Evaluation of intranasal TLR2/6 agonist INNA-051: safety, tolerability and proof of pharmacology

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Early clinical trials of intranasal INNA-051 show its potential as a prophylactic that boosts innate immunity. The TLR2/6 agonist activated local pathways associated with antiviral host defence and accelerated viral clearance after influenza challenge. https://bit.ly/3VdmQpD

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Abstract

Background Local priming of the innate immune system with a Toll-like receptor (TLR)2/6 agonist may reduce morbidity and mortality associated with viral respiratory tract infections, particularly for the elderly and those with chronic diseases. The objectives of the present study were to understand the potential of prophylactic treatment with a TLR2/6 agonist as an enhancer of innate immunity pathways leading to accelerated respiratory virus clearance from the upper airways.

Methods Two randomised, double-blind, placebo-controlled clinical trials were conducted in healthy adult participants. The first dose-escalation study assessed safety, tolerability and mechanistic biomarkers following single and repeated intranasal administrations of INNA-051. The second was an influenza A viral challenge study assessing the impact of treatment on host defence biomarkers and viral load.

Results INNA-051 was well tolerated in both studies, with no dose-limiting toxicities identified. Mechanistic biomarkers assessed in both studies demonstrated the expected engagement of pharmacology, including innate immune pathways. There were lower than anticipated rates of infection. *Post hoc* analysis conducted in laboratory-confirmed infected participants with low or no antibody titre against the challenge virus showed INNA-051 treatment led to a significantly shorter duration of infection and increased expression of genes and pathways associated with host defence responses against influenza.

Conclusions The safety and pharmacology profile of INNA-051 confirms preclinical studies. INNA-051 increased expression of genes and pathways associated with host defence responses against influenza and was associated with a shorter duration of infection. These studies support further clinical assessment in the context of natural viral respiratory tract infections in individuals at increased risk of severe illness.

Introduction

Acute respiratory tract infections, caused by RNA viruses such as coronavirus, influenza virus, respiratory syncytial virus, metapneumovirus and rhinovirus, are a major public health problem and a leading cause of morbidity and mortality worldwide. Those especially at risk for severe complications are the elderly, patients with chronic respiratory diseases such as COPD, and those with suppressed or compromised immune systems who often fail to mount or maintain a sufficient immune response to vaccines [1]. Respiratory viral infections, especially those caused by rhinoviruses, are a major cause of asthma and COPD exacerbations, resulting in a significant impact on patients and global healthcare systems [2]. Consequently, there remains an unmet need for safe, effective and convenient prophylaxis approaches that complement vaccines and reduce disease severity in individuals at risk of serious complications.

Drug development efforts disproportionately focus on viral targets, leading to the development of direct-acting antiviral (DAA) therapies. While this strategy can be highly effective against certain viruses, it has several disadvantages, especially for infections with RNA viruses that often mutate rapidly, creating quasi-species that can escape DAAs [3–6]. Moreover, DAAs require rapid diagnosis and intervention to be effective.

Approaches targeting host defences are attractive alternatives to DAAs, likely to exhibit virus-agnostic activity and have the potential to rapidly address outbreaks of novel viral variants or viral pathogens that have yet to be characterised and for which no vaccine or antiviral exists.

One such strategy is boosting the innate immune system, the first and major line of defence against viral infections. In children, it has been shown that pre-activated airway epithelial cells and innate immune cells, including macrophages, are primed for virus sensing resulting in an increased early innate antiviral response to SARS-CoV-2 infection when compared to adults [7].

Pathogen detection by the innate immune system relies on the engagement of pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-like receptors (RLRs) [8–12]. PRRs' activation mobilises programs of gene and protein expression that include type I and III interferons (IFNs) and various downstream antiviral mechanisms [9, 13, 14]. The interferon response is therefore a target for antiviral therapeutic intervention. Recombinant type I IFN beta [15–17] and type III IFN lambda [18, 19] have been trialled for treating respiratory viral infections with demonstrated clinical benefits. Subcutaneous recombinant IFN lambda treatment led to accelerated SARS-CoV-2 viral clearance in a Phase 2 study [18] and significantly reduced the incidence of hospitalisation or emergency department visits among acute symptomatic COVID-19 outpatients in a Phase 3 study [19]. Interferon lambda responses to infection are compromised in individuals with chronic lung conditions such as asthma, who may benefit from therapeutic augmentation of this response to limit infection severity.

TLRs, the sentinel stimulators of the host immune defence against invading pathogens, are also recognised as potential targets for host-directed, virus-agnostic strategies [10, 20–24]. Synthetic agonists of the viral DNA/RNA-recognising TLR molecules, TLR3, TLR7/8 and TLR9, boosted protective innate immune responses against respiratory viruses [25–28]; however, success in the clinic has been limited, mostly due to the systemic release of pro-inflammatory cytokines and type I IFNs, which produce flu-like symptoms and other side-effects in humans [29–31].

INNA-051 is a novel, potent agonist of cell-surface TLR 2/6 that is expressed by both human nasal epithelial cells and innate immune cells [32]. It was engineered to have limited systemic bioavailability for topical administration [33]. It is being developed for intranasal delivery as most respiratory viruses replicate in nasal mucosa epithelial cells [34, 35]. In animal models, prophylactic administration of INNA-051 and close analogues to the upper respiratory tract was effective against multiple respiratory viruses, including SARS-CoV-2, influenza and rhinovirus [33, 36–39]. INNA-051's close analogues were shown to mediate the upregulation of innate immune defence pathways in airway epithelial cells, defined by early expression of nuclear factor-κB-regulated antimicrobial genes, including Type III interferons (IFN lambda), followed by immune cell recruitment. The direct and downstream effects of TLR2/6 pathways activation on nasal epithelial cells and recruited macrophages, in particular, was associated with reduced virus dissemination to the lungs of infected animals, sustaining protection for up to 7 days [38]. The relatively prolonged effect observed in preclinical studies supports the potential for weekly administration in humans [38, 39].

We report the First in Human (FIH) study designed to assess the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of single ascending (SAD) and multiple ascending (MAD) intranasal doses of INNA-051 in healthy adults.

We also report a randomised double-blind placebo-controlled human influenza challenge study exploring the impact of INNA-051 prophylaxis on the course of viral infection, symptoms and host defence biomarkers.

Methods

Trial design for First in Human (FIH) study

The FIH study (INNA-051-HVT-01) was a randomised, double-blind, placebo-controlled assessment of intranasal INNA-051's safety, tolerability, PK and PD in healthy adults at a single Australian site (Scientia Clinical Research Ltd). It involved five single ascending doses (20, 60, 150, 300 and 600 µg) and three multiple ascending doses (60, 150 and 300 µg) on Days 1, 4, 7 and 10. Participants were enrolled and

randomised by the site in a 3:1 ratio (supplementary data) according to a statistician-generated sequence. Participants were discharged post Day 10 assessments, returning for a final follow-up on Day 18 ±2 days.

Trial design influenza challenge study

The influenza challenge (ERY-CSP-001/INNA-051/-IAV) was a randomised, double-blind, placebo-controlled study to evaluate the prophylactic efficacy, safety and tolerability of INNA-051 in healthy adult participants challenged with influenza A/Perth/16/2009 (H3N2) virus. Conducted at a single UK site (hVIVO Services Ltd), in accordance with hVIVO standard screening practice and the study protocol, participants' eligibility and sero-suitability were determined within 90 days prior to quarantine admission (Day 5) using a haemagglutination inhibition (HAI) assay. The antibody titre against influenza A/Perth/16/2009 (H3N2) used as the cut-off value for sero-suitability for the study was ⩽10 haemagglutination (HAI) units. An additional serology sample was collected upon admission to the unit (on Day −5) and analysed upon study completion. Randomisation (see supplementary material) by the site occurred on Day 4, with participants receiving INNA-051 150 µg, INNA-051 300 µg or placebo in a 1:1:1 ratio on Days −4 and −1. On Day 0, participants received ∼$10^{5.5}$ TCID50/mL (of ∼$10^{6.5}$ $TCID_{50}$/mL) A/Perth/16/2009 (H3N2) liquid formulation based on a total inoculation volume of 100 µL. Study procedures continued until discharge on Day 8, with a final follow-up on Day 28 ±3 days.

The primary and key secondary efficacy end-points included analyses of area under the viral load-time curve (VL-AUC), incidence of infection measured by two quantifiable quantitative real-time PCR samples on 2 consecutive days, peak viral load, duration of quantifiable quantitative real-time PCR measurements, peak symptoms score, total symptom score area under the curve (TSS-AUC) and duration of self-reported symptoms.

IMP manufacture (both studies)

The INNA-051 drug product and matching placebo were manufactured and labelled in accordance with good manufacturing practice and Australian Therapeutic Goods Administration requirements by PCI Pharma Services (Melbourne, Australia). The product was formulated using the INNA-051 drug substance dissolved in 0.9% saline with 0.1% w/w EDTA and administered as an aqueous nasal spray solution *via* the Aptar (Crystal Lake, IL, USA) cartridge pump system nasal spray pump device (100 µL actuation volume).

