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# New human in vitro co-culture model of keratinocytes and sensory neurons like cells releasing substance P with an evaluation of the expression of ZIKV entry receptors: A potent opportunity to test Zika virus entry and to study Zika virus' infection in neurons?

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## Abstract

During the course of acute ZIKV infection, pruritus is a cardinal symptom widely documented in the literature. Its frequent association with dysesthesia and several dysautonomic manifestations, suggests a pathophysiological mechanism involving the peripheral nervous system. The aim of this study was to develop a functional human model to potentially able to be infected by ZIKV: by demonstrating the functionality on a new human model of co-culture of keratinocyte and sensory neuron derived from induced pluripotent stem cells using a classical method of capsaicin induction and SP release, and verify the presence of ZIKV entry receptor in these cells. Depending of cellular type, receptors of the TAMs family, TIMs (TIM1, TIM3 and TIM4) and DC-SIGN and RIG1 were present/detected. The cells incubations with capsaicin resulted in an increase of the substance P. Hence, this study demonstrated the possibility to obtain co-cultures of human keratinocytes and human sensory neurons that release substance P in the same way than previously published in animal models which can be used as a model of neurogenic skin inflammation. The demonstration of the expression of ZIKV entry receptors in these cells allows to considerate the potent possibility that ZIKV is able to infect cells.

## KEYWORDS

itch, neuropeptides, receptors, sensory neurons, virus, Zika

Claire Bocciarelli and Nadège Cordel contributed equally to this study.

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## 1 | BACKGROUND

Zika virus (ZIKV) has been responsible for an extensive epidemic affecting people in more than 56 countries since its emergence in Brazil in 2015.<sup>1</sup> It was declared a public health emergency by the World Health Organization on February 2016 due to its high rate of neurological complications (congenital microcephaly, encephalitis, myelitis, Guillain-Barré syndrome).<sup>2-5</sup> During acute Zika virus infection, pruritus is currently considered as a cardinal symptom since it was described as the second most frequent symptom after exanthema (80%–95%) during the large-scale epidemic in Rio de Janeiro and the following epidemics of the Americas.<sup>6,7</sup> A frequent association of ZIKV pruritus with dysesthesia (52%) possibly associated with dysautonomic symptoms such as dry mouth and eyes, urinary incontinence, heat intolerance and sweating disorders has been reported in the literature suggesting the concomitant existence of a small-fibre neuropathy.<sup>8,9</sup> Pruritus is considered as symptom of small-fibre neuropathies.<sup>10</sup>

The skin is the site of a high viral load during the acute phase of ZIKV infection.<sup>11-13</sup> The main receptors for the entry of the virus into the cells are receptors of the TAMs family: TYRO3 and AXL which are receptor tyrosine kinases, TIMs (T-cell immunoglobulin mucin: TIM1, TIM3 and TIM4) as well as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin). RIG1 (retinoic acid-inducible gene 1) is an essential modulator of ZIKV infection by controlling apoptosis.<sup>13,14</sup> In human, dendritic cells, fibroblasts, keratinocytes and also keratinocyte cell line HaCat express at least one of these receptors and are permissive to ZIKV infection.<sup>11-13,15-19</sup> Associated symptoms of ZIKV infection may be related to an infection of keratinocytes but maybe also the sensory nerve. Until now, the involvement of peripheral nervous system in ZIKV infection was poorly described. The presence of these receptors in peripheral nerve cells, nor in sensory neurons was never studied. Only studies on progenitors of neural crest stem cells (hNCCs) and peripheral neurons (hPNS) have been carried out, finding an increase in cell death after ZIKV infection.<sup>20</sup>

