Gene 927 (2024) 148649

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

Birch pollen-induced signatures in dendritic cells are maintained upon additional cytomegalovirus exposure

Zeinab Fneish [a,1], Jennifer Becker [a,1], Felix Mulenge [a], Firas Fneish [b], Bibiana Costa [a], Claudia Traidl-Hoffmann [c,d,e], Stefanie Gilles [c,d], Ulrich Kalinke [a,f,*]

[a] Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection Research, a joint venture between the Helmholtz Centre for Infection Research and the Hannover Medical School, 30625 Hannover, Germany
[b] Institute of Cell Biology and Biophysics, Department of Biostatistics, Leibniz University Hannover, 30419 Hannover, Germany
[c] Environmental Medicine, Faculty of Medicine, University of Augsburg, 86156 Augsburg, Germany
[d] Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany
[e] Christine-Kühne Center for Allergy Research and Education (CK-Care), 7265 Davos, Switzerland
[f] Cluster of Excellence RESIST (EXC 2155), Hannover Medical School, 30625 Hannover, Germany

ARTICLE INFO

Edited by: Nicola Lopizzo

Keywords:
Aqueous birch pollen extract
Viral infection
Monocyte-derived dendritic cells
RNA-sequencing
Antiviral immunity
Pro-inflammatory cytokines
PI3K signaling

ABSTRACT

During the birch pollen season an enhanced incidence of virus infections is noticed, raising the question whether pollen can affect anti-viral responses independent of allergic reactions. We previously showed that birch pollen-treatment of monocyte-derived dendritic cells (moDC) enhances human cytomegalovirus (HCMV) infection. Here we addressed how in moDC the relatively weak pollen response can affect the comparably strong response to HCMV. To this end, moDC were stimulated with aqueous birch pollen extract (APE), HCMV, and APE with HCMV, and transcriptomic signatures were determined after 6 and 24 h of incubation. Infection was monitored upon exposure of moDC to GFP expressing HCMV by flow cytometric analysis of GFP expressing cells.

Principle component analysis of RNA sequencing data revealed close clustering of mock and APE treated moDC, whereas HCMV as well as APE with HCMV treated moDC clustered separately after 6 and 24 h of incubation, respectively. Communally induced genes were detected in APE, HCMV and APE with HCMV treated moDC. In APE with HCMV treated moDC, the comparably weak APE induced signatures were maintained after HCMV exposure. In particular, NF-κB/RELA and PI3K/AKT/MAPK signaling were altered upon APE with HCMV exposure. Earlier, we discovered that NF-κB inhibition alleviated APE induced enhancement of HCMV infection. Here we additionally found that impairment of PI3K signaling reduced HCMV infection in HCMV and APE with HCMV treated moDC.

APE treated moDC that were exposed to HCMV show a unique host gene signature, which to a large extent is regulated by NF-κB activation and PI3K/AKT/MAPK signaling.

1. Introduction

Observational studies identified the birch pollen season as an environmental risk factor for airway infections with rhinovirus and SARS-CoV-2 (Damialis et al., 2021; Gilles et al., 2020). Nevertheless, the exact effects of pollen exposure on virus infection are still controversially discussed (Hoogeveen et al., 2021). Dendritic cells (DC) are professional antigen presenting cells that are vital orchestrators of antiviral immunity (Cabeza-Cabrerizo et al., 2021), and that are pivotal in mounting a plethora of immune reactions to pollen components (Salazar

Abbreviations: moDC, Monocyte-derived dendritic cells; HCMV, Human cytomegalovirus; APE, Aqueous pollen extract; DC, Dendritic cells; DEG, Differentially expressed genes; NGF, Nerve growth factor; RTK, Receptor tyrosine kinases; EBV, Epstein-Barr virus; PCA, Principle component analysis; PI3K, Phosphatidylinositol-3-kinase; MAPK, Mitogen-activated protein kinase; NF-κB, Nuclear factor kappa B; PBMC, Peripheral blood mononuclear cells; MIEP, Major immediate early promotor; PPI, Protein-protein interaction; ISG, Interferon stimulated gene.

* Corresponding author at: Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection Research, a joint venture between the Helmholtz Centre for Infection Research and the Hannover Medical School, 30625 Hannover, Germany.
E-mail address: ulrich.kalinke@twincore.de (U. Kalinke).

