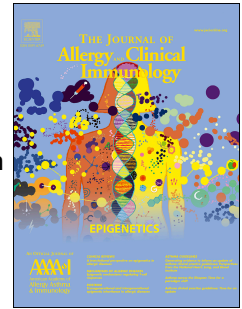


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Induction of innate cytokine responses by respiratory mucosal challenge with R848 in zebrafish, mice and humans

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Word count:1093

Capsule summary

We compared live zebrafish, mouse and human nasal challenge responses to the TLR7/8 agonist resiquimod (R848). We found remarkably similar induction of mediators in the three species, offering novel mucosal models of innate anti-viral immunity.

Keywords

mucosal innate immunity
nasal respiratory mucosa
resiquimod
vaccine adjuvants
comparative cross-species analysis

Abbreviations

ds	double-stranded
il1b	interleukin 1beta
ifn	interferon
poly(I:C)	polyinosinic:polycytidylic acid
R848	resiquimod
SAM	synthetic absorptive matrix
TLR	Toll-like receptor
tnfa	tumor-necrosis-factor alpha

To the editor

Development of new therapies and vaccines to combat respiratory viral infections is slow, partly due to limited understanding on innate immune responses at the respiratory mucosal site of disease. Detailed characterization of such responses may facilitate biomarker definition for respiratory diseases, provide novel mechanistic insights and a platform for the testing of novel therapeutics. Recently, non-invasive serial nasosorption of mucosal lining fluid has been used to study immune responses to experimental live human rhinovirus (1). However, human viral infection models require specialized centres and resources, some studies requiring quarantine of volunteers.

In vivo animal models of innate immune stimulation are useful alternatives, for example mammalian models of airway mucosal polyinosinic:polycytidylic acid (poly(I:C)) challenge (a viral double-stranded (ds) RNA mimetic) are well established and demonstrate the ability of these agents to induce proinflammatory cytokines by respiratory cells (2-4). Many non-infectious models of innate antiviral immunity have used resiquimod (R848; a TLR7/8 agonist, single-stranded (ss) RNA mimetic), which is closely related to imiquimod. R848 causes different vaccine-specific immune responses in minipigs when given intradermally or intranasally, while intranasal R848 had adjuvant activity in macaques (5). Studies using these models in mice, chimpanzees and ferrets have provided valuable insights into mechanisms of immunity to and pathogenesis of respiratory viral infections. However, they are not always practical to use, and they do not always accurately mimic human infection responses. Furthermore, the extent to which these models predict human vaccine efficacy is often unclear (6, 7).

The zebrafish (*Danio rerio*) is an attractive alternative vertebrate species, especially due to similarities with the human innate and adaptive immune system. Recently we have used

zebrafish gills to assess respiratory inflammation and our results suggest zebrafish are a relevant model to study mechanisms of respiratory mucosal innate immune responses (8). We therefore developed parallel live zebrafish, mouse and human challenge models to study the effects of viral RNA-mimic TLR agonists with relevance to respiratory viral infection. These comparative studies allow assessment of cytokine responses at comparable and accessible sites of the respiratory mucosa (Fig. 1A-C).

Human nasal samples were collected serially by nasosorption (using a synthetic absorptive matrix, SAM) following saline and TLR agonist nasal challenge of 9 volunteers (see Supplementary Table 1 for baseline characteristics of participants and Supplementary Table 2 for nasal and systemic observations and clinical symptoms after R848 administration). The mouse nasal cavity is inaccessible for repetitive sampling so we developed a mucosal tissue sampling technique *ex vivo* by applying an absorption approach similar to that used for humans. Zebrafish whole gill tissue was harvested at several similar time points after R848 challenge. Figure 1 shows how remarkably similar cytokine responses were across the three species, especially between zebrafish and human (see Fig. S1 for detailed responses of individual human subjects). An early response was observed for tumor-necrosis-factor alpha (TNF- α), while interferon gamma (IFN- γ) increased later. These results suggest that R848 can be administered to humans as a non-infectious viral-type challenge of the innate immune system, while complementary studies in mice and zebrafish could allow mechanistic insight.

When poly(I:C) was applied, neither the fish gill nor human nose responded (Fig. S2). In contrast, the mouse nasal mucosal response to poly(I:C) was characterized by an early increase of *Tnfa*, *Il6* and *Ifna2* transcript levels and a later increase in *Ifng*. Overall, the R848 and

poly(I:C) challenges demonstrated both matching, and discrepant innate antiviral responses, in the different models.

To further refine the use of zebrafish gills as a model to study viral mimetics, we also established a non-invasive sampling technique using SAM to allow for repetitive sampling and thereby longitudinal studies of individual fish, which also contributes to the 3Rs through refinement and reduction of animal procedures. *Tnfa*, *il1b*, *ifnphi1* and *ifng1.1*, transcripts were successfully detected using this method and significantly increased in gills stimulated by R848 (Fig. S3A-D). Making use of transgenic zebrafish with labelled immune cells, we examined both neutrophil (*Tg(lyz:GFP)*) and lymphocyte (*Tg(lck:eGFP)*) distribution within the gill tissue and found a significant early transient increase in neutrophils (Fig. 2A-B) followed by an increase in lymphocytes (Fig. 2C-D) in the lamella following R848 stimulation. Such cells, but not vascular epithelial cells, were also harvested using SAM (Fig. S3E-I). These data highlight the number of useful features of the zebrafish respiratory mucosal model that permit investigation of mechanistic immune pathways for assessing topical drug effects, viral infections and vaccine adjuvant activity.

