Journal of Virological Methods xxx (xxxx) 114731

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Generation of an A549 ISRE-luciferase stable cell line

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ARTICLE INFO

Keywords:
ISRE-Luciferase
Innate immune response
IFN-β
RIG-I
Viral infection
Influenza A virus

ABSTRACT

With its human lung origin, A549 cell line is a designated cellular model for viral respiratory infections studies. As such infections are known to lead to innate immune responses, various IFN signaling modifications occur in infected cells and have to be considered in respiratory viruses experiments. Here, we describe the generation of an A549 stable cell line that expresses firefly luciferase upon interferon-β stimulation, as well as upon RIG-I transfection and upon influenza A virus infection. Of the 18 clones generated, the first one, namely A549-RING1, demonstrated appropriate luciferase expression in the different conditions tested. This newly established cell line may therefore be used to decipher the impact of viral respiratory infection on innate immune response depending on IFN stimulation, without any plasmid transfection step. A549-RING1 can be provided upon request.

1. Introduction

Human A549 cells are adenocarcinomic human alveolar basal epithelial cells commonly used for a wide range of applications (Giard et al., 1973). Given the tropism of some respiratory viruses, such as Influenza A virus (IAV), for human respiratory epithelial cells, A549 cells represent a particularly adapted model for viral infections experiments and subsequent innate immunity assays.

In an infected cell, foreign and potentially pathogenic materials, namely pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs) localized either on the cell membrane, on the endosomal membrane or in the cytoplasm (Iwasaki and Pillai, 2014). One of the best described cytoplasmic PRRs is retinoic acid-inducible gene I (RIG-I), which is notably involved in IAV genome sensing. At first in an inactive state, RIG-I undergoes conformational changes, directed by poly-ubiquitination, that lead to its tetramerization in the presence of viral genome in the cytoplasm (Thoresen et al., 2021).

PAMPs recognition leads to various signaling pathways activation and type I interferons (IFN-α and IFN-β) and type III interferons (IFN-λ), as well as pro-inflammatory cytokines production. Produced IFNs are secreted in an autocrine and in a paracrine way from the infected cell and bind to different membrane receptors on the infected cell but also on surrounding cells. This binding leads to the JAK/STAT signaling cascade activation and to the translocation of phosphorylated STATs transcription factors complexes (ISGF3) into the nucleus, where they bind to IFN-stimulated response element (ISRE) sequences in interferon-stimulated genes (ISGs) promoters to induce their transcription. In turn, ISGs will establish and maintain an innate immune response. However, this signaling pathway may be subverted by viral proteins, as it was described for IAV non-structural protein NS1, a well-known virulence factor (Lamotte and Tafforeau, 2021; Gack et al., 2009; Talon et al., 2000; Rückle et al., 2012; Wang et al., 2000; Marc, 2014). Innate immune response is therefore a signaling pathway that must be considered in experiments involving respiratory viruses such as IAV.

A549 cells were found to be uneasily infected and transfected with a plasmid at the same time. Therefore, in order to facilitate innate immune response studies under viral infection, we aimed to generate an A549 stable cell line expressing the luciferase reporter gene upon IFN-β stimulation. A549 cells were transfected with a Neo-ISRE-Luc plasmid encoding for the firefly luciferase under the control of a promoter sequence containing five ISRE copies (Lucas-Hourani et al., 2013). Selected clones were tested either upon IFN-β stimulation, upon transfection of an active RIG-I plasmid (NRIG-I), or upon IAV infection, all conditions normally leading to modifications in IFN signaling, detectable through luminescence reading. Among the 18 clones tested, the first one, A549-RING1, demonstrates the most notable variations in luciferase expression, with higher luminescence detection upon IFN-β stimulation and NRIG-I transfection and lower luminescence detection under IAV infection, as compared to mock-treated cells. A549-RING1 cell line may therefore be used to study the impact of viral infection, as well as the impact of proteins of interest, on innate immune response, without the need of reporter gene coding plasmid transfection.

