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

Running title: Immunosuppressive function of Syncytin-2 on T cell

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

Keywords: Syncytin-2, Th1 cytokines, syncytiotrophoblast, placenta, exosomes, immunosuppression

Adjimon G. Lokossou[1,3¶], Caroline Toudic[1,3], Phuong Trang Nguyen[2,3], Xavier Elisseeff[1,3], Amandine Vargas[5], Éric Rassart[1,3], Julie Lafond[1,3], Line Leduc[6], Steve Bourgault[2,3,4], Caroline Gilbert[7], Tatiana Scorza[1,3,4], Jorge Tolosa[8] and Benoit Barbeau[1,3,4]*

[1]Department of Biological Sciences, [2]Department of Chemistry and [3]Centre de recherche BioMed, [4] Centre d'Excellence en Recherche sur les Maladies Orphelines—Fondation Courtois, Montreal, Canada; [5]Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, Canada. Université du Québec à Montréal, Montréal, Canada; [6]CHU Ste-Justine, Montréal, Canada; [7]Centre de Recherche du CHU de Québec, Québec, Canada; [8]Mothers and Babies Research Centre and Hunter Medical Research Institute, The University of Newcastle, Newcastle, Australia

Grant support: This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC; #298527-2013), by the Canadian Institutes of Health Research (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

*To whom correspondence should be addressed: Benoit Barbeau, Department of biological sciences, Université du Québec à Montréal, 141 Avenue du Président-Kennedy, H2X 1Y4, Montréal (Québec), Canada, Tel.: (514) 987-3000 ext. 4576; Fax: (514) 987-4647; EMAIL: barbeau.benoit@uqam.ca

[¶] Actual address : École Polytechnique d'Abomey Calavi, Université d'Abomey, Calavi, Benin

Abstract

Modulation of the activation status of immune cell populations during pregnancy depends on placental villous cytotrophoblast (VCT) cells and the syncytiotrophoblast (STB) layer. 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 in STB formation through its fusogenic properties, but also possesses an active 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 have an immunosuppressive effect, placental exosomes were incubated with activated Jurkat T cells and PBMCs. Upon quantification of Th1 cytokines in the supernatants, T cell activation was severely reduced. Interestingly, exosomes from Syn-2-silenced VCT incubated with PBMCS were less suppressive when compared to exosome derived from VCT transfected with control siRNA. Our results suggest that Syn-2 could be an important immune regulator both locally and at the systemic level, via its association with placental exosomes.

Introduction

During normal pregnancy, the maternal immune system has been thought to be only modulated in order for the immunologically foreign foetus to be protected from rejection [1]. Recent studies have highlighted that placenta-mediated modulation of the activity of the immune responses 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 cells, 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 [5]. Unlike extravillous cytotrophoblasts, villous cytotrophoblasts are non-invasive but fuse to form and maintain the structure of the peripheral syncytiotrophoblast layer through a well-regulated process. A number of studies have demonstrated that envelope 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 has clearly suggested that envelope (Env) Syncytin-1 and Syncytin-2 indeed participate in the formation of the syncytiotrophoblast layer by mediating fusion with underlying cytotrophoblast cells through interaction with their specific receptors [8].

Interestingly, HERV Env proteins 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 have been suggested to be altered in pregnancy disorders, such as pre-eclampsia [17-21]. In all these studies, including those with ISD region from exosgenous retroviruses, a 17 amino acid-long peptide in a dimerized form 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 signalling results in activation of ERK1/2 MAP kinases and other cellular effectors [22]. Studies on the Friend murine leukemia virus have further shown 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 syncytiotrophoblast 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 villous cytotrophoblast-derived extracellular vesicles. 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 SU subunit [26]. Peptides were synthesized by solid phase peptide synthesis and were characterized by LC/MS-TOF. 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 1640 medium (350-000-CL; Wisent, Montreal, Canada) and 10% exosome-free FBS (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 (PBMCs)

This study was approved by the Ethic committees of both Université du Québec à Montréal and The Ste Justine Hospital. Venous blood was collected from 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 villous cytotrophoblasts 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 x 10^5 to 1.8 x 10^6 U) and DNase I (Millipore Sigma; from 15 to 30 mg per digestion) for 30 minutes 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 x 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 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 x 10^7 cells) were transfected by the DEAE-Dextran protocol, as previously described [29]. Plasmids pNFAT-Luc and pNF-κB-Luc have been previously described and respectively contains NFAT and NF-κB-binding sites positioned upstream of a minimal promoter and the luciferase reporter gene [27]. Small interfering RNAs (siRNA) 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 x 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 at 2000g for 15 min and at 10,000g (4°C) for 30 min. Following two subsequent 100,000g centrifugations (4°C), supernatants were discarded and pellets containing exosomes were resuspended in PBS (100 µl). Exosome preparations were stored at -80°C in 50 µl aliquots and analysed by Western blot.

Incubation of Jurkat and PBMCs 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 to 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 x 10^5 cells) were cultured in sodium bicarbonate-free supplemented RPMI 1640 medium (20mM

Hepes, 2mM 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) and cultured for 18 to 48 h before harvesting the supernatant. For exosomes, PBMC or transfected Jurkat cells (5 x 10^5 cells) cultured in sodium bicarbonate-free supplemented RPMI 1640 medium (20mM Hepes, 2mM 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 and harvesting of supernatant). Following incubation, cell viability was determined by Lactate Dehydrogenase Activity Assay (MAK066, Sigma-Aldrich), according to provided instructions (Promega).

Western blot analysis

Cells were resuspended in 2× SDS 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 (1:1000). After overnight incubation at 4°C, membranes were washed three times in PBS-Tween 0.05% and incubated with

horseradish peroxidase (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 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, 100μM 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-α), 5′-TGAGGTACAGGCCCTCTGAT-3′ (reverse TNF- α), 5'-GAAGGTGAAGGTCGGAGTCAA-3' (forward GAPDH) and 5'-GGAAGATGGTGATGGGATTTC-3' (reverse GAPDH).

Quantification of Th1 and Th2 cytokine in Jurkat and PBMC cells

Jurkat supernatants were assayed for TNF-α with the commercial ELISA MAX™ Deluxe kit (#430204**,** Biolegend, San Diego, California). PBMC 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 IL-13, 1pg/ml for IL-2, IFN-γ, TNF-α and 1.1 pg/ml for IL-5, IL-6 and IL-10.

190

Statistics

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

Results

The dimerized Syncytin-2 ISD peptide induces ERK1 and ERK 2 phosphorylation. Previous studies reported that Syncytin-1 and -2 harbour 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, i.e. $(Sync\text{-}2\text{-}ISD)_2$. 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 $(Sync\text{-}2\text{-}ISD)_2$ dimeric peptide on Jurkat cells at a fixed concentration and for different time lengths (Figure 1A). Kinetic experiments showed that $(Sync\text{-}2\text{-}ISD)_2$ 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 signalling 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 normalised for protein levels further confirmed the important induction of phosphorylation in response to treatment with the $(Sync\text{-}2\text{-}ISD)_2$ peptide (Figure 1B). A dose response experiment was next performed and revealed a gradual increment of ERK1/2 phosphorylation in $(Sync\text{-}2\text{-}ISD)_2$-treated Jurkat cells,

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

(Sync-2-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-2-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-2-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 (data not shown). 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-2-ISD)$_2$ inhibits cytokine production in PMA-Ionomycin-activated PBMCs. 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-2-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 analysed following the addition of a pool of biotinylated antibodies recognizing all targeted cytokines. Using this assay, we first compared untreated *vs*. 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. When PBMCs were incubated in the presence of (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 (Sync-2-ISD)$_2$-treated PBMCs (Figure 3B-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 (Sync-2-ISD)$_2$, while a limited impact was observed in (Rev 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.

