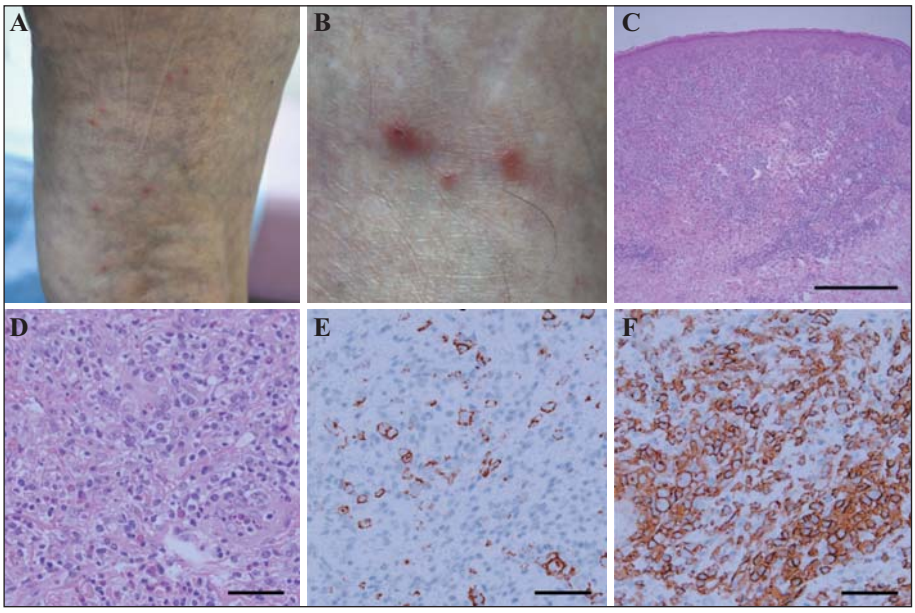

Figure 1. **A**, **B**) Multiple red-to-brown crusted papules on the thighs. **C**, **D**) Wedge-shaped dense diffuse infiltrates in the superficial and middle dermis, consisting of large atypical cells with irregular nuclei, prominent nucleoli and frequent mitoses (haematoxylin and eosin staining; scale bars: 500 μm [**C**] and 50 μm [**D**]). Immunohistochemically, the large cells are CD30-positive (**E**) and CD4-positive (**F**) (scale bars: 50 μm).

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SARS-CoV-2 does not replicate in HaCaT spontaneously immortalized human keratinocytes: implications for the pathogenesis of COVID-19-associated skin manifestations

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)-related cutaneous manifestations have been reported [1], however, the direct or indirect role of SARS-CoV-2 infection in their pathophysiology is still debated [2]. Angiotensin-converting enzyme 2 (ACE2) is highly expressed in keratinocytes, suggesting percutaneous transmission as a potential route for SARS-CoV-2 infection [3]. Currently, the susceptibility of keratinocytes to SARS-CoV-2 has not been investigated. Thus, two HaCaT spontaneously immortalized human keratinocyte cell lines (one from Prof. Boniotto ["HaCaT"], the other from Cell Line Service ["HaCaT CLS"]) were challenged with SARS-CoV-2; viral load was quantified in supernatants and intracellularly and a possible cytopathic effect was evaluated. Vero E6 cells (ATCC-CRL-1586) were used as controls.

Both HaCaT and Vero E6 cells were infected with a SARS-CoV-2 strain, previously isolated on Vero E6 cells, at a multiplicity of infection [MOI] of 0.1 and 0.01 for 1, 2 or 3 hours of viral absorption. Cells were microscopically observed at the fourth and seventh day post-infection to detect possible cytopathic effects. RNA viral load was assessed in the supernatants (at Days 0, 4 and 7) and intracellularly (at Day 7) based on an RT-PCR assay, as previously described [4]. Immunofluorescence staining for the nucleocapsid protein was performed with anti-SARS/SARS-CoV-2 coronavirus nucleocapsid antibody (1:100, MA1-7403, Thermo Fisher Scientific) and anti-mouse Alexa Fluor 488 secondary antibody (1:500, A11029, Thermo Fisher Scientific) on cells grown on Primo screening Black plate and fixed on the seventh day post-infection [5].

ACE2, *TMPRSS2* and *ACTB* (as an endogenous calibrator) gene expression was assessed with iTaq Universal SYBR Green Supermix (Bio-rad) by RT-PCR. Raw data were analysed with the Relative Quantification manager software. The Kruskal Wallis test was used to evaluate the difference in RNA viral load between Days 0 and 7 for each cell line. No morphological alterations were observed in HaCaT cells, while Vero E6 cells showed a SARS-CoV-2-induced cytopathic effect (*figure 1*).