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https://doi.org/10.1016/j.jviromet.2023.114731
Received 11 January 2023; Received in revised form 5 April 2023; Accepted 9 April 2023
0166-0934/© 20XX

2. Material and methods

2.1. Plasmids

Neo-ISRE-Luc plasmid was provided by Dr. P.-O. Vidalain (VIRal Infection Metabolism Immunity team, CIRI, Lyon, France). This plasmid encodes for a firefly luciferase reporter gene under the control of a promoter containing five ISRE copies and has neomycin resistance gene. The ISRE-Luc sequence is derived from pISRE-Luc (Strategene, Ref 219089) and was cloned into a pCi-neoΔCMV-gw vector (Lucas-Hourani et al., 2013) (Fig. S1). NRIG-I plasmid was provided by Dr. G. Caignard (UMR 1161 Virologie, INRAE, Maisons-Alfort, France) and encodes for an constitutively active form of RIG-I protein (Yoneyama et al., 2004).

2.2. Mammalian cell culture

Human A549 cells (ATCC #CCL-185) were grown in DMEM (Dulbecco's Modified Eagle Medium, PAN-Biotech) supplemented with 10% decomplemented Fetal Bovine Serum (FBS, Gibco), and 1% of antibiotics (10 000 units/mL penicillin and 10 000 µg/mL streptomycin, Gibco) (DMEM + FBS + Ab medium) at 37 ˚C and 5% CO_2. The HEK-293 cell line stably expressing the ISRE-Luciferase reporter gene after Neo-ISRE-Luc plasmid transfection (STING-37 cells) (Lucas-Hourani et al., 2013) was provided by Dr. P.-O. Vidalain and was grown in the same conditions as A549 cells.

2.3. Antibiotic kill curve

A549 cells were diluted in DMEM + FBS + Ab medium, seeded in 96-wells plates at a density of 1×10^4 cells/well, and plates were incubated at 37 ˚C and 5% CO_2. After 24 h, medium was discarded and replaced by DMEM + FBS + Ab medium supplemented with geneticin (G418, Thermo Scientific) in different concentrations (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2 mg/mL). Cell viability was assessed by using Cell Counting Kit-8 (CCK-8, Sigma). 48 h and 7 days after incubation, 10 µL of CCK-8 were pipetted into each well. After a 2 h incubation, the absorbance was read at 450 nm by using a plate reader (Fig. S2).

2.4. A549 cells transfection and colonies isolation

A549 cells were seeded in 6-wells plates at a density of 3×10^5 cells/well. After 24 h, A549 cells were transfected with 3 µg of neo-ISRE-Luc plasmid using jetPEI DNA Transfection Reagent (Polyplus). Non-transfected A549 cells were also cultivated as control. 24 h post-transfection, cells were split at 3 different dilutions into 10 cm dishes in DMEM + FBS + Ab medium. After 24 h, the medium was discarded and replaced by fresh medium containing 0.8 mg/mL of G418 (Fig. S2). The medium with G418 was renewed once a week until large colonies appeared. Colonies were detached and isolated in wells of a 96-wells plate containing medium with 0.8 mg/mL of G418.

2.5. IFN-ß stimulation, NRIG-I transfection and IAV infection

Each A549-ISRE-Luc clone was seeded in white opaque 96-wells plate (Greiner Bio-One) at a density of 2×10^4 cells/well, in DMEM + FBS + Ab medium (without G418). Three conditions were tested for each clone: IFN-β stimulation at either 100 IU/mL or 1000 IU/mL, NRIG-I transfection and IAV infection. STING-37 and non-modified A549 cells served as controls in each condition.

For IFN-β stimulation, culture medium of each clone was replaced 48 h after seeding by non-supplemented DMEM (D0) containing either 100 IU/mL or 1000 IU/mL recombinant IFN-β (Human IFN-Beta 1a mammalian, PBL Assay Science).

For NRIG-I transfection, the culture medium was replaced with D0 24 h after clones seeding, and cells were transfected with 100 ng of NRIG-I plasmid by using jetPRIME transfection reagent (Polyplus).

