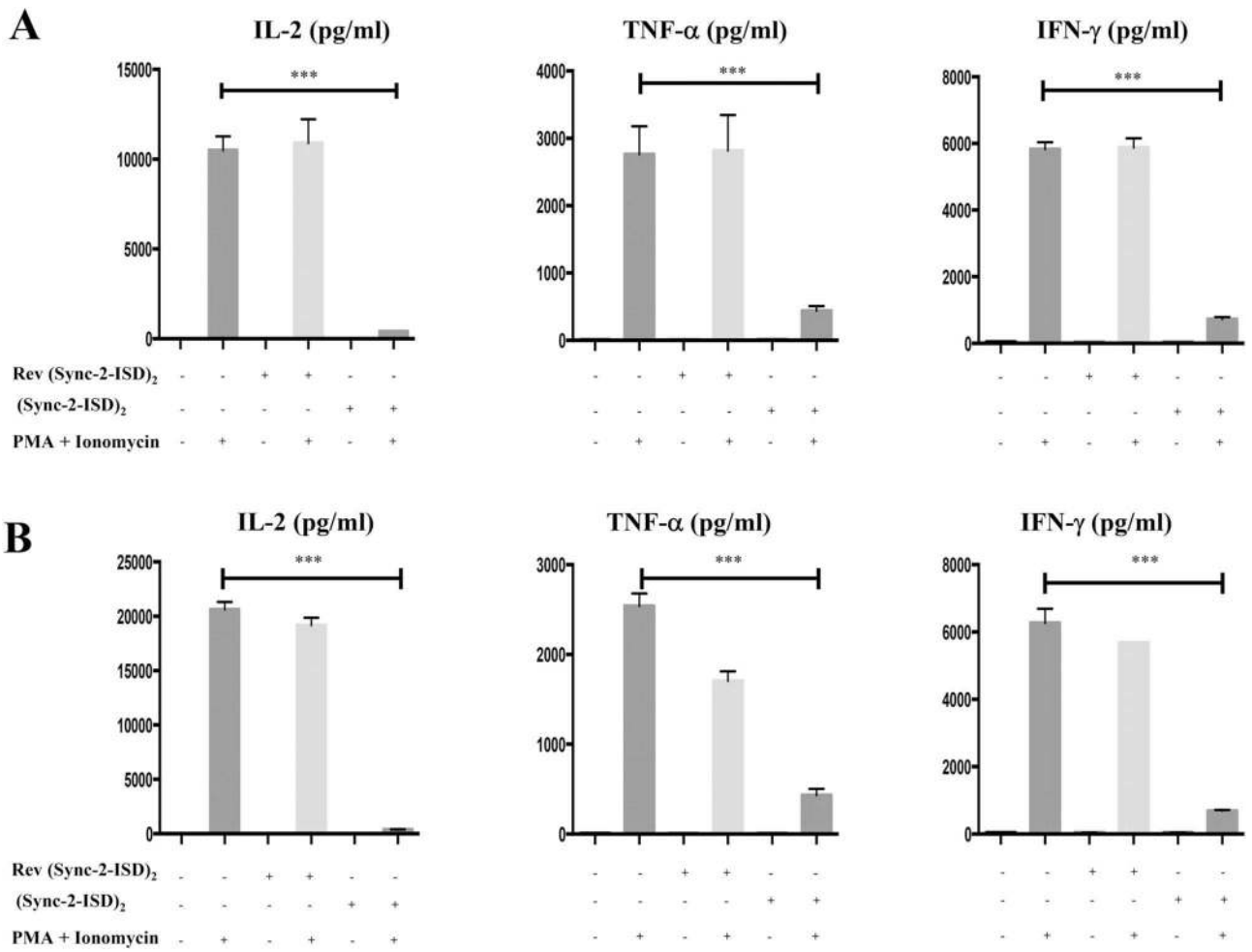

Figure 5. Quantification of Th1 cytokine levels in PMA/ionomycin-stimulated PBMCs following treatment with $(Sync\text{-}2\text{-}ISD)_2$. Peripheral blood mononuclear cells were incubated with $(Sync\text{-}2\text{-}ISD)_2$ versus control Rev $(Sync\text{-}2\text{-}ISD)_2$ (60 μM). After 2 h, cells were stimulated with PMA (20 ng/ml)/ionomycin (1 μM) and cytokines were measured at 24 h (A) and 48 h (B) post-treatment. Quantification of cytokine levels from Figures 3 and 4 are presented in pg/ml and are based on standard curves generated for each cytokine. Means ± SE were calculated for each measured cytokine and results are representative of two independent experiments. $^{***}P < 0.001$.

(1:1000). After overnight incubation at 4 °C, membranes were washed three times in PBS-Tween 0.05% and incubated with HRP-conjugated goat anti-rabbit/anti-mouse antibodies (1:5000) for 1 h at room temperature. After three washes, signals were then revealed with the ECL (enhanced chemiluminescence) Western blotting reagent (RPN2106, Millipore Sigma). Membranes were scanned with the Fusion FX5 system (Montreal Biotech Inc., Dorval, Canada).

RT-PCR analysis

Total cellular RNA was isolated from cells using RNeasy mini kit (#74106, Qiagen, Mississauga, Canada) according to the supplier's protocol. Briefly, RNA (0.5 μg) was incubated in the presence of oligo (dT) (25 ng/μl), 10 mM DTT, 100 μM dNTP, 10 U SuperScript II reverse transcriptase (18064-014, Thermofisher Scientific, Waltham MA), and 20 U SUPERase-In (AM2696, Invitrogen Canada) at 37 °C for 45 min. The resulting cDNAs (1 μL) were then PCR amplified in the presence of 1 U Taq DNA polymerase (Thermofisher Scientific), 1X Taq buffer, 100 μM dNTP, and 0.5 μM of each primer. PCR conditions were as follows: a first step of denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation (30 s at 95 °C), annealing (30 s from 55 °C), and extension (30 s at 72 °C) with a final cycle of 10 min at 72 °C. The following primers were used: 5′-AGCCCATGTTGTAGCAAACC-3′ (forward TNF (tumor necrosis factor)-α), 5′-TGAGGTACAGGCCCTCTGAT-3′ (reverse TNF- α), 5′-GAAGGTGAAGGTCGGAGTCAA-3′ (forward GAPDH (glyceraldehyde-3-phosphate dehydrogenase)) and 5′-GGAAGATGGTGATGGGATTTC-3′ (reverse GAPDH).