[1] These authors contributed equally.

https://doi.org/10.1016/j.gene.2024.148649
Received 8 December 2023; Received in revised form 15 May 2024; Accepted 4 June 2024
Available online 7 June 2024

and Ghaemmaghami, 2013; Smole et al., 2015). In a previous study, we demonstrated that stimulation of DC with birch pollen extract resulted in a pro-inflammatory gene signature controlled by the transcription factor NF-κB, which was responsible for enhanced human cytomegalovirus (HCMV) replication in the cells (Fneish et al., 2022). In line with that, we also showed that in DC activation of stimulator of interferon signaling (STING) enhances HCMV infection presumably via NF-κB activation (Costa et al., 2024).

Several studies investigated how birch pollen modulates innate immune cells by proteomic approaches (Strasser et al., 2017; Wisgrill et al., 2022). Although such studies emphasize that birch pollen exposure modulates innate immune pathways, it still is largely unknown how pollen skews the immune response of DC in the presence of viruses. Moreover, the transcriptional profile of DC shaped by the concomitant treatment with pollen and virus has not been investigated, yet.

In this study, we addressed how gene signatures induced by aqueous birch pollen extract (APE), which is a weak stimulus, influence those induced by the relatively strong stimulus, HCMV, in moDC. Our experiments highlight that APE treatment of moDC induces a subset of genes that remains stable in the presence of HCMV infection. Furthermore, relative to HCMV infection alone, co-exposure of moDC to APE and HCMV skewed the cells' overall response to one controlled by PI3K/AKT/MAPK signaling, which was essential for efficient HCMV infection.

2. Methods

2.1. Human moDC generation

Blood from healthy donors with unknown atopy and HCMV seropositivity status was obtained from DRK-Blutspendedienst NSTOB gGmbH (Germany). Peripheral blood mononuclear cells (PBMC) were obtained following density gradient centrifugation of blood. PBMC were incubated with anti-CD14 coupled magnetic microbeads (Miltenyi), following the manufacturer's instructions, and $CD14^+$ monocytes were isolated. To obtain immature monocyte-derived dendritic cells (moDC), monocytes were incubated with a mixture of 500 U/mL IL-4 (Miltenyi) and 500 U/mL GM-CSF (Miltenyi) at 37 °C for 5 days.

2.2. Aqueous pollen extract (APE) preparation

Birch pollen grains collected in the Augsburg area in Germany were used to prepare APE, as described previously (Beck et al., 2013). Briefly, a pool of pollen samples from six birch trees located at different sites in the greater region of Augsburg was used to create the aqueous pollen extract (APE). In brief, 30 mg pollen grains were suspended in 1 mL of sterile PBS and incubated for 30 min with repeated vortexing. The suspension was centrifuged (30 min, 1000*g*) and the supernatant was passed through a 0.2 μm pore-size sterile filter to remove residual pollen grains. The pollen-free supernatant was frozen at −80 °C until further usage. The total protein content of APE was 100 μg/mL (Coomassie blue assay), and the Bet v 1-content was 20 μg/mL, whereas the LPS content was < 0.01 EU/mL, i.e., below the detection limit of the Limulus amebocyte lysate (LAL)-assay. For the stimulation of monocyte derived dendritic cells, APE was diluted 10-fold in the cell culture medium. Accordingly, the final pollen concentration in the cell culture assays corresponds to 10 μg/mL of total pollen-derived protein and 2 μg/mL Bet v 1.

2.3. Treatment of moDC

moDC were treated with 3 mg/mL APE, or they were infected with recombinant human cytomegalovirus (HCMV) expressing GFP under the control of the major immediate early promotor (MIEP) at MOI 3, or they were simultaneously treated with 3 mg/mL APE and HCMV at MOI 3 (APE with HCMV). To increase the efficiency of HCMV infection, cells were subsequently centrifuged for 30 min at 300*g*. For PI3K inhibition experiments, moDC were incubated with 1 μM, 5 μM and 10 μM ZSTK474 (MedChemExpress), or they were mock treated with the solvent dimethyl sulfoxide (DMSO) devoid of the inhibitor for 1 h prior to stimulation with HCMV alone or APE with HCMV for 24 h.

2.4. RNA extraction and sequencing

Untreated, APE, HCMV, and APE with HCMV treated moDC derived from monocytes of a total of four healthy donors (two male and two female donors) from two independent experiments, were lysed and kept at −80 °C. RNA was then extracted using the RNA midi extraction kit (Machery-Nagel) according to the manufacturer's instructions. RNA sequencing and analysis was performed as previously described (Fneish et al., 2022).