For IAV infection, cells were exposed to the virus 24 h after their seeding. Briefly, medium was discarded, and cells were washed with D0. Cells were then infected at a multiplicity of infection (MOI) of 10^{-4} with IAV $H1N1_{WSN}$ (strain A/WSN/1933) diluted in D0.

After 24 h of IFN-β treatment, and 48 h of NRIG-I transfection and IAV infection, firefly luciferase activity was determined by using the One-Glo reagent according to manufacturer's recommendations (Promega) and luminescence was detected on a microplate reader (Glo-Max Navigator System, Promega).

3. Results

A549 cells were transfected with neo-ISRE-Luc plasmid and were then grown in medium containing 0.8 mg/mL of G418 selective antibiotic (Fig. S2). After one month of culture, 18 clones were recovered. These 18 A549-ISRE-Luc clones were then exposed to three conditions modulating IFN pathway, in order to determine the proper activation of ISRE promoter and subsequent luciferase expression. To note, no differences in cell growth were noticed between the 18 clones and the original A549 cells after their isolation.

Firstly, A549-ISRE-Luc clones were directly stimulated with IFN-β, at both 100 IU/mL and 1000 IU/mL. A549-ISRE-Luc clone 3 and clone 8 showed an unusual diminution of luminescence signal in the presence of 100 IU/mL IFN-β, while the same concentration improves ISRE-Luc expression in clones 1, 2 and 17, with a 24-fold, 27-fold and 2-fold induction, respectively. Stimulation of these three clones with 1000 IU/mL IFN-β also promotes a high luciferase expression, even though no significant differences were noticeable between the use of the two IFN-β concentrations. In STING-37 cells, increasing dose of IFN-β leads to a high luciferase expression (p-value: 0.0017) (Fig. 1A).

Cytoplasmic sensors RIG-I and MDA5, but also Toll-like receptors found in endosomal vesicles (TLR3 and 7), can detect IAV RNA in host cells (Killip et al., 2015). However, it was shown that IFN response to IAV infection is mostly mediated by RIG-I (Baum et al., 2010; Kato et al., 2006; Loo et al., 2008; Yoneyama et al., 2004). We therefore aimed to investigate A549-ISRE-Luc clones response to NRIG-I plasmid transfection. In the presence of the constitutively active form of RIG-I, 10 of the 18 A549-ISRE-Luc clones showed a significant increase in luciferase expression, demonstrating a higher IFN production promoted by RIG-I in these cells. This induction is 6-fold, 5-fold and 8-fold higher in clones 1, 2 and 17, respectively, than in non-transfected cells (Fig. 1B).

Finally, A549-ISRE-Luc clones were infected with influenza A virus, which is known to modulate innate immune response, by either promoting antiviral state through its presence or subverting IFN activation through its virulence proteins (Lamotte and Tafforeau, 2021). Upon infection, only A549-ISRE-Luc clone 1 and clone 2 showed a significant difference in ISRE-Luc expression compared to mock condition. Indeed, infection leads to a decreased luciferase signal of 0.37-fold in clone 1 and 0.31-fold in clone 2, supporting the phenomenon of innate immune response subversion by IAV (Fig. 1C).

4. Discussion

From the 18 A549-ISRE-Luc clones generated, the clone 1 gave expected results for luciferase expression in all conditions tested. Indeed, IFN-β stimulation and NRIG-I transfection promote a significant higher expression of luciferase than in non-treated cells, with respective 24-fold and 6-fold signal increase. However, there was no significant difference between the use of IFN-β at 100 IU/mL or 1000 IU/mL.

