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Pluripotent stem cells are insensitive to the cytotoxicity of TNFα and IFNγ

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Abstract

Recent studies have demonstrated that embryonic stem cells (ESCs) have an underdeveloped innate immune system, but the biological implications of this finding are poorly understood. In this study, we compared the responses of mouse ESCs (mESCs) and mESC differentiated fibroblasts (mESC-FBs) to tumor necrosis factor α (TNFα) and interferons (IFNs). Our data revealed that TNFα, IFNα, IFNβ, or IFNγ alone do not cause apparent effects on mESCs and mESC-FBs, but the combination of TNFα and IFNγ (TNFα/IFNγ) showed toxicity to mESC-FBs as indicated by cell cycle inhibition and reduced cell viability, correlating with the expression of inducible nitric oxide synthase (iNOS). However, none of these effects were observed in mESCs that were treated with TNFα/IFNγ. Furthermore, mESC-FBs, but not mESCs, are vulnerable to cytotoxicity resulting from lipopolysaccharide (LPS)-activated macrophages. The insensitivity of mESCs to cytotoxicity in all cases is correlated with their lack of responses to TNFα and IFNγ. Similar to mESCs, human ESCs (hESCs) and iPSCs (hiPSCs) do not respond to TNFα and are not susceptible to the cytotoxicity of TNFα, IFNβ, or IFNγ alone or in combination that significantly affects human foreskin fibroblast (hFBs) and Hela cells. However, unlike mESCs, hESCs and hiPSCs can respond to IFNγ, but this does not cause significant cytotoxicity in hESCs and hiPSCs. Our findings in both mouse and human PSCs together support the hypothesis that attenuated innate immune responses could be a protective mechanism that limits immunologic cytotoxicity resulting from inflammatory and immune responses.

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Introduction

Embryonic stem cells (ESCs), the pluripotent stem cells (PSCs) experimentally derived from preimplantation stage embryos, retain the capacity to differentiate into various cell lineages and have unlimited ability to proliferate under proper *in vitro* conditions. These properties have led to intensive studies of these cells as a promising source for cell-based regenerative medicine. Interestingly, recent studies have demonstrated that both human and mouse ESCs (hESCs and mESCs) and induced PSCs (iPSCs) lack or have attenuated innate immune responses to pathogenic agents and inflammatory cytokines in comparison with differentiated somatic cells. This finding has led to the conclusion that the underdeveloped innate immune system is a common feature of PSCs (Pare & Sullivan 2014, Guo *et al.* 2015), but the biological implications of this phenomenon are poorly understood.

The innate immunity provides quick responses to a broad range of pathogens and is presumably developed in most, if not all, types of mammalian cells (Sen 2001, Kawai & Akira 2011). The innate immune system includes different forms of nonspecific defense mechanisms, but antiviral, antibacterial, and inflammatory responses constitute the central parts of this defense system. The attenuated innate immune responses in ESCs raise intriguing questions about the rationale for ESCs to not have a fully developed innate immune system that serves somatic cells so well. Innate immune and inflammatory responses are elicited by molecules known as pathogen-associated molecular patterns (PAMPs) derived from microbial pathogens (Newton & Dixit 2012). Through interactions with their specific cellular receptors, PAMPs activate several transcription factors, mainly NFκB and IRFs, leading to the expression of interferons (IFNs) and inflammatory cytokines that participate in different aspects of immune responses (Samuel 2001, Kawai & Akira 2011).

A series of our recent studies and those of other investigators have demonstrated that ESCs and iPSCs are unable to express type I IFNs and lack response to lipopolysaccharide (LPS, a bacterial endotoxin) and inflammatory cytokines TNFα and IL1β (Guo *et al.* 2015). Although the underlying molecular basis is not completely understood, the attenuated innate immune

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responses in ESCs can be explained by the findings that the receptors for viral RNA, LPS, and TNFα are expressed at low levels or not functional (Zampetaki *et al.* 2006, 2007, Chen *et al.* 2010, Wang *et al.* 2013, 2014*a*, D'Angelo *et al.* 2017). The lack of NFκB activation in ESCs by immune stimuli provides the explanation at the transcriptional level for an overall underdeveloped innate immune system in ESCs since NFκB is a master transcription factor commonly used by various PAMPs and inflammatory cytokines (Napetschnig & Wu 2013). Diverging from the conventional perspective as an in-born property of somatic cells, apparently, innate immunity is not (or at least not completely) innate to ESCs but is acquired by somatic cells during differentiation as we have demonstrated in mESC-FBs, which acquired the ability to express IFNβ and to respond to TNFα after *in vitro* differentiation (Wang *et al.* 2014*b*, D'Angelo *et al.* 2016).

Based on the cellular origin and cellular receptors, IFNs are classified into types I, II, or III (Samuel 2001). They use different versions of signaling mechanisms and have some cell-specific functions, but all IFNs exhibit antiviral activity and modulate the function of immune systems. Through autocrine and paracrine signaling mechanisms, IFNs bind to their cell surface receptors and trigger the activation of the Janus kinases-STAT (JAK-STAT) pathways, leading to the expression of IFN-stimulated genes (ISGs) that promote the cell to enter an 'antiviral state' (Samuel 2001). Therefore, the IFN system includes the cellular capacity to produce and respond to IFNs. Although ESCs are deficient in expressing IFNα and IFNβ (type I), they do have limited responsiveness to these cytokines (Ruffner *et al.* 1993, Whyatt *et al.* 1993, Wang *et al.* 2014*b*, D'Angelo *et al.* 2016). In this study, we report that mESCs do not respond to IFNγ, but hESCs and hiPSCs are able to express IFNγ-induced genes typically seen in differentiated somatic human cells. However, all PSCs share a similarity in that they all lack response to TNFα and are insensitive to TNFα and IFN cytotoxicity that negatively affects differentiated somatic cells.

The biological implications of the underdeveloped innate immune system as a unique property of ESCs have been speculated from different perspectives as we have recently discussed (Guo *et al.* 2015, Guo 2017, 2019). Immune response is viewed as a double-edged sword: while it serves as a critical part of the defense mechanism, it can also cause collateral damage to tissue cells since IFNs and inflammatory cytokines negatively impact cell proliferation and viability of many types of tissue cells (Hertzog *et al.* 1994, Samuel 2001, Garcia *et al.* 2007). It is conceivable that immunologic cytotoxicity resulting from immune and inflammatory responses could be tolerated by tissues of developed organisms, but it could be detrimental to ESCs in an early embryo. From this point of view, we hypothesize that attenuated immune responses in ESCs could be an adaptive mechanism that allows them to avoid immunological cytotoxicity at early stages of embryogenesis (Guo 2019). In this study, we demonstrated that ESCs are indeed refractory to the immunologic cytotoxicity and elucidated the underlying molecular mechanisms.

Materials and methods

Cell culture and cell treatment

The immunological properties of mESCs and mESC-FBs have been investigated with two mESC cell lines and their differentiated cells (D3, DBA252, D3-FBs, and DBA252-FBs) in our previous studies (Wang *et al.* 2014*b*, D'Angelo *et al.* 2016). Since the two sets of mESCs and mESC-FBs were similar in all properties tested, the experiments in this study were mainly performed with D3 mESCs and mESC-FBs differentiated from D3 cells, but the results from key experiments were confirmed with DBA252 ESCs. mESC-FBs at passages 10–35 were used in the study. RAW264.7 cells (RAW, a murine macrophage cell line) and human foreskin fibroblasts (hFBs) were obtained from ATCC. mESCs were maintained in standard mouse ESC medium (Wang *et al.* 2013). mESC-FBs and RAW cells were routinely cultured in DMEM with 10% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin. hESCs (H9) were from WiCell (Madison, WI) and hiPSCs (AICS-0037-172) were obtained from Coriell Institute for Medical Research (Camden, NJ). Both were cultured in Essential 8 Medium on Geltrex-coated plates (Thermo Fisher Scientific). All cells were cultured at 37°C in a humidified incubator with 5% CO_2.

