Evidence of increased Cathepsin B/L and decreased TMPRSS2 usage for cell entry by the SARS-CoV-2 Omicron variant

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ABSTRACT

The SARS-CoV-2 Omicron variant harbors a large number of mutations in its spike protein compared to the original SARS-CoV-2 strain, which may alter its ability to enter cells. To quantify this effect, we analysed recent datasets on SARS-CoV-2 pseudovirus entry into cells *in vitro* using a mathematical model. SARS-CoV-2 can enter cells via two pathways, one using the host proteases Cathepsin B/L and the other using the host protease TMPRSS2. We found enhanced entry efficiency of the Omicron variant in cells where the original strain preferentially used Cathepsin B/L and reduced efficiency where it used TMPRSS2, indicating that the Omicron variant had evolved to use the Cathepsin B/L pathway better but at the expense of its ability to use the TMPRSS2 pathway for entry. Applying our model, we estimated >4-fold enhanced efficiency of the Omicron variant in entry via the Cathepsin B/L pathway and >3-fold reduced efficiency in entry via the TMPRSS2 pathway compared to the original or other strains. This differential entry pathway usage may explain the altered cell tropism of the Omicron variant compared to other strains and have implications for its infectivity, transmissibility, and interventions.

1. INTRODUCTION

The new SARS-CoV-2 variant Omicron (B.1.1.529), declared a variant of concern on 25th November 2021, is fast becoming the dominant strain in several countries. It harbours as many as 37 mutations in the spike protein compared to the original SARS-CoV-2 strain [1] and is able to evade neutralising antibodies generated by previously infected or vaccinated individuals [1-3], possibly leading to its increased transmissibility and rapid global spread. The spike protein facilitates the entry of the virus into cells [4, 5]. Indeed, emerging data indicates that the high number of mutations in the Omicron variant affects its viral entry properties and cell tropism [2, 3, 6, 7], which in turn may influence its ability to establish infection post-exposure and the severity of the subsequent symptoms. How the mutations influence entry, however, remains to be elucidated.

Early studies showed that the original SARS-CoV-2 (Wuhan-Hu-1) strain displayed broad cell tropism, with viral entry a key determinant of the tropism both *in vitro* [4] and *in vivo* [8]. Intriguingly, multiple recent *in vitro* studies suggest that the Omicron variant's cell tropism may be altered and its entry efficiency different from the original and other variants in a cell line-dependent manner [2, 3, 6, 7]. For instance, SARS-CoV-2 pseudotyped virus bearing either the B.1 or the Delta variant spike protein showed higher entry efficiency than the Omicron pseudotyped virus in Caco-2 (human, colon) and Calu-3 (human, lung) cells, whereas the Omicron pseudotyped virus entered Vero (African green monkey, kidney) and 293T (human, kidney) cells more efficiently than the other variants [3]. Similar trends were observed in live SARS-CoV-2 virus infection assays [6, 7]; the Delta variant infection spread was significantly greater than the Omicron variant in Calu-3 cells [6, 7], whereas the spread of the two variants was similar in VeroE6 cells [6]. What causes the relative efficiency of the Omicron variant to be higher in some cells and lower in others than other variants?

2. HYPOTHESIS: DIFFERENTIAL ENTRY PATHWAY USAGE

The first step in SARS-CoV-2 entry into target cells is the binding of the viral spike protein, S, with the host cell surface receptor angiotensin-converting enzyme 2 (ACE2). Cell tropism is thus expected to be affected by ACE2 expression levels [4, 5, 8]. The Omicron spike protein binds soluble human ACE2 strongly [1, 3]. Further, ACE2 is necessary for the entry of all SARS-CoV-2 variants [2-4, 6, 7]. Thus, any variation in ACE2 expression across cell types is likely to have similar effects on the entry efficiencies of all the variants. The cell type-dependent variation in the entry efficiency of the Omicron variant is therefore unlikely to arise from the variations in the ACE2 expression level across cell types. Omicron spike protein incorporation into pseudotyped virus appears to be compromised compared to the Delta and Wuhan D614G strains [7]. However, this reduction in spike protein density is expected to decrease the Omicron entry efficiency across all cell types, thus ruling it out as a potential cause of the differential entry efficiency observed.