Keratinocytes and peripheral sensory nerve fibres are strongly involved in pruritus, pain and neurogenic skin inflammation. Substance P (SP) and the transient receptor potential vanilloid 1 (TRPV1) are implicated and considered as important mediators in the genesis and maintenance of pruritus and pain associated to CNI.<sup>21-25</sup> They are present on both keratinocytes and sensory neuron from DRG. Different works showed by in vitro tests that stimulation of co-culture by a solution of capsaicin, an agonist of TRPV1, induces a calcium response and a significant TRPV1-specific release of SP, allowing that these models are functional and could be used for the study a part of CNI and pruritus mechanism.<sup>26,27</sup> These models were adapted to study mechanism linked independently to inflammation, sensitive skin, itch or pain.<sup>27-30</sup> The limitation of these models is that sensory neurons were issued from animals. Recent research allows the obtention of sensory neurons of human through the differentiation of induced pluripotent stem cells (iPS).<sup>31-38</sup>

Hence, after validation of the model by induction of SP release we were interested in the expression of ZIKV entry receptors in keratinocytes and in human sensory nerve endings that are in the first line of external environment.

## 2 | QUESTION ADDRESSED

The aim of the study was to propose a human functional in vitro model composed by keratinocytes and sensory neurons potentially useable to study the cellular consequences of infection by ZIKV by validation of the expression of receptor implicated in ZIKV entry in cells. More precisely, we developed a co-culture model using human primary keratinocytes and human sensory neurons differentiated from iPS. This model was compared, with a focus of TRPV1 activation and SP release, with previously developed co-culture models constituted by animals' cells. Finally, we studied the expression of receptors permitting the entry of ZIKV on the cells of the model.