For cell treatment, mESCs, hESCs, and hiPSCs were seeded at 30–40% confluence and mESC-FBs, hFBs, and Hela cells were seeded at 60–70% confluence. For co-culture experiments, mESCs (10–15% of total cells) were mixed with mESC-FBs before seeding. Seeded cells were usually cultured for 24 h before experiments. To determine cellular response to cytokines, cells were treated with TNFα and different IFNs, individually or in combinations as stated in individual experiments. For most experiments, TNFα and IFNγ were used at the concentration of 20 ng/mL. IFNα and IFNβ were used at the concentrations of 500 and 5000 U/mL, respectively, or as stated in individual experiments. All cytokines used in this study are mouse or human recombinant cytokines. Mouse IFNα was purchased from eBioscience (San Diego, CA). All other cytokines were purchased from Peprotech (Rocky Hill, NJ).

Preparation of conditioned medium (CM) from LPS-activated RAW cells

The method for preparing a conditioned medium (CM) has been previously described (D'Angelo *et al.* 2018). Briefly, RAW cells were treated with LPS (100 ng/mL, isolated from *Escherichia coli* O111:B4) (Sigma-Aldrich) for 4 h. The medium was removed, and cells were thoroughly washed with PBS before being cultured in fresh medium for an additional 24 h. The CM was collected and designated as LPS/CM. CM prepared from RAW cells without treatment was used as a

control (Con/CM). The CM was diluted with fresh medium at a 1:1 ratio for cell treatment.

Cell proliferation, viability, and cell cycle analysis

Cytotoxicity associated with immune and inflammatory responses (referred to as immunologic cytotoxicity) can be shown in different forms. In this study, cytotoxicity is defined by its effects on cell proliferation, viability, and/or cell cycle progression. Cell proliferation and viability were determined by the number of viable cells after toluidine blue staining as we previously described (Wang *et al.* 2013). The absorbance at 630 nm of the stained cells was measured with a plate reader. The values, which correlate with the number of viable cells, were used as an indirect measurement of cell proliferation or viability. Cell viability was also routinely monitored with a phase-contrast microscope during the time course of treatment. The images were acquired with a digital camera mounted on the microscope. Cell cycle analysis by flow cytometry was performed with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) after the cells were stained with 50 μg/mL propidium iodide as previously described (Wang *et al.* 2013). Cell gating was performed by selecting the cell population from forward vs side scatter (FSC vs SSC) dot plots to exclude debris. The cell cycle profiles were generated with CFlow software (BD Biosciences, San Jose, CA).

Protein analysis by Western blot and flow cytometry

Protein analysis by Western blot and flow cytometry was performed according to our published methods (Wang *et al.* 2013). The antibodies against β-actin, ICAM1, STAT1, iNOS, IFNγR1, JAK1, p16, and p21 were purchased from Santa Cruz Biotechnology and IFNγR2 antibodies were from BD Biosciences (San Jose, CA). For flow cytometry, fixed cells were incubated with the antibodies against the specific proteins to be analyzed; the cells were then incubated with secondary antibodies conjugated with FITC and examined with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Cell gating was performed as described in the cell cycle analysis. The histograms were generated from median FITC fluorescence intensity of each sample with CFlow software (BD Biosciences, San Jose, CA).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRI-reagent (Sigma-Aldrich). cDNA was prepared using Moloney murine leukemia virus reverse transcriptase (Promega Corp.). RT-qPCR was performed using SYBR green ready mix (Bio-Rad) on a MX3000P RT-PCR system (Agilent Technologies) as previously described (Guo *et al.* 2007). The mRNA levels from RT-qPCR were calculated using the comparative Ct method (Pfaffl 2001). β-Actin was used as a calibrator for the calculation of relative mRNA levels of the tested genes. As specified in individual experiments, the mRNA levels were either expressed as fold-activation, where the values in the controls were designated as 1, or expressed as relative levels normalized to β-actin. The sequences of the primer sets utilized for RT-qPCR are listed in Table 1.

Luciferase reporter assay

To determine the transcription activity of STAT1, plasmids encoding a *Stat1* responsive firefly luciferase reporter (m67-Luc reporter) (Liddle *et al.* 2006) were transfected into mESCs or mESC-FBs in 24-well dishes (0.5 μg plasmid per well). At 24 h after transfection, the cells were treated with IFNγ for 6 h or 12 h. Cell lysates were prepared from treated cells and used for reporter activity assay with a luciferase assay system (Promega Corp.). The intensity of luminescence was measured with a SpectraMax M3 plate reader (Molecular Devices, San Jose, CA). The NFκB transcription activity was determined with a plasmid construct encoding an *Nfkb* luciferase reporter (LgK-IFN-Luc reporter, Addgene, Watertown, MA) following the same protocols as described for m67-Luc reporter.

Statistical analysis

For statistical analysis, data are presented as the mean ± S.D. derived from either three independent experiments or from a representative experiment performed in triplicate that was performed at least twice with similar results. Statistical analysis was performed with Microsoft Excel using a two-tailed and unpaired Student's *t*-test. Differences are considered statistically significant when $P < 0.05$.

Table 1 The primer sequences used for RT-qPCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-Actin	CATGTACGTAGCCATCCAGGC	CTCTTTGATGTCACGCACGAT
iNos	CAGCACAGGAAATGTTTCAGC	TAGCCAGCGTACCGGATGA
Isg15	AGGTCTTTCTGACGCAGACTG	GGGGCTTTAGGCCATACTCC
Stat1	GCTGCCTATGATGTCTCGTTT	TGCTTTTCCGTATGTTGTGCT
Irf1	ATGCCAATCACTCGAATGCG	TTGTATCGGCCTGTGTGAATG
Ifngr1	CTGGCAGGATGATTCTGCTGG	GCATACGACAGGGTTCAAGTTAT
Ifngr2	TCCTCGCCAGACTCGTTTTC	GTCTTGGGTCATTGCTGGAAG
Jak1	ACGCTCCGAACCGAATCATC	GTGCCAGTTGGTAAAGTAGAACC
Jak2	TTGTGGTATTACGCCTGTGTATC	ATGCCTGGTTGACTCGTCTAT
Oct4	AGTTGGCGTGGAGACTTTGC	CAGGGCTTTCATGTCCTGG
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGTC
Sox2	GACAGCTACGCGCACATGA	GGTGCATCGGTTGCATCTG

Results

mESC-FBs, but not mESCs, are sensitive to cytotoxicity of TNFα and IFNγ

mESCs and mESC-FBs represent cells from the same origin with different levels of innate immunity (Wang *et al.* 2014*b*, D'Angelo *et al.* 2016). They were treated with TNFα, IFNα, IFNβ, and IFNγ individually or in indicated combinations. None of these cytokines alone showed a significant effect on cell viability of mESCs or mESC-FBs within a 2- to 3-day treatment period. In mESC-FBs, TNFα alone or TNFα in the presence of IFNα caused a slight decrease of cell number, but the combination of TNFα with IFNγ (TNFα/IFNγ) reduced the number by nearly 50% (Fig. 1A) and caused cell death (Fig. 1B) that eventually led to cells detaching from the culture dish. On the other hand, mESCs under the same treatment conditions did not show any of these signs. Similar results were observed in a different mESC line (DBA 252 ESCs, data not shown). The selective cytotoxicity of TNFα/IFNγ to mESC-FBs was further illustrated in a co-culture system where mESCs and mESC-FBs were seeded in the same dish. mESCs grown in colonies (Fig. 1B, areas circled with dotted lines) can be easily distinguished from the large individual mESC-FBs. After treatment with TNFα/IFNγ for 72 h, only a small population of mESC-FBs survived while mESC colonies were mostly intact (Fig. 1B), further demonstrating that mESC-FBs, but not mESCs, are sensitive to cytotoxicity of TNFα/IFNγ.