Safety assessments for FIH study

Safety assessments included reporting adverse events (AEs), vital signs, clinical laboratory evaluations (haematology, coagulation profile, clinical chemistries and urinalysis), peak nasal inspiratory flow (PNIF), peak expiratory flow (PEF) and visual analogue score (VAS) scoring of nasopharyngeal symptoms.

Safety assessments for influenza challenge study

Safety assessments included reported AEs, physical examinations, vital signs and clinical laboratory evaluations (haematology, clinical chemistries, coagulation profile and urinalysis).

PK analysis for FIH study

Plasma PK concentrations of INNA-051 were measured in plasma samples using a validated analytical method based on solid phase extraction followed by liquid chromatography-mass spectrometry analysis. The lower limit of quantitation (LLOQ) of the INNA-051 assay was 100 $pg \cdot mL^{-1}$. For the PK assessment, plasma samples were collected on Day 1 within 10 min prior to investigational product administration and at 15 min, 30 min and 1, 2, 4, 6, 8, 12, 18 and 24 h post-dose.

Sample collection (FIH study)

Nasosorption™ FX·i sampling devices (Mucosal Diagnostics – Hunt Developments, UK) were used to absorb mucosal lining fluid and cells from both nostrils at pre- and post-dosing timepoints [40]. For the SAD arm, samples for cytokine analysis were collected 24 h pre-dose and 6, 12, 24 and 48 h and on Day 7 post-dosing; samples for RNA analysis were collected 8 h post-dosing. For the MAD arm, samples were collected for cytokine analysis 24-hours pre-dose and 6, 12, 24, and 48 h post-dose and on Day 18; samples for RNA analysis were collected 24 h pre-dose and 8 h after each dose.

Blood for serum isolation was collected pre-dose, 6, 24 and 48 h post-dose and 7 days post final dose).

Sample collection (Influenza Challenge Study)

Nasopharyngeal swab samples were collected twice daily starting 2 days post-viral challenge (Day 2) up to discharge from quarantine (Day 8) for quantitative real-time PCR viral load assays and PD analysis. Additional nasal samples were collected at Day −1 (post second IMP/placebo treatment) and 24 h post-challenge (Day 1) for PD analysis.

Blood for serum isolation was collected at quarantine admission (Day −5) and at the follow-up visit (Day 28 ±3 days) to confirm seroconversion. Seroconversion in the protocol was defined as a four-fold increase in serum HAI titre between the Day −5 and Day 28 samples, where the follow-up HAI titre is ⩾20.

Cytokine analyses (FIH study)

The concentrations of interleukin (IL)-1β, macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, IFN-α2a, IFN-γ, IL-10 and GROα in serum and nasal-lining secretions for MIP-1α, MCP-1 and IFN-α2a were determined using MSD plates. Samples were analysed on the MS 2400 imager according to the manufacturer's instructions. Standards and samples were measured in duplicate. One participant in SAD cohort 1 was excluded from analysis due to high cytokine concentrations at baseline.

RNA analyses (FIH study)

RNA extraction and concentration along with analysis with the NanoString nCounter Human PanCancer Immune Profiling Assay were completed as previously described [41–43].

Statistics

Cytokine and VAS data were plotted in GraphPad Prism v9. Statistical analysis was performed on baseline subtracted data (after Log10 transformation for cytokine concentrations) by mixed-effects model fit using restricted maximum likelihood (REML), with Geisser–Greenhouse correction. Adjusted p-values <0.05 by Dunnett's (>2 groups) or Sidak's (2 groups) correction for multiple comparisons to placebo were considered statistically significant.

Individual viral load values were compared using a repeated measures model with treatment group and visit/time point as fixed effect, and the interaction between study visit/time point and treatment group using a compound symmetry covariance structure. The parameter estimates (least square means, estimated differences between the least squares means and the associated confidence intervals and p-values) at each visit/time point were calculated using REML method, and the Kenward–Roger's method was used to determine the degrees of freedom. To account for multiple comparisons, the confidence intervals and p-values were adjusted based on the Dunnett–Hsu method as a sensitivity analysis.

Duration of infection and symptoms were compared between treatment and placebo groups by Wilcoxon rank-sum test, with the Benjamini–Hochberg procedure used to adjust for multiple comparisons.

For RNA analyses, quality control and normalisation was undertaken utilising nSolver 4.0 (NanoString Technologies, Seattle, WA, USA) with gene expression analysis completed using a combination of limma for differential gene expression [44] in the R computing environment [45], and nSolver 4.0 for pathway scoring [46] and cell type profiling [41–43]. Raw gene expression data were normalised against a set of six positive and six negative controls to account for background noise and platform-associated variation. Data were filtered to exclude genes expressed below a minimum detection threshold of 20 in 90% of samples in the post-treatment timepoints. Reference gene normalisation was performed using geNorm [47]. Algorithm geNorm calculates a normalisation factor based on a pairwise comparison of the housekeeping genes and takes those genes with the lowest variance. Principal component analysis was performed using DEGreport v1.32 [48]. Differentially expressed genes were assessed with a Benjamini–Yekutieli adjusted t-test with significance accepted at p<0.05, and Log2 fold-change >1 or <−1.

Results

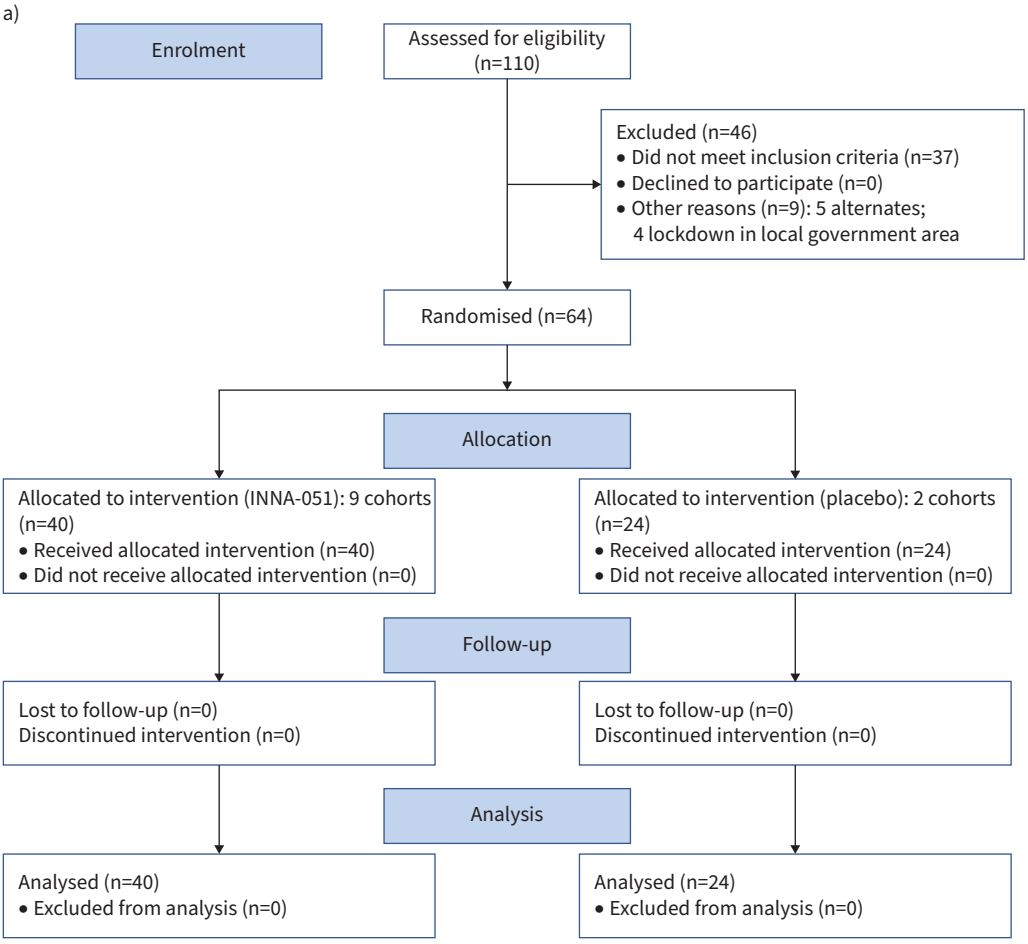
FIH study: population, dosing and disposition

64 participants aged 19–55 years were enrolled and randomised to receive INNA-051 or placebo in one of the SAD (20–600 µg) or MAD cohorts (60–300 µg; figure 1a). Each cohort included eight participants who received INNA-051 (n=6) or placebo (n=2). Participant demographics and disposition are shown in supplementary table S1.