(Sync-2-ISD)$_2$ inhibits cytokine production in anti-CD3/anti-CD28-activated PBMCs 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 IFN-γ and TNF-α in these graphs) were again strongly induced but severely hampered upon the addition of the (Sync-2-ISD)$_2$ peptide. In contrast, incubation with (Rev Sync-2-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-2-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 villous cytotrophoblasts and then stimulated with the PMA/Ionomycin combination (Figure 8). Following analyses of by RT-PCR and normalisation with GAPDH, 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-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 for villous cytotrophoblasts, act negatively on the activation of T cells.

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 FACS-based approach described above, we first quantified production of IFN-γ and TNF-α (vs. 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 (Figure 9; 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 focussed 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 were tested, as we had previously noted efficient repression of expression by this approach [26, 30]. As expected, when isolated villous cytotrophoblasts were transfected with Syncytin-2 siRNAs, after 24 and 48 h, isolated exosomes showed limited levels of Syncytin-2 as compared to exosomes from control (scrambled) siRNA-transfected cytotrophoblasts (Figure 10B).

We then compared normal vs. Syncytin-2-deficient exosomes for their immunosuppressive potential on anti-CD3/anti-CD28-activated PBMCs. As depicted in Figure 10A, exosomes derived from villous cytotrophoblasts 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 (day 1 and 2) of culture of transfected villous cytotrophoblasts 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.

Discussion

In this study, we have analysed 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 immunosuppression domain (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 $(Sync\text{-}2\text{-}ISD)_2$.

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 demonstrated that the Syncytin-2 ISD was able to inhibit the production of Th1 cytokines (TNF-α, IFN-γ, IL-2 and IL-6), 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 ERV from other species) harbour 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 to immunity related to cancer development [2], it can also be argued that tumours 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 syncytiotrophoblast 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 foeto-maternal tolerance [24, 35, 36]. In this study, we have confirmed that exosomes inhibited the production of Th1 cytokines (TNF-α, IFN-γ, 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* tumour rejection assay in which tumour cells expressing different Env proteins acquired the capacity to proliferate in resistant mice [11-13, 37, 38]. This immunosuppressive function of HERV Env proteins in this context might depend on the action of Env-containing exosomes released from tumour 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, i.e. 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 study by Eksmond *et al*. (2017) 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 [42]. 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 modulated immune response and which immune cell populations are targeted by the HERV immunosuppressive domain.

In conclusion, our data suggest that Syncytin-2 via its ISD domain, and particularly through its association to exosomes, contribute in the modulation of the microenvironment surrounding the foetus by acting on the Th1 response. Since placenta-derived-extracellular

vesicles of pre-eclamptic 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 pre-eclamptic 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 pre-eclampsia. More studies will be needed to address this possibility.

ACKNOWLEDGMENT

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.

References

1. Warning JC, McCracken SA, Morris JM. A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system. Reproduction 2011; 141:715-724.
2. Mor G, Aldo P, Alvero AB. The unique immunological and microbial aspects of pregnancy. Nat Rev Immunol 2017; 17:469-482.
3. Lash GE. Molecular Cross-Talk at the Feto-Maternal Interface. Cold Spring Harb Perspect Med 2015; 5.
4. Erkers T, Stikvoort A, Uhlin M. Lymphocytes in Placental Tissues: Immune Regulation and Translational Possibilities for Immunotherapy. Stem Cells Int 2017; 2017:5738371.
5. Morrish DW, Dakour J, Li H. Functional regulation of human trophoblast differentiation. J Reprod Immunol 1998; 39:179-195.
6. Denner J. Expression and function of endogenous retroviruses in the placenta. APMIS 2016; 124:31-43.
7. Costa MA. Scrutinising the regulators of syncytialization and their expression in pregnancy-related conditions. Mol Cell Endocrinol 2016; 420:180-193.
8. Lokossou AG, Toudic C, Barbeau B. Implication of human endogenous retrovirus envelope proteins in placental functions. Viruses 2014; 6:4609-4627.
9. Haraguchi S, Good RA, Day NK. Immunosuppressive retroviral peptides: cAMP and cytokine patterns. Immunol Today 1995; 16:595-603.
10. Takahashi A, Day NK, Luangwedchakarn V, Good RA, Haraguchi S. A retroviral-derived immunosuppressive peptide activates mitogen-activated protein kinases. J Immunol 2001; 166:6771-6775.

11. Schlecht-Louf G, Renard M, Mangeney M, Letzelter C, Richaud A, Ducos B, Bouallaga I, Heidmann T. Retroviral infection in vivo requires an immune escape virulence factor encrypted in the envelope protein of oncoretroviruses. Proc Natl Acad Sci U S A 2010; 107:3782-3787.

12. Mangeney M, de Parseval N, Thomas G, Heidmann T. The full-length envelope of an HERV-H human endogenous retrovirus has immunosuppressive properties. J Gen Virol 2001; 82:2515-2518.

13. Mangeney M, Renard M, Schlecht-Louf G, Bouallaga I, Heidmann O, Letzelter C, Richaud A, Ducos B, Heidmann T. Placental syncytins: Genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins. Proc Natl Acad Sci U S A 2007; 104:20534-20539.

14. Morozov VA, Dao Thi VL, Denner J. The transmembrane protein of the human endogenous retrovirus--K (HERV-K) modulates cytokine release and gene expression. PLoS One 2013; 8:e70399.

15. Hummel J, Kammerer U, Muller N, Avota E, Schneider-Schaulies S. Human endogenous retrovirus envelope proteins target dendritic cells to suppress T-cell activation. Eur J Immunol 2015; 45:1748-1759.

16. Tolosa JM, Schjenken JE, Clifton VL, Vargas A, Barbeau B, Lowry P, Maiti K, Smith R. The endogenous retroviral envelope protein syncytin-1 inhibits LPS/PHA-stimulated cytokine responses in human blood and is sorted into placental exosomes. Placenta 2012; 33:933-941.

17. Keith JC, Jr., Pijnenborg R, Van Assche FA. Placental syncytin expression in normal and preeclamptic pregnancies. Am J Obstet Gynecol 2002; 187:1122-1123; author reply 1123-1124.

18. Knerr I, Huppertz B, Weigel C, Dotsch J, Wich C, Schild RL, Beckmann MW, Rascher W. Endogenous retroviral syncytin: compilation of experimental research on syncytin and its possible role in normal and disturbed human placentogenesis. Mol Hum Reprod 2004; 10:581-588.

19. Langbein M, Strick R, Strissel PL, Vogt N, Parsch H, Beckmann MW, Schild RL. Impaired cytotrophoblast cell-cell fusion is associated with reduced Syncytin and increased apoptosis in patients with placental dysfunction. Mol Reprod Dev 2008; 75:175-183.

20. Chen CP, Chen LF, Yang SR, Chen CY, Ko CC, Chang GD, Chen H. Functional characterization of the human placental fusogenic membrane protein syncytin 2. Biol Reprod 2008; 79:815-823.

21. Vargas A, Toufaily C, LeBellego F, Rassart E, Lafond J, Barbeau B. Reduced expression of both syncytin 1 and syncytin 2 correlates with severity of preeclampsia. Reprod Sci 2011; 18:1085-1091.

22. Haraguchi S, Good RA, Day-Good NK. A potent immunosuppressive retroviral peptide: cytokine patterns and signaling pathways. Immunol Res 2008; 41:46-55.

23. Adam S, Elfeky O, Kinhal V, Dutta S, Lai A, Jayabalan N, Nuzhat Z, Palma C, Rice GE, Salomon C. Review: Fetal-maternal communication via extracellular vesicles - Implications for complications of pregnancies. Placenta 2017; 54:83-88.

24. Gohner C, Plosch T, Faas MM. Immune-modulatory effects of syncytiotrophoblast extracellular vesicles in pregnancy and preeclampsia. Placenta 2017; 60 Suppl 1:S41-S51.

25. Holder BS, Tower CL, Forbes K, Mulla MJ, Aplin JD, Abrahams VM. Immune cell activation by trophoblast-derived microvesicles is mediated by syncytin 1. Immunology 2012; 136:184-191.