The effects of TNFα and IFNγ on cell cycle and pluripotency marker expression

Inflammatory cytokines can exert their cytotoxicity by causing cell death, cell cycle inhibition, or loss of cell-specific functions. We analyzed the effects of TNFα and IFNγ on the cell cycle of mESCs and mESC-FBs. Under normal conditions, mESCs have a large cell population in the S and G2/M phases (Fig. 2A), which is the basis for their rapid proliferation rate. The cell cycle profile of mESCs was not affected by either TNFα or IFNγ alone or TNFα/IFNγ after 24 h (Fig. 2A, mESC). mESC-FBs are predominantly in G1 phase and have a much lower proliferation rate than mESCs. In control cells, about 16% of cells were in G2/M phase, and this was not affected by TNFα or IFNγ alone. However, mESC-FBs treated with TNFα/IFNγ had only 5% of cells in G2/M phase and G1 phase cells were increased to 87% (Fig. 2A, mESC-FB, 87% vs 75% in Con), which indicate a slowed cell cycle progression. Consistent with the results from cell cycle analysis, the protein levels of P16 and P21 (two major cell cycle inhibitors) in mESCs and mESC-FBs were not affected by TNFα or IFNγ alone, but TNFα/IFNγ treatment resulted in 1.9- and 2.4-fold increase of P16 and P21, respectively, in mESC-FBs. Such effect was not observed in mESCs (Fig. 2B). Therefore, TNFα/IFNγ can negatively affect the cell viability as well as cell cycle progression of mESC-FBs, whereas mESCs are able to avoid these negative effects. In addition, the mRNA levels of three major mESC pluripotency markers, *Oct4*, *Nanog*, and *Sox2*, were not affected by IFNγ or TNFα/IFNγ (Fig. 2C).

mESCs are insensitive to TNFα and have limited response to IFNγ

We have previously reported that mESCs do not respond to TNFα due to their lack of TNFα receptor expression and deficiency in NFκB activation, but differentiated mESC-FBs become responsive to TNFα (D'Angelo *et al.* 2016, 2017). The functionality of the signaling pathway that mediates the effects of IFNγ in mESCs and mESC-FBs has not been investigated. We first compared IFNγ-stimulated expression of inducible nitric oxide synthase (*iNos*) and *Isg15*. As shown in Fig. 3A, IFNγ at 20 ng/mL induced significant mRNA increases of both genes in mESC-FBs (230- and 62-fold, respectively, at 24 h). TNFα induced about 25-fold induction of iNOS mRNA, but its combination with IFNγ (TNFα/IFNγ) dramatically potentiated the induction of iNOS mRNA and, to a lesser extent, ISG15 mRNA in mESC-FBs (Fig. 3A). On the other hand, IFNγ-induced mRNA of iNOS and ISG15 in mESCs were induced less than three-fold, with a slight increase in the cells treated with TNFα/IFNγ (Fig. 3A). Dose–response analysis indicated that IFNγ induced significant mRNA increases of iNOS and ISG15 at concentrations as low as 5 ng/mL in mESC-FBs (Fig. 3B). Although IFNγ-induced iNOS mRNA increased in a dose-dependent manner in mESCs, the induction level was about eight times less than in mESC-FBs, even at 20 ng/mL. The induction of ISG15 mRNA was not induced by IFNγ in mESCs at all concentrations tested (Fig. 3B). At the protein level, as determined by flow cytometry, IFNγ- and TNFα/IFNγ-induced iNOS expression paralleled its mRNA level in mESC-FBs. However, the limited mRNA induction of iNOS did not result in a detectable increase of iNOS protein in mESCs (Fig. 3C). Taken together, our results revealed a close correlation between the cellular sensitivity to the cytotoxicity of TNFα/IFNγ and the magnitude of cellular response to the two cytokines in mESCs and mESC-FBs.

mESCs are less vulnerable than mESC-FBs to the cytotoxicity resulting from LPS-activated macrophages

To test if mESCs can avoid cytotoxicity caused by other inflammatory insults, we used an *in vitro* macrophage-induced inflammation model. This method is based on the fact that macrophages, the tissue-resident immune cells, secrete a large amount of inflammatory cytokines when activated by LPS, mimicking bacterial infection (Cameron & Churchill 1980, Mosser & Edwards 2008). The conditioned medium (CM) collected from LPS-stimulated RAW cells (LPS/CM), which contains various

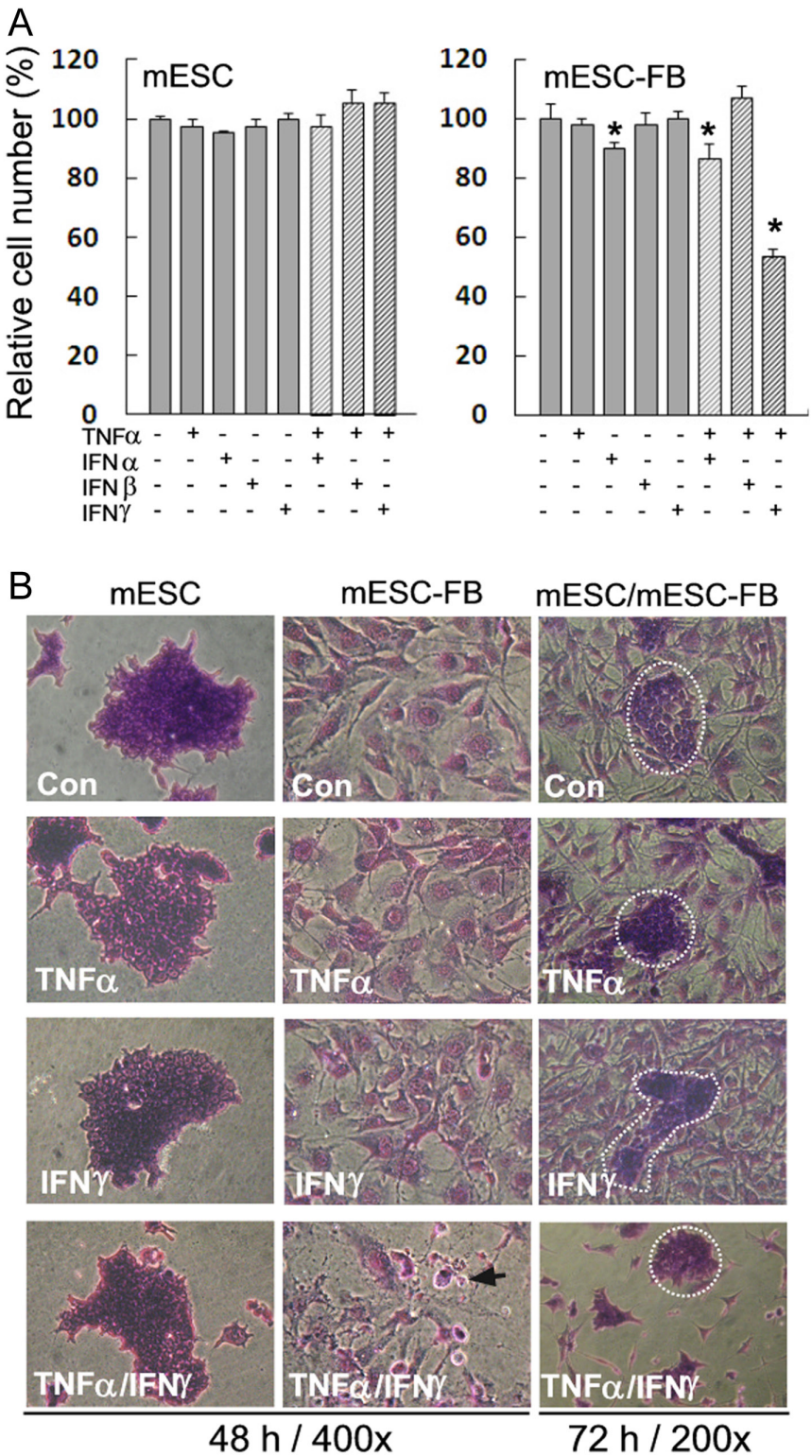

Figure 1 Effects of TNFα and different IFNs on the viability of mESCs and mESC-FBs. (A) Cells were treated with TNFα, IFNα, IFNβ, or IFNγ alone or with indicated combinations. After 48 h, cell viability was determined by toluidine blue staining assay. The cell number in control (cells without any treatment) was defined as 100%. Values are mean ± S.D. of a representative experiment that was performed in triplicate. *$P < 0.05$, compared with the control. (B) mESCs and mESC-FBs grown in separate culture dishes or in co-culture (mESC/mESC-FB) were treated under the indicated conditions. Cells were fixed and stained with toluidine blue and then they were examined under a phase-contrast microscope and photographed with a digital camera. The arrow denotes a dead cell and the dotted circle indicates a mESC colony. Con represents control cells without any treatment.