Quantification of Th1 and Th2 cytokine in Jurkat and peripheral blood mononuclear cells

Jurkat supernatants were assayed for TNF-α with the commercial ELISA MAX Deluxe kit (#430204, Biolegend, San Diego, California). Peripheral blood mononuclear cell supernatants were assayed for Th1 and Th2 cytokines using commercial LEGENDplex Human Th1/Th2 Panel (8-plex) (#740013, Biolegend, San Diego, California). The sensitivity of the assays was 0.8 pg/ml for interleukin (IL)-13, 1 pg/ml for IL-2, interferon (IFN)-γ and TNF-α, and 1.1 pg/ml for IL-5, IL-6, and IL-10.

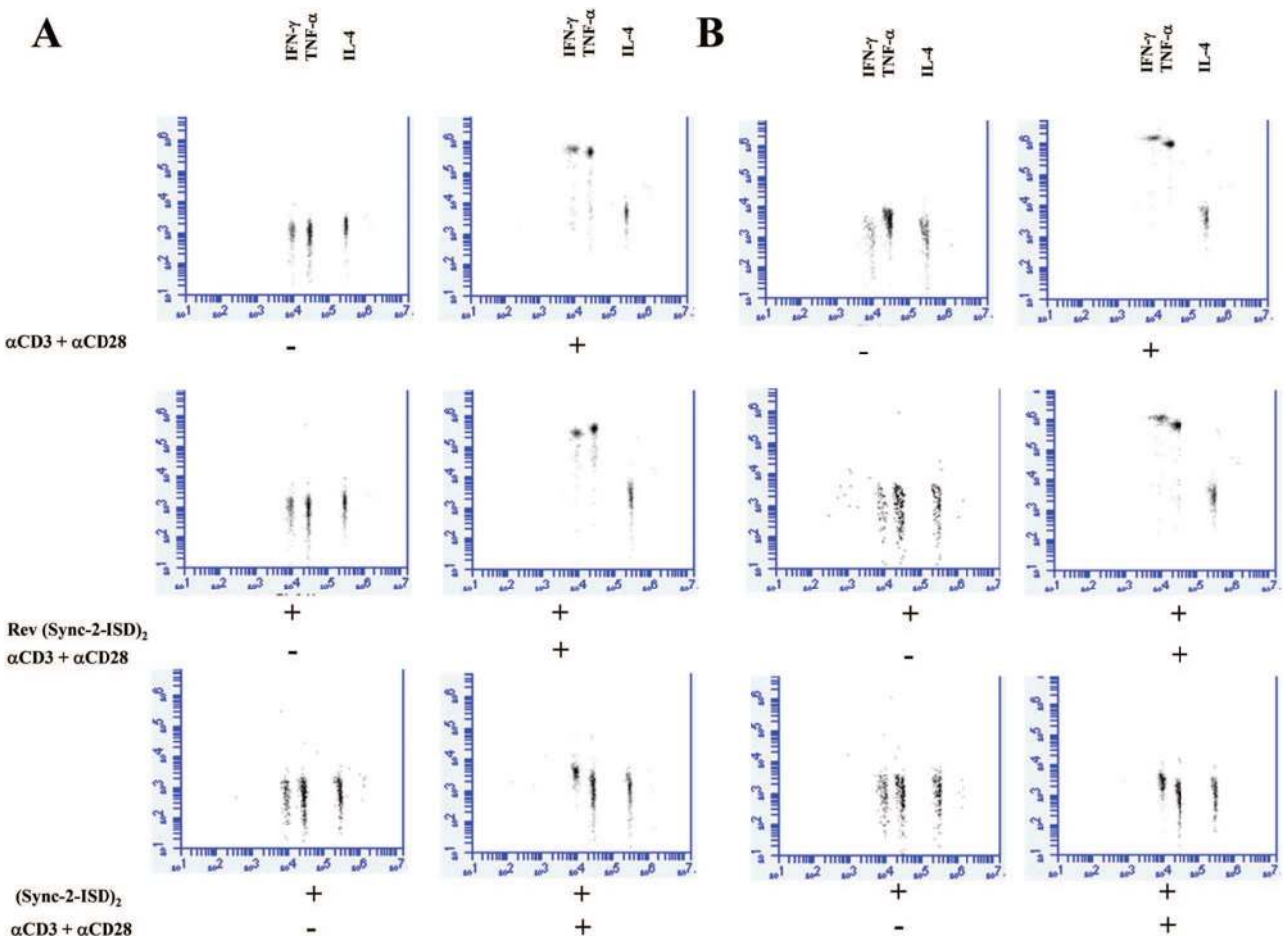

Figure 6. (Sync-2-ISD)$_2$ inhibits Th1 cytokine production in anti-CD3/anti-CD28-activated PBMCs. Peripheral blood mononuclear cells were incubated with 60 μM (Sync-2-ISD)$_2$ or Rev (Sync-2-ISD)$_2$. After 2 h, cells were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) antibodies and cytokines were measured in supernatants after 24 (A) and 48 h (B) through the LEGENDplex Human Th1/Th2 Panel kit.

Statistics

All experiments were performed in triplicates, except for certain experiments using LEGENDplex, which were representative of two independent experiments (indicated in legends of Figure 5, 7, 10 and 11.). Error bars in the graphic data represent means ± SEM. Data were analyzed by a 1-tailed or paired Student's t test. Statistical analyses were performed with the GraphPad Prism software (San Diego, CA).