26. Vargas A, Moreau J, Landry S, LeBellego F, Toufaily C, Rassart E, Lafond J, Barbeau B. Syncytin-2 plays an important role in the fusion of human trophoblast cells. J Mol Biol 2009; 392:301-318.

27. Fortin JF, Barat C, Beausejour Y, Barbeau B, Tremblay MJ. Hyper-responsiveness to stimulation of human immunodeficiency virus-infected CD4+ T cells requires Nef and Tat virus gene products and results from higher NFAT, NF-kappaB, and AP-1 induction. J Biol Chem 2004; 279:39520-39531.

28. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF, 3rd. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986; 118:1567-1582.

29. Larocque E, Andre-Arpin C, Borowiak M, Lemay G, Switzer WM, Duc Dodon M, Mesnard JM, Barbeau B. Human T-cell leukemia virus type 3 (HTLV-3) and HTLV-4 antisense-transcript-encoded proteins interact and transactivate Jun family-dependent transcription via their atypical bZIP motif. J Virol 2014; 88:8956-8970.

30. Vargas A, Zhou S, Ethier-Chiasson M, Flipo D, Lafond J, Gilbert C, Barbeau B. Syncytin proteins incorporated in placenta exosomes are important for cell uptake and show variation in abundance in serum exosomes from patients with preeclampsia. FASEB J 2014; 28:3703-3719.

31. Cianciolo GJ, Copeland TD, Oroszlan S, Snyderman R. Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. Science 1985; 230:453-455.

32. Denner J. Immunosuppression by retroviruses: implications for xenotransplantation. Ann N Y Acad Sci 1998; 862:75-86.

33. Bolze PA, Mommert M, Mallet F. Contribution of Syncytins and Other Endogenous Retroviral Envelopes to Human Placenta Pathologies. Prog Mol Biol Transl Sci 2017; 145:111-162.

34. Grandi N, Tramontano E. HERV Envelope Proteins: Physiological Role and Pathogenic Potential in Cancer and Autoimmunity. Front Microbiol 2018; 9:462.

35. Redman CW, Sargent IL. Circulating microparticles in normal pregnancy and pre-eclampsia. Placenta 2008; 29 Suppl A:S73-77.

36. Mincheva-Nilsson L, Nagaeva O, Chen T, Stendahl U, Antsiferova J, Mogren I, Hernestal J, Baranov V. Placenta-derived soluble MHC class I chain-related molecules down-regulate NKG2D receptor on peripheral blood mononuclear cells during human pregnancy: a possible novel immune escape mechanism for fetal survival. Journal of immunology 2006; 176:3585-3592.

37. Mangeney M, Heidmann T. Tumor cells expressing a retroviral envelope escape immune rejection in vivo. Proc Natl Acad Sci U S A 1998; 95:14920-14925.

38. Schlecht-Louf G, Mangeney M, El-Garch H, Lacombe V, Poulet H, Heidmann T. A targeted mutation within the feline leukemia virus (FeLV) envelope protein immunosuppressive domain to improve a canarypox virus-vectored FeLV vaccine. Journal of virology 2014; 88:992-1001.

39. Cheynet V, Ruggieri A, Oriol G, Blond JL, Boson B, Vachot L, Verrier B, Cosset FL, Mallet F. Synthesis, assembly, and processing of the Env ERVWE1/syncytin human endogenous retroviral envelope. J Virol 2005; 79:5585-5593.

40. Renard M, Varela PF, Letzelter C, Duquerroy S, Rey FA, Heidmann T. Crystal structure of a pivotal domain of human syncytin-2, a 40 million years old endogenous retrovirus fusogenic envelope gene captured by primates. J Mol Biol 2005; 352:1029-1034.

41. Gong R, Peng X, Kang S, Feng H, Huang J, Zhang W, Lin D, Tien P, Xiao G. Structural characterization of the fusion core in syncytin, envelope protein of human endogenous retrovirus family W. Biochem Biophys Res Commun 2005; 331:1193-1200.

42. Eksmond U, Jenkins B, Merkenschlager J, Mothes W, Stoye JP, Kassiotis G. Mutation of the Putative Immunosuppressive Domain of the Retroviral Envelope Glycoprotein Compromises Infectivity. J Virol 2017; 91.

Figure legends

Figure 1: The dimerized Syncytin-2 ISD peptide induces ERK1/2 phosphorylation in Jurkat cells. A-B. Jurkat cells were either left untreated or treated with monomeric Sync-2-ISD, dimeric $(Sync\text{-}2\text{-}ISD)_2$, monomeric Reverse Sync-2-ISD or dimeric Reverse $(Sync\text{-}2\text{-}ISD)_2$ (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 $(Sync\text{-}2\text{-}ISD)_2$-treated cells (**B**). Data represents fold increase of pERK1/2 (normalized with signals for total ERK1/2) compared to 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 $(Sync\text{-}2\text{-}ISD)_2$ for 1 h. Western blot analysis was performed as indicated in **A-B**. Results are representative of three independent experiments.

Figure 2: $(Sync\text{-}2\text{-}ISD)_2$ inhibits TNF-α production in Jurkat T cells. Jurkat cells were incubated with 60 μM of monomeric or dimerized Sync-2-ISD vs. control Rev Sync-2-ISD peptides. After 2 h, cells were stimulated with PMA (20ng/ml)/Ionomycin (1 μM) and TNF-α was measured in the supernatant after 24 h (**A**) and 48hrs (**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. PBMCs 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 (20ng/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.

Figure 4: (Sync-2-ISD)$_2$ inhibits Th1 cytokine production in PMA/Ionomycin-activated PBMC. PBMCs 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 (20ng/ml)/Ionomycin (1 μM) and cytokines were measured in supernatants after 48 h through the LEGENDplex™ Human Th1/Th2 Panel kit.

Figure 5: Quantification of Th1 cytokine levels in PMA/Ionomycin-stimulated PBMCs following treatment with (Sync-2-ISD)$_2$. PBMCs were incubated with (Sync-2-ISD)$_2$ vs. control Rev (Sync-2-ISD)$_2$ (60 μM). After 2 h, cells were stimulated with PMA (20ng/ml)/Ionomycin (1 μM) and cytokines were measured. Quantification of cytokine levels from Figure 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. *** $p < 0.001$.

Figure 6: (Sync-2-ISD)$_2$ inhibits Th1 cytokine production in anti-CD3/anti-CD28-activated PBMCs. PBMCs were incubated with 60 μM (Sync-2-ISD)$_2$ or Rev (Sync-2-ISD)$_2$. After 2 h,

cells were stimulated 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.

Figure 7: Quantification of cytokine levels in anti-CD3/anti-CD28-stimulated PBMCs following treatment with (Sync-2-ISD)$_2$. PBMCs were incubated with (Sync-2-ISD)$_2$ vs. Rev (Sync-2-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 *** $p < 0.001$.

Figure 8: Inhibition of TNF-α production in Jurkat T cells by trophoblast-derived exosomes. Jurkat cells were incubated with different concentrations of exosomes (0, 5 or 10 μg/ml) in triplicates. After 24 h, cells were stimulated with PMA (20ng/ml)/Ionomycin (1 μM) and analysed for TNF-α expression by RT-PCR (6 h post-treatment, **A**) and TNF-α secretion by ELISA in harvested supernatants (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 normalisation with GAPDH. Results are representative of three different experiments**. C-D**. Jurkat cells transfected with pNF-κB-Luc (C) or pNFAT-Luc (C-D) were stimulated with PMA (20ng/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 **** $p < 0.0001$.

Figure 9: Villous cytotrophoblast-derived exosomes inhibit Th1 cytokine production in anti-CD3/anti-CD28-activated PBMCs. PBMCs were incubated in the presence of 50 µg/ml of exosomes isolated from freshly purified day 1 villous cytotrophoblasts (CT-DE). 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.

Figure 10: Reduced levels of Syncytin-2 on the surface of cytotrophoblast-derived exosomes decrease their inhibitory capacity over Th1 cytokine production. PBMCs 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 Syncytin-2-specific siRNA (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.