Following ACE2 engagement, successful entry requires that the spike protein be cleaved into subunits by host proteases, either by the transmembrane serine protease TMPRSS2 at the plasma membrane or the cysteine proteases Cathepsin B and Cathepsin L in the endosomal vesicles. The proteases TMPRSS2 and Cathepsin B/L are thought to work independently, facilitating SARS-CoV-2 entry through two independent pathways [5, 9] (Fig. 1A). We recently analysed data on SARS-CoV-2 pseudotyped virus infection *in vitro* [4] using a mathematical model of SARS-CoV-2 entry and found that the relative usage of the TMPRSS2 and Cathepsin B/L entry pathways by the original SARS-CoV-2 strain varied widely across cell lines [9]. For example, Vero cells predominantly admitted entry through the Cathepsin B/L pathway, whereas Calu-3 cells allowed entry via the TMPRSS2 pathway. Vero cells overexpressing TMPRSS2 permitted entry via both pathways [4]. Importantly, the original strain displayed the ability to use either pathway, with the preferred pathway possibly based on

the relative expression levels of the two proteases [5]. We reasoned that the Omicron variant might use these pathways differently from the original (or the Delta) strain, possibly underlying its differential entry efficiency and altered cell tropism.

3. EMPIRICAL EVIDENCE: CORRELATION OF ENTRY EFFICIENCY WITH PATHWAY USAGE

To test our hypothesis, we examined data from the recent experiments of Hoffmann *et al*. [3], who measured the extent of infection of cells *in vitro* by the Omicron variant relative to other variants in different cell types. Interestingly, we found a correlation between the entry efficiency of the Omicron variant and the relative usage of the two entry pathways by the original strain (Fig. 1B). With cell lines (Calu-3 and Caco-2) where the usage of the TMPRSS2 entry pathway by the original strain was dominant, the Omicron pseudotyped virus entry was significantly less efficient than the B.1 and delta strains. By contrast, the Omicron virus entry was more efficient in cell lines (293T and Vero) where the Cathepsin B/L entry pathway usage by the original strain was dominant (Fig. 1B). One possible interpretation of this relationship is that the Omicron variant entry is relatively less effective through the TMPRSS2 pathway and more effective via the Cathepsin B/L pathway than the other strains. Consistent with this notion, camostat mesylate, a TMPRSS2 inhibitor, was less potent against the Omicron variant than the Delta variant in blocking live virus infection of VeroE6 cells overexpressing TMPRSS2, which allows entry via both pathways [6]. Moreover, syncytium formation in a cell-cell fusion assay, which requires TMPRSS2 but not Cathepsins, was severely impaired for the Omicron variant compared to both the Delta [6, 7] and the Wuhan-Hu-1 D614G [7] strains. The increased efficiency of Cathepsin B/L usage together with the reduced efficiency of TMPRSS2 usage by the Omicron variant may explain its altered cell tropism relative to the original strain or other variants.

4. MATHEMATICAL MODEL OF SARS-CoV-2 ENTRY

To quantify the increased efficiency of the Cathepsin B/L pathway usage and reduced TMPRSS2 usage by the Omicron variant, we developed a mathematical model and applied it to analyse multiple recent datasets. We considered cell culture experiments where target cells expressing TMPRSS2 and Cathepsin B/L were exposed to pseudotyped viral particles bearing the spike protein, S, of either the original SARS-CoV-2 strain or a variant. In the experiments, the target cells, T, would get infected by pseudotyped virions, V, to produce infected cells, I, depending on the expression levels of TMPRSS2 and Cathepsin B/L. The following equations described the ensuing dynamics:

$$\frac{dT}{dt} = (\lambda - \mu)T - k\overline{S_{tc}}TV \qquad (1)$$

$$\frac{dI}{dt} = k\overline{S_{tc}}TV - \delta I \qquad (2)$$

$$\frac{dV}{dt} = -c_V V \qquad (3)$$

Here, target cells proliferate and die with rate constants λ and μ, respectively. They get infected by free virions, V, with the maximal rate determined by the second order rate constant k. Cells can have different susceptibilities to infection based on the expression levels of the host factors involved. Following a previous formalism [9], we defined the average susceptibility of a cell population to entry via the Cathepsin B/L pathway as $\overline{S_c}$ and that via the TMPRSS2 pathway as $\overline{S_t}$. These susceptibilities increase with the expression levels of the respective proteases and saturate to unity when the levels are in excess. For a given cell type, a viral variant that has a higher $\overline{S_c}$ (or $\overline{S_t}$) would be more efficient at entry via the Cathepsin B/L (or TMPRSS2) pathway than a variant with a lower $\overline{S_c}$ (or $\overline{S_t}$). Assuming entry via the two pathways to be independent, the overall susceptibility of the population to entry can be written as $\overline{S_{tc}} = \overline{S_t} + \overline{S_c} - \overline{S_t}\,\overline{S_c}$. This susceptibility determines the rate of target cell infection relative to the

maximal rate k. The latter, maximal rate occurs when the proteases are in excess and do not limit entry. Infected cells die with a rate constant δ. Free virions are cleared with the rate constant c_V. Pseudotyped virions are replication-incompetent and do not produce new viral particles.

Experiments with pseudotyped virions typically last less than 24 h. It is reasonable to assume that cell proliferation and death do not affect the infection dynamics significantly during this period. Therefore, we ignored the proliferation and death terms in Eqs. [1-2], which allowed us to derive an analytical expression for the time evolution of the population of infected cells: $I(t) = T_0\left(1 - e^{\frac{k\overline{S_{tc}}V_0}{c_V}(e^{-c_V t}-1)}\right)$, where T_0 and V_0 are the initial target cell density and viral concentration, respectively. Using this expression, we obtained the fraction of cells infected by the pseudotyped virions, f_a, at time t_d post-infection as

$$f_a = \frac{I(t_d)}{T_0} = \left(1 - e^{\vartheta\overline{S_{tc}}V_0}\right) \qquad (4)$$

where $\vartheta = \frac{k}{c_V}(e^{-c_V t_d} - 1)$ is a constant for a given t_d.

We fit this expression to measured data from recent assays and estimated the efficiency of the usage of the two pathways by the Omicron variant relative to the original or other strains.

5. QUANTITATIVE ESTIMATES OF RELATIVE ENTRY EFFICIENCY

We considered data from cell types where the original strain nearly exclusively entered via either the Cathepsin B/L or the TMPRSS2 pathways, so that we could estimate the relative efficiency of the usage of either pathway by the Omicron variant. We thus examined first data from Garcia-Beltran *et al*. [2], who measured the fraction of 293T-ACE2 cells infected by pseudotyped virus bearing the wild-type or the Omicron spike proteins at different viral concentrations, V_0 (Fig. 2A). 293T-ACE2 cells predominantly permit original strain entry via

the Cathepsin B/L pathway [9]. Given the relatively poor efficiency of TMPRSS2 usage by the Omicron variant (Fig. 1B), we made the approximation $\overline{S_{tc}} \approx \overline{S_c}$ in Eq. (4). Using the resulting expression, we fit predictions of f_a as a function of V_0 to the corresponding experimental data [2] (Fig. 1C) and estimated the composite parameter $\vartheta\overline{S_c}$ for the wild-type (wt) and the Omicron variant. Our model provided good fits to the data (Fig. 2A). Taking ratios of the estimated composite parameter and recognizing that ϑ is unlikely to be strain dependent, we obtained $\overline{S_c^{Omicron}}/\overline{S_c^{wt}}$~4.6, indicating an ~4.6-fold increased efficiency of the usage of the Cathepsin B/L pathway by the Omicron variant in 293T-ACE2 cells relative to the original strain.