secreted inflammatory cytokines including TNFα and IL-1β (D'Angelo *et al.* 2018), was used to treat mESCs and mESC-FBs. Within a 2-day treatment period, LPS/CM caused a ~40% reduction of the viable cells in mESC-FBs. Addition of IFNγ further potentiated the cytotoxic effect of LPS/CM and, to a lesser extent, Con/CM (CM collected from RAW cells without LPS stimulation). These effects were not observed in mESCs (Fig. 4A). The mRNA levels of iNOS induced by the aforementioned conditions in mESCs and mESC-FBs (Fig. 4B) showed a

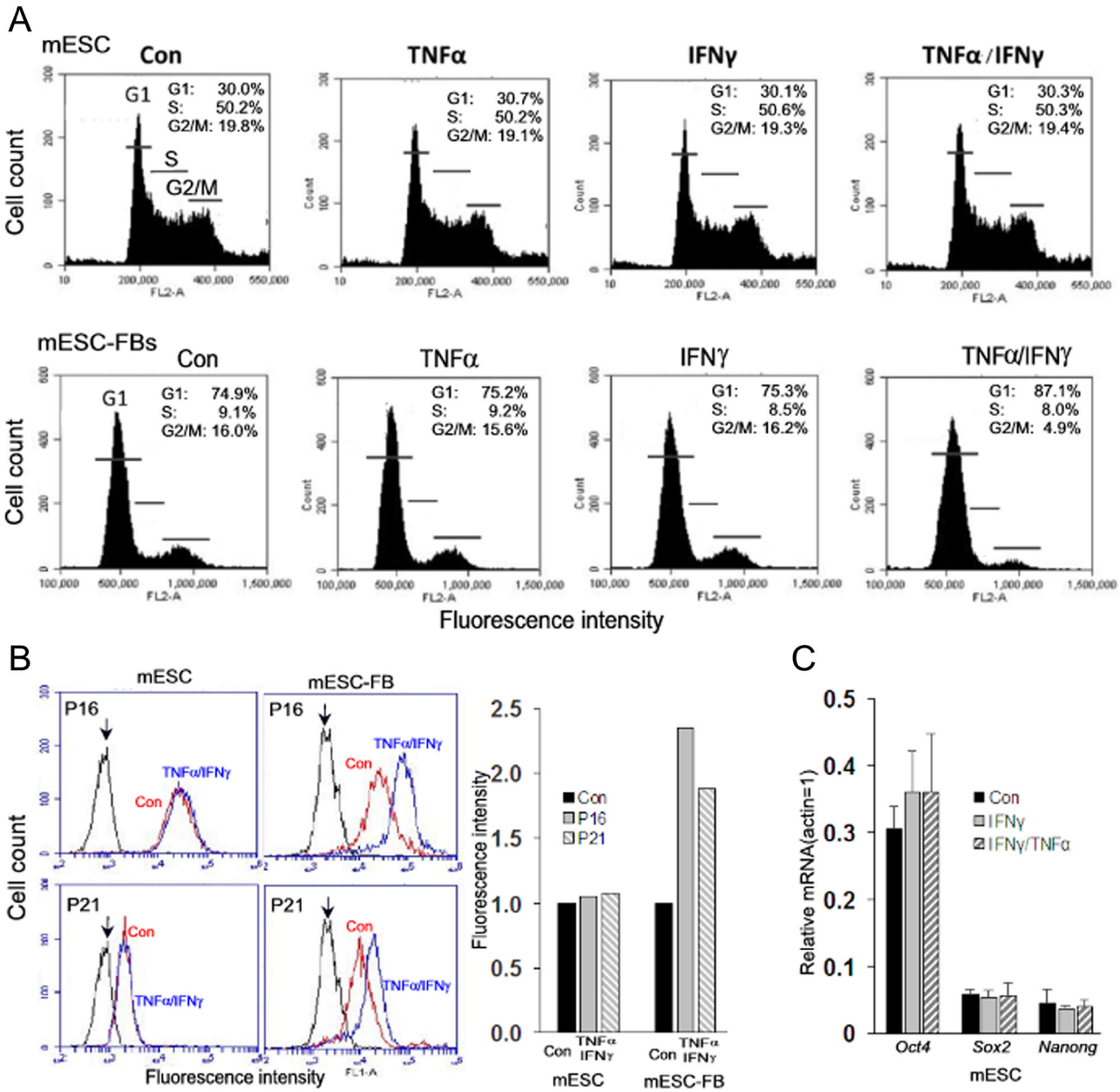

Figure 2 Effects of TNFα and IFNγ on the cell cycle and expression of pluripotency markers. Cells were treated with TNFα, IFNγ, or TNFα/IFNγ for 24 h and were analyzed for: (A) Cell cycle analysis by flow cytometry (insets show percentages of cell populations in different phases). (B) The protein expression levels of *p16* and *p21* analyzed by flow cytometry (the arrows in histograms denote the cells stained only with secondary antibodies as a negative control and used for cell gating). The bar graph shows the average of median fluorescence intensities of tested samples from two experiments. The value of median fluorescence intensity in the control experiment (Con, the cells without any treatment) is defined as 1. (C) mRNA expression of pluripotency markers determined by RT-qPCR (the values are mean ± S.D. of three independent experiments).

similar pattern to the cells treated with IFNγ and TNFα/IFNγ as described in the previous experiments (Fig. 3A), correlating with the levels of cellular cytotoxicity. We do not know what other components in CM in addition to TNFα contribute to its toxicity, but clearly, exogenously adding IFNγ to the CM mimics the experiments performed with IFNγ and TNFα/IFNγ.

Relative expression levels of IFNγ signaling molecules in mESCs and mESC-FBs

Unlike the TNFα signaling pathway that is nearly completely inactive in mESCs (D'Angelo *et al.* 2017), the IFNγ signaling pathway seems to be functional, although only marginally, since IFNγ was able to elicit limited