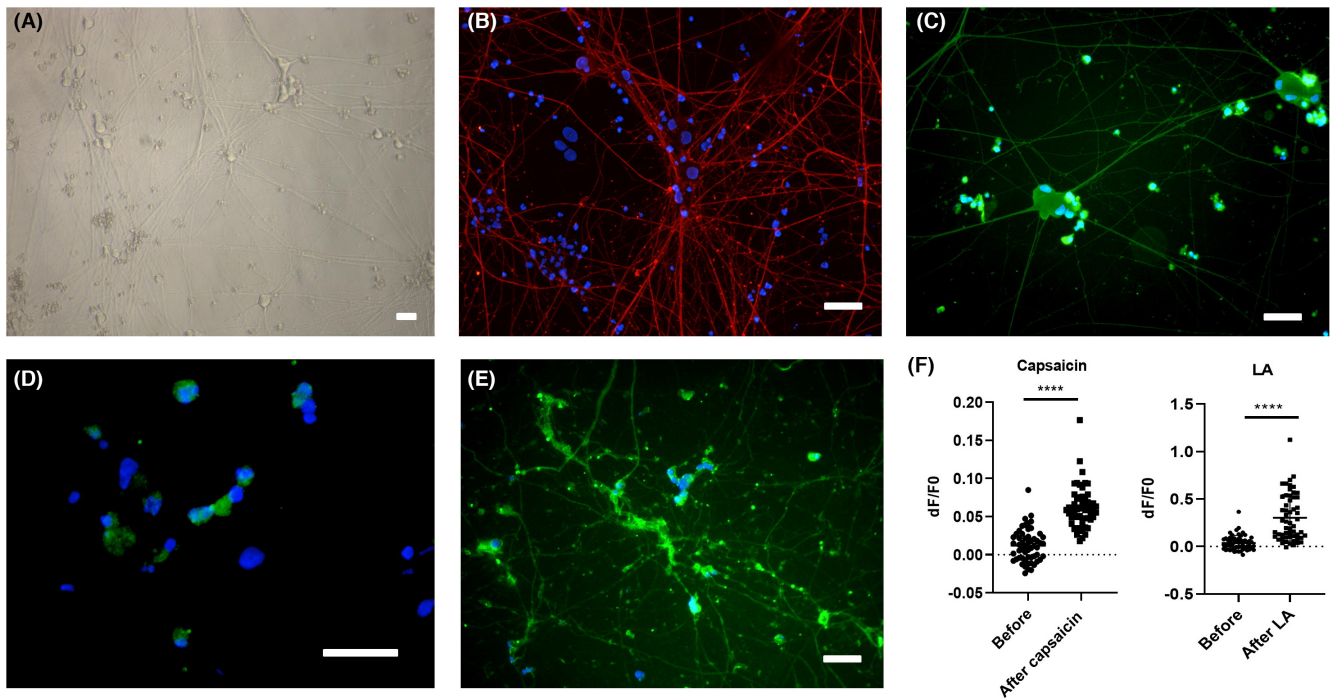
### 2.1 | Experimental design

The in vitro model was constituted by human keratinocytes from abdominoplasties seeded in the same well with sensory neurons from human iPS or rodent DRG. Firstly, neuronal and sensory marker of neurons from iPS cells were checked. Hence, to validate the functionality of the cells and the model, capsaicin was used to induce calcium flux and SP release as previously described in other models.<sup>27,28,30</sup> The expression or not of ZIKV entry receptors (DC-SIGN, AXL, TYRO3, TIM1, RIG1) in iPS differentiated cells into sensory neurons was evaluated by qPCR and/or immunochemistry. Sensory neurons from iPS cells were sourced from AXOL Bioscience (Ax0055, Cambridge, UK) and shipped frozen. The cells were seeded and cultured according manufacture's instruction.

## 3 | RESULTS

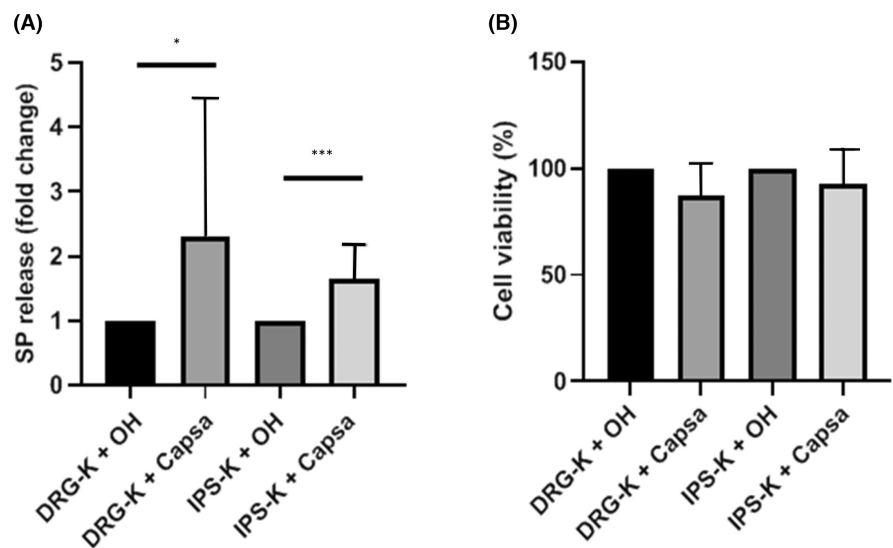
After more than 6 weeks after seeded, the iPS cells presented a neuronal morphology with (Figure S1) or without (Figure 1A) and a dense nerve fibre network was observable in conformity with the supplier (Figure 1A). In complement with the provider, qPCR analysis showed the expression of BRN3A, a sensory expression marker, with a Ct of  $27.86 \pm 2.2$  ( $N=4$ ) and TRPV1 with a Ct of  $31.9 \pm 2.03$  ( $N=4$ ). Immunostaining showed the expression of neurofilament M (neuronal marker), peripherin (peripheric neuronal marker), BRN3A and TRPV1 confirming sensory neuronal differentiation (Figure 1B-E). The functionality of cells was assessed by calcium imaging. Capsaicin and lactic acid induced a calcium flux (Figure 1F).