Animal models are central to our understanding of innate anti-viral immunity. However, the translation of these studies to human disease can be limited. This can result in the need for primate models of disease which are ethically, financially and logistically challenging. Here, we establish parallel methods for the administration of TLR ligands directly onto the respiratory mucosa in three species, with measurement of local inflammation using simple and reproducible sampling methods. The development of a human nasal mucosal model is of special interest since the nose is the portal for viral respiratory infections, that cause widespread winter morbidity and

mortality, and there are advantages in studying a complex multicellular mucosal system directly in man.

Using these approaches we demonstrate remarkably analogous IFN and inflammatory cytokine production following R848 stimulation in the human and mouse nasal mucosa and zebrafish gill tissue, which was evident despite the functional inactivity of mouse TLR8 (9). By contrast humans and zebrafish did not respond to poly(I:C), demonstrating that this common mouse model of viral innate immune activation may have limited translation to the human mucosal response to dsRNA in viruses. This study demonstrates that respiratory challenge with R848 may offer a novel mucosal model of anti-viral immunity in humans. This human challenge model may be particularly suited to understanding differences in innate anti-viral responses in allergic and respiratory diseases such as asthma and COPD, where viral infections are major exacerbation triggers. As such, mechanistic studies employing poly(I:C) challenge of mice may lack direct translation to humans. Instead, the R848 model can confidently be extended to mice and zebrafish, where the analogous response to R848 allows more detailed mechanistic insights, relatable to those seen in humans. Overall, these novel parallel *in vivo* mucosal models offer a platform for translational studies and trials of novel anti-viral therapies, vaccines and adjuvants.

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Author contributions

FP, AJ, MW, RST, SM designed and performed experiments. FP, AJ, LB, RST wrote the initial draft of the manuscript. RJS, CJ, PJO, LB, TTH, MJD edited the manuscript. LB, TTH, MJD conceived the study.