2.5. Flow cytometry analysis of HCMV infection

HCMV exposed moDC were analyzed after 24 h of incubation by quantifying the percentage of cells expressing the virally encoded GFP by flow cytometry. GFP expression of HCMV and APE with HCMV treated moDC was measured using the ID7000 spectral analyzer (SONY Biotechnology). Data analysis was performed with FlowJo software (version 10.7.2).

2.6. Data analysis

Statistical analysis of data was performed with Graph Pad Prism 9 software. The Wilcoxon signed rank test without correction for multiple testing was used to compare between treatments in the experiments using the PI3K inhibitor ZSTK474.

Differentially expressed genes (DEG) were identified based on the following cutoff values for gene expression: absolute log2-fold change > 2 (except for Fig. 4A, in which an absolute log2-fold change > 1.5 was used) and P-value < 0.05. Gene expression in heatmaps was represented by a Z-score. As donor-to-donor variation can be a potential source of noise that may confound the identification of differentially expressed genes, donor effects were modelled as 'batch' prior to performing differential analysis. The test p-values of DEG were adjusted to multiple comparisons by using the Benjamini-Hochberg procedure. Gene ontology enrichment analysis was performed using the Molecular Signatures Database (MSigDB).

Cytoscape software was used for protein-protein interaction (PPI) network analysis with a confidence of 0.6. The TRRUST database was used for transcription factor analysis.

3. Results and discussion

3.1. Characterization of genes that are differentially expressed after APE, HCMV, and APE with HCMV treatment of moDC

moDC were treated with APE, HCMV, or they were co-treated with APE and HCMV at MOI 3 (APE with HCMV) and after 6 and 24 h of incubation, total RNA was isolated and RNA-sequencing was performed (Fig. 1**A**). moDC from a total of 4 donors were treated using the described settings, whereby stimulation of the cells was carried out in two rounds, with moDC derived from two donors in each round. Although there were variations in the magnitude of responses between donors, principal component analysis showed that untreated and APE treated samples clustered closely together, whereas HCMV and APE with HCMV treated samples clustered separately in a time point dependent manner (Fig. 1**B**). These data confirmed that upon exposure of moDC to APE and HCMV, strong variations in gene expression were mainly induced by the HCMV treatment, whereas APE exposure caused only slight changes in the overall transcriptional profile of moDC.

To inspect the breadth of transcriptional changes induced by each treatment, we determined the number of genes that were differentially

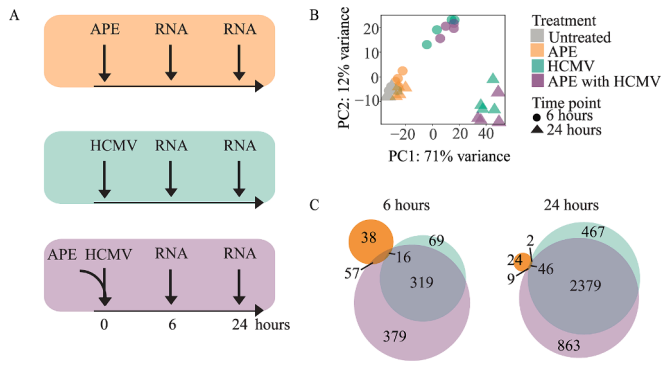

Fig. 1. Characterization of differentially expressed genes (DEG) induced in APE, HCMV, and APE with HCMV treated moDC. (A) Schematic depiction of the experimental settings. (B) Principal component analysis (PCA) of samples. (C) Venn diagrams of differentially expressed genes (DEG) induced by each treatment, relative to the mock-treated condition, at 6 and 24 h of incubation.

expressed (DEG) in APE, HCMV, and APE with HCMV treated moDC in relation to mock-treated samples. HCMV exposed moDC induced relatively more DEG at 6 and 24 h than APE treated moDC. Interestingly, APE with HCMV treatment induced unique sets of DEG at 6 and 24 h post incubation and had a more dramatic effect on moDC transcription than APE or HCMV treatment alone **(**Fig. 1**C).** Our results signify that although APE treatment of moDC induces a relatively weaker response than HCMV, APE with HCMV co-treatment triggers a characteristic gene signature with unique features that are not detected after separate treatment with either APE or HCMV.