Results

The dimerized Syncytin-2 immunosuppressive domain peptide induces ERK1 and ERK 2 phosphorylation

Previous studies reported that Syncytin-1 and -2 harbor a functional ISD domain [13, 16]. Based on former analyses of this domain, we were interested in more closely examining the inhibitory function of the Syncytin-2 ISD. Since it was reported that CKS-17, a 17 amino acid-long immunosuppressive peptide corresponding to the equivalent domain of Feline and Murine Leukemia Viruses, triggered activation of ERK 1 and ERK 2 MAP kinases in the monocytic THP-1 cell line [10], we first tested if comparable effects were induced in Jurkat CD4+ T cells. As previously reported, the peptide was tested as a monomer, termed Sync-2-ISD, or as its dimerized form, that is, (Sync-2-ISD)$_2$. Jurkat cells were cultured in serum starved-medium for 2 h and stimulated with different concentrations of these peptides and for different time lengths (Figure 1). We first assessed the impact of the (Sync-2-ISD)$_2$ dimeric peptide on Jurkat cells at a fixed concentration and for different time lengths (Figure 1A). Kinetic experiments showed that (Sync-2-ISD)$_2$ induced ERK1/2 phosphorylation in a time-dependent manner, as demonstrated by Western blot. In sharp contrast, no induction (or very minimal levels) of ERK1/2 phosphorylation was noted in Jurkat cells treated with monomeric/dimeric version of Rev Sync-2-ISD or the Sync-2-ISD monomer, demonstrating the specificity of the signaling potential of the Syncytin-2 ISD region. Levels of total ERK remained generally stable in these experiments. Furthermore, resulting densitometry analyses of the phosphorylation status of ERK1/2 normalized for protein levels further confirmed the important induction of phosphorylation in response to treatment with the (Sync-2-ISD)$_2$ peptide (Figure 1B). A dose response experiment was next performed and revealed a gradual increment of ERK1/2 phosphorylation in (Sync-2-ISD)$_2$-treated Jurkat cells, which leveled off at 30 μM (Figure 1C). No similar induction was noted in Jurkat cells treated with the control non-dimerized form of the peptide (data not shown).

These results thereby indicated that the dimerized representative peptide of the ISD domain of Syncytin-2 specifically induced ERK1/2 phosphorylation in Jurkat cells in a comparable manner to the

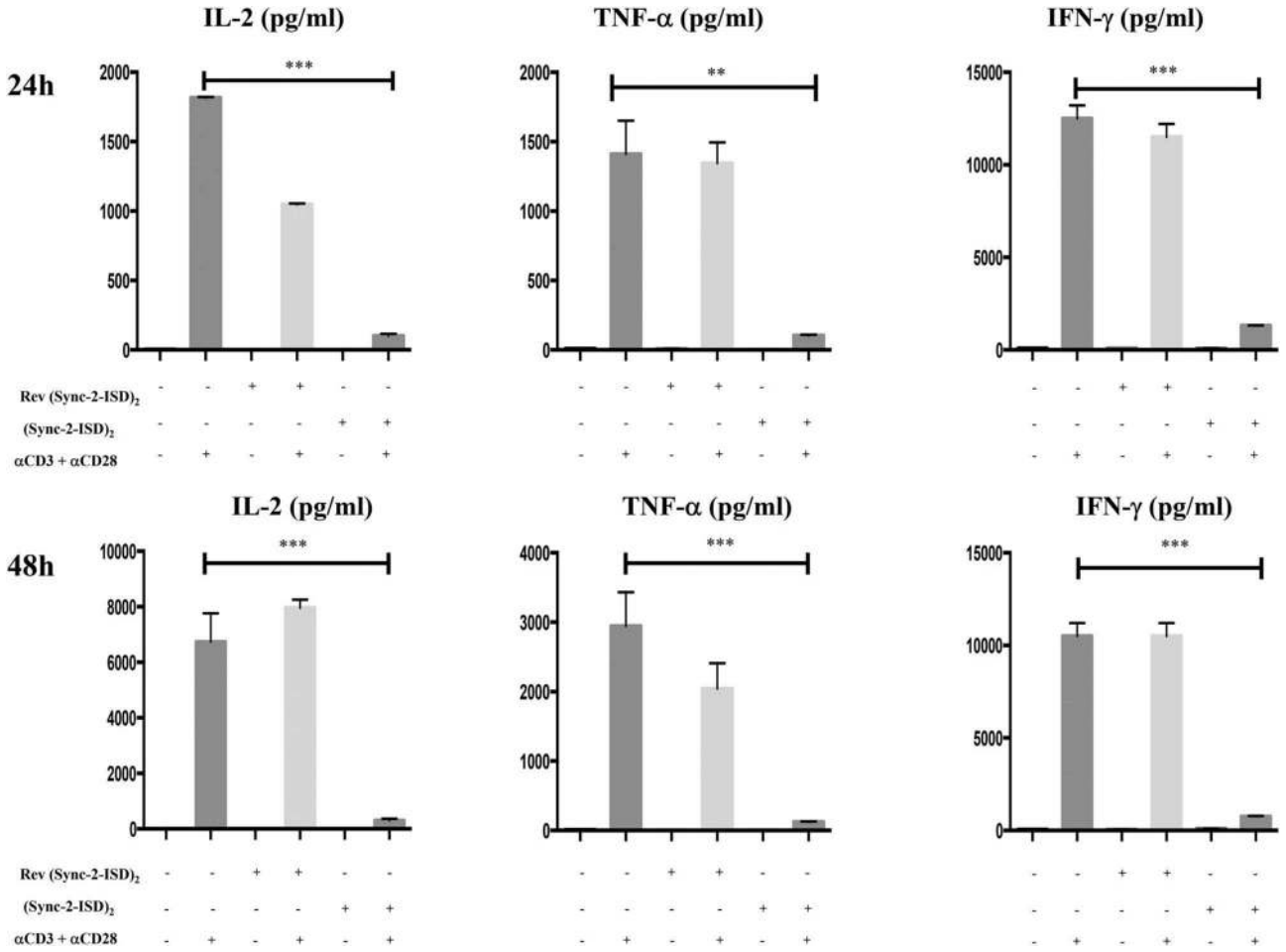

Figure 7. Quantification of cytokine levels in anti-CD3/anti-CD28-stimulated PBMCs following treatment with $(Sync\text{-}2\text{-}ISD)_2$. Peripheral blood mononuclear cells were incubated with $(Sync\text{-}2\text{-}ISD)_2$ versus Rev $(Sync\text{-}2\text{-}ISD)_2$ (60 μM). After 2 h, cells were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) antibodies and cytokines were measured in supernatants after 24 and 48 h. Quantification of cytokine levels from Figure 6 are presented in pg/ml. Means ± SE were calculated for each measured cytokine and results are representative of two independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

induction observed with the ISD domain of envelope proteins from different retroviruses.