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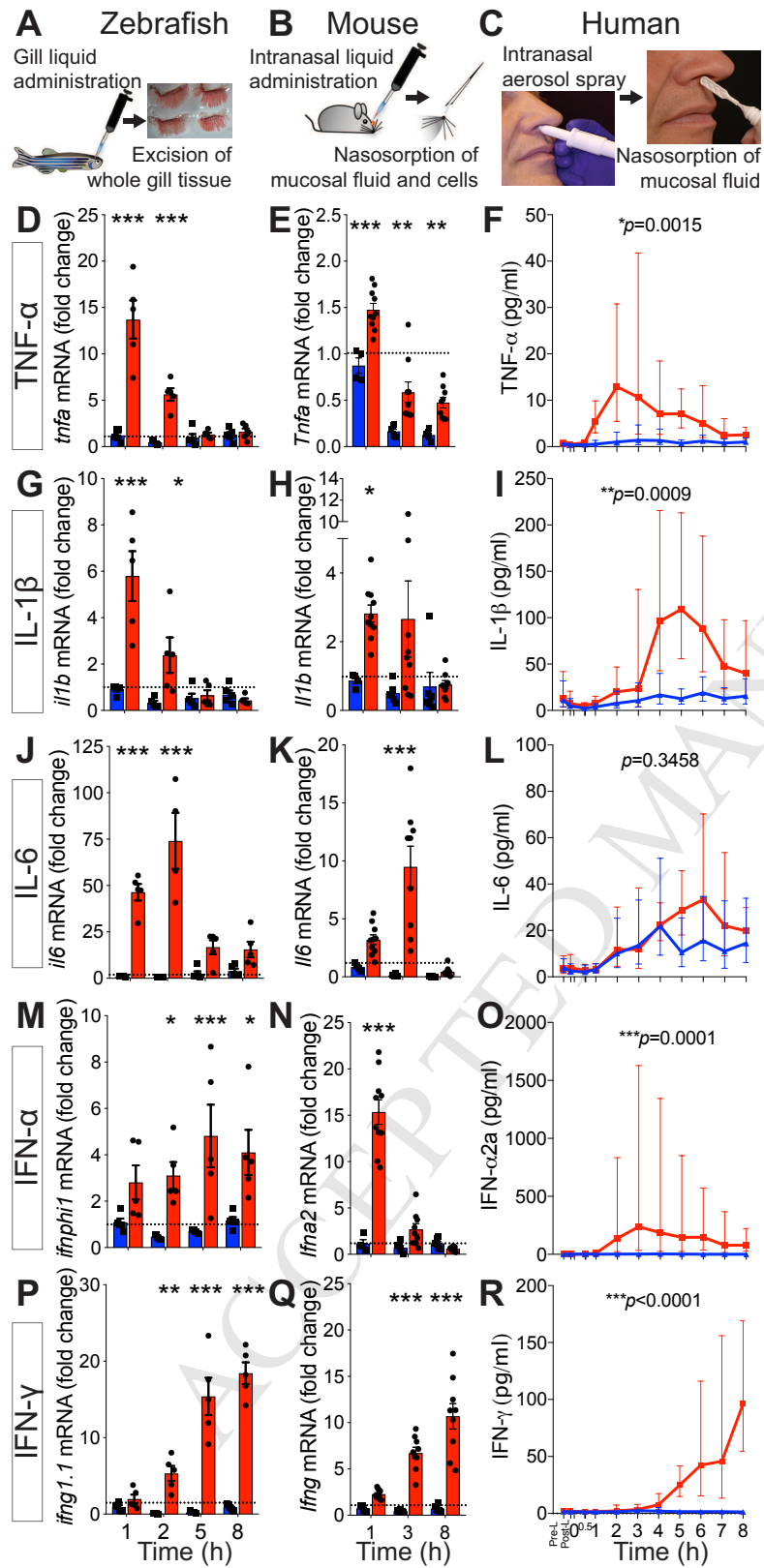
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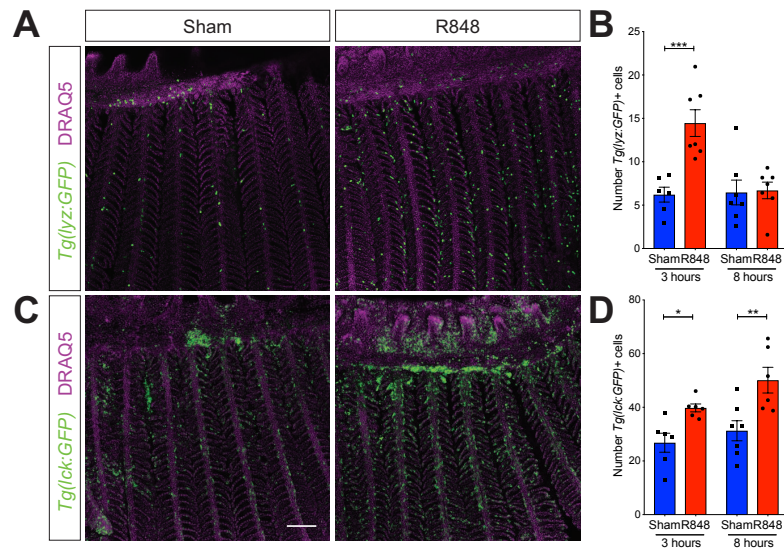
Figure 1: Kinetic profile of mucosal pro-inflammatory cytokine responses after zebrafish gill, mouse and human nasal stimulation with R848.

(A - C) Schematics showing mucosal administration of TLR agonists and sampling of mucosal tissue/ fluids to assess responses. (D, G, J, M, P) qRT-PCR analysis of zebrafish gill (n=5, representative of 3 experiments). Mean $\pm$ SEM. Two-way ANOVA followed by Sidak's multiple comparison test. (E, H, K, N, Q) qRT-PCR analysis of mouse nasal mucosa (n=4-10 pooled from two independent experiments). Mean $\pm$ SEM. Two-way ANOVA followed by Sidak's multiple comparison test. (F, I, L, O, R) Soluble protein mediator analysis of human nasal samples (n=9). Geometric mean and 95% CI. Paired t-test on $\log_{10}$ transformed AUC values.

Figure 2: R848 induces lymphocyte migration in zebrafish gills.

(A&C) Maximum z-stack projections of (A) *Tg(lyz:GFP; neutrophils)* and (B) *Tg(lck:GFP; lymphocytes)* gills following treatment with water or R848 for (A) 3 hours and (C) 8 hours. N=7. Scale bars=100 μ m. (B&D) Average number of GFP+ cells in the first 20 lamellae of each filament, each dot=average counts per individual fish (n $\geq$ 6). Mean $\pm$ SEM. Two-way ANOVA followed by Sidak's multiple comparison test.





Human studies

Participants

This human study was approved by the London-Harrow Research Ethics Committee [clinicaltrials.gov identifier NCT02090374]. Healthy participants were enlisted from a database of previous volunteers as well as recruitment via newspaper adverts. Subjects were aged between 18-60 years and were current non-smokers with a history of <5 pack years. Participants had no history of asthma, respiratory illness or any other major systemic disease and were required to be off nasal, inhaled and systemic steroids. Participants with allergic rhinitis were permitted. Written, informed consent was obtained from all volunteers prior to enrolment.