We next analysed the experiments of Hoffmann *et al*. [3] mentioned above (Fig 1B). We focussed on Calu-3 cells, which appear to allow entry of the original strain nearly exclusively via TMPRSS2. Here, we therefore made the approximation $\overline{S_{tc}} \approx \overline{S_t}$ in Eq. (4). Unlike the above dataset, measurements here were available at a single initial viral load. Further, measurements were available of the fractions of cells infected by viruses pseudotyped with the Omicron variant relative to those with the B.1 or Delta variants. Note that B.1 has spike proteins identical to the original strain except for the D614G mutation. Because the overall infection of Calu-3 cells is small, Taylor series expansion of Eq. (4) yielded $f_a \approx \vartheta\overline{S_t}V_0$. Accordingly, the susceptibility of cells to entry via the TMPRSS2 pathway of the Omicron variant relative to the B.1 strain would be given by the ratio of the fractions of cells infected in the two assays: $\overline{S_t^{Omicron}}/\overline{S_t^{B.1}} = f_a^{Omicron}/f_a^{B.1}$. Using the latter expression and the corresponding data yielded $\overline{S_t^{Omicron}}/\overline{S_t^{B.1}}$~0.28 and $\overline{S_t^{Omicron}}/\overline{S_t^{Delta}}$~0.12. Thus, we estimated ~3.6-fold and ~8.3-fold decreased efficiency of the Omicron variant in using TMPRSS2 for entry in Calu-3 cells compared to the B.1 and Delta strains, respectively (Fig. 2B).

6. CONCLUSION AND DISCUSSION

The Omicron variant thus appears to have evolved to use Cathepsin B/L more efficiently and TMPRSS2 less efficiently than other variants for cell entry. This switch in the Omicron variant's preference for entry pathways may have clinical implications. Drugs targeting TMPRSS2, such as camostat mesylate [4], and Cathepsin B/L pathway, such as hydroxychloroquine [10], are in clinical development. The effectiveness of TMPRSS2 inhibitors is likely to decrease and that of Cathepsin B/L inhibitors is likely to increase against the Omicron strain. Furthermore, our mathematical model previously predicted synergy between TMPRSS2 and Cathepsin B/L inhibitors against SARS-CoV-2 infection [9]. Because the Omicron variant's preference for the TMPRSS2 pathway is reduced but not eliminated, combination treatment with TMPRSS2 and Cathepsin B/L inhibitors may exhibit limited but possibly useful synergy against Omicron variant infection of cells that admit both entry pathways. Future studies may establish these implications quantitatively. Our present approach offers a route for such quantification via analysis of *in vitro* experiments.

Implications also follow for our understanding of the cell tropism of the Omicron variant. Because of its preferred and more efficient usage of the Cathepsin B/L pathway, it is likely to preferentially infect cells expressing high levels of Cathepsin B/L. It would be interesting to test whether such cells are present in greater abundance in the upper respiratory tract, facilitating greater transmission of the Omicron variant than other variants. Similarly, future studies may test whether the reduced usage of TMPRSS2 results in less severe disease compared to the other variants.

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COMPETING INTERESTS

The authors declare that no conflicts of interests exist.

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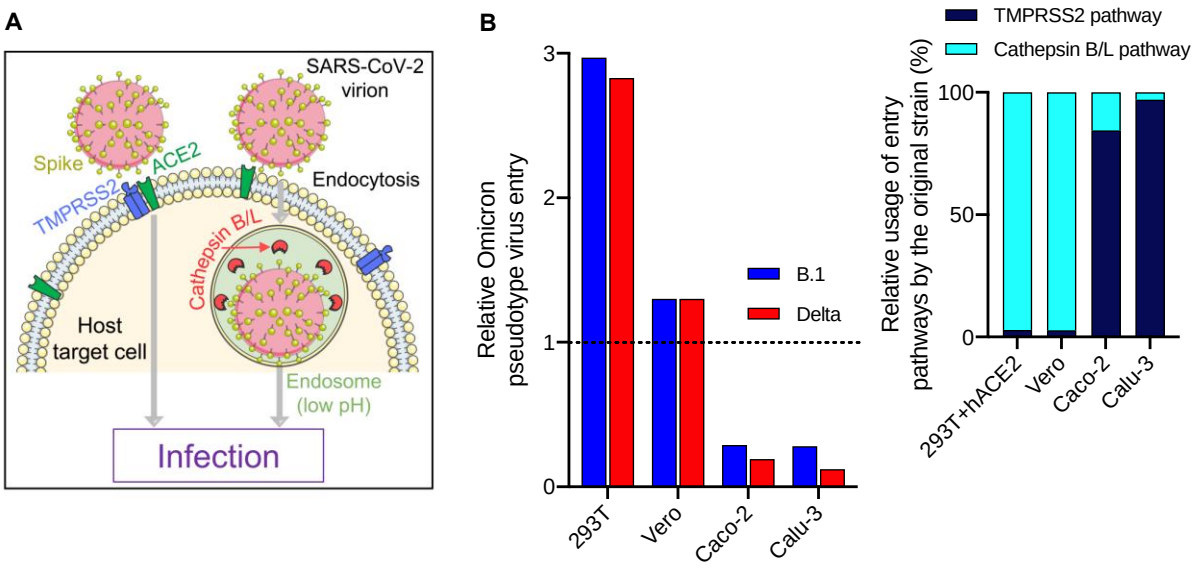