$(Sync\text{-}2\text{-}ISD)_2$ inhibits TNF-α production in Jurkat cells

In order to determine if the previously reported immunosuppressive capacity of Syncytin-2 could also affect activated T cells [13], monomeric/dimeric forms of Sync-2-ISD or Rev Sync-2-ISD were incubated in the presence of Jurkat T cells. After addition of PMA/ionomycin, cell supernatants were quantified for TNF-α production. As presented in Figure 2, $(Sync\text{-}2\text{-}ISD)_2$ inhibited TNF-α production in response to PMA/ionomycin stimulation of Jurkat cells, whereas its monomeric form or reverse peptide controls did not show a similar important reduction. Of note, the dimerized reverse Sync2-ISD did show significant reduction at 24 h post-treatment, but at a lower extent than $(Sync\text{-}2\text{-}ISD)_2$ treated Jurkat cells and not at the 48 h time point. As expected, TNF-α levels were undetectable in Jurkat cells treated with peptides in absence of PMA/ionomycin. Importantly, these treatments had no impact on cell viability, based on measurement of lactate dehydrogenase activity (data not shown).

These data thereby confirmed that the ISD domain of Syncytin-2 demonstrated a specific inhibitory effect on T cell activation and that only the dimeric form was capable of inducing this inhibition.

$(Sync\text{-}2\text{-}ISD)_2$ inhibits cytokine production in PMA-ionomycin-activated peripheral blood mononuclear cells

In order to address the immunosuppressive potential of the Syncytin-2 ISD in a more physiologically relevant manner and to evaluate a wider range of cytokines, we next tested whether the $(Sync\text{-}2\text{-}ISD)_2$ peptide modulates the cytokine profile of PBMCs. Freshly isolated PBMCs were thus stimulated or not with PMA/ionomycin following pre-incubation with dimerized Syncytin-2 ISD peptide or respective controls. Th1 and Th2 cytokine levels were next quantified in supernatants from PBMCs through the Biolegend multiplex analysis kit. This assay relies on the use of beads of two different sizes and with variation in their fluorescence. Each set of beads are bound to antibodies against a specific cytokine and, upon binding to their specific cytokine, can be detected and analyzed following the addition of a pool of biotinylated antibodies recognizing all targeted cytokines. Using this assay, we first compared untreated versus activated PBMCs (24 h) for the production of Th1 and Th2 cytokines (Figure 3A). Results showed that Th1 cytokines, IL-2, TNF-α, and IFN-γ were responsive to activation as depicted in our flow cytometry data. As opposed to this induction, only weak stimulation of Th2 cytokines was observed at 24 h post-stimulation.

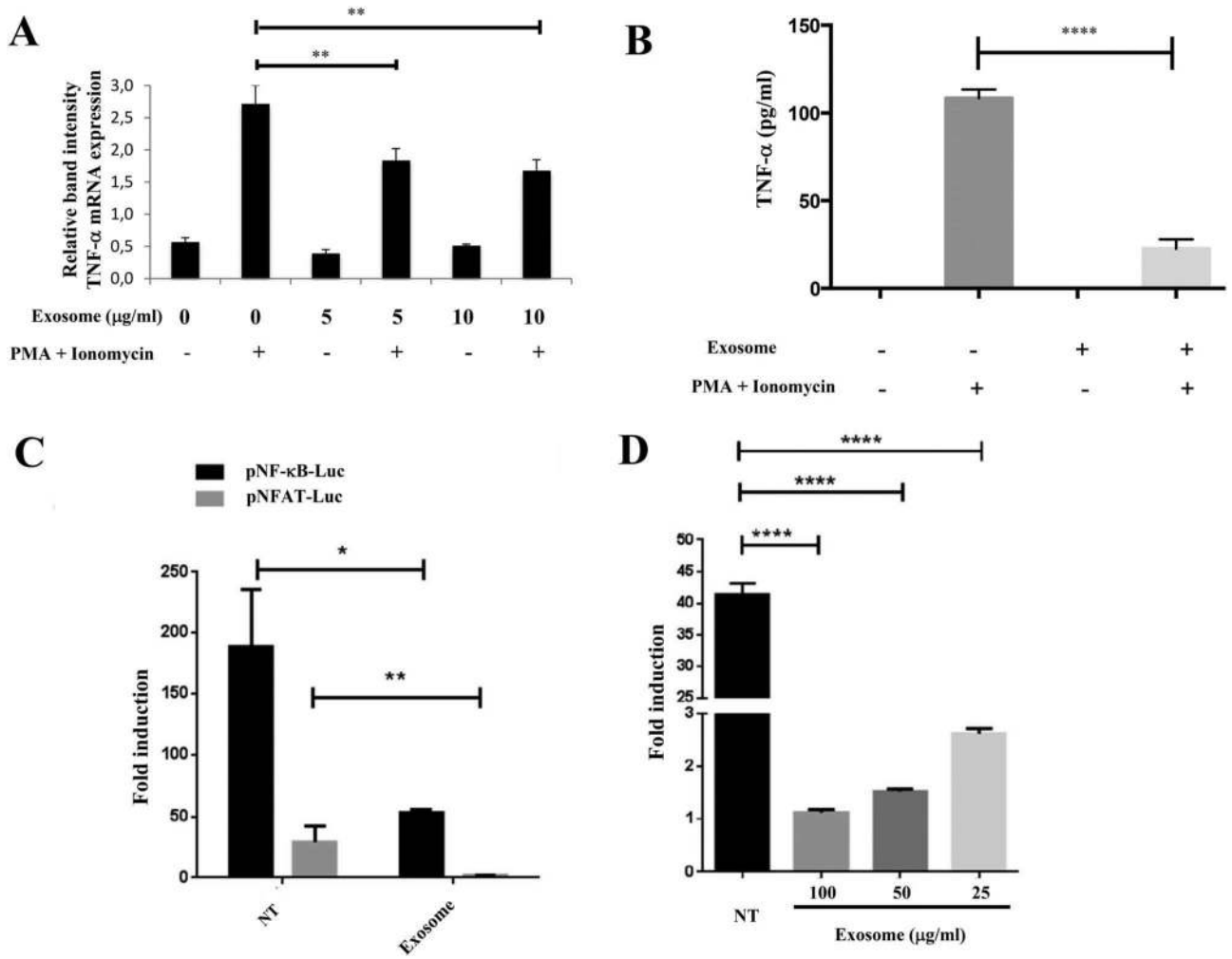

Figure 8. Inhibition of TNF-α production in Jurkat T cells by trophoblast-derived exosomes. (A and B) Jurkat cells were incubated with different concentrations of exosomes (5, or 10 μg/ml) in triplicates. After 24 h, cells were stimulated with PMA (20 ng/ml)/ionomycin (1 μM) and analyzed for TNF-α expression by RT-PCR (6 h post-treatment, A) and TNF-α secretion by ELISA (18 h post-treatment, B). For RT-PCR analysis, GAPDH signals were amplified in parallel and signals were scanned for densitometric analyses. Results are presented following normalization with GAPDH. Results are representative of three different experiments. (C and D) Jurkat cells transfected with pNF-κB-Luc (C) or pNFAT-Luc (C and D) were stimulated with PMA (20 ng/ml)/ionomycin (1 μM) for 8 h and incubated in the presence of fixed (50 μg/ml) (C) or varying concentrations (D) of isolated exosomes. Luciferase activity was measured in three independent transfection samples and fold induction are presented as a mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