3.2. APE treatment of moDC induces several genes that are also induced after exposure to HCMV

HCMV infection results in a simultaneous up- and downregulation of host cellular factors, which facilitates virus replication in the cells. Analysis of consensus genes regulated by APE, HCMV, and APE with HCMV treated moDC **(**Fig. 2**A)** at 6 and 24 h post incubation revealed an abundance of interferon stimulated genes (ISG), genes implicated in TSLP signaling (*CRLF2*) as well as genes involved in pro-inflammatory cytokine signaling (*IL6, IL32*) **(**Fig. 2**B).** Interestingly, NF-κB target genes *TNIP3* and *IL6* that were communally upregulated in APE or HCMV treatment alone were further elevated upon APE with HCMV co-treatment of moDC, suggesting an additive effect of both treatments on the induction of those genes **(**Fig. 2**C).** Thus, our analysis shows that APE drives the transcription of core genes that are similarly regulated upon HCMV infection and that are further enhanced by APE with HCMV co-treatment.

Such an observation is in accordance with our previous work, which highlighted at the protein level, that APE with HCMV treated moDC mounted considerably higher IFN-α and IL-6 cytokine responses than HCMV only treated moDC (Fneish et al., 2022). Notably, epithelial cells exposed to birch pollen extract similarly showed enhanced *IL6* and *TNIP3* expression (Candeias et al., 2021), which further supports the pro-inflammatory characteristics of birch pollen in immune and epithelial cells. Interestingly, cytokines such as IL-6 activate expression of HCMV immediate early (IE) genes and thus can potentially promote HCMV reactivation (Dupont et al., 2019). Similarly, pro-inflammatory responses induced in moDC by APE treatment can be exploited by HCMV to proceed with its infection cycle.

3.3. APE treatment induces distinct gene signatures in moDC that are largely unaffected by additional HCMV exposure

A subset of genes triggered in moDC by APE treatment were also induced by APE with HCMV treatment, but not by HCMV exposure alone **(**Fig. 3**A).** Interestingly, the expression profile of such APE-related genes was similar in APE and APE with HCMV treated moDC, whereas in HCMV treated moDC, their expression resembled that of the untreated condition **(**Fig. 3**B).** Accordingly, we found a strong correlation in the expression of those genes between APE and APE with HCMV treated moDC at 6 and 24 h post incubation **(**Fig. 3**C).** When analyzing the function of these genes, we found that they predominantly belonged to nerve growth factor (NGF) and receptor tyrosine kinase (RTK) signaling **(**Fig. 3**D).** This shows that APE treatment induces gene signatures, which are not masked by HCMV infection. Moreover, NGF activation has been shown to be associated with LPS induced Th2 polarization as well as in Th2 stimulation of allergic asthma in murine models (Qin, 2014; Tan, 2016). Unlike APE and APE with HCMV treatments, HCMV only exposed moDC did not show altered expression of such genes that are implicated in conferring a Th2 bias. $CD4^+$ T-cells primed by HCMV infected moDC are characterized by a Th1 bias during lytic infection (Jackson Sarah et al., 2017). Thus, APE induced gene signatures that are maintained upon APE with HCMV treatment and that are involved in a Th2 bias might influence T-cell responses to HCMV infection.

3.4. APE treatment alters the overall moDC response to HCMV infection

APE with HCMV co-exposure of moDC resulted in a unique set of DEG that were not induced by APE or HCMV treatments alone **(**Fig. S1A). To corroborate the functional importance of this unique gene set, we ran a protein–protein interaction (PPI) network analysis on the combined DEG induced 6 and 24 h post incubation. Our analysis revealed a network, which contained a chemokine/chemokine receptor rich cluster. Interestingly, we found a notable presence of integrins, growth factors and growth factor receptors (such *as ITGA10, ITGB7, PDGF, FGF2, FGFR4*) in the network, which primarily belonged to PI3K/AKT/MAPK signaling pathways **(**Fig. S1B). We therefore conclude that APE with HCMV co-exposure triggers distinctive immune reactions in moDC that are characterized by growth factor induced signaling and cell survival pathways.

To investigate how APE treatment changes the overall HCMV-induced gene expression profile in more detail, we identified DEG for APE with HCMV treated cells, relative to HCMV only treated cells. This analysis revealed a unique set of up- and down-regulated genes at 6 and 24 hpi, which was overall consistent in all four donors analyzed **(**Fig. 4**A).** The PPI network analysis of these DEG from both time-points showed that clusters that included chemokines and PI3K/AKT/MAPK target genes were again the most represented in the network **(**Fig. 4**B).** Moreover, NF-κB and RELA were identified as the main upstream regulators of genes that were altered by APE with HCMV treatment in moDC **(**Fig. 4**C).** This shows that APE treatment skews the overall gene expression induced by HCMV infection and results in unique host gene signatures that seem to be mainly regulated by NF-κB activation and PI3K/AKT/MAPK signaling.