Influenza challenge study: population, dosing and disposition

123 participants aged 19–53 years were enrolled and randomly assigned to receive INNA-051 or placebo. The intent-to-treat participants (ITT) included 40 participants in the placebo, 38 in the INNA-051 150 µg and 41 in the 300 µg groups (supplementary figure S1 and table S2). Participants were given two doses of INNA-051 or placebo on Days −4 and −1 and inoculated on Day 0 with Influenza A/Perth/16/2009 (H3N2).

a)


Enrolment
Assessed for eligibility (n=110)
Excluded (n=46)
• Did not meet inclusion criteria (n=37)
• Declined to participate (n=0)
• Other reasons (n=9): 5 alternates; 4 lockdown in local government area
Randomised (n=64)
Allocation
Allocated to intervention (INNA-051): 9 cohorts (n=40)
• Received allocated intervention (n=40)
• Did not receive allocated intervention (n=0)
Allocated to intervention (placebo): 2 cohorts (n=24)
• Received allocated intervention (n=24)
• Did not receive allocated intervention (n=0)
Follow-up
Lost to follow-up (n=0)
Discontinued intervention (n=0)
Lost to follow-up (n=0)
Discontinued intervention (n=0)
Analysis
Analysed (n=40)
• Excluded from analysis (n=0)
Analysed (n=24)
• Excluded from analysis (n=0)

b)

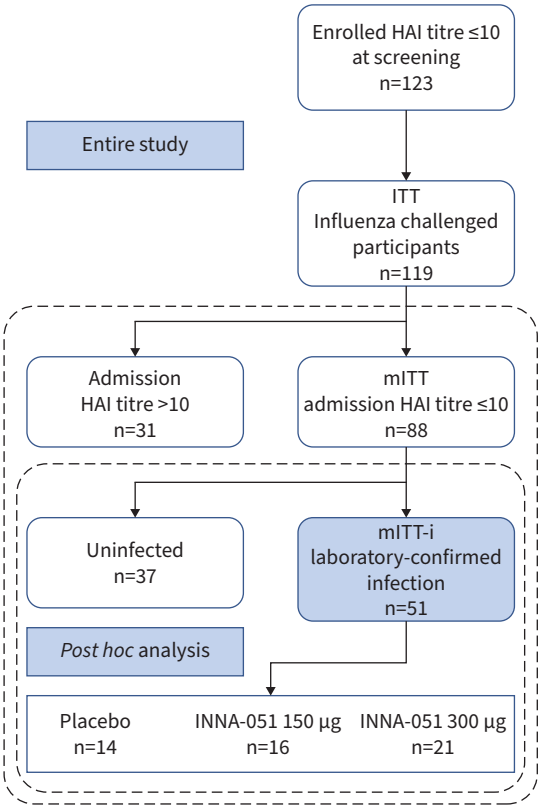

Enrolled HAI titre ≤10 at screening n=123
Entire study
ITT Influenza challenged participants n=119
Admission HAI titre >10 n=31
mITT admission HAI titre ≤10 n=88
Uninfected n=37
mITT-i laboratory-confirmed infection n=51
Post hoc analysis
Placebo n=14
INNA-051 150 µg n=16
INNA-051 300 µg n=21

FIGURE 1 CONSORT diagrams. a) Healthy volunteer dose escalation study. Single ascending dose (SAD) and multiple ascending dose (MAD) pooled, with active or placebo as allocated interventions. b) Healthy volunteer influenza challenge study of infected modified intent-to-treat (mITT) and modified intent-to-treat – laboratory-confirmed infection (mITT-i) population included in *post hoc* analysis (full study population in the online supplementary material). HAI: haemagglutinin inhibition.

FIH study: safety assessments

Treatment-emergent AEs (TEAEs) consisted predominantly of those localised to the nasopharynx, including nasal congestion, nasal discomfort, nasal inflammation and rhinorrhoea, all of which were mild in severity (SAD 90.1%, MAD 96.3%) and self-limiting (supplementary table S3). There was a trend towards a higher incidence of these TEAEs in the INNA-051 groups relative to placebo.

Descriptive analysis of VAS found that nasal blockage and rhinorrhoea trended higher in the INNA-051-treated MAD cohorts relative to placebo for the first dose administered (supplementary figure S2). Notably, for the subsequent doses the peak mean VAS scores for nasal blockage and rhinorrhoea were considerably lower than for the first dose. There were no numerical increases in VAS scores for any of the symptoms with subsequent dosing.

There was no dose-related trend in the severity of TEAEs reported nor a cumulative effect on the intensity of nasal symptoms with repeated dosing. There were no severe TEAEs reported in the study. Full details on safety and tolerability outcomes assessed are provided in the online supplementary material.

Influenza challenge study: safety assessments

Overall, the INNA-051 two-dose regimen and subsequent challenge virus administration with influenza A/Perth/16/2009 (H3N2) were considered safe and well tolerated.

The proportion of participants with any TEAEs was 49% in the placebo arm and 80.5% in both INNA-051 arms. The majority of TEAE events (70%) were considered related to the study treatment (39.5% in placebo, 76.5% in the INNA-051 150 µg group and 74.5% in the INNA-051 300 µg group).

The most frequently reported TEAEs in the placebo, INNA-051 150 µg, and INNA-051 300 µg arms included nasal congestion, rhinorrhoea, headache, sneezing and oropharyngeal pain and were more common in the INNA-051 arms relative to placebo. Most of these TEAEs were generally mild and self-limited, with events moderate in severity being infrequent among the INNA-051 recipients (supplementary table S4). Further details on TEAEs recorded can be found in the online supplementary material.

Clinical laboratory safety results generally remained stable over time, were comparable between the treatment arms and no trends in changes in laboratory results were identified. There were no notable changes over time or differences between the treatment arms for physical examination, vital signs and concomitant medications.

There were no serious AEs reported in either study.

FIH study: nasal cytokine assessments

The concentrations of macrophage chemoattractants MCP-1 and MIP-1α, identified in non-clinical pharmacology studies as mechanistic PD biomarkers for INNA-051 treatment [38], were assayed in nasosorption samples collected before and after INNA-051 administration in trial participants (figure 2a,d). Reflecting the key role played by macrophages in the mechanism of action of INNA-051 in preclinical mechanistic studies, MCP-1 and MIP-1α were identified in these studies as indicators for the biological response induced by INNA-051 treatment [36].

Significantly increased concentrations of MCP-1 and MIP-1α were observed in most SAD cohorts (figure 2b,c) peaking at 6–24 h post-dose and in MAD cohorts 1–3 compared to placebo (figure 2e,f) after each of the four treatments of INNA-051, with no apparent dose–response to treatment observed. MCP-1 and MIP-1α levels remained elevated during the treatment period when compared to placebo and to baseline levels. Measurements taken on study day 18 (8 days after final dose) showed a return to baseline levels for these two biomarkers (figure 2e,f).

Nasal concentrations of IFN-α2a were below the LLOQ (34.86 $pg \cdot mL^{-1}$) in all samples (data not shown), confirming non-clinical pharmacology studies demonstrating that INNA-051 does not directly stimulate the release of type I interferons.