When PBMCs were incubated in the presence of $(\text{Sync-2-ISD})_2$, an important inhibition of the induced IL-2, IFN-γ, and TNF-α levels was noted, while no such strong impact was apparent in Rev $(\text{Sync-2-ISD})_2$-treated PBMCs (Figure 3B and C). Similar analyses were conducted on PBMCs stimulated for 48 h (Figure 4). Th1 cytokine production was again importantly induced, although Th2 cytokines showed more important increment in their levels in PBMC supernatant, most notably for IL-13 (Figure 4A). Importantly, induced cytokines were severely reduced when cells were incubated with $(\text{Sync-2-ISD})_2$, while a limited impact was observed in (Rev $\text{Sync-2-ISD})_2$-treated cells.

Through internal controls and standard curves provided in the assay, values of cytokine concentration for data presented in Figures 3 and 4 were estimated and are depicted in Figure 5. Again, as demonstrated above, the dimerized Syncytin-2 ISD peptide was clearly shown to confer an inhibitory capacity toward Th1 cytokine production in activated PBMCs. Only limited inhibition by the control dimeric peptide was noted in treated PBMCs. In these experiments, no reduction in cell viability was noted in any of the tested treatment (data not shown).

These data thereby further supported the immunosuppressive function of the Syncytin-2 ISD region and indicated that Th1 cytokines were consequently blocked in their production.

$(\text{Sync-2-ISD})_2$ inhibits cytokine production in anti-CD3/anti-CD28-activated peripheral blood mononuclear cells

To corroborate the above results and to test more representative T cell-specific activators, PBMCs were treated with a combination of anti-CD3/anti-CD28 antibodies prior to their exposure to the peptides. As shown in Figure 6A and B, analyses of supernatant of PBMCs harvested at 24 and 48 h after activation by the multiplex analysis kit again revealed that Th1 cytokines (solely depicted by

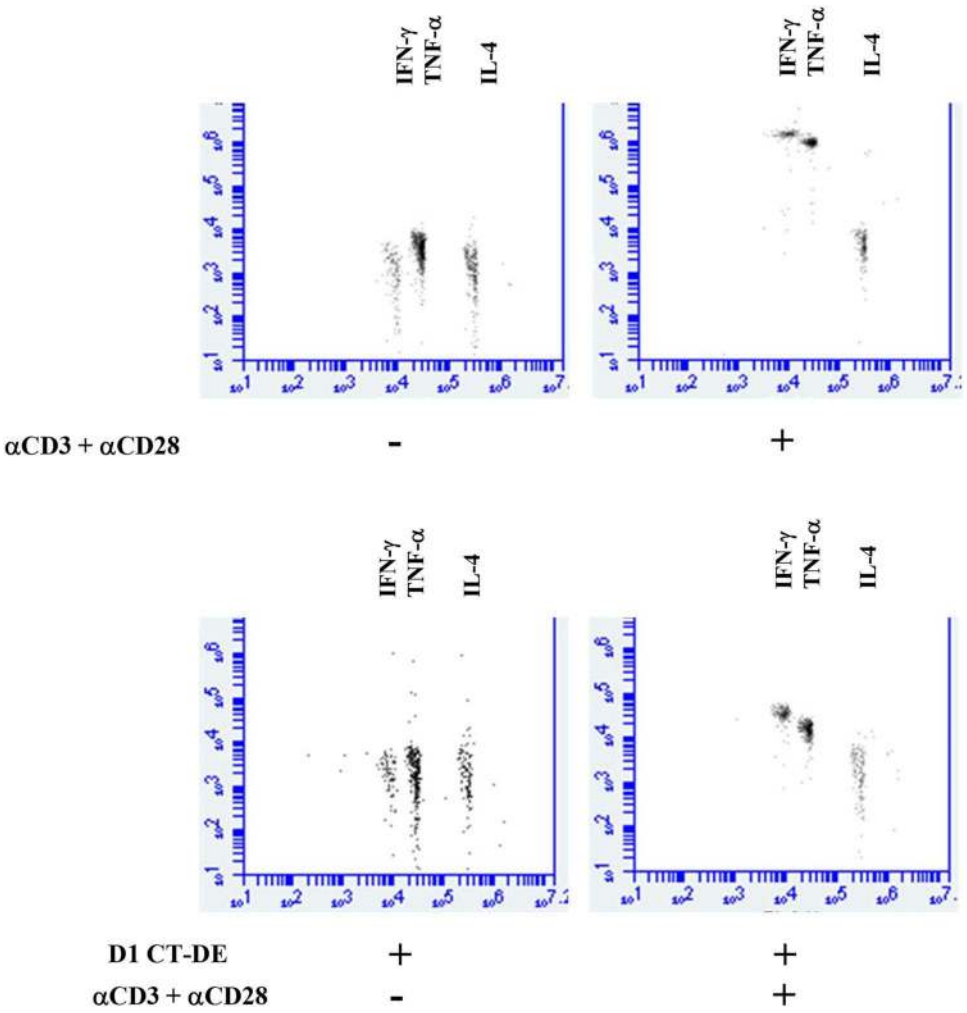

Figure 9. VCT-derived exosomes inhibit Th1 cytokine production in anti-CD3/anti-CD28-activated PBMCs. Peripheral blood mononuclear cells were incubated in the presence of 50 μg/ml of exosomes isolated from freshly purified day 1 VCTs (CT-DE: cytotrophoblast-derived exosomes). After 24 h, cells were stimulated in the presence of anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) antibodies and cytokines were measured in supernatant after 24 h through the LEGENDplex Human Th1/Th2 Panel kit.