We previously identified NF-κB as a pro-viral host factor, which was responsible for APE mediated enhancement of HCMV infection in moDC (Fneish et al., 2022). To address whether PI3K similarly contributes to enhancing HCMV infection following APE treatment, we pre-treated moDC with the pan PI3K competitive inhibitor ZSTK474 (ZST), then treated the cells with HCMV or APE with HCMV for 24 h, and finally quantified the percentages of cells expressing virally encoded GFP. PI3K inhibition by ZST effectively reduced expression of viral genes after HCMV and APE with HCMV treatment of moDC in a dose dependent manner **(**Fig. 4**D).** Remarkably, even in the presence of high concentrations of ZST, expression of viral genes was still higher following APE with HCMV treatment of moDC than with HCMV treatment alone **(**Fig. 4**E).** Of note, in the presence of 1 μM and 5 μM ZST HCMV infection was still significantly enhanced upon co-treatment with APE, whereas in the presence of 10 μM ZST the effect of APE on enhancing HCMV infection was still detectable, but no longer statistically significant.

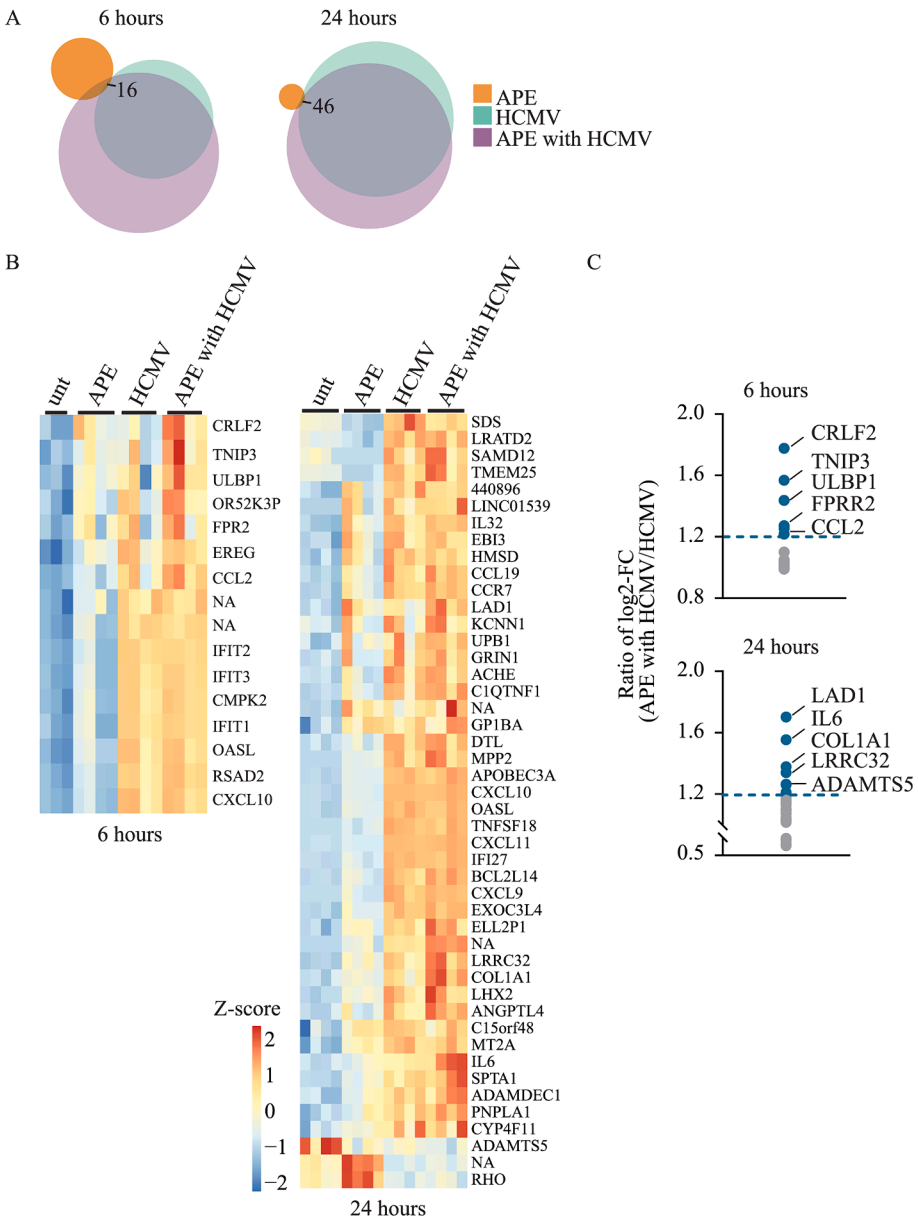

Fig. 2. APE treatment of moDC induces several genes that are also induced upon HCMV infection. (A) Venn diagrams depicting numbers of DEG communally regulated in moDC treated with APE, HCMV, or APE with HCMV after 6 and 24 h of incubation. (B) Heat maps showing DEG as presented in (A) after 6 and 24 h of incubation. Columns represent data from individual donors. (C) DEG shown in (B) were analyzed for their expression in moDC treated with APE with HCMV relative to treatment with HCMV alone. Symbols in blue represent genes that are upregulated in a statistically relevant manner in APE with HCMV vs HCMV treated moDC, i.e., a fold-change higher than 1.2, whereas the grey symbols represent genes that have not changed.

Thus, PI3K signaling is vital for efficient HCMV infection, but it does not seem to be responsible for APE induced augmentation of HCMV infection.