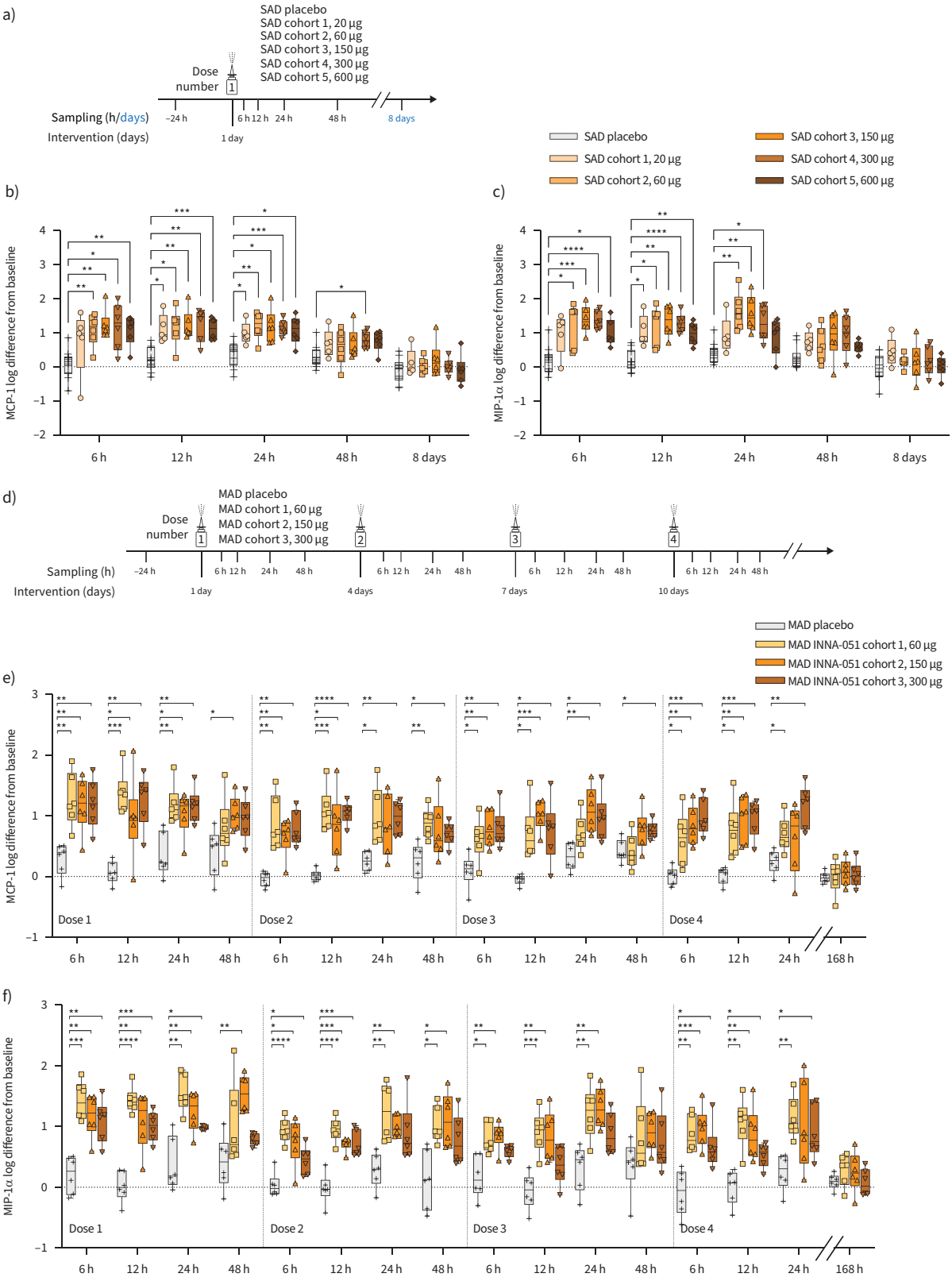


FIGURE 2 Nasal monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α after treatment with INNA-051 or placebo in single ascending dose (SAD) and multiple ascending dose (MAD) cohorts 1–3. a–c) Participants in SAD study received a single dose of either 20, 60, 150, 300 or 600 µg of INNA-051 (n=6/cohort) or placebo (pooled placebos n=10). d–f) Participants in MAD study received 60, 150 or 300 µg of INNA-051 (n=6/cohort) or placebo (pooled placebos n=6) four treatment doses spaced 3 days apart. Nasal samples taken using

Nasosorption FX-I devices at 6, 12, 24 and 48 h and 8 days post-final dose were analysed by MSD ELISA for nasal secretions of SAD cohorts b) MCP-1 and c) MIP-1α, or MAD cohorts e) MCP-1 and f) MIP-1α. Statistical analysis was performed on log10 transformed and baseline subtracted data by mixed-effects model (restricted maximum likelihood) with Dunnett's correction for comparisons to the placebo. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

There was no evidence of an increased systemic release of storm cytokines (IL-6, IFN-γ, TNF-α or IL-1β) after single or multiple doses of INNA-051 (data not shown) nor of the anti-inflammatory cytokine IL-10. This finding is consistent with the inability to detect INNA-051 in plasma (levels below LLOQ 0.1 $ng \cdot mL^{-1}$), suggesting a lack of systemic drug exposure. Small and transient changes from baseline of MIP-1α and MCP-1 were observed in various SAD cohorts and for MAD cohort 3 in relation to MIP-1α (supplementary figure S3).

FIH study: immune gene expression analysis from nasal samples

Changes to the expression of immune response associated genes across study arms were analysed in nasosorption samples collected at baseline, 8 h post-treatment and for MAD cohorts 48 h after treatments 1–3 using the 730-gene NanoString Human Pan Cancer Immunology panel (figure 3a).

In the SAD study, principal component analysis (PCA) revealed separation of baseline from 8 h post-administration, with samples from INNA-051-treated participants clustering distinctly from baseline and placebo samples. Gene expression from each of the 8-h post-dose differences clustered together, indicating similarity in the pattern of gene expression after each dose (figure 3b; MAD 3 shown).

Administration of INNA-051 was associated with increased expression of immune genes from pretreatment to all four 8-h time points (Day 1, Day 4, Day 7 and Day 10) in the MAD cohorts, with increases in key innate immune response genes that mediate pathogen sensing and host defences that include inflammasome molecules *NLRP3*, *CASP1* and *IL1B*, TLR pathway genes *TLR2* and *TLR4*, *MYD88*, *NFKB1* and *IRF7*, the genes encoding RIG-I (*RIGI*) and MDA5 (*IFIH1*), chemokines *CCL2*, *CCL3*, *CCL20* and adhesion molecules *ITGAL*, *CEACAM1* and *ICAM1*. ∼60% of the genes that were highly expressed at post-dose time points were consistently found after each of the four doses (153 genes differentially expressed after all doses, out of 255 upregulated in total). Pathway scoring showed increased innate immune functions, including pathogen defence, TLR signalling, macrophage, natural killer (NK) and chemokines and cytokines (figure 3c), maintained at a consistent level following repeat doses. Placebo treatment had no effect on these pathways at any of the timepoints (data not shown). Additionally, INNA-051 administration increased the inferred abundance of $CD45^+$-expressing immune cells (figure 3d). The presence of $CD45^+$ immune cells was inferred from *PTPRC* mRNA abundance. Danaher *et al.* [49] acknowledged that CD45 is expressed by a number of immune cell types and so it is a generic marker of immune cell abundance in a sample. Further cell typing using cell-specific gene co-expression patterns demonstrated a significant increase in the abundance of innate immune cells, including macrophages, cytotoxic cells and neutrophils (supplementary figure S4). The pattern of change in immune pathway gene expression and cell abundance scores in the MAD 2 (150 µg) compared with the MAD 3 cohorts (300 µg) demonstrates dose–response.

Target engagement by INNA-051 was demonstrated by increased expression of genes in the TLR signalling KEGG pathway [50]. The majority of TLRs and downstream signal transduction genes included in the NanoString panel showed increased expression in all MAD cohorts. The greatest enrichment of TLR pathway gene expression enhancement was observed in the MAD 3 cohort (figure 4).

Influenza challenge study: pharmacodynamics and efficacy assessments

The incidence of infection measured by two quantifiable quantitative real-time PCR samples on 2 consecutive days was lower than anticipated. Laboratory-confirmed infections were 18 out of 40 (45%) in the placebo group, 20 out of 38 (52.6%) for the 150 µg group and 24 out of 41 (58.5%) for the 300 µg group (intent-to-treat (ITT), figure 1b). In addition, 31 (26%) of the intent-to-treat infected (ITT-i) participants across all groups were subsequently found to have circulating antibodies (HAI titre >10) against the challenge virus at Day −5 (clinic admission), despite being found to have HAI titre ≤ 10 during the screening process, including eight participants in the placebo group, 14 participants in the 150 µg group and nine participants in the 300 µg group (table 1). In both ITT and ITT-i groups, no significant treatment differences were observed for any of the primary and key secondary clinical end-points assessed relative to placebo.