To demonstrate that the co-culture response is close to the previously established and published model with animal cells, we prepared DRG-keratinocyte co-culture incubated with capsaicin  $10 \mu\text{M}$  for 15 min. Capsaicin led to a significant increase of the SP



**FIGURE 1** Characterization of iPS cells after more 6 weeks of culture. (A) Picture of the iPS cells after more 6 weeks of culture observed in phase contrast microscope. Immunostaining of the neurofilaments M (mature neuronal marker) (B), TRPV1 (C), BRN3A (sensory neuronal marker) (D), peripherin (peripheric neuronal marker) (E). TRPV1, peripherin, BRN3A were revealed in green, neurofilaments M in red and nuclei in blue by DAPI. (F) Calcium imaging experiment. Calcium flux were recorded on individual cells before and after application of capsaicin or lactic acid. The graph reports the amplitude of each individual cell response and mean values ( $\pm$ standard deviation) of a representative experiment. Each dot represent one cells. \*\*\*\*, Wilcoxon test,  $n = 56$ .

**FIGURE 2** Cell response after capsaicin induction. (A) Release of substance P after activation of co-cultures by capsaicin (Capsa) with the ethanol (OH) control. The graph reports the mean values ( $\pm$ standard deviation) of the 7 (with DRG cells) or 3 (with iPS cells) independent experiments in fold change to the control ( $N = 7$ ,  $*p < 0.05$ ,  $N = 3$ ,  $***p < 0.001$  [Mann-Whitney test]). (B) Cytotoxic effect of capsaicin on co-cultures (Capsa) with ethanol (OH) as a control. The graph reports the mean values considering the control as 100% viability ( $\pm$ standard deviation) of the independent three experiments.



release by 2.3-fold higher than the control (SD: 0.8,  $N = 7$ ,  $p < 0.05$ ) (Figure 2A).<sup>27</sup> Capsaicin incubated with the sensory neurons from iPS and keratinocyte co-cultures resulted in a significant increase of SP release by 1.6-fold higher than the ethanol control (SD: 0.5,  $N = 3$ ,  $p < 0.001$ ). Incubation with capsaicin does not induce modification of cell viability in the DRG-keratinocytes and sensory neurons from iPS-keratinocytes co-cultures compared to the ethanol control ( $N = 3$ ) (Figure 2B). Capsaicin could therefore be used at this

concentration without causing any bias related to cellular toxicity or detachment of cells under our conditions.

All transcript of the receptors identified as allowing the entry of ZIKV were detected by qPCR on proliferating human keratinocytes ( $N = 2$ ). TIM1 was expressed with a mean Ct of 17.24 (SD: 3.83). TYRO3 was expressed with a Ct of 25.23 (SD: 1.83). The DC-SIGN and AXL were very weakly expressed with a Ct of 34.1 (SD: 1.48) and 33.45 (SD: 1.05) respectively. All mRNA were detected