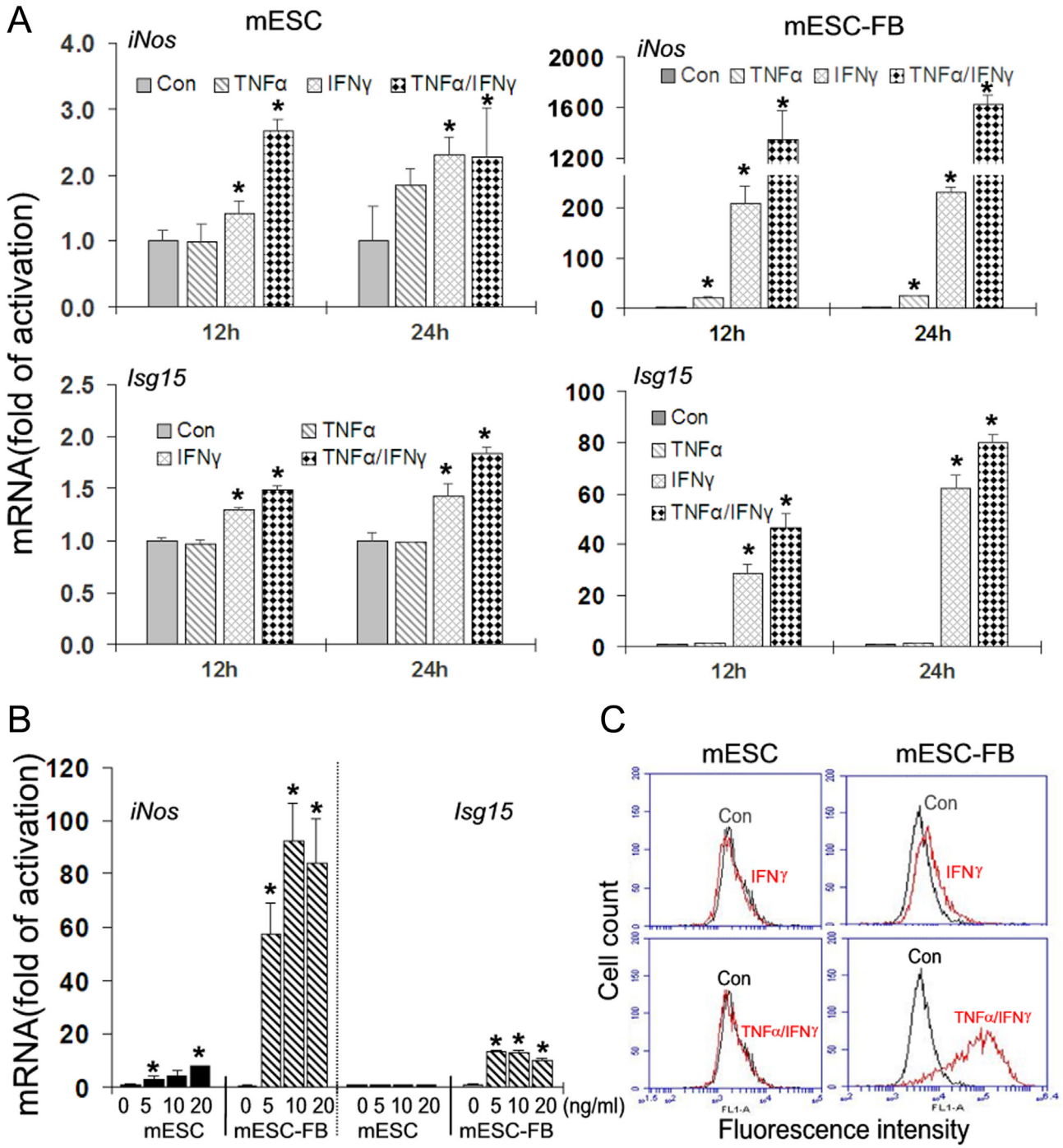

Figure 3 IFNγ-induced gene expression in mESCs and mESC-FBs and the effect of TNFα. Cells were treated with IFNγ, TNFα, or TNFα/IFNγ under specified conditions. (A) The mRNA levels of *iNos* and *Isg15* were determined by RT-qPCR in the cells treated with 20 ng/mL IFNγ, TNFα, or TNFα/IFNγ. (B) Dose–response in the cells that were treated with IFNγ at the indicated concentration for 24 h. mRNA levels of *iNos* and *Isg15* were determined by RT-qPCR. (C) Flow cytometry analysis of *iNos* in the cells that were treated with 20 ng/mL IFNγ or TNFα/IFNγ for 24 h. For RT-qPCR analysis, the values are mean ± S.D. of representative experiments performed in triplicate. *$P < 0.05$, compared with the control (Con). Flow cytometry data are from representative experiments that were performed three times with similar results.

but detectable iNOS mRNA (Fig. 3). We compared the expression levels of the major signaling components that mediate the effect of IFNγ in mESCs and mESC-FBs. IFNγ binds to its receptor complex consisting of IFNγR1 and IFNγR2, which in turn activates tyrosine kinases JAK1 and JAK2 leading to STAT1-regulated gene expression. As shown in Fig. 5, these genes were expressed at comparable levels in mESCs and

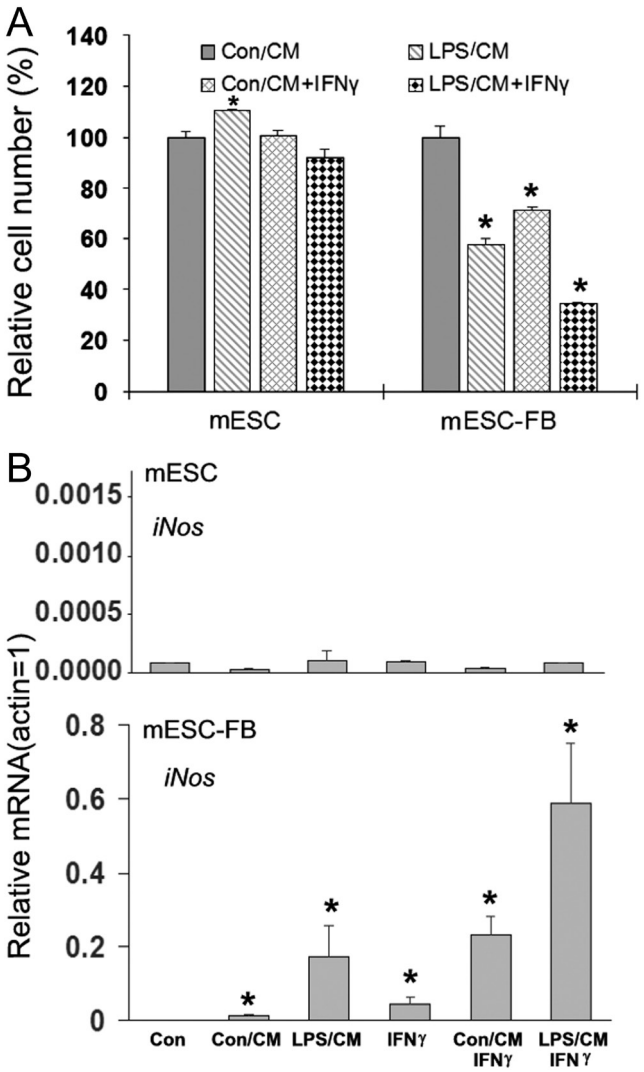

Figure 4 Effects of CM prepared from RAW cells in the presence or absence of IFNγ on mESCs and mESC-FBs. (A) Cells were treated with conditioned medium from untreated RAW cells (Con/CM) or LPS-stimulated RAW cells (LPS/CM) in the presence or absence of IFNγ (5 ng/mL). After 48 h treatment, cell viability was determined by toluidine blue staining. The cell number in control (Con) was defined as 100%. The values are mean ± S.D. of a representative experiment that was performed in triplicate. (B) The mRNA level of *iNos* was determined by RT-qPCR in cells treated for 24 h under the conditions described in A. Values are mean ± S.D. of a representative experiment that was performed in triplicate. **P* < 0.05, compared with Con.