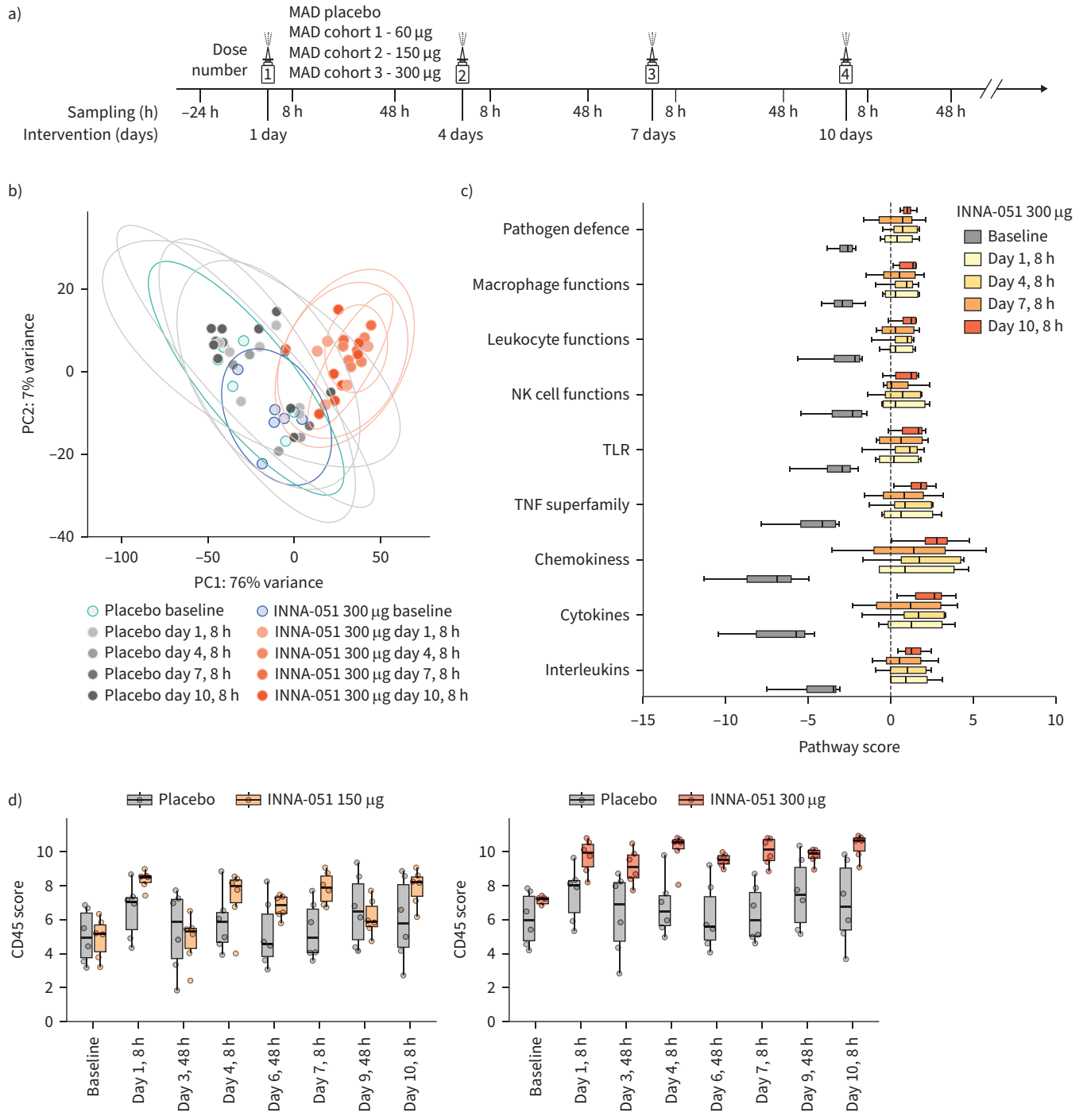


FIGURE 3 Increased immune gene expression in nasal samples after each of four doses of INNA-051. a) Participants in INNA-051 300 µg multiple ascending dose (MAD) 3 cohort (n=6) and placebo (n=6) received four treatment doses spaced 3 days apart. Nasal samples were taken at baseline and at 8 and 48 h after each dose using Nasosorption FX-I devices. Immune gene expression analysis was performed using the NanoString Counter Human Pan Cancer Immune Profiling panel. b) Principal component analysis (PCA) of NanoString immune gene expression data from pre- and 8 h post-treatment placebo and INNA-051 MAD 300 µg cohorts. First two principal components of each sample with ellipses for each group are plotted. c) Pathway scores representing the first principal component of genes within immune pathways (x-axis) were calculated using the NanoString Pathway Module. d) Immune cell profiling using cell-specific gene co-expression patterns in MAD 2 (150 µg) and MAD 3 (300 µg) cohorts, showing inferred abundance of $CD45^+$ expressing-immune cells. NK: natural killer; TLR: Toll-like receptor; TNF: tumour necrosis factor.

Subsequent results presented are based on *post hoc* analysis in a subset of the population designated as the mITT-i (modified ITT – infected) population (figure 1b). As pre-existing immunity against the challenge viral strain is known to impact the rate, duration and severity of infection [51], only participants with HAI

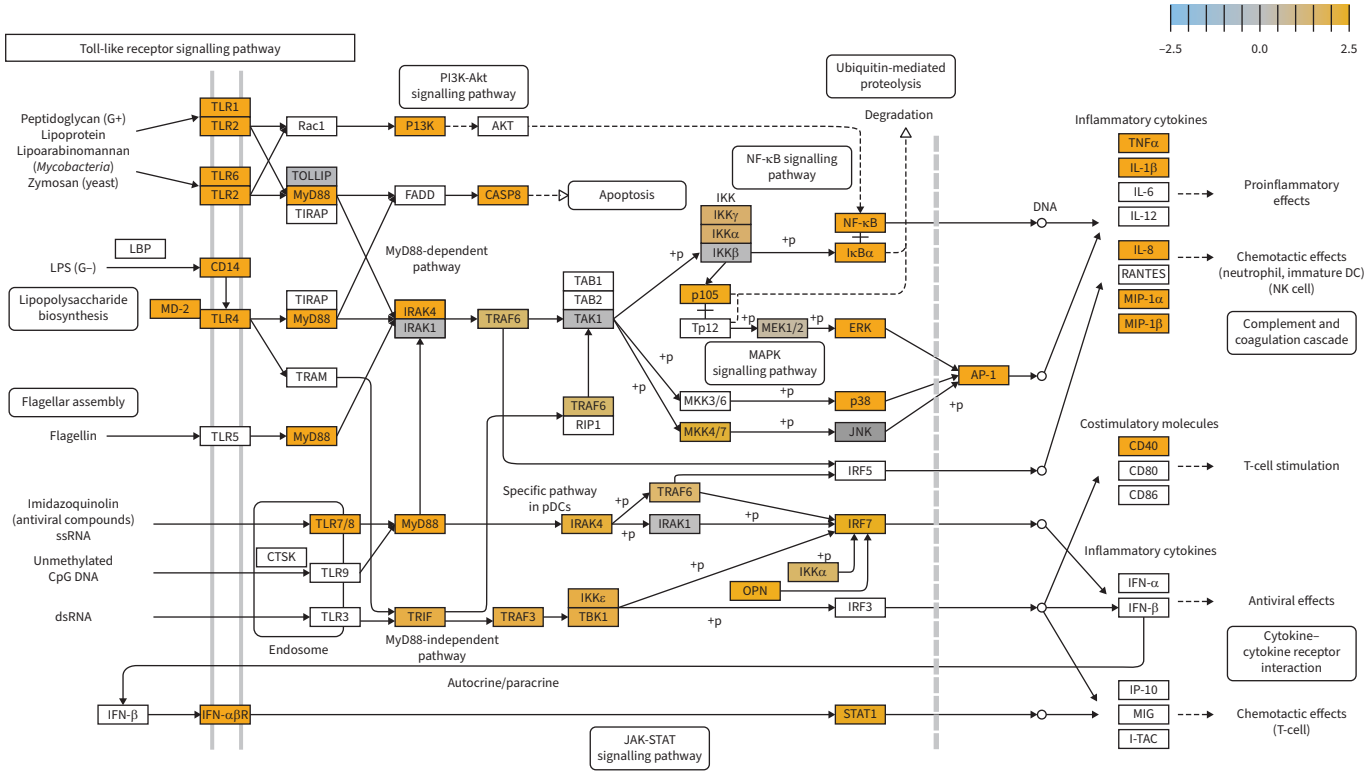


FIGURE 4 Genes from the Toll-like receptor signalling pathway remain differentially expressed 8 h post 4th dose in MAD cohorts. A KEGG (Kyoto encyclopaedia of genes and genomes) diagram is a computerised representation of a biological pathway and its components. Genes and gene families within the plot are represented within the Pan Cancer Immune Profile Panel shown in colour or are in grey. Genes and gene families known to be involved in the pathway but not represented within the panel are shown in white. Genes and gene families that are overexpressed in the KEGG pathway are shown in shades of orange (log2 fold-differences 0 to 2.5). Representative figure for MAD cohort 3 (300 mg dose) D-10 (8 h post-dose). Data on KEGG graph rendered in Pathview.

antibody titres $\leq$ 10 against the challenge virus at quarantine (Day −5) and laboratory-confirmed infection by PCR were included in the mITT-i population (table 1).

Duration of infection was measured as quantifiable quantitative real-time PCR viral RNA detected in mITT-i participants (figure 5b). The duration of infection was ~26 h shorter for the 300 µg group (mean duration of 106.1 h) when compared to placebo (mean duration of 132.1 h, adjusted p=0.0173). Viral load (in $\log_{10}$ copies·mL^{-1} by quantitative real-time PCR) was significantly lower for INNA-051 300 µg when compared to placebo from Day 6 post-challenge (figure 5c).

The estimated treatment differences (effect size) and the adjusted (accounting for multiple comparisons) confidence intervals and the associated p-values are presented in figure 5d. A significant difference was observed on Day 7, where INNA-051 300 µg mean value was lower than the placebo mean values (adjusted p-value=0.0465).