Figure 11: Quantification of cytokine levels in anti-CD3/anti-CD28-stimulated PBMCs following exposure to cytotrophoblast-derived Syncytin-2- vs. Syncytin-2+ exosomes. PBMCs 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 Syncytin-2-specific siRNA (siSync2) or scrambled control siRNA (siScr.) (one or two-day cultures: D1- D2). After 24 h, cells were stimulated anti-CD3 (0.5 μg/ml) and anti-CD28 (1.5 μg/ml) 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 *** $p < 0.001$.

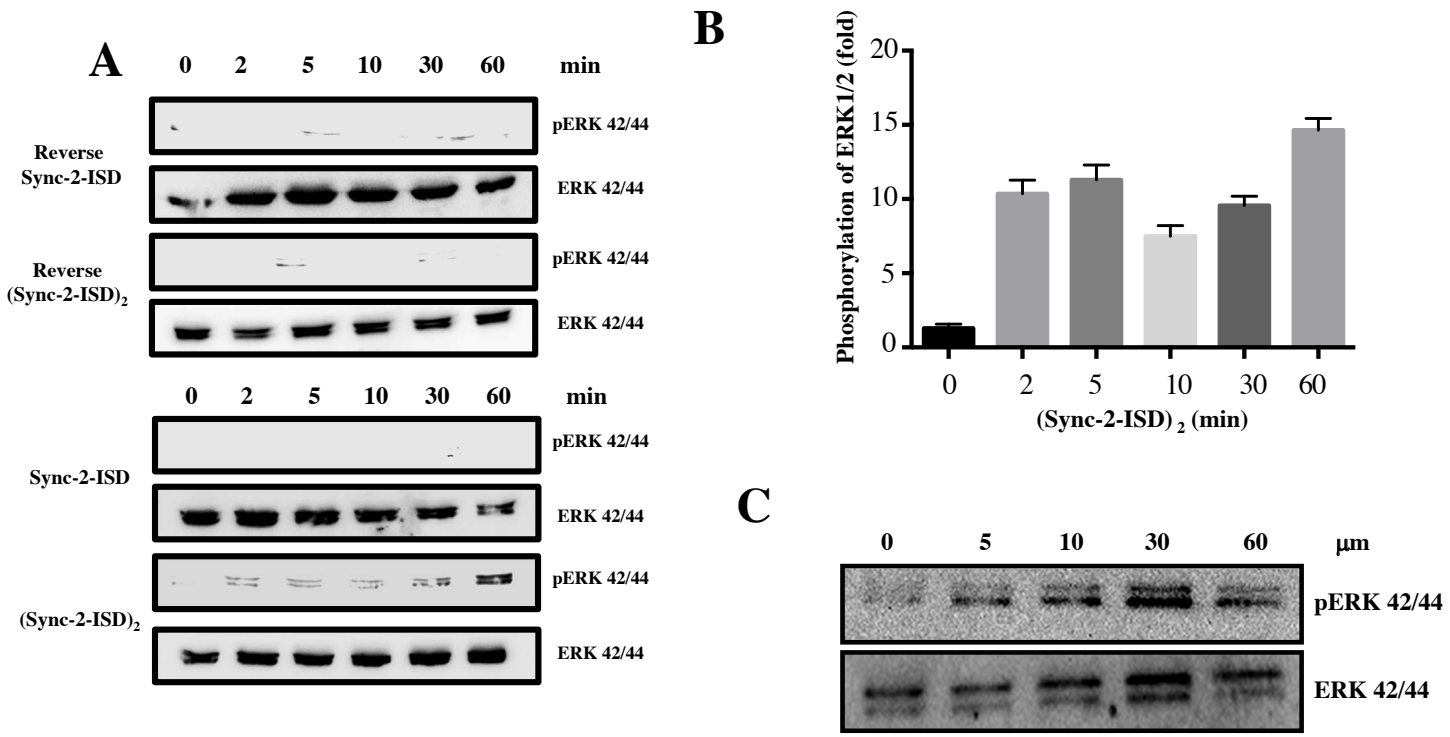

Figure 1

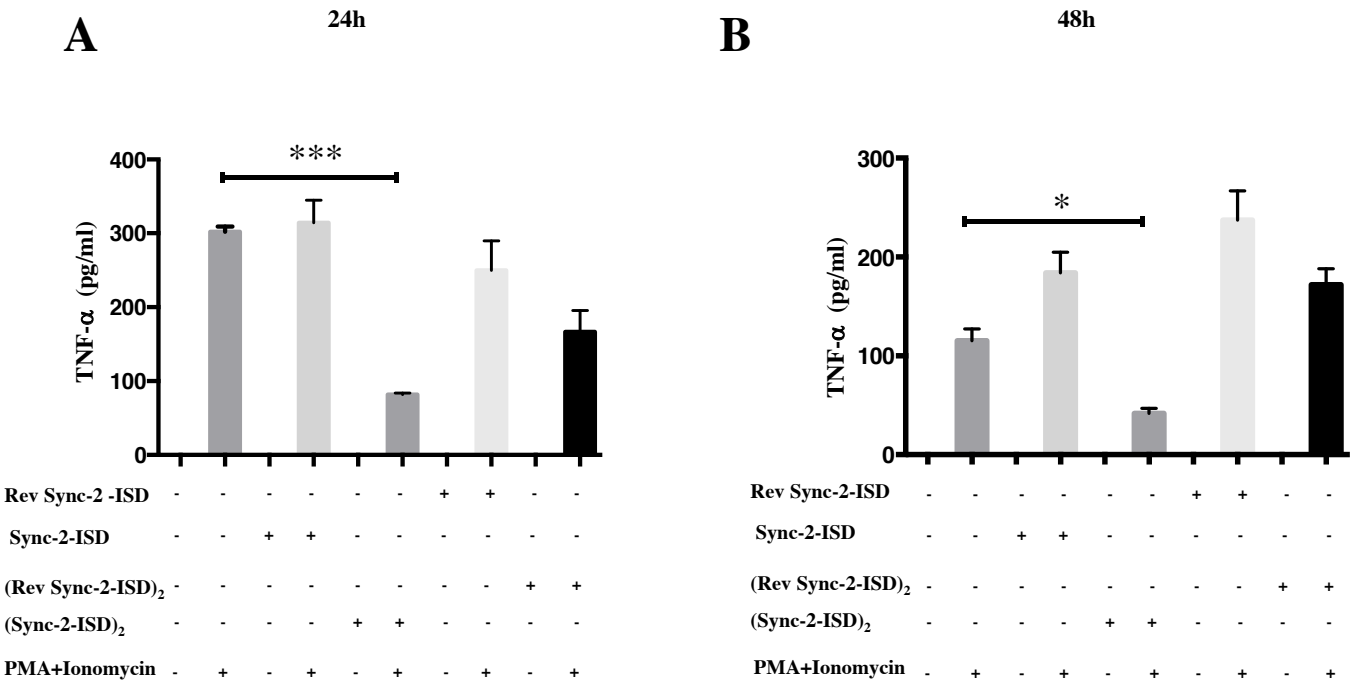

Figure 2

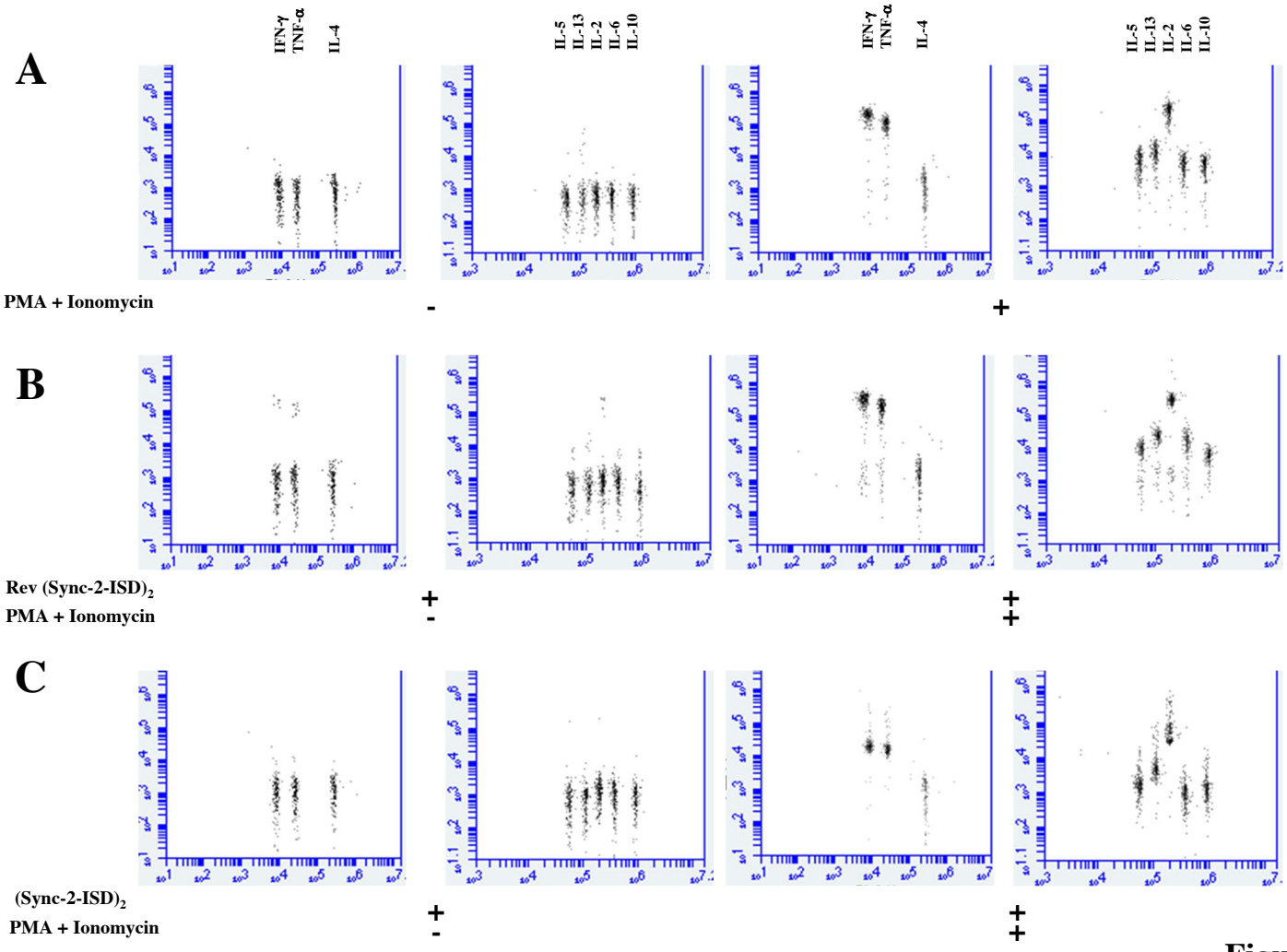