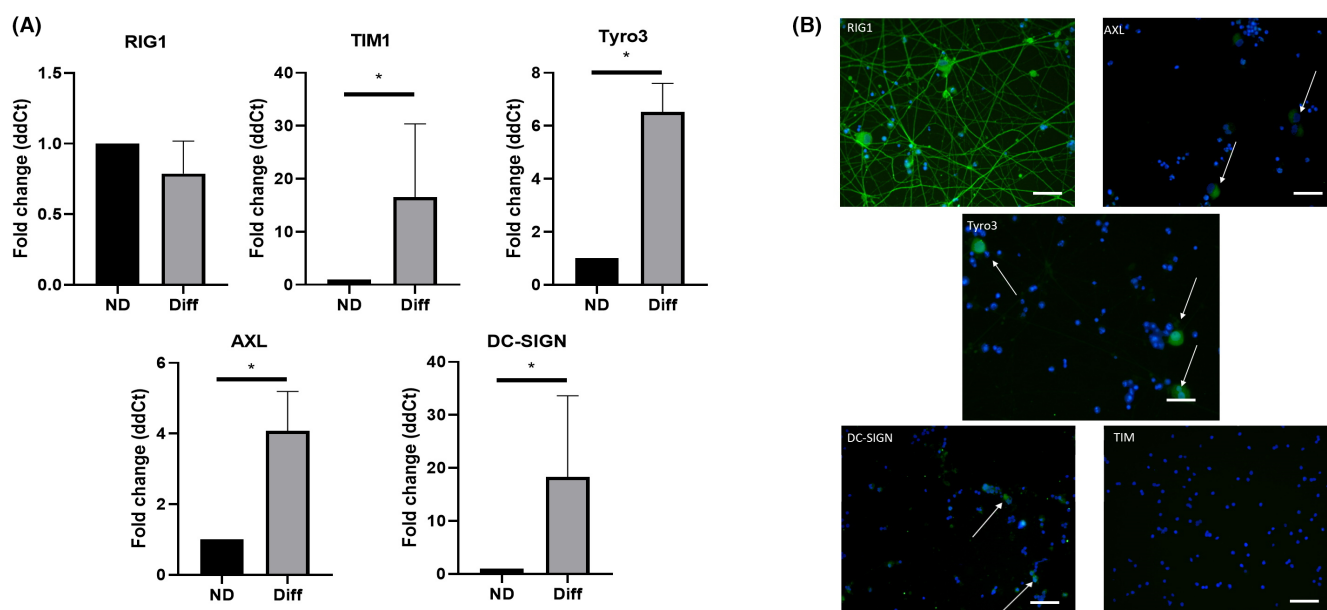
by qPCR on the DRGs ( $N=2$ ) with the exception of DC-SIGN. TYRO3 was the most expressed receptor with a Ct of 24 (SD: 0.1), followed by AXL with a Ct of 27.1 (SD: 0.5) and TIM1 with a Ct of 30.2 (SD: 0.7). All mRNA were detected in sensory neurons differentiated from iPS cells. The most expressed were Tyro3 with a Ct of 26.3 (SD: 1.24,  $N=4$ ), followed by DC-SIGN with a Ct of 28.9 (SD: 3.99,  $N=4$ ). RIG1 and TIM1 receptors were weakly expressed with a Ct of 31.2 and 30.7 respectively (respective SD: 1.24,  $N=3$  and 0.37,  $N=4$ ). AXL was very weakly expressed with a Ct of 34.8 (SD: 0.89). Comparatively with pre-differentiated iPS cells (at the seeding) mRNA of Tyro3, AXL, TIM1 and DC-SIGN types but not RIG1 were significantly more expressed after 6 weeks of maturation (Figure 3A). RIG1 was highly expressed on iPS cells differentiated in sensory neurons as revealed by immunostaining. RIG1 was expressed on cellular body and nerve fibres. AXL, TYRO3, DC-SIGN and TIM1 were very weak or not found (Figure 3B). DC-SIGN, AXL and Tyro3 were identified uniquely on cellular body and in a small number of cells.

#### 4 | CONCLUSION AND PERSPECTIVES

This study showed that a co-culture of human keratinocytes and iPS differentiated into peripheral sensory neurons could be used for in vitro studies to mimic a part of neurogenic skin inflammation and maybe test the infection of these cells by ZIKV. Firstly, we validated the sensory characteristic of differentiated iPS cells by the expression by immunostaining of neurofilaments M (neuronal marker), TRPV1, BRN3A (sensory neuronal marker), peripherin

(peripheric neuronal marker). By calcium imaging we confirmed the functionality of cells. Concerning the co-culture model, in a review of skin-nerve models Schutte et al. presented in phase contrast microscopy a picture of a co-culture of human sensory neurons with keratinocytes.<sup>39</sup> In our work, a significant increase of the SP release after stimulation by capsaicin was observed. Results were similar when we used a heterologous model (neurons from rat DRG and human keratinocytes) and the homologous model (neurons issued from human iPS and human keratinocytes). This response is comparable to the results obtained by Pereira et al. using co-culture between cells from pig DRGs and pig keratinocytes, which enabled them to create a model for the “study of neurogenic skin inflammation”.<sup>27</sup> Significant release of SP after activation of TRPV1 with resiniferatoxin and non-significant release with capsaicin (10  $\mu$ M) was previously observed in sensory neurons from iPS.<sup>40</sup> Our results on sensory neurons differentiated from iPS cultured with human keratinocytes showed a significant SP release after capsaicin incubation (10  $\mu$ M) attesting the participation of keratinocytes. Alternatively, the use of sensory neurons from skin derived precursors could be evaluated to avoid the use of OGM (iPS).<sup>41</sup>