Genes that belong to the PI3K/AKT/MAPK pathway such as integrins and fibroblast growth factors and their receptors were shown to be associated with pathological lung disease in asthma patients (Tan et al., 2020). As these genes are also important for HCMV entry in cells (Feire et al., 2004), this could potentially account for the enhanced levels of HCMV and Epstein Barr virus (EBV) in the lungs of asthma patients (Choi et al, 2021) who are also allergically sensitized to pollen (Osborne et al., 2017; Kitinoja et al., 2020).

This and our previous study show that the weak moDC responses to birch pollen can significantly affect HCMV infection of moDC. It is noteworthy to mention that a birch pollen peak can result in up to 20,000 pollen grains/m^3 or even more, suggesting that the concentration of pollen extract we used in this study, i.e., a final concentration of 10 μg/mL total pollen protein containing 2 μg/ mL Bet v 1, presumably corresponds to *in vivo* relevant concentrations. Additionally, such a concentration is broadly used in other studies analyzing stimulation of immune cells with pollen allergens (Smole et al., 2015; Wambre et al., 2011). Nevertheless, in the kind of *in vitro* experiments we performed it is challenging to estimate physiologically relevant concentrations of pollen. This is due to ambiguity regarding whether pollen grains accumulate on the nasal mucosa, e.g., in virus-induced aphta or in blocked sinuses. Furthermore, it is not clear how fast pollen grains are cleared by cilial beating, as data on the local concentrations of pollen-derived substances in the nose is lacking. To have a better understanding of these processes, experiments in primary human nasal epithelial cell (HNEC) air–liquid-interface cultures exposed to pollen aerosols would be needed. In such cultures, the coordinated cilial beats lead to the gradual accumulation of pollen grains in the center of the wells. This could result in locally massively enhanced extraction of pollen-derived