Study design

The study consisted of two nasal challenges: initially with saline, followed 1 to 4 weeks later by a TLR agonist (either R848 or poly(I:C)). On the day of the challenge visit, nasal mucosal lining fluid was sampled at baseline using nasosorption (Nasosorption FX-i, Hunt Developments, Midhurst, West Sussex, UK) for a duration of one minute before and after nasal lavage as described previously (10). After nasal challenge, nasosorption was performed at 30 minutes and then at hourly intervals for up to 8 hours in all subjects. Clinical parameters of nasal obstruction were assessed using total nasal symptom scores (TNSS) and peak nasal inspiratory flow (PNIF) as described elsewhere (11).

Nasal challenge with TLR agonists

TLR agonists utilized in the study included, high molecular weight poly(I:C) (InvivoGen) and VacciGrade R848 (InvivoGen). Saline and TLR agonists were administered using a Bidose nasal delivery device (Thermo-Fisher, Epsom, UK), which emits 100 µl per actuation. Poly(I:C) has been previously tolerated in another study at doses of up to 800 µg per nostril over a 24-hour period with no adverse events reported (12). Therefore, poly(I:C) was given at a dose of 500 µg per nostril in five 100 µl instalments using the Bidose over a one-hour period. R848 was delivered at a dose of 10 µg per nostril utilising one actuation of the Bidose device

Processing of samples

Mucosal lining fluid obtained from volunteers was eluted as described previously (10) and stored in assay buffer at -80°C as well as culture supernatant from PBMC experiments. These were thawed out prior to assay of chemokines and cytokines. This was performed using the MSD platform (Mesoscale Diagnostics, MSD, Gaithersburg, Md, USA) using Proinflammatory Panel 1 (IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α) and an IFNα2a kit.

Mouse studies

Mice

C57BL/6 mice were purchased from Charles River or Harlan Laboratories, Inc. All mice were maintained in pathogen-free conditions, and gender and age-matched mice aged 8-10 weeks were used for each experiment. All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) within Imperial College London and approved by the UK Home Office in accordance with the Animals (Scientific procedures) Act 1986 and the ARRIVE guidelines.

Intranasal stimulation with TLR agonists, mouse nasosorption and qRT-PCR

For exposure to TLR ligands, mice were lightly anaesthetised and instilled intranasally with poly(I:C) HMW or resiquimod (R848: Vaccigrade), both from Invivogen, at 2.5 µg per g of mouse weight in 100 µl. Mice were sacrificed at 1, 3 and 8 hours post-exposure by intraperitoneal administration of pentobarbital. To gain access to the nasal cavity, the snout and cartilage were removed using scissors. To sample the mucosa a small piece of SAM was inserted into both nostrils using fine tweezers. RNA from the SAM was extracted using Trizol and the PureLink™ RNA Micro Kit (*Life Technologies*) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA and qRT-PCR was carried out as described below. The following Taqman primer and probes were used: *Tnfa* (Mm00443260_g1), *Il1b* (Mm00434228_m1), *Il6* (Mm_00446190_m1), *Ifng* (Mm00801778_m1), *Ifna2* (Mm00833961_s1), *Ifnb1* (Mm00439546_s1), *Hprt* (Mm00446968_m1). Ct values obtained were normalized to *Hprt* and calibrated to the median control sample for relative quantification using the comparative Ct method.

Zebrafish studies

Zebrafish care

Wild type zebrafish used in this study were reared and maintained according to standard practices, at 28.5°C on a 14 h light/ 10 h dark cycle. All procedures conformed to UK Home Office requirements (ASPA 1986). The transgenic zebrafish lines, *Tg(lyz:GFP)* (13), *Tg(mpx:GFP)* (*Tg(mpx:GFP)i114*) (14), *Tg(lck:GFP)* (15) and *Tg(fli:GFP)* (16) were used.

Zebrafish gill treatment with TLR ligands and gillsorption

Zebrafish were anaesthetized in 0.17 mg/ml Tricaine Methanesulfonate (MS-222, Sigma) solution and positioned laterally under a dissecting microscope on a Petri dish. Any water was carefully removed and the right gill side of the fish gently dabbed with tissue. R848 (Invivogen) was applied unilaterally to the gill for 5 minutes. Fish were returned to fresh system water. To analyze transcript changes following stimulation, zebrafish were either euthanized in MS-222 (4 mg/ml) and their gills dissected or anaesthetized in MS-222 (0.17 mg/ml), their gill tissues gently dabbed to remove excess water and a piece of synthetic absorptive matrix (SAM; Fibrous Polyester; Hunt Developments, Midhurst, West Sussex, UK adapted from (10)) applied for 5 seconds before returned to fresh system water.