IFN-γ and TNF-α in these graphs) were again strongly induced but severely hampered upon the addition of the $(Sync\text{-}2\text{-}ISD)_2$ peptide. In contrast, incubation with $(Rev\ Sync\text{-}2\text{-}ISD)_2$ led to a modest impact on levels of Th1 cytokines. Estimated concentrations of Th1 cytokines, including IL-2 (Figure 7) further confirmed this specific inhibitory action of $(Sync\text{-}2\text{-}ISD)_2$ in comparison to the control dimerized peptide.

Using two different combinations of activators, our data thus demonstrated that the ISD domain of Syncytin-2 possessed immunosuppressive activity on activated PBMCs, mainly toward a Th1 response.

Placental exosomes down-regulate TNF-α expression and activation of Jurkat cells

Given the immunosuppressive potential of the Syncytin-2 ISD domain, we next examined the contribution of Syncytin-2 to the inhibitory capacity of placental exosomes on T cell activation. We first confirmed that indeed these exosomes were capable of negatively modulating T cell activation. Jurkat cells were thus incubated in the presence of increasing quantities of exosomes isolated from cultured VCTs and then stimulated with the PMA/ionomycin combination (Figure 8). Following analyses by RT-PCR and normalization with GAPDH, results indicated that TNF-α expression was induced following PMA/ionomycin treatment, while addition of trophoblast exosomes inhibited this induction (Figure 8A). Quantification of supernatant-associated TNF-α confirmed that placental exosomes reduced TNF-α secretion in Jurkat cells (Figure 8B). In order to further demonstrate the inhibitory capacity of cytotrophoblast-derived exosomes on T cells, Jurkat cells were transfected with constructs containing luciferase reporter gene under the regulation of either NF-κB or NFAT, two transcription factors of high importance in activation of T cells. As shown in Figure 8C and D, activation of both transcription factors by PMA/ionomycin was severely affected following incubation with exosomes. This effect was further demonstrated to be dose-dependent (Figure 8D).

These results hence corroborated former studies showing that placental exosomes, in this case produced from VCTs, act negatively on the activation of T cells.

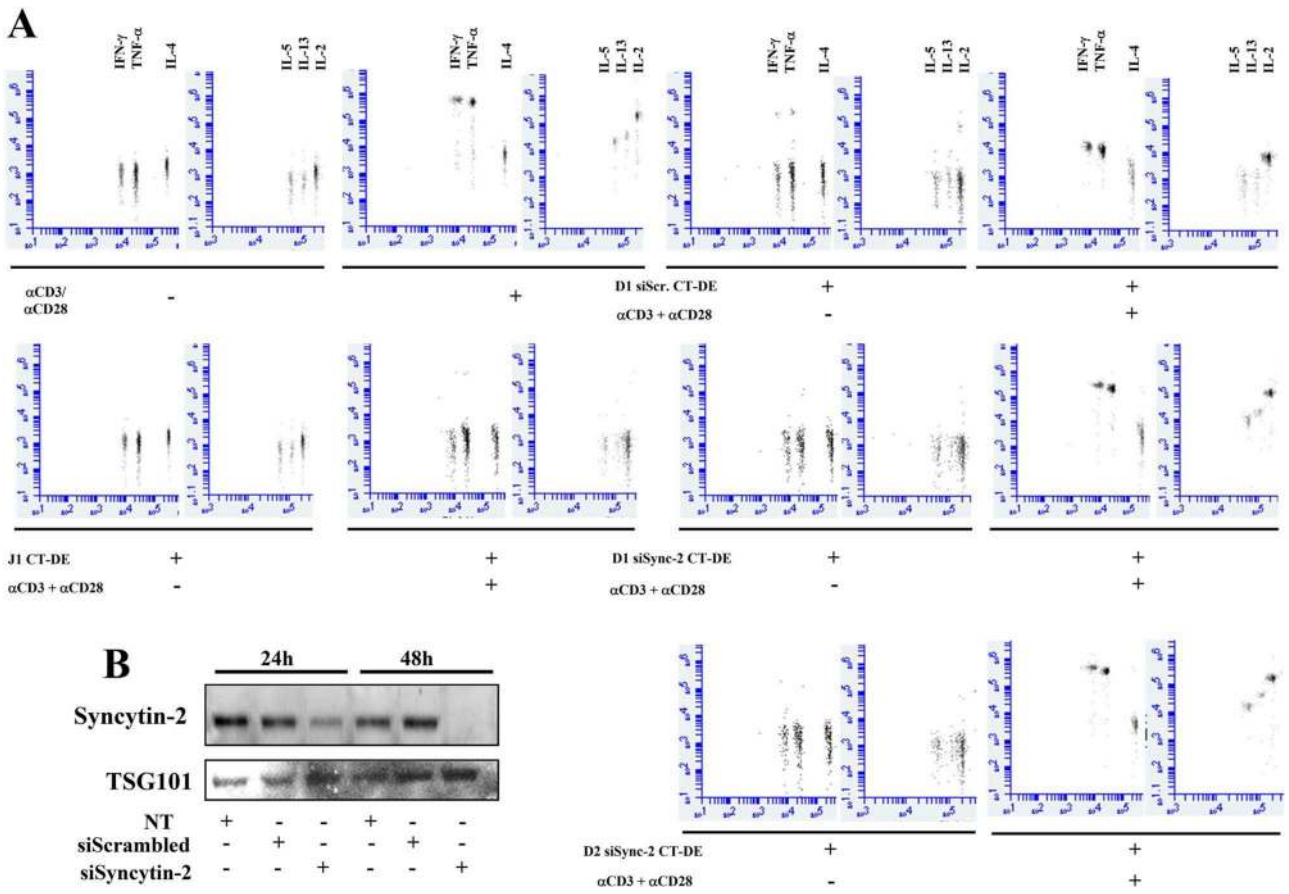

Figure 10. Reduced levels of Syncytin-2 on the surface of cytotrophoblast-derived exosomes decrease their inhibitory capacity over Th1 cytokine production. Peripheral blood mononuclear cells were incubated in the presence of 50 μg/ml of exosomes (CT-DE) isolated from untransfected primary cytotrophoblast cells or from primary cytotrophoblast cells transfected with siSync2 or scrambled control siRNA (siScr) (one or two-day cultures: D1–D2). After 24 h, cells were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) and cytokines were measured in harvested supernatants after 24 h through the LEGENDplex Human Th1/Th2 Panel kit (A). Western blot analyses were performed on extracts from cytotrophoblast-derived exosomes from each transfection condition using anti-Syncytin-2 and anti-TSG101 antibodies at 24 and 48 h after transfection (B). These results are representative of two independent experiments.