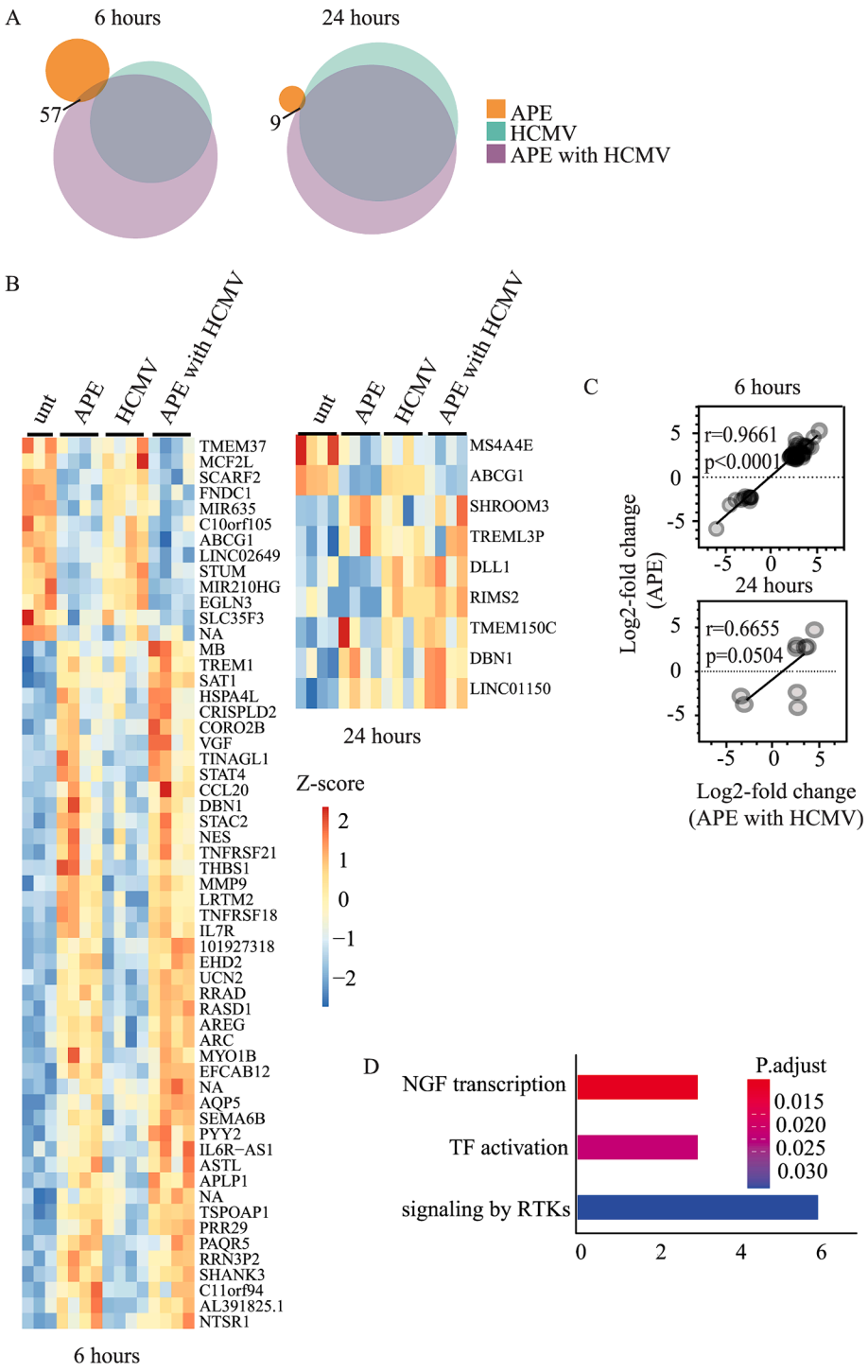

Fig. 3. APE induced gene profiles remain stable upon additional HCMV infection of moDC. (A) Venn diagrams depicting DEG uniquely shared between APE and APE with HCMV treated moDC. (B) Heat maps showing DEG shown in (A). Columns represent data from individual donors. (C) Scatter plots of absolute log_2-fold change values in APE and APE with HCMV treated moDC, of DEG shown in (B). (D) Gene ontology (GO) analysis of DEG shown in (B).

compounds, as presumably similarly taking place under *in vivo* conditions in the nasal cavity, when the nasal passage is blocked and the pollen-containing mucus is not efficiently cleared. Thus, concentrations of pollen-derived substances that are extracted locally by the nasal fluid and taken up by resident antigen presenting cells might be rather high. In conclusion, calculations addressing the question whether concentrations of pollen proteins used in *in vitro* experiments reflect *in vivo* conditions after pollen exposure are based on many assumptions and therefore have a certain degree of uncertainty. Moreover, future studies will have to be performed to address whether pollen from other plants can induce similar effects.

Here we studied the impact of APE treatment on HCMV infection because observational studies detected enhanced reactivation of herpesviruses during the pollen season, and because HCMV is an obligatory human specific virus that coevolved with the human species and developed a plethora of immune evasion mechanisms. During the pollen season it is possible that individuals are first exposed to the ever-present pollen in the environment and then become infected with a virus. In case