RNA extraction, cDNA synthesis and qRT-PCR

Dissected gill tissue from adult zebrafish were homogenised using a pestle in lysis buffer and processed for RNA using the MagMAX-96 Total RNA Isolation Kit, according to the manufacturer's instructions. To extract RNA from cells attached to SAM following gillsorption, the SAM was placed in Trizol and then separated from the Trizol by centrifugation using Corning® Costar® Spin-X® centrifuge tube filters (without membrane) followed by RNA processing using the PureLink™ RNA Mini Kit (*Life Technologies*) according to the manufacturer's instructions. The quantity and quality of RNA was assessed spectrophotometrically using a Thermo Scientific NanoDrop™ 1000. 125 ng of total RNA were used for reverse transcription using High-Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. qRT-PCR was performed with 2% of complementary DNA (cDNA) generated using Taqman fast universal 2 x PCR Master Mix (Applied Biosystems) and Taqman primer and probes assays for 18S (4319413E), zebrafish *il1b* (Dr03114368_m1), *tnfa* (Dr03126850_m1), *il6* (FAM/MGB-NFQ custom TaqMan RNA assay: TGGAGGCCATAAACAGCCAGCTGCA), *ifnphi1* (Dr03100938_m1), *ifng1.1* (Dr03109489_g1). All reactions were performed in duplicate using a 7500 Fast Real-time PCR system (Applied Biosystems). Cycle threshold (Ct) values

obtained were normalized to 18S (for zebrafish) or *Hprt* (for mice) and calibrated to the median control sample for relative quantification using the comparative Ct method.

Whole mount immunostaining

Dissected gills were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Gills were washed twice in PBS, once in deionised water, incubated in acetone at -20°C for 10 mins, and rinsed in deionised water and twice in PBST (PBS, 0.05% Triton X, 0.05% Tween-20). Gills were then incubated in blocking buffer (PBST, 1% DMSO, 5% donkey serum (Gibco)) for 30 mins followed by incubation with 1:1000 polyclonal chicken anti-GFP (Abcam, ab13970) in block buffer at 4°C overnight. Gills were rinsed four times in PBST and incubated in 1:250 polyclonal donkey anti-chicken-AF488 (Jackson ImmunoResearch, 703-545-155) in block buffer for 4 hrs at room temperature. Gills were rinsed in PBST and PBS before incubating in DRAQ5 (1:1000, ThermoFisher Scientific) and rinsing further in PBST. Gills were imaged on a Leica SP5 inverted confocal microscope and images processed with ImageJ software. Cells were counted using Icy software in the first 20 lamellae, from the arch, of each undamaged or unobstructed filament. Counts were performed in a blinded manner.

Immunohistochemistry of gillsorption

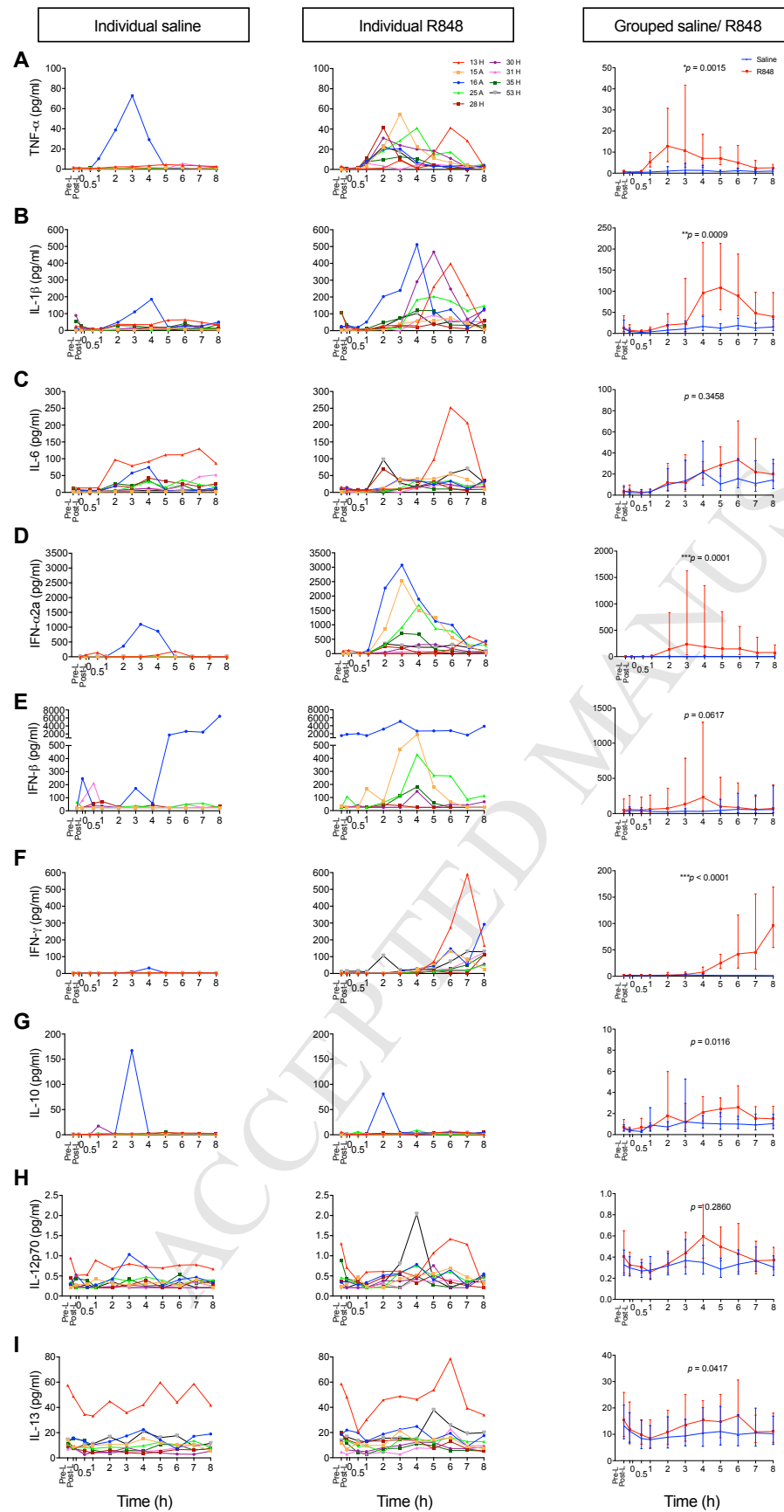
Following application on the gills, the SAM was fixed in 4 % paraformaldehyde (PFA, Sigma) for 1 hour at room temperature. After briefly washing in PBS and deionised water (dH₂O), the SAM was placed in acetone at -20°C for 5 minutes, followed by brief washes dH₂O and PBST (PBS, 0.05% Triton-X, 0.05% Tween-20) and blocking with 5% goat or 5% donkey serum (Sigma) in PBST + 1% DMSO, for 30 minutes at room temperature (RT). The SAM was incubated with primary antibodies (rabbit anti-GFP (1:1000, Life Technologies, A-11122), mouse anti-cytokeratin antibody (1:100, Thermo Scientific, MA1-82041), rabbit anti-zebrafish L-plastin antibody (1:500) in blocking solution for 1 hour at RT, followed by washes in PBST and incubation in secondary antibodies (donkey anti-rabbit-AF488 (1:500, Life Technologies, A-21206), donkey anti-rabbit-AF555 (1:500, Life Technologies, A-21428), goat anti-mouse-IgG-AF633 (1:1000, Life Technologies; A21052) for 30 minutes at RT followed by washes in PBST and PBS before incubation in DRAQ5 (1:1000, Thermo Fisher Scientific, 62254) in PBS for 15 minutes at RT. Imaging was performed using an inverted Leica SP5 confocal microscope and image processing was carried out using Fiji.