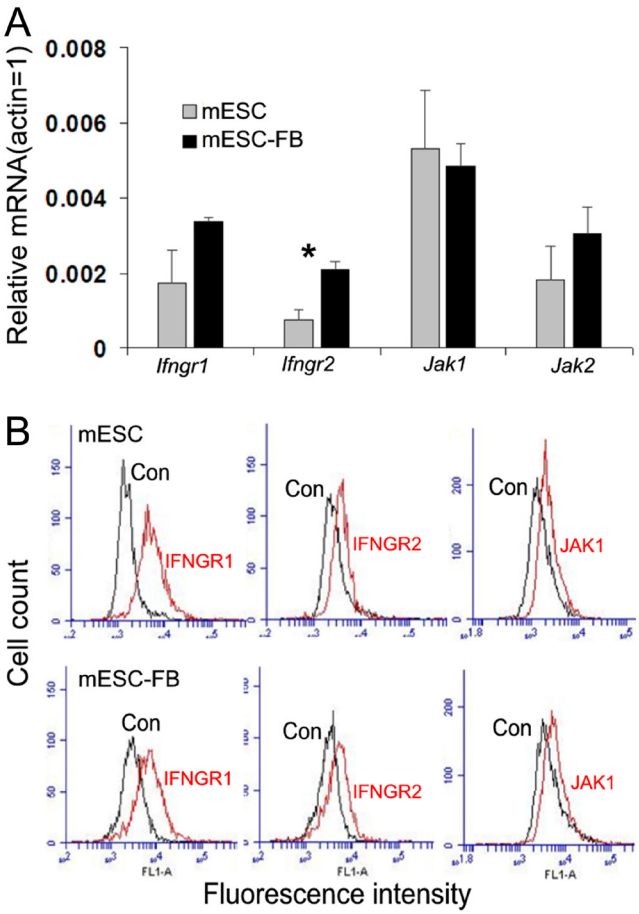

Figure 5 Expression levels of *Ifngr* and *Jak* in mESCs and mESC-FBs. (A) RT-qPCR analysis of the relative mRNA levels of the indicated genes in mESCs and mESC-FBs. Values are mean ± S.D. of results from three independent experiments. **P* < 0.05, compared between mESC and mESC-FBs. (B) Flow cytometry analysis of the relative protein levels of the indicated genes in mESCs and mESC-FBs. Data are representative profiles of experiments that were performed three times with similar results. Con represents the negative control in which the cells were only incubated with secondary antibodies.

mESC-FBs as determined by flow cytometry, although IFNγR2 mRNA was expressed at a lower level in mESCs than in mESC-FBs. While these results suggest that mESCs may have the molecular basis to initiate and transduce IFNγ signals at the cell surface, the functionality of these molecules is difficult to judge by their expression since their activities are regulated at multiple levels by different mechanisms. Therefore, we examined the transcription activity of STAT1, which is downstream of the IFNγR-JAK complex.

IFNγ induces limited transcription activity of STAT1 in mESCs

STAT1 is the key transcription factor that initiates the expression of IFNγ-induced genes. Its transcription activity depends on phosphorylation of Tyr residues by JAK1/2. Phosphorylated STAT1 forms a dimer that translocates to the nucleus where it activates the target genes. The expression of STAT1 itself is also upregulated by IFNγ. In addition, IRF1 is another transcription factor controlled by STAT1. The newly synthesized IRF1 and STAT1 from the initial response start a second wave of transcription of IFNγ-induced genes, such as *iNos* (Holtzman *et al.* 2002). This mechanism, characterized by 'two waves' of responses, was readily demonstrated in mESC-FBs, in which IFNγ induced rapid mRNA transcription of IRF1 and STAT1 (Fig. 6A). Interestingly, unlike its dramatic potentiation effect on IFNγ-induced

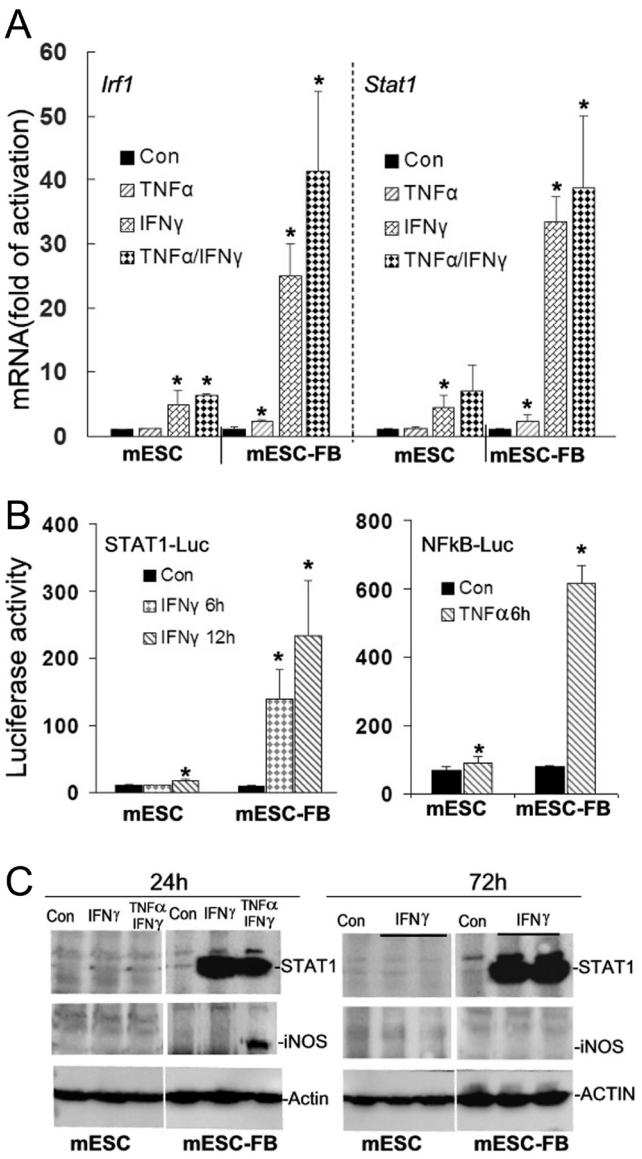

Figure 6 IFNγ-induced expression of *Irf1* and *Stat1* in mESCs and mESC-FBs. (A) Cells were treated with IFNγ, TNFα, or TNFα/IFNγ for 12 h. The mRNA levels of *Irf1* and *Stat1* were determined by RT-qPCR. The values are mean ± s.d. of a representative experiment that was performed in triplicate. **P* < 0.05, compared with Con. (B) Luciferase reporter assay was used in the cells transfected with plasmids encoding a *Stat1* or *Nfkb* luciferase reporter gene. The results are expressed as fold-activation where the luciferase activity in the control experiments (Con, cells without treatment with IFNγ or TNFα) is designated as 1. The values are mean ± s.d. of a representative experiment performed in triplicate. **P* < 0.05, compared with Con. (C) Western blot analysis of STAT1 and iNOS proteins in the cells treated with IFNγ or TNFα/IFNγ for 24 h (left panels) and in the cells treated with IFNγ alone for 72 h (right panels). The two lanes of IFNγ-treated mESCs or mESC-FBs for 72 h represent the samples from two independent experiments. STAT1 and iNOS were detected with their specific antibodies. ACTIN was used as a loading control.

iNOS expression in mESC-FBs (Fig. 3A), TNFα only slightly increased IFNγ-induced expression of IRF1 and STAT1 (Fig. 6A). In mESCs, the same treatments induced a marginal increase of the mRNA of IRF1 and STAT1 in comparison with mESC-FBs (~5-fold in mESCs vs 25- to 30-fold in mESC-FBs, Fig. 6A). Therefore, IFNγ-induced initial transcription activity of STAT1 is detectable but rather low in mESCs. To confirm this result, we analyzed the transcription activity of STAT1 by a luciferase reporter gene ectopically expressed from a plasmid. As shown in Fig. 6B, IFNγ-induced luciferase activity, representing STAT1 transcription activity, paralleled the cellular transcription activity of STAT1 as measured by IFNγ-induced expression of IRF1 and STAT1 (Fig. 6A). Similarly, high-level luciferase activity driven by a TNFα-activated NFκB reporter was detected in mESC-FBs, but luciferase activity was marginal in mESCs (Fig. 6B), consistent with the same conclusion that mESCs do not respond to TNFα as demonstrated by experimental approaches (D'Angelo *et al.* 2016, 2017).