TABLE 1 Number of intent-to-treat (ITT) participants with haemagglutination inhibition (HAI) titre >10 at admission (day −5)

	ITT participants, n	ITT participants with HAI titre >10, n (%)
Placebo	40	8 (20)
INNA-051 150 µg	38	14 (37)
INNA-051 300 µg	41	9 (22)
Total	119	31 (26)

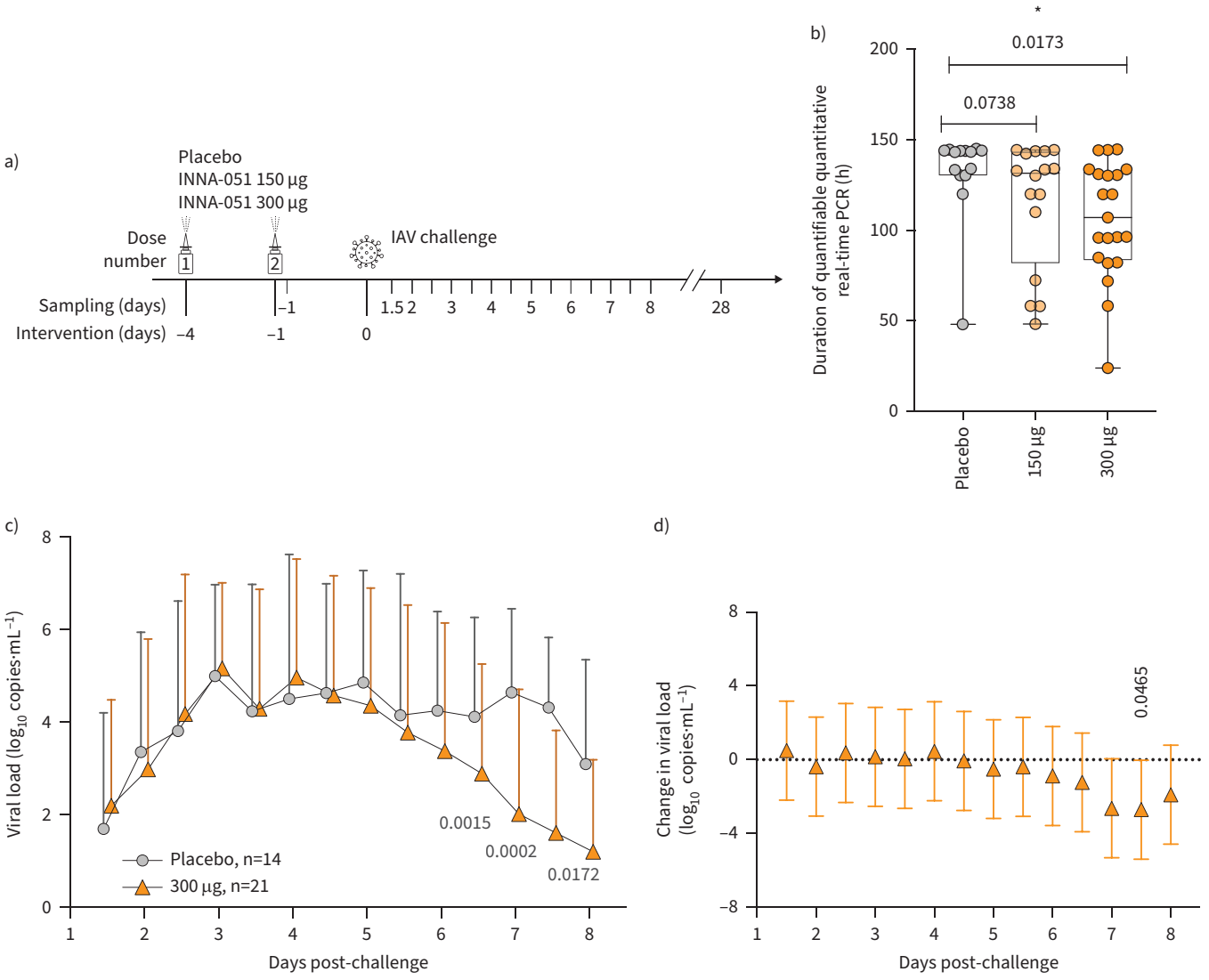


FIGURE 5 *Post hoc* analysis of influenza infection course in modified intent-to-treat – laboratory-confirmed infection (mITT-i) placebo and INNA-051 administration cohorts. a) Participants in influenza challenge study received two treatment doses of placebo (n=14), 150 µg (n=16) or 300 µg (n=21) of INNA-051 spaced 3 days apart then challenged with IAV 24 h after the second administration. Nasal samples were taken prior to challenge twice daily after day 1 post-challenge, to day 8 post-challenge using nasal swabs for measurement of viral RNA. b) Total duration of quantifiable viral RNA detection by quantitative real-time PCR in mITT-i population. c) Least squares mean viral RNA quantity for placebo and 300 µg INNA-051 mITT-i administration cohorts shown over time. Statistically significant differences ($p<0.05$) between cohorts as determined by the restricted maximum likelihood (REML) method and the Fisher's unadjusted least significant difference test are indicated. Error bars represent standard deviation. d) As a sensitivity analysis, the estimated difference in least squares mean viral RNA quantity in the INNA-051 300 µg mITT-i cohort compared to placebo mITT-i cohort was determined with p-values adjusted by the Dunnett–Hsu method to account for multiple comparisons (indicated where $p<0.05$). Error bars represent 95% CI. b) INNA-051 150 µg and 300 µg mITT-i administration cohorts were compared to placebo by Wilcoxon rank-sum test, with the Benjamini–Hochberg procedure used to adjust for multiple comparisons. Adjusted p-values are shown.

While not statistically significant, there was a dose-related trend towards a reduced duration of influenza symptoms for participants who had at least a total symptom score of ⩾2 on any day, when comparing the INNA-051 300 µg and 150 µg dose groups to placebo (supplementary figure S5).

Immune gene expression in the influenza challenge study

Similar to the FIH study, we observed changes to the expression of immune genes induced by INNA-051 administration in nasosorption samples. Prior to influenza challenge, treatment with 300 µg of INNA-051 increased expression of 371 out of 730 genes (50.8%) tested compared to the placebo cohort (194 genes (26.6%) in the 150 µg dose), including cytokine/chemokine and signal transduction genes which were also

observed in the FIH study (*e.g. CCL2*, *CCL3*, *CCL20*, *CXCL1*, *IL6*). Of the 371 genes, 54 were members of the KEGG influenza host–response pathway (out of 81 genes from this pathway included in the panel). 24 of these genes were also upregulated in the 150 µg group (figure 6). Gene transcription from multiple facets of the host response to influenza [12] was amplified in the INNA-051 300 µg cohort, including members of the TLR/NF-κB pathways (*TLR1/2/4/6/8/10*, *NFKBIA*, *NFKB2*, *RELA*, *IRF4/7*, *MYD88*),