The entry of ZIKV in skin cells was previously demonstrated.<sup>11,12</sup> It is consistent with our observation by qPCR of the expression of arbovirus receptors in cultures of human primary keratinocytes. Moreover, we observed the presence of arbovirus transcripts entry receptors AXL, TYRO3, RIG1 and TIM1 on rat DRGs cells by qPCR. Volpi et al. reported the possibility of ZIKV infection of DRGs from rats deficient in alpha/beta interferon (Ifnar1-KO) in vitro.<sup>42</sup> Swartwout et al. showed the possibility of ZIKV infection of DRGs



**FIGURE 3** Expression profile of pre-differentiated and after 6 weeks of maturation into sensory neurons of iPS cells. (A) RT-qPCR analysis of mRNA expression encoding the RIG1, TIM1, AXL, DC-SIGN and TYRO3 genes, in pre-differentiated (ND) and after maturation of iPS cells (Diff). The graph reports the mean values ( $\pm$ standard deviation) of the 4 independent experiments ( $N=4$ ). \* $p < 0.05$ , Mann-Whitney test). (B) Immunostaining of RIG1, AXL, TIM1, DC-SIGN and Tyro3 on differentiated iPS cells after 6 weeks. RIG1, AXL, DC-SIGN, TIM1 and Tyro3 was revealed in green (FITC), nuclei in blue by DAPI. Scale bar = 50  $\mu$ m.

from immunocompetent mice in vitro but with a high multiplicity of infection (MOI) of 30.<sup>43</sup>

Lee et al. showed the capability of iPS to be infected by varicella-zoster virus.<sup>32</sup> More interestingly, Oh et al. demonstrated the direct infection of peripheral neuron by ZIKV but without identify the entry receptor.<sup>20</sup> By immunostaining we showed than RIG is expressed on peripheral sensory nerve fibre, it is maybe the receptor implicated in the entry of ZIKV. In in vivo context, or in vitro, it is possible too that ZIKV penetrate in the cells by keratinocytes or dendritic cells and penetrate in the neurons via nerve-skin cells interactions. Exact mechanism of ZIKV infection and interaction with skin cells need to be investigated more specifically.

We developed a human homologous functional co-culture model functionally available for the study of a part of pruritus and neurogenic cutaneous inflammation mechanism linked to substance P. Keratinocytes and differentiated iPS cells express receptors required for the entry of ZIKV. Furthermore, different work showed the possibility of ZIKV to infect in vitro keratinocytes or peripheral neurons differentiated from iPS suggesting the interest of the model to study pruritus and neurogenic inflammation generated by ZIKV. Due to high expression of RIG1 in nerve fibres but also in keratinocytes, it is a very interesting candidate to permit the entry of ZIKV in the skin.

#### AUTHOR CONTRIBUTIONS

Claire Bocciarelli wrote the original draft of the manuscript and realized most of the experiments. Nadège Cordel participated to take in place the project, participated to write the manuscript, review and edit the article and found financial support. Raphael Leschiera, Matthieu Talagas, Christelle Le Gall-Ianotto participated to the experimentation. Weiguo Hu provided skin sample. Pascale Marcorelles participated to take in place the project. Joachim Fluhr participated to write the publication and brought important suggestions. Laurent Misery corrected the manuscript and participated to take in place the project. Nicolas Lebonvallet led the project and participated in all of these aspects. Gaëlle Bellemere and Stéphanie Bredif provide important data from an independent collaboration. All authors have read and approved the final manuscript.

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#### CONFLICT OF INTEREST STATEMENT


Authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

### Supplementary material S1

### Supplementary material S2

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