Figure 1. Omicron variant entry efficiency relative to B.1 and Delta strains correlates with the relative usage of the TMPRSS2 and Cathepsin B/L pathways. (A) Schematic of the two independent entry pathways accessible to SARS-CoV-2. **(B)** The Omicron entry efficiency relative to either the B.1 (blue) or the Delta (red) variant across cell types. Data was taken from Hoffmann *et al*. [3]. *Inset*, the relative usage of the entry pathways by the original strain. Data was taken from Padmanabhan *et al*. [9].

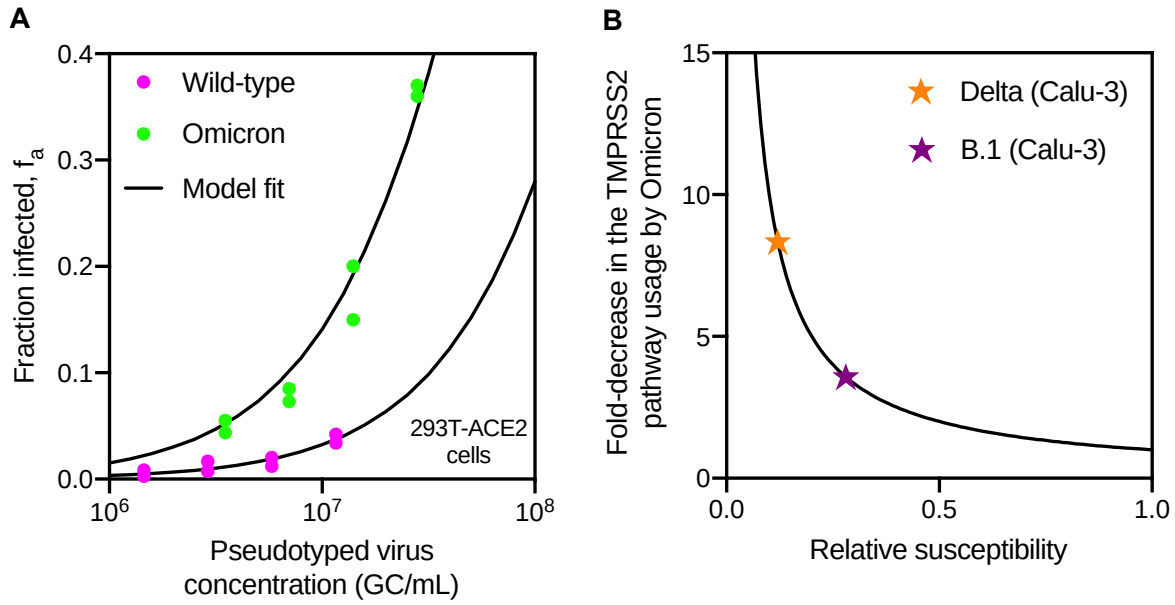

Figure 2. Estimation of entry pathway usage by the Omicron variant. (A) Fits of model predictions (lines) to the experimental data (symbols) taken from Garcia-Beltran *et al*. [2] measuring the fraction of cells infected by pseudotyped virus bearing either the wild-type or the Omicron spike proteins. Data was fit using the tool NLINFIT in MATLAB R2017b. **(B)** The fold-decrease in Omicron entry efficiency through the TMPRSS2 pathway as a function of relative susceptibility to entry. Symbols place experimental data of relative infection taken from Hoffmann *et al*. [3] on the relative susceptibility curve predicted (see text) so that the fold decrease can be read off. In A and B, experimental data was extracted using Engauge Digitizer 12.1.