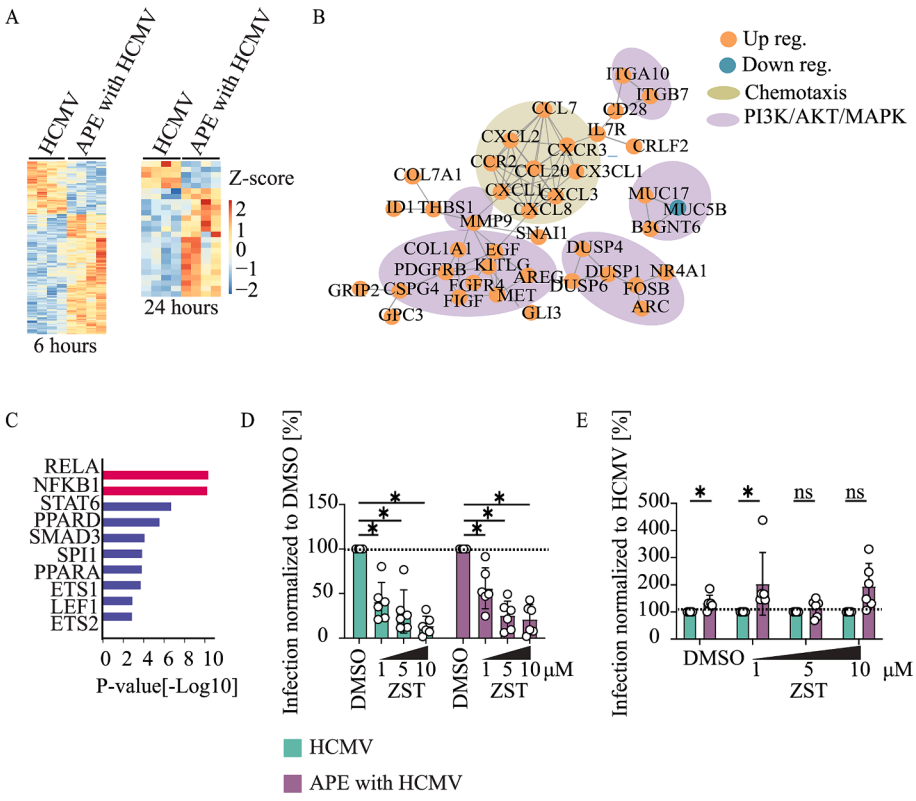

Fig. 4. APE with HCMV treatment of moDC induces a response distinct from that induced by HCMV exposure alone. (A) Heat maps of DEG between HCMV and APE with HCMV treatment of moDC. Columns represent data from individual donors. (B) PPI network of DEG shown in (A). Colored ellipses represent GO, orange and blue circles represent up and downregulated genes, respectively (C) Transcription factor analysis of combined DEG shown in (A). moDC were exposed to the PI3K inhibitor ZSTK474 (ZST) 1 h prior to HCMV or APE with HCMV treatment. The HCMV variant used here expressed GFP under the control of the viral MIEP and GFP expression was used as a surrogate for HCMV infection of moDC. (D) Percentage of change of GFP expressing cells from DMSO treated moDC or (E) from moDC treated with HCMV alone in the presence of each ZST concentration. *p = 0.0312, ns = not significant, data represent mean ± SD.

of HCMV, many individuals are already latently infected and the pollen season might influence the reactivation of HCMV. We believe that our study provides a detailed view of how co-treatment with APE can affect virus infection. This is a first step towards later studies addressing implications of different pre-, co– and post-treatment regiments of APE. Moreover, it will be of interest to address whether pollen can affect infections with viruses that have not existed since too long in the human population. In this context, SARS-CoV-2 would be particularly interesting because it is an airborne virus that infects the host via the air stream that may contain high loads of pollen.

4. Conclusion

Although birch pollen is a weaker stimulus for moDC than HCMV, moDC simultaneously exposed to APE and HCMV show an overall skewed response when compared with moDC treated with HCMV alone. moDC treated with APE and HCMV show responses that are controlled by PI3K and NF-κB signaling, which are pathways that typically support HCMV infection.

Funding

This study was supported by funding from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; Project ID 398367752 – FOR 2830, to U.K.; Project ID 398066876- GRK 2485/1 (VIPER), to U.K.; Project ID 158989968 – SFB 900-B2, to U.K.), by funding from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy—EXC 2155 "RESIST"—Project ID 39087428 to U.K., and by funding from the Helmholtz Association [Zukunftsthema "Immunology & Inflammation" (ZT-0027)] to C.T.-H. and U.K.

CRediT authorship contribution statement

Zeinab Fneish: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Jennifer Becker:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Felix Mulenge:** Formal analysis. **Firas Fneish:** Formal analysis. **Bibiana Costa:** Formal analysis. **Claudia Traidl-Hoffmann:** Resources. **Stefanie Gilles:** Resources. **Ulrich Kalinke:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We are grateful to Berenike Lange and Kira Baumann for excellent technical assistance. We thank Christian Sinzger and Kerstin Laib-Sampaio for generating and providing recombinant human cytomegalovirus expressing GFP. We thank the DRK-Blutspendedienst NSTOB gGmbH (Germany) for providing the buffy coats.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.148649.

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