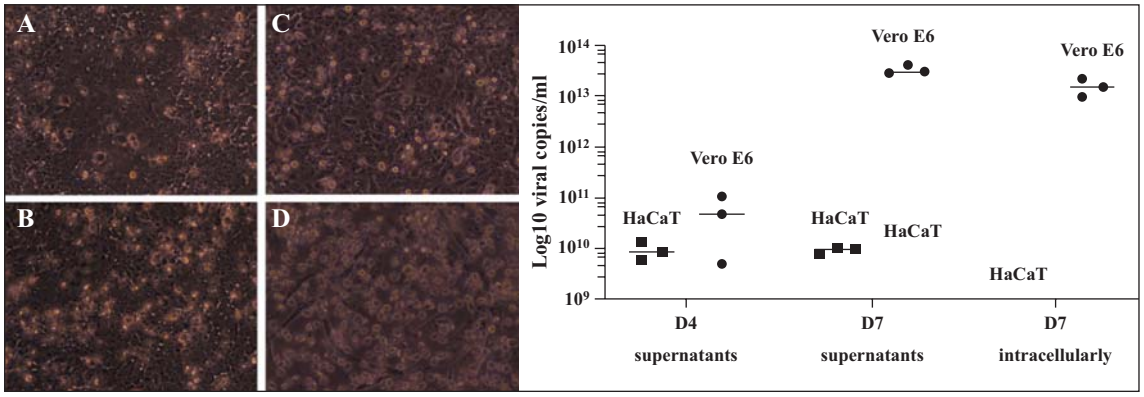

Figure 1. Representative microscopic images of the cytopathic effect of SARS-CoV-2 infection on the seventh day post-infection. Non-infected cells are also shown for comparison (magnification: x40). **A**) Non-infected HaCaT cells. **B**) HaCaT cells infected with SARS-CoV-2. **C**) Non-infected Vero E6 cells. **D**) Vero E6 cells infected with SARS-CoV-2.

RNA viral load in the supernatant from Day 0 to day 7 remained almost the same in the two HaCaT cells line, with a slight decrease at Day 7 for all the tested conditions, with a virus challenge of 0.1 MOI (∼8 Log_{10}) and 0.01 MOI (∼7 Log_{10}). A lack of increase in supernatant viral load may indicate a lack of SARS-CoV-2 replication within these types of cells (*supplementary figure 1*).

In Vero E6 cells, RNA viral level was detected at ∼ 10 Log_{10} on Day 4 and 7 for the higher MOI (0.1) and at ∼ 9 and ∼ 10 Log_{10} respectively on Day 4 and 7 for the lower MOI (0.01) for all the absorption times ($p< 0.05$ for all the tested conditions). At Day 0, the viral load was closer to that measured in HaCaT cells, however, at this time (a few hours after infection), it is not possible for the virus to replicate to any significant level. A lack of increase between the fourth and seventh day could be ascribed to a plateau of viral amplification that was reached on Day 4 (*supplementary figure 1*).

Intracellularly, the level of viral RNA was higher in Vero E6 cells, (∼9 Log_{10}) than HaCaT cells (∼5 Log_{10}), confirming the lack of viral amplification in the latter (*supplementary figure 2*).

SARS-CoV-2 virions were unable to enter and replicate within HaCaT cells. SARS-CoV-2 entry mostly depends on the interaction between the viral spike proteins and ACE2 and TMPRSS2 [6]. HaCaT cells strongly express the *ACE2* gene (as reported in the Human Protein Atlas) but lack *TMPRSS2* gene expression [7]. These data are in accordance with our gene expression results. Indeed, *ACE2* was expressed by HaCaT cells ($\Delta CT_{ACTB\text{-}ACE2}$ ∼ 4), but *TMPRSS2* was only weakly expressed ($\Delta CT_{ACTB\text{-}TMPRSSE2}$ ∼10).

SARS-CoV-2 RNA and proteins have been detected in skin biopsies of different COVID-19-related cutaneous manifestations [1], mainly localized in epithelial cells of eccrine sweat glands and in the endothelium of small dermal vessels [8, 9]. These findings indicate that keratinocytes are not permissive to virus entry, thus corroborating our results. Moreover, the presence of SARS-CoV-2 in both endothelial and eccrine cells indicates that the virus may disseminate to the skin via the blood and that sweat could be considered as a route of transmission [10]. It might be hypothesized that indirect immune-mediated mechanisms are responsible for most COVID-19-associated cutaneous manifestations. ■

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1684/ejd.2022.4206.
Figure 1. SARS-CoV-2 RNA viral load in the supernatant of HaCaT cells (**A**, **B**) and Vero E6 cells (**C**) at Day 4 and 7 post infection. The viral load is expressed as Log_{10} viral copies/mL.
Figure 2. Intracellular RNA viral load of SARS-CoV-2 in the HaCaT and Vero E6 cell lines at Day 7 post-infection. The viral load is expressed as Log_{10} viral copies.

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