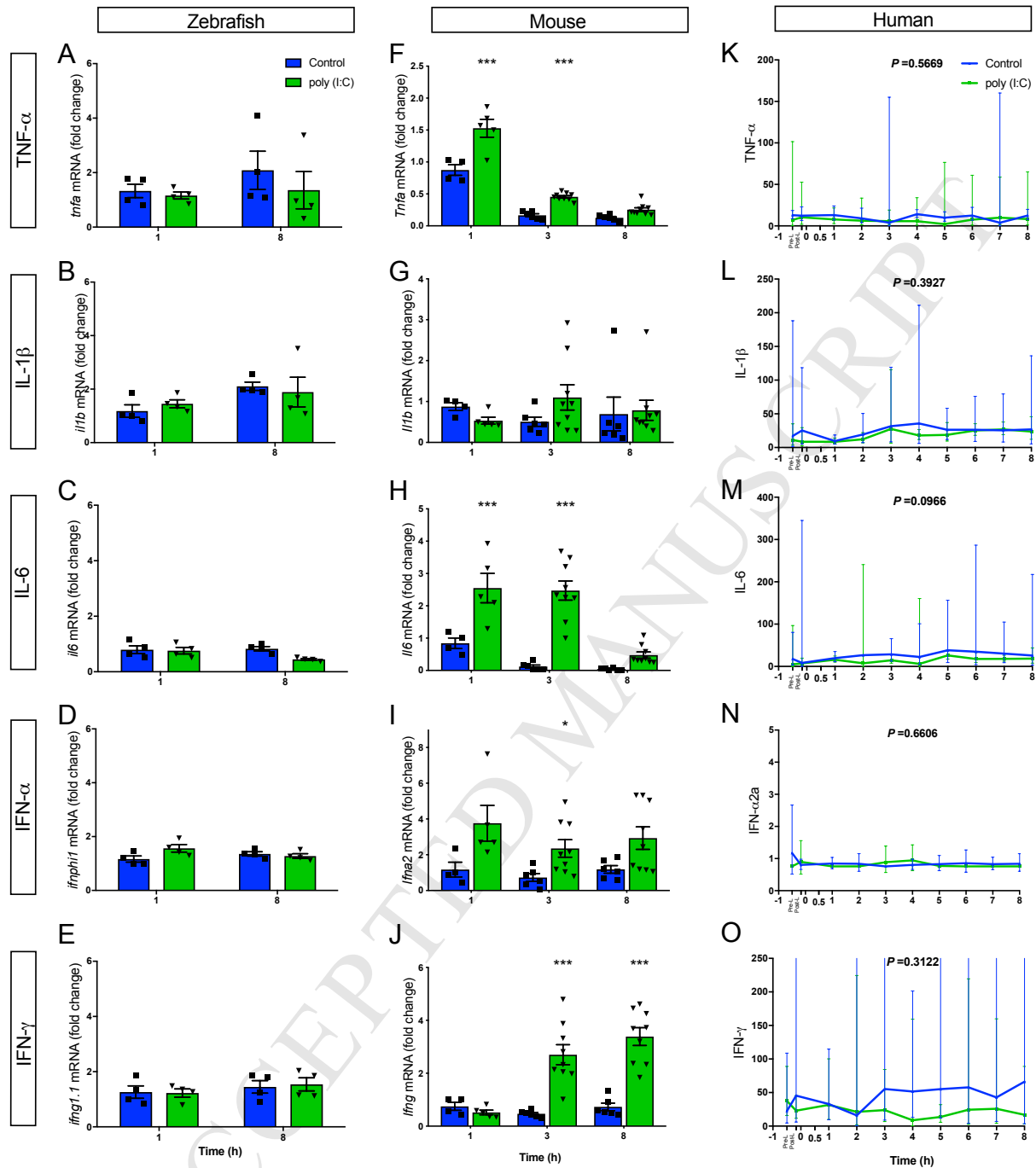
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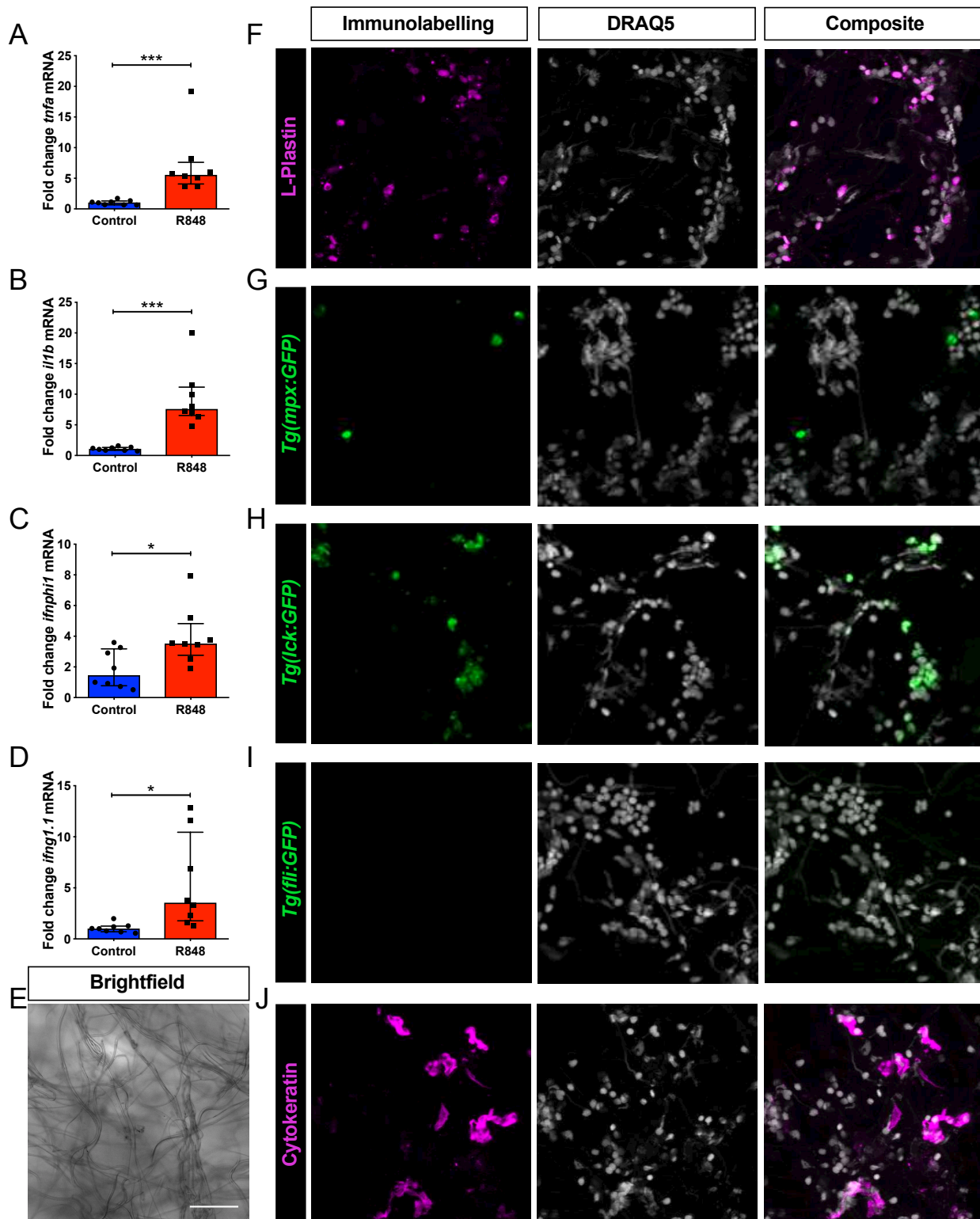
All statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad software, CA, USA). Normality distribution was tested with the D'Agostino–Pearson omnibus test. When comparing two groups unpaired two-tailed t-tests (followed by Welch's correction test for non-equal standard deviations) and Mann-Whitney tests were used for parametric and non-parametric datasets, respectively. Two-way ANOVA followed by a Sidak's multiple comparison test was used for time-course datasets. P values of less than 0.05 were deemed statistically significant with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. For the human studies, paired t-test of AUC of log₁₀ transformed values between 0-8 hours was initially calculated for each mediator in individual subjects (n=9) after nasal challenge with saline and R848. Subsequently, AUC values were compared between groups using a paired t-test. The baseline parameter for AUC was set at the lower limit of detection (LLOD) for each mediator.