To test the long-term effects of IFNγ and TNFα/IFNγ on STAT1 expression, we treated mESCs and mESC-FBs for 24 and 72 h and analyzed STAT1 protein by Western blot. As shown in Fig. 6C, IFNγ and TNFα/IFNγ did not induce the expression of STAT1 or iNOS in mESCs. Apparently, the limited increase of STAT1 mRNA stimulated by IFNγ and TNFα/IFNγ in mESCs (Fig. 6A) did not result in a detectable STAT1 protein. On the contrary, IFNγ alone caused accumulation of a large amount of STAT1 in mESC-FBs, consistent with its mRNA levels determined by RT-qPCR (Fig. 6A). Although TNFα/IFNγ did not induce more STAT1 expression than IFNγ alone, only the combination of the two cytokines induced iNOS expression in mESC-FBs (Fig. 6C, 24 h), consistent with flow cytometry analysis (Fig. 3C). While IFNγ-induced STAT1 lasted for at least 72 h in mESC-FBs, nonetheless, it is not sufficient to induce iNOS (Fig. 6C) or cause cytotoxicity as described in Fig. 1.

hESCs and hiPSCs share similarity with mESCs in lacking responsiveness to TNFα and are insensitive to cytokine cytotoxicity

To determine whether the observations that we have made in mouse cells also apply to human cells, we analyzed the effects of TNFα and IFNs on hESCs and hiPSCs and compared these effects with naturally differentiated human foreskin fibroblasts (hFBs). Similar to mESCs, the proliferation rate, cell viability, and colony morphology of hESCs and hiPSCs were not affected by TNFα, IFNβ, or IFNγ alone or in indicated combinations (Fig. 7A). However, unlike mESC-FBs, where only TNFα/IFNγ caused significant cytotoxicity (Fig. 2B), in hFBs, TNFα/IFNγ, TNFα/IFNβ, and IFNβ alone showed significant

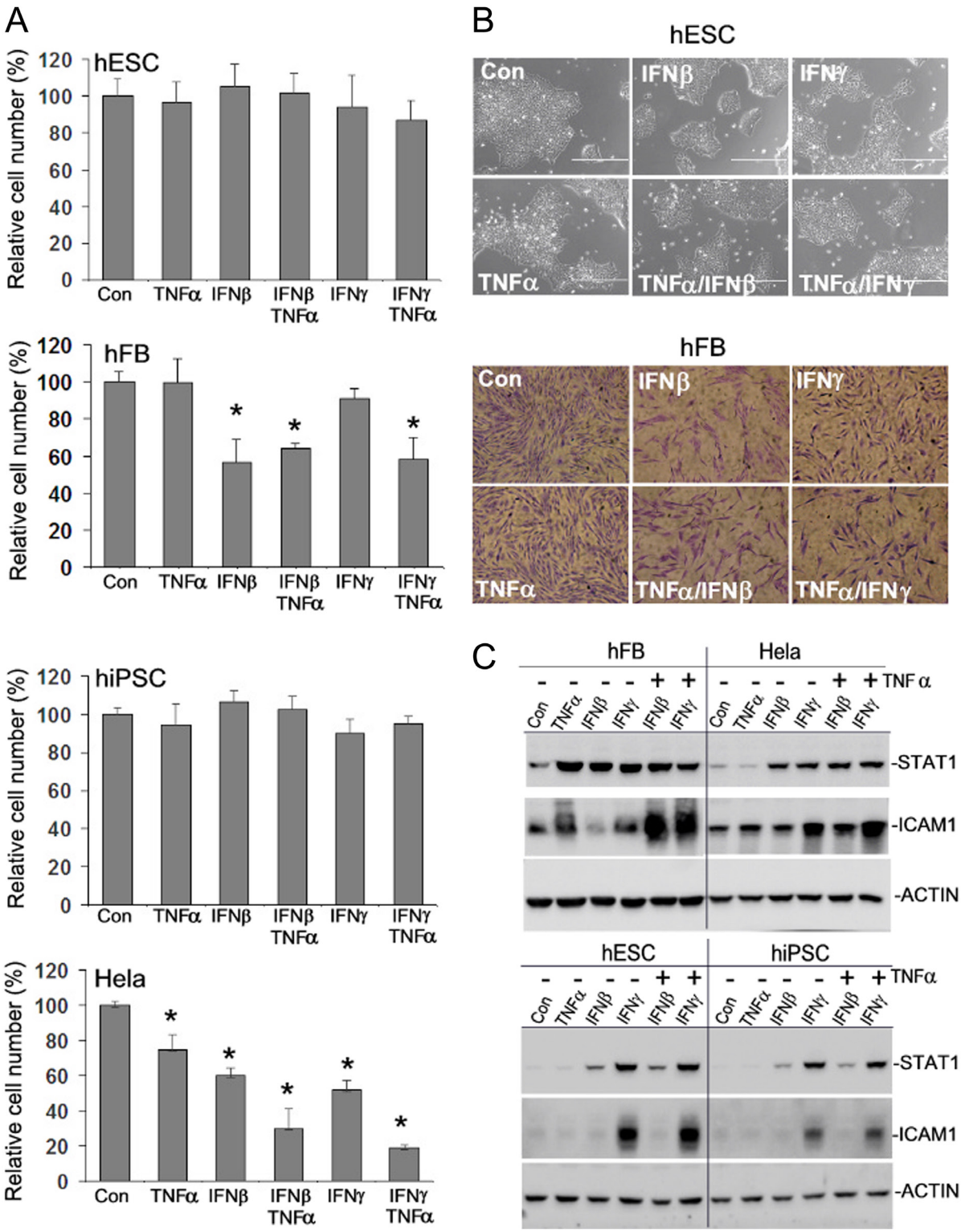

Figure 7 Effects of different TNFα and IFNs on hESCs, hiPSCs, hFBs, and Hela cells. (A) Cells were treated with TNFα, IFNβ, or IFNγ alone or with indicated combinations. After 72 h, cell viability was determined by toluidine blue staining assay. The cell number in control (Con, cells without any treatment) was defined as 100%. Values are mean ± S.D. of a representative experiment that was performed in triplicate. *$P < 0.05$, compared with Con. (B) hFBs and hESCs were treated with indicated cytokines. Cells fixed and stained with toluidine blue (hFBs) or live cells (hESCs) were examined under a phase-contrast microscope and photographed with a digital camera. (C) Western blot analysis of STAT1 and ICAM1 expression. Cells were treated with the cytokines under the same conditions as described in B. STAT1 and ICAM1 were detected with their specific antibodies. ACTIN was used as a loading control.

inhibitory effects on cell proliferation (Fig. 7A), but none of these treatments caused apparent cell death (Fig. 7B). We further tested the response of Hela cells, a human cancer cell line originated from epithelial cells. Not only did TNFα/IFNγ show the strongest cytotoxic effects, but TNFα, IFNβ, and IFNγ alone all also significantly reduced cell number to different degrees. Apparently, TNFα/IFNγ synergistically cause cytotoxicity in all differentiated human and mouse cells tested, whereas the cytotoxicity of individual cytokines is cell type or species dependent.

In mouse cells, iNOS can be induced by TNFα and IFNγ and is synergistically upregulated by TNFα/IFNγ since the promoter of the *iNos* gene has binding sites for both NFκB and STAT1 (Korhonen *et al.* 2005). However, its expression in human cells is regulated quite differently in a cell-type-specific manner. In particular, *iNOS* is not induced by TNFα/IFNγ in normal human fibroblast cell lines (Ganster *et al.* 2001), which is also the case in our experiments with hFBs (data not shown). Therefore, we analyzed the expression of *ICAM1*, which is another gene known to be regulated by TNFα and IFNγ (Ohmori *et al.* 1997). As shown in Fig. 7C, in both hFBs and Hela cells, ICAM1 protein was induced by TNFα and by IFNγ alone and was synergistically potentiated by TNFα/IFNγ (Fig. 7C). In the case of STAT1, its expression was stimulated by IFNβ and IFNγ in both hFBs and Hela cells as expected. STAT1 was also upregulated by TNFα in hFBs, but not in Hela cells. This is not surprising since TNFα-induced STAT1 has been reported in some cells, which could take place indirectly through the induction of IFNs (Hong *et al.* 2001, Yarilina & Ivashkiv 2010). In hESCs and hiPSCs, ICAM1 was induced by IFNγ, while STAT1 was induced by IFNγ and by IFNβ, but at lower levels than those observed in hFBs and Hela cells. However, TNFα did not show any effects on hESCs and hiPSCs, in contrast to hFBs and Hela cells (Fig. 7C). Therefore, the lack of response to TNFα is a common feature shared by hESCs, hiPSCs, and mESCs, and this is likely the key factor that limits the cytotoxicity caused by the synergistic action of TNFα and IFNγ.