Impact of exosome-associated Syncytin-2 on the immunosuppressive capacity of placental exosomes

Since we and others have previously demonstrated that both Syncytin-1 and Syncytin-2 are integrated at the surface of placental extracellular vesicles (including exosomes) [16, 25, 30], we were thus interested in determining the contribution of these proteins in exosome-mediated immunosuppression. Using the fluorescence-activated cell sorting (FACS)-based approach described above, we first quantified production of IFN-γ and TNF-α (versus IL-4) by PBMCs activated by the addition of anti-CD3/anti-CD28 antibodies and incubated in the presence of placental exosomes. Placental exosomes indeed showed an important inhibitory effect on the induced production of Th1 cytokines (Figures 9 and 10A). The addition of exosomes to PBMCs did not lead to higher cell death in comparison to untreated cells (data not shown).

Based on these results, we focused on Syncytin-2 for its contribution in the immunosuppressive capacity of placental exosomes. To selectively deplete Syncytin-2 from these exosomes, Syncytin-2-specific siRNAs (siSync2s) were tested, as we had previously noted efficient repression of expression by this approach [26, 30]. As expected, when isolated VCTs were transfected with Syncytin-2 siRNAs, after 24 and 48 h, isolated exosomes showed limited levels of Syncytin-2 as compared with exosomes from control (scrambled) siRNA-transfected cytotrophoblasts (Figure 10B).

We then compared normal versus Syncytin-2-deficient exosomes for their immunosuppressive potential on anti-CD3/anti-CD28-activated PBMCs. As depicted in Figure 10A, exosomes derived from VCTs transfected with control (scrambled) siRNAs showed strong immunosuppressive abilities, as demonstrated by IFN-γ, IL-2, and TNF-α levels present in the supernatant of treated PBMCs. However, importantly, Syncytin-2-depleted exosomes harvested at two different days (days 1 and 2) of culture of transfected VCTs demonstrated a lesser inhibitory effect on IFN-γ, IL-2, and TNF-α production. Results of the quantification of the different Th1 cytokines from these FACS analyses are summarized in Figure 11 for both 24 and 48 h conditions. These data indeed strongly argued that placental exosomes showed an immunosuppressive property on Th1 cytokine secretion and that depletion of Syncytin-2 from their surface hampered this inhibitory potential.

Overall, these data demonstrated that Syncytin-2 contributed to the immunosuppression mediated by placental exosomes on activated PBMCs by reducing levels of secreted Th1 cytokines.

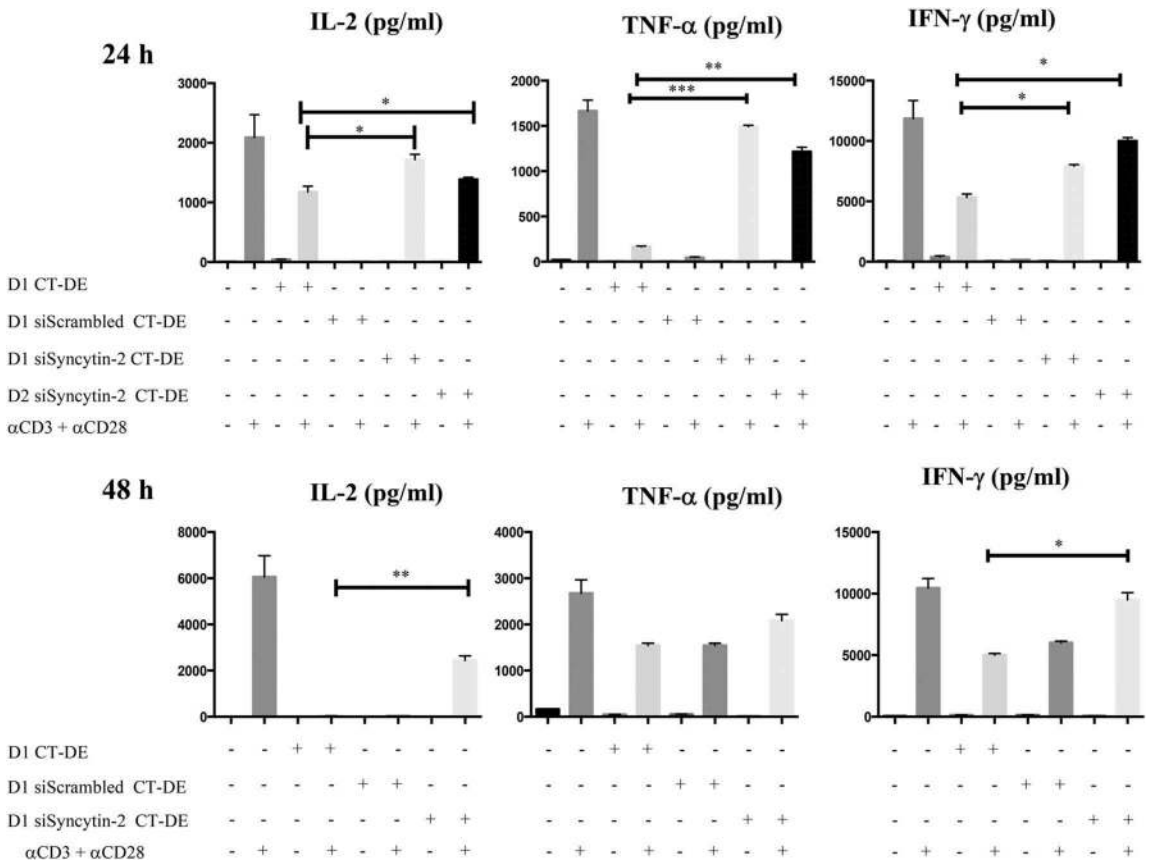

Figure 11. Quantification of cytokine levels in anti-CD3/anti-CD28-stimulated PBMCs following exposure to cytotrophoblast-derived Syncytin-2^{-} versus Syncytin-2^{+} exosomes. Peripheral blood mononuclear cells were incubated in the presence of 50 µg/ml of exosomes (CT-DE) isolated from untransfected primary cytotrophoblast cells or from primary cytotrophoblast cells transfected with siSync2 or siScr (one or two-day cultures: D1–D2). After 24 h, cells were stimulated with anti-CD3 (0.5 µg/ml) and anti-CD28 (1.5 µg/ml) antibodies and cytokines were measured in supernatants after 24 and 48 h through the LEGENDplex Human Th1/Th2 Panel kit. Quantification of cytokine levels from Figure 10 are presented in pg/ml. Means ± SE were calculated for each measured cytokine and results are representative of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Discussion