The selection of IFN-β in our experiments was leading by the description of a HEK-293-ISRE-Luc stable cell line generation by using the same Neo-ISRE-Luc plasmid, as described here (Lucas-Hourani et al.,

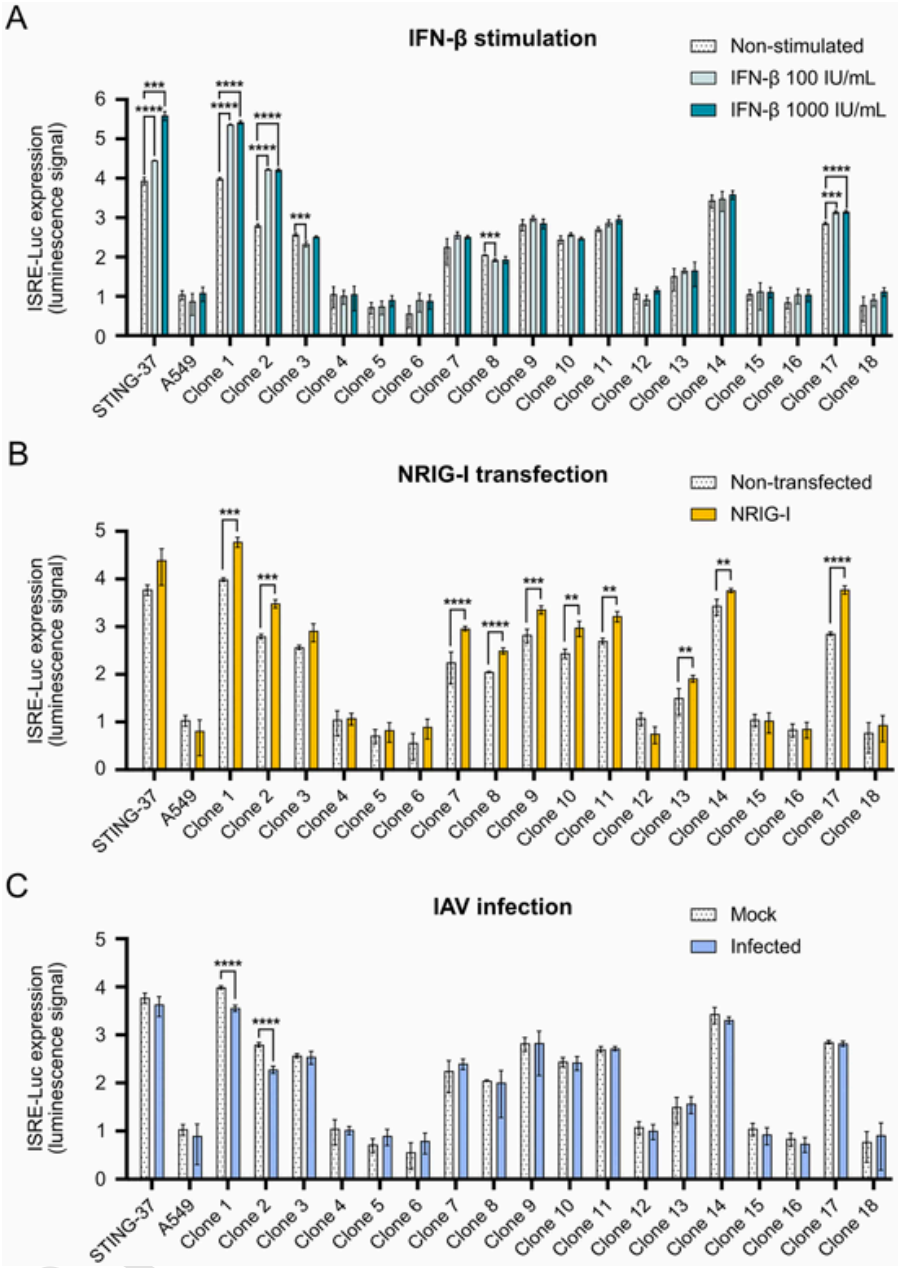

Fig. 1. ISRE-Luc expression in the 18 generated A549-ISRE-Luc clones (A) upon IFN-β stimulation at 100 IU/mL or 1000 IU/mL, (B) upon NRIG-I transfection, and (C) upon IAV infection ($H1N1_{WSN}$, MOI 10^{-4}). ISRE-Luc expression is represented as Log10 of luminescence signal. STING-37 and classical A549 cells served as controls in each condition. IAV: influenza A virus. Each value represents the mean of four independent experiments + SD, and p-values were calculated with multiple unpaired t-tests, * <0.05, * * < 0.01, * ** <0.001, * ** * < 0.0001.