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Supplementary figure legends

Supplementary Figure S1: Kinetic immune response of nasal mediators induced by R848 nasal challenge in individual subjects.

Immune response of individual subjects (n=9) after saline and R848 challenge (A-I). Of interest, the three volunteers with atopy had relatively enhanced type 1 IFN responses, although this study was not specifically powered to address this question. Grouped data (A-I) displayed as geometric mean and 95% CI; blue line=saline, red line=R848. Paired t-test on AUC of log10 transformed values between 0-8 hours in individual subjects after nasal challenge. IFN- β values n=7. Abbreviations: H, Healthy; A, Allergic rhinitis volunteer

Supplementary Figure S2: Kinetic profile of mucosal pro-inflammatory cytokine responses after zebrafish gill, mouse and human nasal stimulation with poly(I:C).

(A-E) qRT-PCR analysis of zebrafish gills (n=4, representative of 2 experiments) following gill treatment. Mean $\pm$ SEM. Two-way ANOVA followed by Sidak's multiple comparison test. (F-J) qRT-PCR analysis of mouse nasal mucosa (n=4-10 pooled from two independent experiments) sampled with SAM following intranasal stimulation. Mean $\pm$ SEM. Two-way ANOVA followed by Sidak's multiple comparison test. (K-O) Soluble protein mediator analysis of human nasal MLF (n=8) as measured using the nasosorption technique following challenge. Geometric mean and 95% CI. AUC analyses.

Supplementary Figure S3: Gillsorption as a non-invasive sampling tool to monitor mucosal pro-inflammatory cytokine and interferon responses after zebrafish gill stimulation with R848

(A-D) qRT-PCR analysis of adult zebrafish gills sampled with synthetic absorptive matrix (SAM) at 1 hour following gill treatment with water (control, blue bars) or R848 (0.5 mg/ml, red bars) for 5 minutes. Dot plots show relative expression values obtained for individual fish (n=8), which were normalized to 18S and expressed as fold change relative to the median control sample. Mean $\pm$ SEM. * P <0.05, ** P <0.01, *** P <0.001. Mann-Whitney test. (E) Representative brightfield image of SAM corresponding to J. (F-J) Representative images (maximum projection of confocal z-stack) of cells absorbed by the SAM following application on the gill tissue of live WT zebrafish stained with (F) a L-Plastin antibody (red) or transgenic zebrafish (G) *Tg(mpx:GFP)*, (H) *Tg(lck:GFP)* and (I) *Tg(fli:GFP)* stained with an anti-GFP antibody (green) or (J) a cytokeratin antibody (red). SAM was co-stained with DRAQ5 (cyan).

Subject Code	Age	Gender	Grass pollen skin prick test positive?	Eosinophil Count ($\times 10^9/L$)	Serum Total IgE
13	45	Male	N	0	23.5
15	29	Female	Y	0.1	67.5
16	49	Female	Y	0.2	1085
25	35	Female	Y	0.2	142
28	34	Female	N	0.3	54.1
30	27	Female	N	0	32.8
31	47	Female	N	0	4.69
35	45	Female	N	0.1	-
53	32	Female	N	^	2.12

Supplementary Table 1: Baseline characteristics of participants undergoing R848 nasal challenge. ^ data unavailable

Subject Code	Nasal symptoms	Fever $>37.2^{\circ}\text{C}$	Myalgia	Fatigue	Headache	Comments
13	Nil	N	N	N	N	Asymptomatic
15	Nil	N	Y	Y	Y	Fatigue for up to 36 hours post challenge
16	Blocked nose	Y	N	N	N	Nose blocked at 3hr post challenge for 1 hour. Temp increase from 36.9 to 37.4 Asymptomatic
25	Blocked + Itchy nose	Y	Y	Y	Y	Shivering soon after administration of R848 for 1.5hr. Flu-like symptoms overnight with nasal symptoms.
28	Nil	N	N	N	N	Asymptomatic
30	Nil	N	N	N	N	Asymptomatic
31	Nil	N	N	N	N	Asymptomatic
35	Nil	N	N	N	N	Asymptomatic
53	Nil	Y	Y	Y	Y	Flu-like symptoms for 24 hours after challenge Temp increased to 39.2C Mild/Moderate headache

Supplementary Table 2: Nasal and systemic symptoms after R848 nasal challenge.

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