Discussion

Tumor necrosis factor α (TNFα) is a potent proinflammatory cytokine that is involved in infectious and inflammatory conditions. It was initially identified for its cytotoxicity that leads to apoptosis or necrosis of certain tumor cells or infected cells (Sedger & McDermott 2014). In a previous study, we reported that TNFα by itself did not cause cytotoxicity in mESCs or mESC-FBs, but it caused cell death of mESC-FBs, but not mESCs, in the presence of transcription inhibitor actinomycin D (D'Angelo *et al.* 2017). This was the first indication that mESCs are resistant to TNFα cytotoxicity. In this study, we demonstrate that not only mESCs but also hESCs and hiPSCs are insensitive to the cytotoxicity associated with TNFα and IFNs that otherwise negatively affects proliferation and viability of differentiated somatic cells. This finding is of great importance from the perspectives of reproductive immunology since it suggests that the attenuated immunologic responses could be an adaptive advantage for ESCs during implantation where the blastocysts are exposed to an inflammatory environment (Mor *et al.* 2017, Robertson *et al.* 2018).

Although TNFα and IFNs have certain cell-specific functions for some physiological processes, including embryo implantation (Mor *et al.* 2017), they are primarily produced for the purpose of immune responses and are known to negatively affect cell proliferation and viability when produced excessively (Hertzog *et al.* 1994). In fact, studies from both clinical and animal models have identified TNFα and IFNγ as 'embryotoxic cytokines' for their contributions to impeded embryo development and pregnancy complication (Hill *et al.* 1992, Cameo *et al.* 1999, Jenkins *et al.* 2000, Chin 2014, Robertson *et al.* 2018). The synergistic effect between TNFα and IFNγ in causing cytotoxicity has been known in human and mouse somatic cells (Clemens 2003), as we demonstrate in mESC-FBs, hFBs, and Hela cells in this study. The crosstalk between TNFα and IFNγ signaling pathways can affect many cellular events via different signaling molecules in a cell-type-dependent manner. In mouse cells, iNOS can be induced by TNFα and IFNγ and is synergistically upregulated by TNFα/IFNγ since the promoter of *iNos* has binding sites for both NFκB and STAT1 (Korhonen *et al.* 2005). Excessive amounts of nitric oxide (NO) produced by iNOS acts as a free radical to cause cytotoxicity (Song *et al.* 2000, Clemens 2003, Korhonen *et al.* 2005). Using an *iNos* knockout mouse model, a recent study provided definitive evidence for iNOS as a key mediator of TNFα/IFNγ cytotoxicity in mouse bone marrow stem cells (mBM-MSCs) (Li *et al.* 2019). This is likely the case in mESC-FBs since they share major properties with mBM-MSCs as we previously reported (D'Angelo *et al.* 2018). Furthermore, TNFα/IFNγ-induced iNOS expression patterns directly paralleled with TNFα/IFNγ cytotoxicity in mESC-FBs (D'Angelo *et al.* 2018). In response to IFNγ, mESC-FBs displayed a well-established 'two wave response pattern' seen in other differentiated cells (Holtzman *et al.* 2002), but this mechanism is apparently not functional in mESCs as we demonstrated by multiple experimental approaches. The IFNγ signaling pathway can be negatively regulated by protein tyrosine phosphatases and suppressors of cytokine signaling (Schroder *et al.* 2004). Whether or not these mechanisms are responsible for and/or contribute to restricting STAT1 transcription activity in mESCs remains to be investigated. However, it is clear that the insensitivity of mESCs to the cytotoxicity of TNFα and IFNγ can be rationally explained by their lack of response to the two cytokines and, therefore, the lack of activation of NFκB and STAT1 in mESCs.

Similar to mESCs, hESCs and hiPSCs do not respond to TNFα and are insensitive to the cytotoxicity of

TNFα/IFNγ, as we reported previously in hESCs (D'Angelo *et al.* 2017) and in this study. However, unlike mESCs, they are able to respond to IFNγ and express IFNγ-induced genes (Drukker *et al.* 2002, Pick *et al.* 2012). Although the exact reason underlying this difference between mESCs and hESCs is not clear, it could be due to their different pluripotency states in which mESCs are believed to be in the 'naive state', whereas hESCs are in the 'primed state' which is developmentally more advanced (Takahashi *et al.* 2018). It is further noted that mESCs (Wang *et al.* 2014*b*), hESCs, and hiPSCs (this study) are also weakly responsive to IFNβ, but neither IFNγ nor IFNβ alone, nor their combination with TNFα showed cytotoxicity to these cells. This is the key feature of hESCs and hiPSCs that distinguish them from Hela cells and hFBs, which show different levels of susceptibility to the cytotoxicity of TNFα, IFNβ, or IFNγ. However, unlike in mESC-FBs where iNOS is synergistically induced by TNFα/IFNγ, iNOS in hFBs is not induced by the two cytokines (data not shown). It has been demonstrated that cytokine-induced iNOS is cell-type dependent in human cells (Ganster *et al.* 2001). However, the synergistic effect of TNFα and IFNγ in both hFBs and Hela cells is clearly demonstrated by *ICAM1*, which is another gene that contains binding sites for NFκB and STAT1 in its promoter region (Roebuck & Finnegan 1999). It is important to point out that the synergy between TNFα and IFNγ in transcriptional activation is mediated by cooperation between NFκB and STAT1 (Ohmori *et al.* 1997), but TNFα/IFNγ cytotoxicity is mediated by different effector molecules. iNOS in mouse cells is only one such molecule that has been better characterized. In human cells, several mechanisms have been reported depending on cell type, including p53 induction and Fas expression, ROS production, and caspase activation (Wright *et al.* 1999, Kimura *et al.* 2003, Kim *et al.* 2005). The cell type and species differences in their sensitivity to cytokine cytotoxicity are also reflected in mESC-FBs, hFBs, and Hela cells. While determining the underlying molecular mechanisms for these differences is out of the scope of this study, the data presented here clearly demonstrate that the lack of response to TNFα in hESCs and hiPSCs is sufficient to allow them to avoid cytokine cytotoxicity as in mESCs where both TNFα and IFNγ signaling pathways are deficient. Therefore, the lack of response to TNFα in PSCs is the key factor that limits the cytotoxicity when TNFα and IFNγ are present.

In summary, the findings in this study provide strong evidence from a physiological context to support the hypothesis that the attenuated response to inflammatory cytokines in ESCs, in particular TNFα, could be an adaptive mechanism that limits the potential damage from immunological cytotoxicity. However, this hypothesis only makes sense if the attenuated immune responses in ESCs do not compromise the cells' immune defense capacity. Thus, it would be rational for ESCs to use alternative defense mechanisms that differ from differentiated somatic cells. In support of this possibility, recent studies suggest that ESCs may utilize a subset of preexisting (intrinsic) ISGs (Pare & Sullivan 2014, Wu *et al.* 2018). Together with these findings, this study provides a rational interpretation of the attenuated immune responses in ESCs from perspectives of reproductive immunology and developmental biology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

B C contributed to the conception and design, collection and assembly of data, data analysis and interpretation. C G and J G performed experiments and contributed to collection and analysis of data. Y-L G and C K contributed to the conception, data analysis/interpretation, manuscript preparation, and financial support.

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