Figure 3

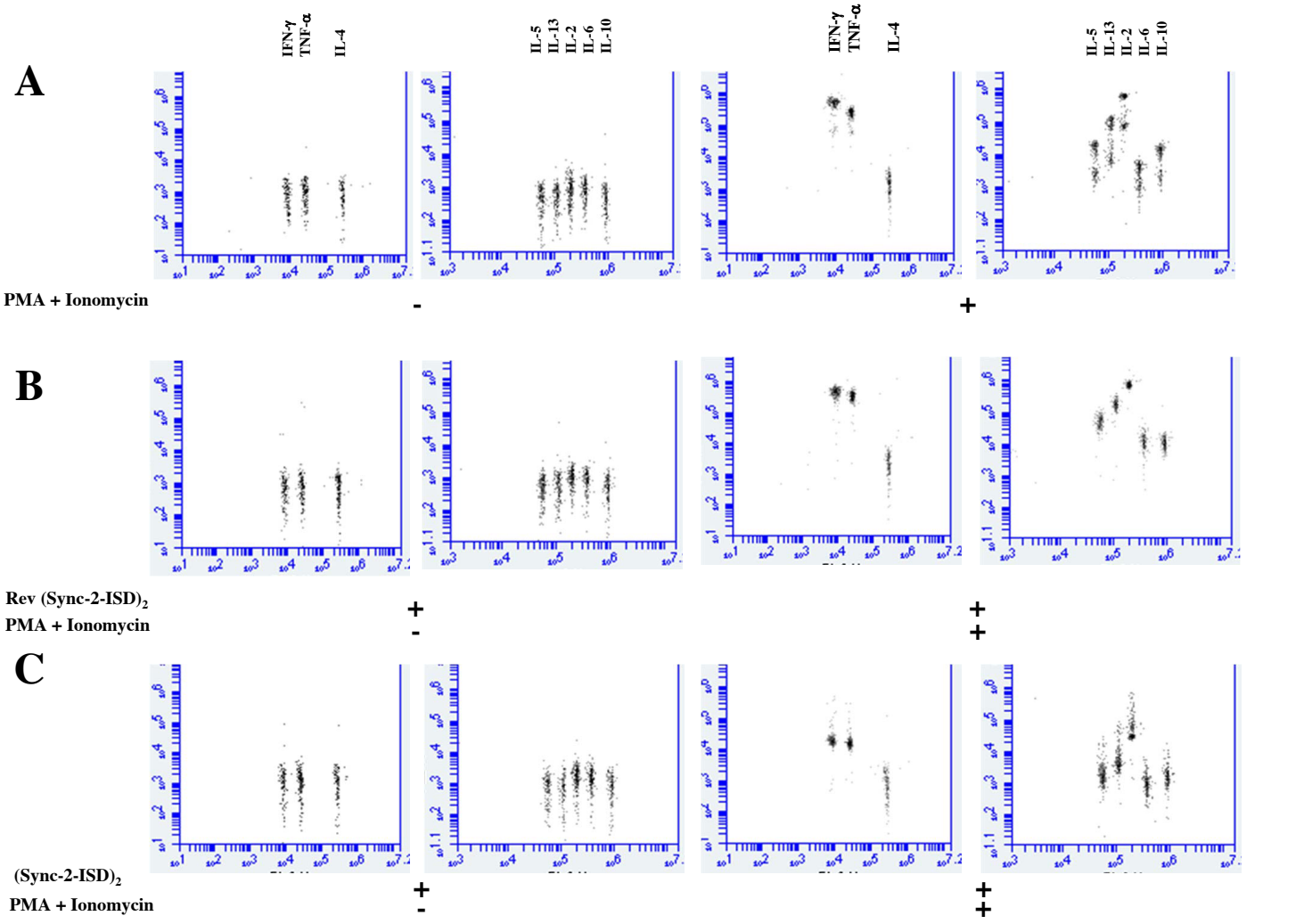

Figure 4

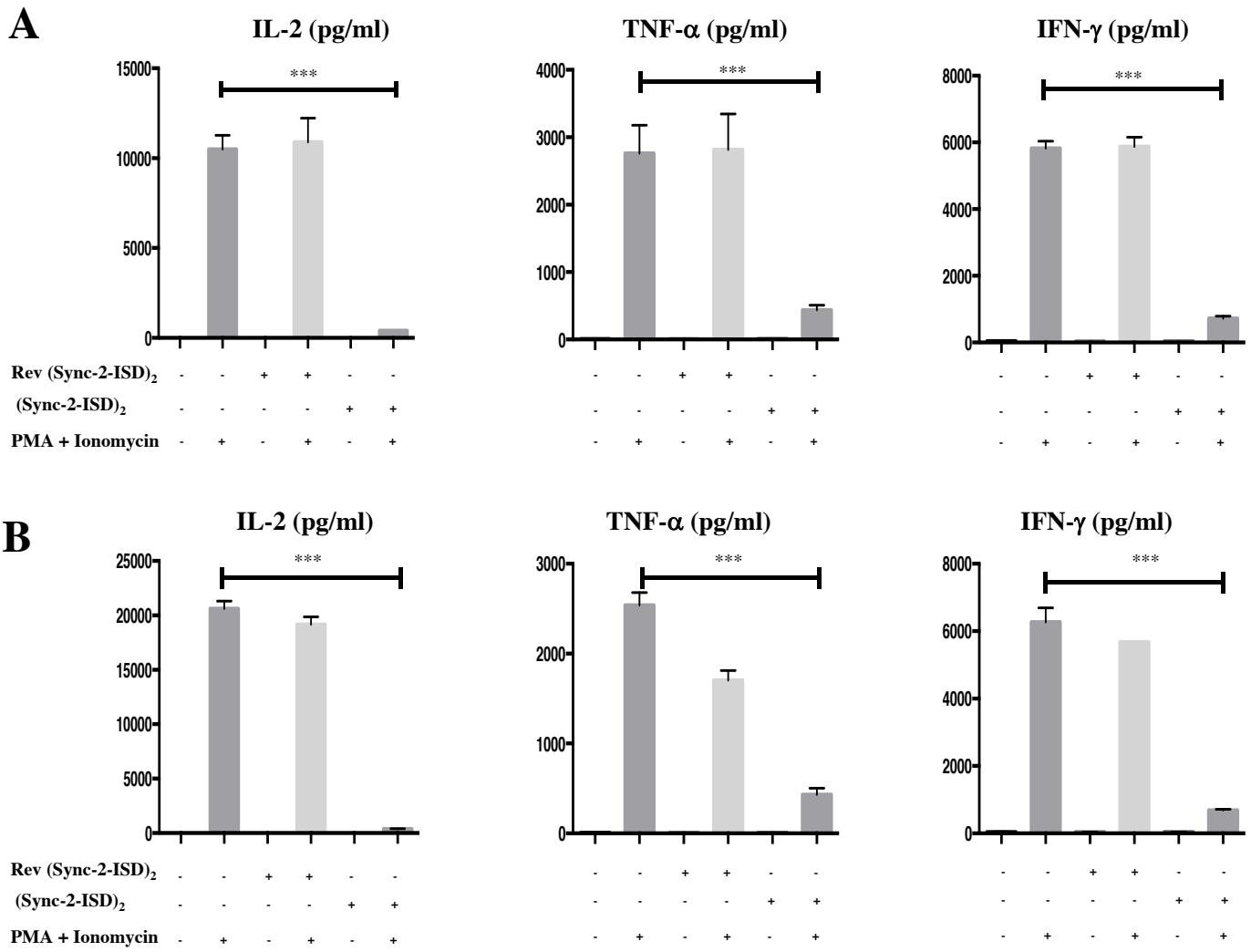

Figure 5

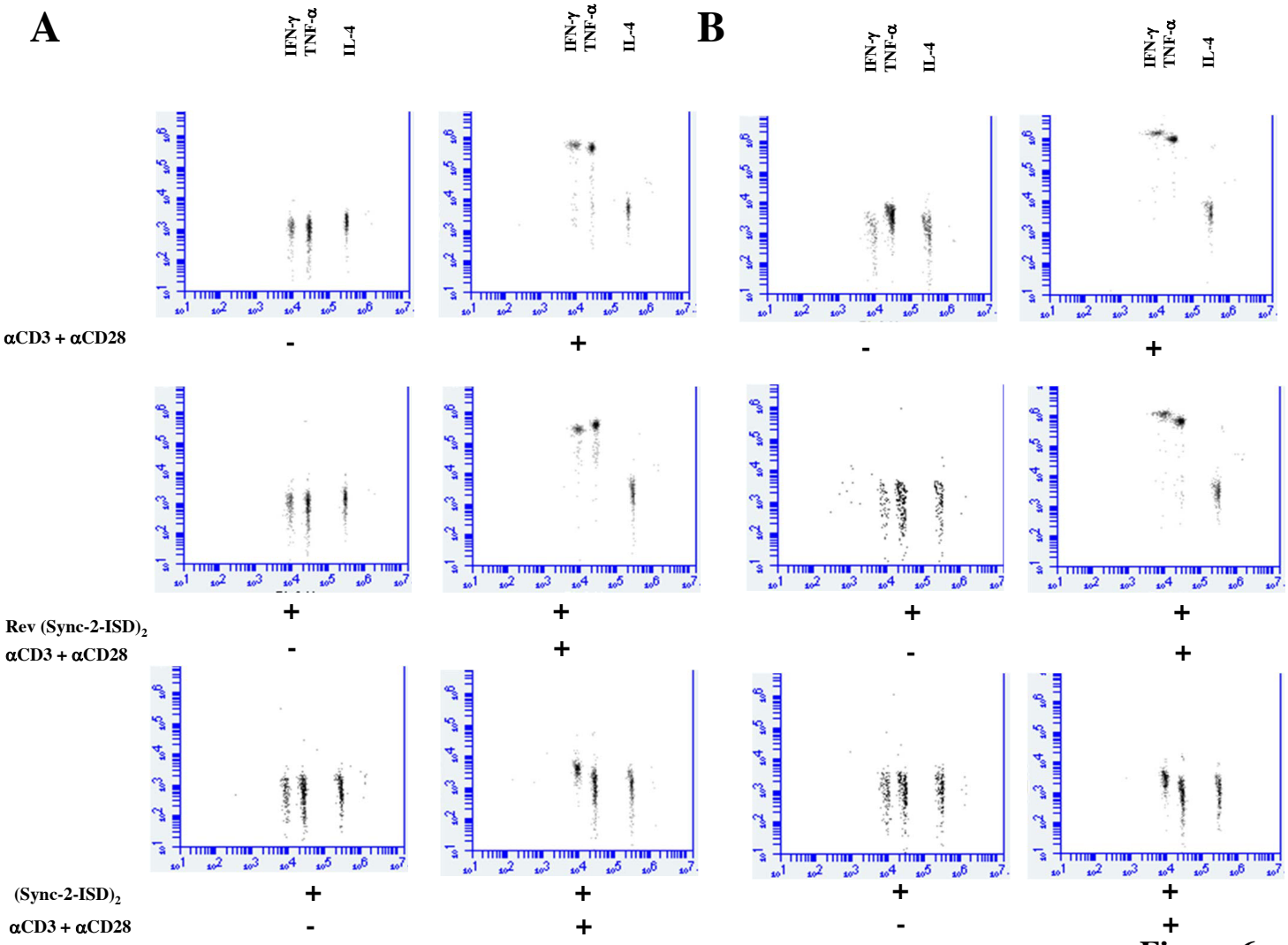

Figure 6

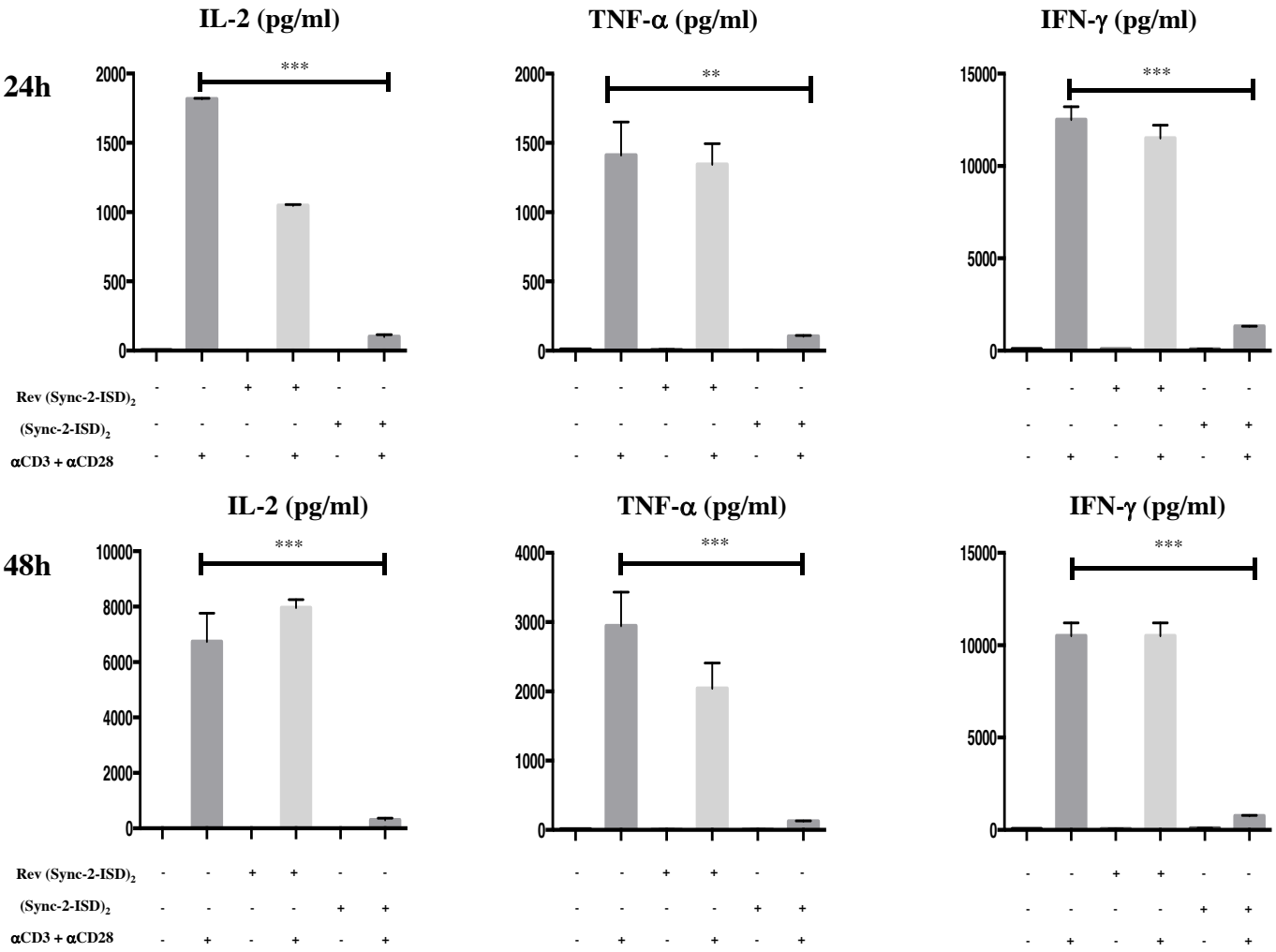

Figure 7