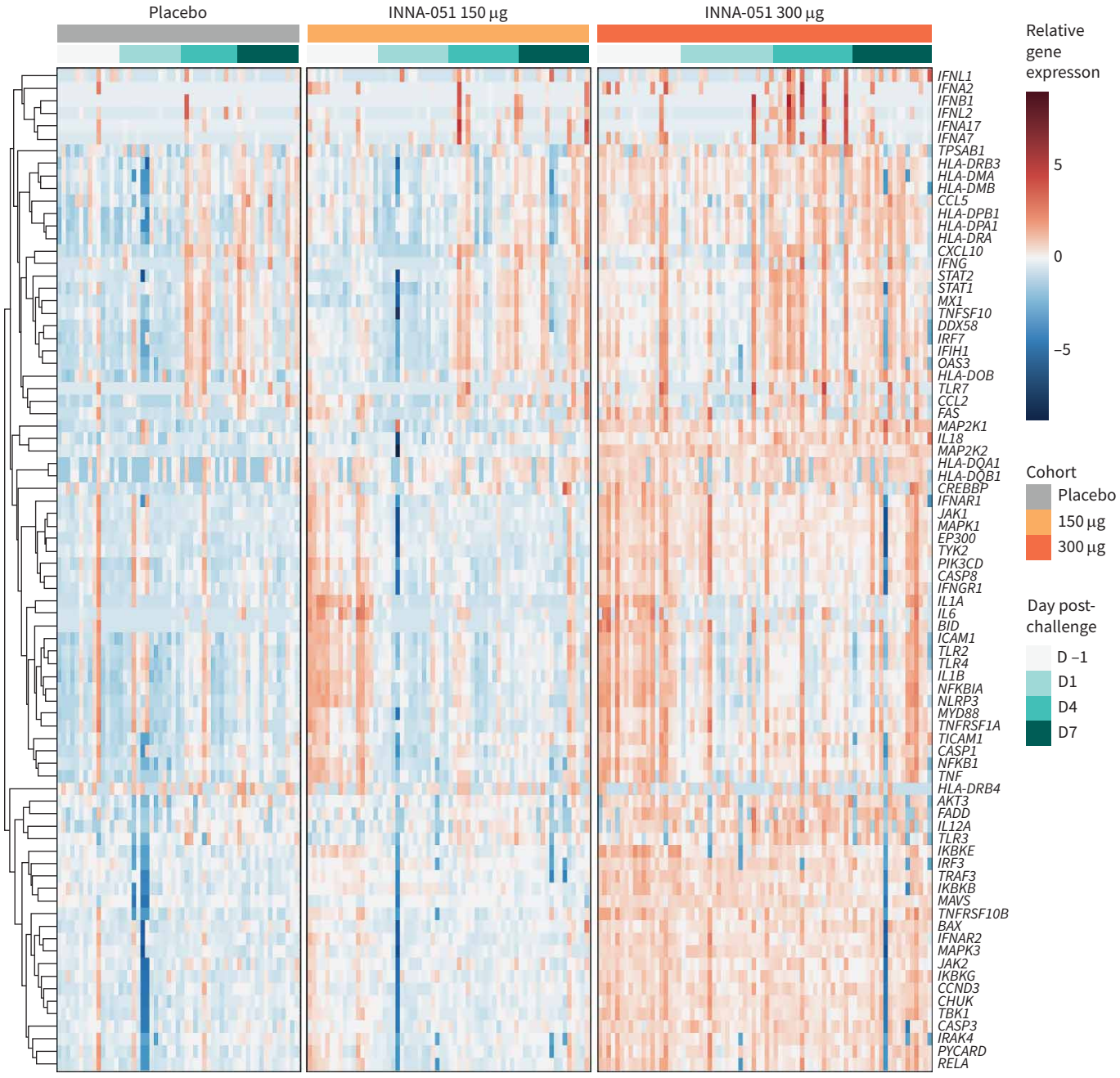


FIGURE 6 Heatmap of influenza response gene expression in nasal samples of modified intent-to-treat – laboratory-confirmed infection (mITT-i) participants from influenza challenge study. Nasosorption samples from mITT-i placebo (n=14), 150 µg (n=16) and 300 µg (n=21) INNA-051 treated participants were analysed for immune gene expression using the panCancer Immune NanoString panel on Day 1 prior to challenge (Day −1; after dose 2), and Days 1, 4 and 7 post-challenge. Genes in heatmap are those in the panel that are present in influenza KEGG pathway hsa05164, with the addition of *TLR2*, *IFNL1* and *IFNL2*. Red–blue colour gradient represents Z-score across study participants of the normalised mRNA log count for each gene, with red indicating higher relative expression and blue lower relative expression. Genes are arranged by hierarchical clustering.

RIG-I (*RIGI/DDX58*, *IFIH1*), inflammasome (*NLRP3*, *IL1B*), JAK/STAT (*MX1*, *OAS3*, *STAT1/3*, *IFITM1/2*, *ISG15*) and MAPK (*MAPK1*, *MAPKAPK2*) pathways. There was a smaller magnitude of change in gene expression in response in the 150 µg group compared to the 300 µg group, with all but one of the genes in this cohort also being differentially expressed in the higher dose cohort.

At day 1 post-challenge, 311 genes, out of 730 genes (42.6%) tested, were significantly highly expressed in the INNA-051 300 µg compared to placebo, of which 43 were present in the influenza KEGG pathway. By this time, the early chemokine transcriptional response to INNA-051 was declining (figure 7c). The most highly expressed genes included IL-1R pathway kinase genes *IRAK1*, *MAPK8* (JNK) and mitochondrial antiviral signalling protein *MAVS*. Influenza pathway genes *MX1* and *OAS3* remained elevated at 1 day post-infection, with *IFITM1/2* and *ISG15* also showing an increased trend over time when compared to the placebo-infected group (figure 7b). Post-infection, the transcriptional changes in the 150 µg group compared to placebo were minimal, with only three genes (*MFGE8*, *PVR* and *IKBKB*) more highly expressed in the treated group; all three were also in the top 30 upregulated genes observed in the 300 µg group.

By Day 4, NOD1 and type I and III IFN encoding genes were more highly expressed in the 300 µg INNA-051 cohort. No changes to the expression of genes encoding type I or III interferons were observed prior to influenza infection (figure 7a). However, at Day 4 post-challenge, *IFNA7* and *IFNL2* were more highly expressed in the INNA-051 300 µg treated group compared to placebo (Log2 fold-change >1 and FDR=0.0069, 0.048 respectively; figure 7a). *IFNB1*, *IFNA2* and *IFNL1* were also significantly increased in expression (FDR <0.05) to a smaller degree (Log2 fold-change=0.84, 0.64 and 0.91 respectively). No significant differences compared to placebo were observed in the 150 µg cohort at Days 4 and 7, while a small group of 21 genes were elevated at Day 7 in the 300 µg cohort.

Discussion

Based on preclinical data [33, 36, 38, 39], we tested the hypothesis that intranasal INNA-051 would be well tolerated in humans at doses that would locally activate innate immunity pathways and would impact the course of infection in a human viral challenge model. In both studies, intranasal INNA-051 was well tolerated, with only mild, self-limiting nasal TEAEs likely related to tissue-localised innate immune responses. Despite the use of a large viral inoculum in the human influenza challenge study, intranasal administration of INNA-051 did not enhance viral replication, nor was it associated with unexpected treatment-related AEs or exacerbated symptoms caused by experimental viral infection.

Gene expression analysis in the FIH and the human influenza challenge study demonstrated local TLR2/6 mediated activation of innate immunity pathways (figures 3, 4 and 7). INNA-051 treatment upregulated expression of TLR signal transduction genes, increased the abundance of $CD45^+$ immune cell scores, including macrophages, cytotoxic cells and neutrophils, and increased expression of genes involved in pathogen defence, macrophage and leukocyte functions, and other innate immune pathways in nasal samples. The induction of MIP-1α and MCP-1 in response to treatment is consistent with the murine mechanism of action studies, which demonstrated that intranasal administration of INNA-051 or close analogues was associated with infiltration of macrophages into the nasal epithelium which is believed to play a key role in influenza virus clearance [38]. It is also consistent with the presence of macrophages that are primed for virus sensing and also neutrophils in the upper airways of children, previously linked to an effective, early innate antiviral response to SARS-CoV-2 infection when compared to adults [7].

In the FIH study, INNA-051 treatment stimulated a local innate immune response as measured by MCP-1 and MIP-1α protein biomarkers but no dose–response relationship was observed. This is consistent with data observed in small animal studies with analogues of INNA-051 [38] and human studies with intranasally administered TLRs, such as the TLR7 agonist GSK2245035 [52].

Findings in both studies demonstrate that transcription of genes encoding multiple pathogen detection and antiviral effector proteins with relevance to influenza infection is heightened by INNA-051 300 µg treatment. Influenza virus infection is detected by epithelial and mucosal immune cells through the sensing of viral RNA by TLRs 3,7 and 8, and RIG-I and the sensing of cellular damage through NLRP3 [12]. Sensing of infection triggers cells to produce type I and III interferons [53–55], as well as chemokine release that leads to the recruitment of immune cells such as monocytes and neutrophils, and the deployment of counter-measures targeting different stages of the viral life cycle [12, 56]. These include the expression of proteins that restrict viral entry (IFITM proteins), genome transport (MxA), protein synthesis (PKR), genome integrity (OAS and RNaseL), protein function (ISG15) and virion release (viperin) [12, 53, 57–59]. In the influenza challenge, TLR2/6 agonist (INNA-051) dosing resulted in increased expression of genes in those pathogen-sensing pathways (*TLR*, *RIG-I* and *NLRP3*), as well as genes