2013). These namely STING-37 cells were tested under the same conditions as A549-ISRE-Luc clones and depict same tendencies as clone 1.

Interestingly, A549 cells can also be stimulated by IFN-λ (Gao et al., 2019), as well as produce this type III IFN (Hsu et al., 2016). Furthermore, IAV infection induces IFN-λ production in A549, through RIG-I-dependent pathway. By activating SOCS1 expression, IAV then inhibits IFN-λ response (Wei et al., 2014). It can thus be inferred that IFN-λ stimulation may also promote luciferase expression in our first A549-ISRE-Luc clone.

In A549-ISRE-Luc clone 1, IAV infection significantly decreased luciferase expression to 0.37-fold compared to mock-treated cells. This diminution may be mostly explained by innate immune response subversion due to IAV virulence factors, such as NS1 protein (Lamotte and Tafforeau, 2021). Indeed, NS1 is described as the main IAV virulence factor and IFN antagonist (Ayllon and García-Sastre, 2015; Hale et al., 2008; Marc, 2014). NS1 was notably shown to directly inhibit RIG-I (Guo et al., 2007; Mibayashi et al., 2007), as well as its positive regulators TRIM25 and Riplet (Gack et al., 2009; Rajsbaum et al., 2012), but also other IFN activators such as IKK, TRAF3 and IRF3 (Gao et al., 2012; Qian et al., 2017; Talon et al., 2000). Moreover, downstream innate immune response factors, such as IFNAR receptors, JAK1, and antiviral ISGs, were also shown to be targeted by NS1 and other IAV proteins (Ayllon and García-Sastre, 2015; Du et al., 2020; Xia et al., 2016). In this way, different studies have shown that an IAV lacking NS1 protein promotes higher IFN responses in infected IFN-β-competent cells (García-Sastre et al., 1998; Kochs et al., 2007; Wang et al., 2000), and that infection with IAV $H1N1_{WSN}$ first induces IFN-β expression which then decreases after 6 h post-infection (Seitz et al., 2010). Furthermore, infection with IAV $H1N1_{WSN}$ induces lower levels expression of some IFN-regulated genes in A549 as compared to non-infected cells (Geiss et

al., 2002). Therefore, it is not surprising that IAV H1N1$_{WSN}$ infection induces a decreased luciferase expression in some A549-ISRE-Luc clones, especially after 48 h of infection.

Although A549-ISRE-Luc clone 2 showed similar tendencies as clone 1, the ratio of luminescence signals was found to be lower in two of the conditions tested. The stability of the clone 1 was not directly assessed, but the clone was maintained in culture for more than 10 passages, allowing us to perform functional experiments.

Classical A549 cells were found to be poorly transfected with a plasmid, and we evaluated that their transfection rate with linear polyethylenimine derivate or cationic polymer-based molecule reagents is from 15% to 20%. Moreover, once transfected, these cells can no more be infected with viruses such as IAV. The generation of an A549 stable cell line expressing an ISRE-Luciferase therefore gives the opportunity to study innate immune response modifications in infected respiratory cells, by avoiding reporter plasmid transfection issues.

5. Conclusion

All in all, A549-ISRE-Luc clone 1 looks like the best candidate for further innate immune response studies under viral respiratory infection without requiring plasmid transfection. This cell line was renamed as A549-RING1 and may be provided upon request.

CRediT authorship contribution statement

Laurie-Anne Lamotte : Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Lionel Tafforeau :** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Pierre-Olivier Vidalain for providing the Neo-ISRE-Luc plasmid and STING-37 cell line. We thank Gregory Caignard and Damien Vitour for providing the NRIG-I plasmid and for helpful discussions. This work was supported by the Fonds de la Recherche Scientifique-FNRS under CDR grant J.0198.20 and by the Fonds pour la Recherche Médicale dans le Hainaut-FRMH. L.-A. L. is supported by a FRIA PhD fellowship.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114731.

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