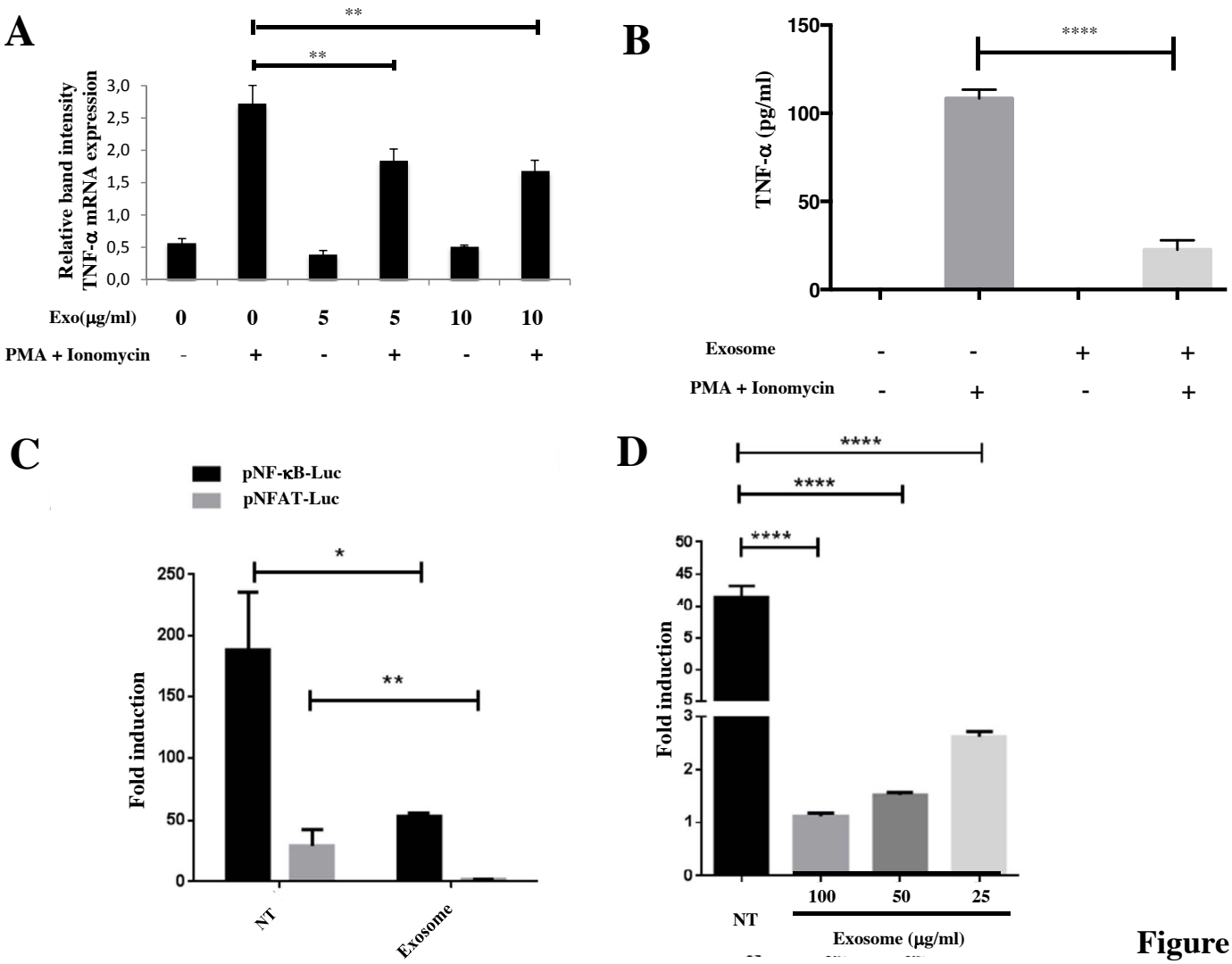

Figure 8

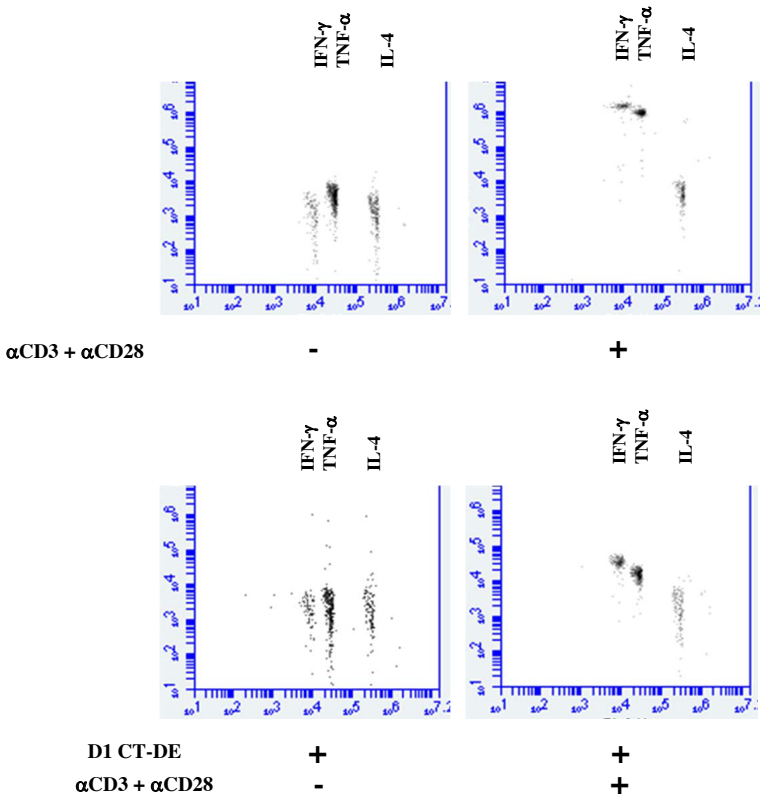

Figure 9

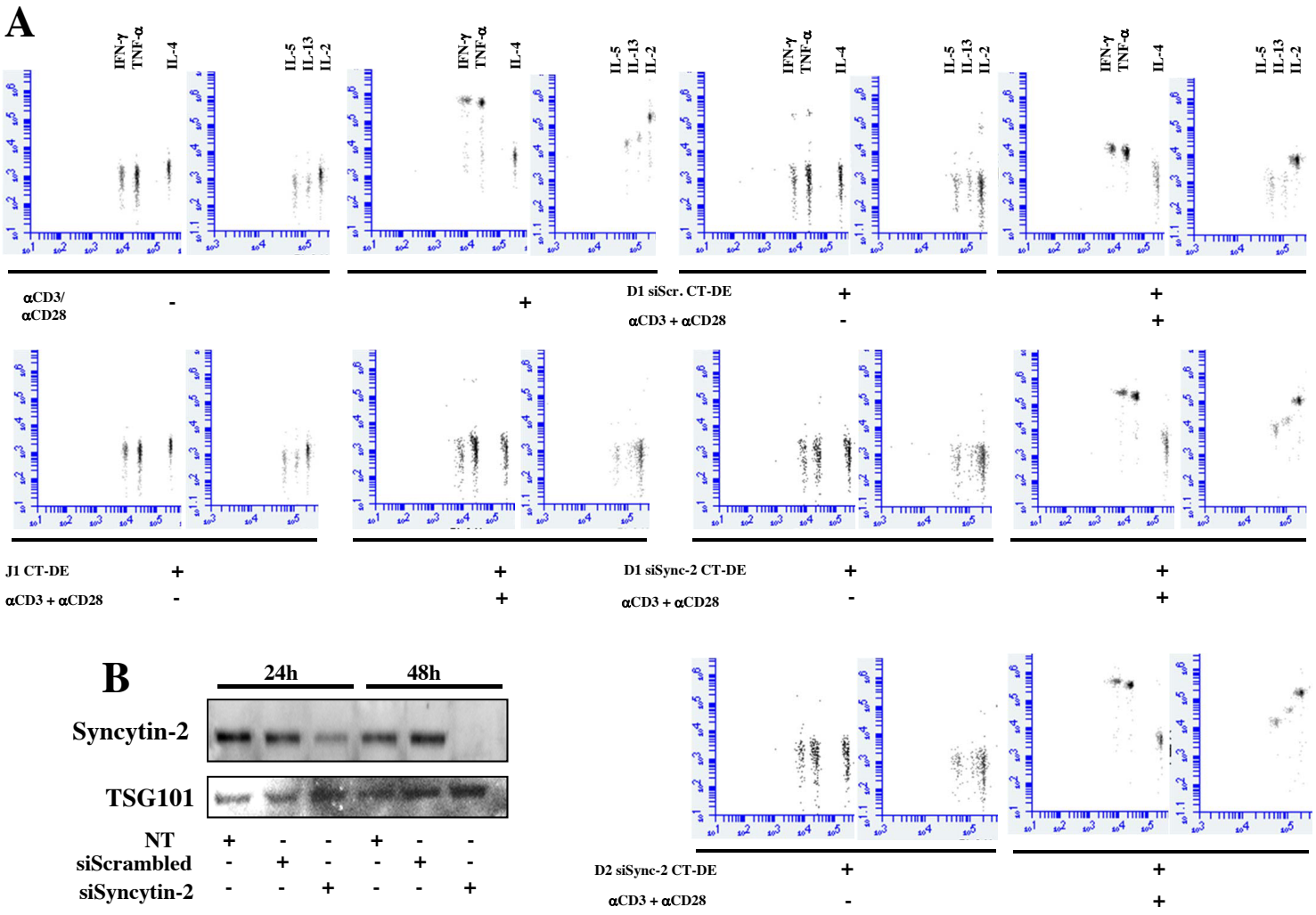

Figure 10

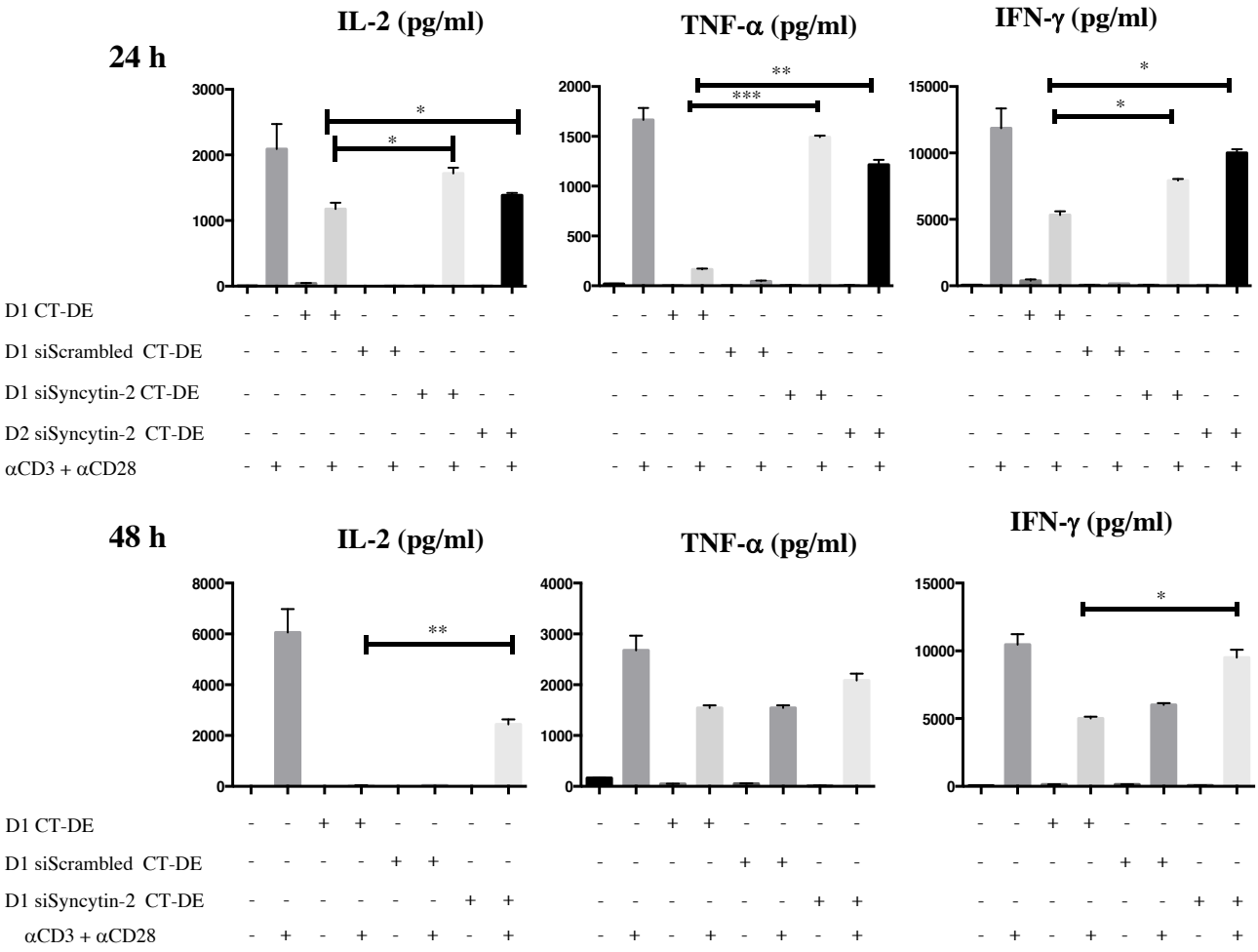

Figure 11