In this study, we have analyzed the immunosuppressive properties of Syncytin-2. It is now established that retroviral infections often promote severe immunosuppression in both animals and humans through a retroviral ISD [22, 31]. Based on our results, we have demonstrated that Syncytin-2 ISD, similarly to peptide CKS-17 and the Syncytin-1 ISD, activates mitogen-activated protein kinase (MAP kinase) and leads to inhibition of cytokine production in PBMCs activated by PMA/ionomycin or by anti-CD3/anti-CD28 [10, 16]. Furthermore, an equal inhibition was demonstrated in Jurkat T cells, in which TNF-α synthesis was specifically reduced following exposure to $(\text{Sync-2-ISD})_2$.

During normal pregnancy, the second trimester is characterized by a shift toward production of Th2 cytokines and inhibition of the Th1 immune response [2]. Our results demonstrate that the Syncytin-2 ISD is able to inhibit the production of Th1 cytokines (TNF-α, IFN-γ, and IL-2), which extends the observation of the impact of Syncytin-1 ISD on the inhibition of Th1 cytokine secretion [16]. These results concur with many studies showing that HERV envelope proteins (like for several exogenous retroviruses and endogenous retrovirus (ERV) from other species) harbor functional ISD domain that allowed viruses to achieve infection in its hosts before being co-opted [32]. Interestingly, a previous study had indicated that the ISD region of HERV-K envelope inhibited the proliferation of activated PBMCs and that this same peptide increased IL-10 production [14]. We have not been able to demonstrate consistent induction of IL-10 secretion in ISD-treated unstimulated or stimulated PBMCs and this could be attributed to concentration of the peptide and variation in time kinetics used to measure cytokine production. Clearly, however, an early impact on Th1 cytokine is apparent and does not seem to rely on IL-10 production, although this cytokine might intervene at later time points in regulating Th1 response. The function of HERV Env ISD needs further investigation but, in the context of pregnancy, numerous studies including our own, provide strong evidence for its role in regulating the immune response [33]. As pregnancy-associated immunity has been compared with immunity related to cancer development [2], it can also be argued that tumors associated with high expression levels of HERV Env might also benefit from a functional ISD in escaping the immune response, although other roles attributed to overexpressed HERV Env proteins have been suggested to contribute to the development of cancer [34].

The placenta, most specifically the STB layer, produces an important amount of extracellular vesicles, which, through their capacity to mediate intercellular communication and to carry miRNA, proteins, and diverse molecules, are thought to act in different

physiological and pathological processes [23]. Several reports have suggested that different components of placental extracellular microvesicles promote feto-maternal tolerance [24, 35, 36]. In this study, we have confirmed that exosomes inhibited the production of Th1 cytokines (TNF-α, IFN-γ, and IL-2) and that Syn-2-negative exosomes lost this Th1 cytokine-suppressing capacity. These results are also very similar to those obtained for the Syncytin-2 ISD peptide and suggest a similar mode of action. They also provide a mechanism by which Syncytin-2 through its incorporation into extracellular microvesicles could modulate the immune response at a distance and in different regions surrounding the placenta. Previous studies on the immunosuppressive function of retroviral envelope proteins have relied on an in vivo tumor rejection assay in which tumor cells expressing different Env proteins acquired the capacity to proliferate in resistant mice [11–13, 37, 38]. The immunosuppressive function of HERV Env proteins in this context might depend on the action of Env-containing exosomes released from tumor cells. In this view, it is also tempting to speculate that, given that exosomes share certain features with retroviruses, such as size and biogenesis, they could also act similarly to retroviruses by generating an immunosuppressive state via surface-associated proteins, including Syncytin-2. In fact, it is highly likely that the distribution of Syncytin-2 on the surface of exosome mimics typical trimeric complexes formed by Env proteins at the surface of exogenous retroviruses, and suggested for Syncytin-1 and Syncytin-2 [39–41]. Such a Syncytin-2 trimer should also adopt the typical six-helix bundle preceding the fusion step. These different structures, that is, trimers and six-helix bundles, might optimize the inhibitory potential of Syncytin-2 and would thereby be displayed appropriately on the surface of exosomes. Such a mechanism of action might in fact explain why only dimerized forms of ISD peptides are inhibitory, as opposed to their monomeric equivalent.

A recent studyby Eksmond et al. [42] had suggested that mutations known to affect ISD function was also severely impacting Friend murine leukemia virus replication and that the role played by the ISD domain during infection might not be related to an immunosuppressive function. However, in our system, we have used a Syncytin-2 ISD peptide and exosome-associated Syncytin-2 to address the immunosuppressive capacity of this HERV Env protein, which therefore allowed us to avoid being constrained by a possible effect of the ISD region on viral replication. The current mechanisms of action of the ISD domain should involve cell surface factor, which are likely different from the identified receptors needed for HERV envelope-mediated fusion. More mechanistic studies are needed to better understand how this envelope region can modulate immune response and which immune cell populations are targeted by the HERV ISD.

In conclusion, our datasuggest that Syncytin-2 via its ISD domain, and particularly through its association to exosomes, contribute in the modulation of the microenvironment surrounding the fetus by acting on the Th1 response. Since placenta-derived extracellular vesicles of preeclamptic patients are altered in their content and number in comparison to those of normal pregnant women, it has been suggested that they might contribute to the development of the disorder [24]. Reduced Syncytin-2 levels in exosomes from preeclamptic patients [30] might therefore play a determinant role in altering immunomodulatory capacity of these vesicles and could be important in the appearance of the symptoms related to preeclampsia. More studies will be needed to address this possibility.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Acknowledgments

We are thankful toward Sophie Perreault and Lise-Angela Ouellet (CHU Sainte-Justine Hospital) for having taken care of patient recruitment. We are also thankful toward Dr Sylvie Girard (Université de Montréal) for her helpful comments on the manuscript.

Conflict of interest

The authors have declared that no conflict of interest exists.

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