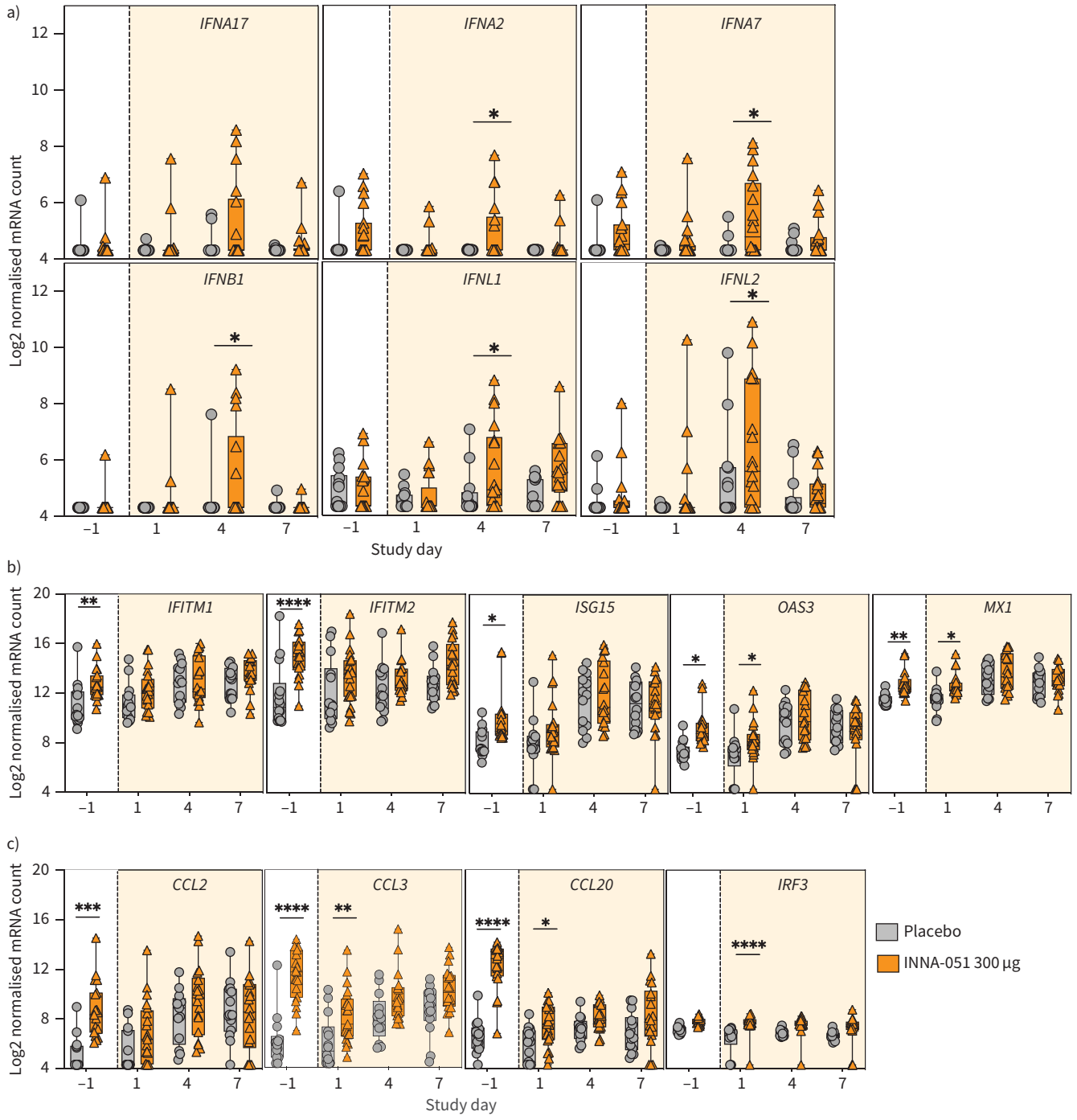


FIGURE 7 Antiviral gene induction by INNA-051 treatment and amplified type I and III interferon (IFN) in response to influenza infection in INNA-051 modified intent-to-treat – laboratory-confirmed infection (mITT-i cohort). a) Log2 normalised counts of type I and III IFN genes present in the panCancer Immune NanoString panel. b) Log2 normalised counts of antiviral genes *IFITM1*, *IFITM2*, *ISG15*, *OAS3* and *MX1*. c) Expression of chemokine genes *CCL2*, *CCL3*, *CCL20* and *IRF3*. Log2 normalised counts of indicated genes are represented for study participants from mITT-i placebo and INNA-051 300 µg groups at study day −1 (post-administration number 2, prior to challenge), and Days 1, 4 and 7 post-challenge (post-challenge time points have a shaded background). Placebo n=14; INNA-051 300 µg n=21. Asterisks indicate false discovery rates (FDR) <0.05: *: FDR <0.05; **: FDR <0.01; ***: FDR <0.001; ****: FDR <0.0001.

associated with chemokine expression and immune cell recruitment. Moreover, increased expression of genes encoding multiple inhibitors of viral replication such as *Mx1* (encoding MxA), *OAS3*, *IFITM1*, *IFITM2*, *ISG15* and members of the type I IFN signal transduction pathway was also observed.

While no evidence of interferon gene or protein expression was found in response to INNA-051 treatment alone, the synergistic effect of INNA-051 prophylaxis with influenza infection boosted transcription of *IFNA7* and *IFNL2* on Day 4 post-challenge when compared to infected individuals in the placebo cohort (figure 7a).

We hypothesise that increased expression of some interferon-stimulated genes (ISGs) after INNA-051 administration may be regulated independently of interferon receptor signalling, as observed in neomycin-treated nasal mucosa of mice lacking receptors for type I or III IFNs [60]. Supporting this hypothesis, preclinical prophylaxis studies with a close analogue of INNA-051 demonstrated no difference in protection in wild-type compared to interferon-α receptor knockout mice [36]. IFN-independent ISG regulation has also previously been observed in murine lungs and was attributed to activities of the transcription factor IRF7 [61, 62].

In the influenza challenge study, INNA-051 300 µg dose was associated with significantly accelerated viral clearance in laboratory-confirmed infected participants that had a HAI antibody titre ⩽10 to the challenge virus (H3N2 A/Perth/16/2009). INNA-051 did not prevent infection in this model confirming results observed following INNA-051 treatment in the *in vivo* ferret SARS-CoV-2 challenge model [33]. The accelerated clearance in humans is further supported by detailed molecular analysis of innate immune genes and genes encoding for known antiviral effector proteins in nasal samples taken from the mITT-i population in the influenza challenge study. Together with the reduced viral load findings, the biomarker signature induced in response to INNA-051 treatment and influenza challenge suggests an enhanced antiviral innate immune response. The observed impact on IFNs and PAMP sensing genes highlights a potential for emulating effects observed in SARS-CoV-2 and community infections clinical trials with topical nebulised IFN biologics [16, 17].

A key limitation of the influenza challenge study was the unexpected low rate of infection observed which necessitated a *post hoc* analysis of infected participants. Pre-existing immunity against the challenge viral strain used is known to impact the rate, duration and severity of infection, the study was designed to include only healthy adult participants that had low or no antibody titre against challenge strain haemagglutinin (HAI). By including uninfected participants based on the pre-specified analysis plan the study failed to achieve its original primary end-point.

Human challenge studies cannot determine the efficacy of treatment in preventing severe disease. Analysing the treatment effect on disease severity was limited by the mild symptoms score observed across all study cohorts, with no correlation observed between viral levels and total symptom scores. Naturally occurring infections with influenza A (H3N2) virus strains have been shown to induce more severe illness [63] compared to the mild symptom scores observed in this challenge study. Mild symptom scores were also previously reported by another human challenge study undertaken with the same A/Perth/16/2009 viral strain [64]. In healthy adults, symptoms of mild upper respiratory tract infection are primarily driven by the response to infection rather than by viral replication; whereas high viral loads, replication in the lower respiratory tract and the associated inflammatory responses are drivers of severe disease [54, 65–67]. It is not possible in a human challenge study to determine the efficacy of treatment in preventing severe disease; however, the reduced duration of infection and viral load at later time points in the INNA-051 treated cohort in this study are consistent with observations in animal challenge models in which INNA-051 reduced viral loads and replication in lungs [33, 38, 39].

In summary, treatment with INNA-051 demonstrated the potential of a TLR2/6 agonist to prime the innate immune response thereby boosting local pathways associated with virus-agnostic host defence responses. Additional studies to test the potential of TLR2/6 agonists in natural community infection settings are needed to fully assess their potential as seasonal prophylactic agents in individuals at risk due to age, occupation and/or comorbidities.

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Data availability: The registration process included the submission of the study protocol and study results.

The First in Human study (INNA-051-HVT-01) is registered at https://anzctr.org.au with identifier number ACTRN12621000607875 and the influenza challenge study (ERY-CSP-001/INNA-051-IAV) is registered at www.ClinicalTrials.gov with identifier NCT05255822.

Ethics statement: The First in Human study (INNA-051-HVT-01) was approved (2021–04–454) by Bellberry Human Research Ethics Committee. The influenza challenge study (INNA-051-IAV-HC-01) was approved by West Midlands – Egbaston Research Ethics Committee and the Medicines and Healthcare products Regulatory Agency. The studies were performed in accordance with the approved protocol, the guidelines of Good Clinical Practice (International Council for Harmonisation E6) and the ethical principles that have their origin in the Declaration of Helsinki (World Health Organization, 2013). All participants gave written informed consent prior to the performance of any study-related procedures.

Author contributions: S. White, C. Demaison, F.A. Mercuri, G. McLachlan, N. Kruger, R. Tal-Singer, B.E. Miller and C. Lemech conceptualised the project and designed the clinical protocols. C. Lemech and R. Hari recruited patients, collected clinical samples and acquired data for the First in Human study. N.P. West and F.A. Mercuri designed experimental protocols. P. Zhang and N.P. West performed experiments and conducted data analysis. S. White, H.A. McQuilten, S. Mynhardt and F.A. Mercuri conducted data analysis. S. White, N. Kruger, C. Lemech and F.A. Mercuri supervised the project. G. McLachlan and N. Kruger coordinated and supervised manufacturing of clinical material. S. White, C. Demaison, R. Tal-Singer, B.E. Miller, N.P. West, H.A. McQuilten and F.A. Mercuri wrote the manuscript. All authors reviewed and edited the manuscript and approved the final version.

Conflict of interest: F.A. Mercuri, C. Demaison and G. McLachlan are employees of ENA Respiratory Pty Ltd, and receive an annual salary and other benefits. In addition, they have shares and share options and are named inventors on numerous granted or pending patent applications controlled by ENA. H.A. McQuilten receives payments as a contract researcher from ENA Respiratory Pty Ltd. S. White, R. Tal-Singer, B.E. Miller and N. Kruger receive payments as paid consultants for ENA Respiratory Pty Ltd, and as part of their compensation have been awarded participation in the company share option plan. R. Tal-Singer is a retiree and shareholder of GSK, and reports personal fees from AstraZeneca, Roche, Vocalis Health, Teva, ImmunoMet, Renovion, Samay Health, GSK and ItayAndBeyond. S. Mynhardt, N.P. West, P. Zhang, C. Lemech and R. Hari receive payments to their institutions for carrying out research.

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