

Pollen induces reactivation of latent herpesvirus and differentially affects infected and uninfected murine macrophages

To the Editor,

Aeroallergens including plant pollen are continuously inhaled and deposited in the respiratory tract and, together with their soluble components, actively interact with a variety of cells in the airways.¹ Besides epithelial cells, B cells,² dendritic cells,³ and macrophages⁴ play a prominent role in these interactions. Apart from causing allergic diseases, pollen exposure might also affect host-pathogen interactions. Both, pollen and pathogens including viruses, may be encountered in the respiratory tract at the same time and by the same cells. For example, it has been shown that pollen exposure weakens the innate defense against respiratory viruses.⁵ Other viruses that are omnipresent in humans are herpesviruses, due to their capability to persist lifelong in the host.⁶ Therefore, a co-occurrence of pollen

exposure and herpesvirus infection is likely. We have recently shown that cells persistently infected with gammaherpesviruses responded to environmental nanoparticle exposure by reactivation of latent virus.⁷ Therefore, we hypothesized that additional airborne factors like plant pollen might reactivate latent herpesvirus too.

To answer this question, persistently murine gammaherpesvirus 68 (MHV-68)-infected macrophages (ANA-1-MHV-68)⁷ were incubated with pollen grains or aqueous pollen extracts (APEs), and lytic virus was determined in the supernatant. As shown in Figure 1, both pollen grains (Figure 1A) and APEs (Figure 1B) resulted in an increase of lytic virus in the supernatant, when compared to the control, with significant increases after stimulation with Amb-pollen grains and all tested APEs.

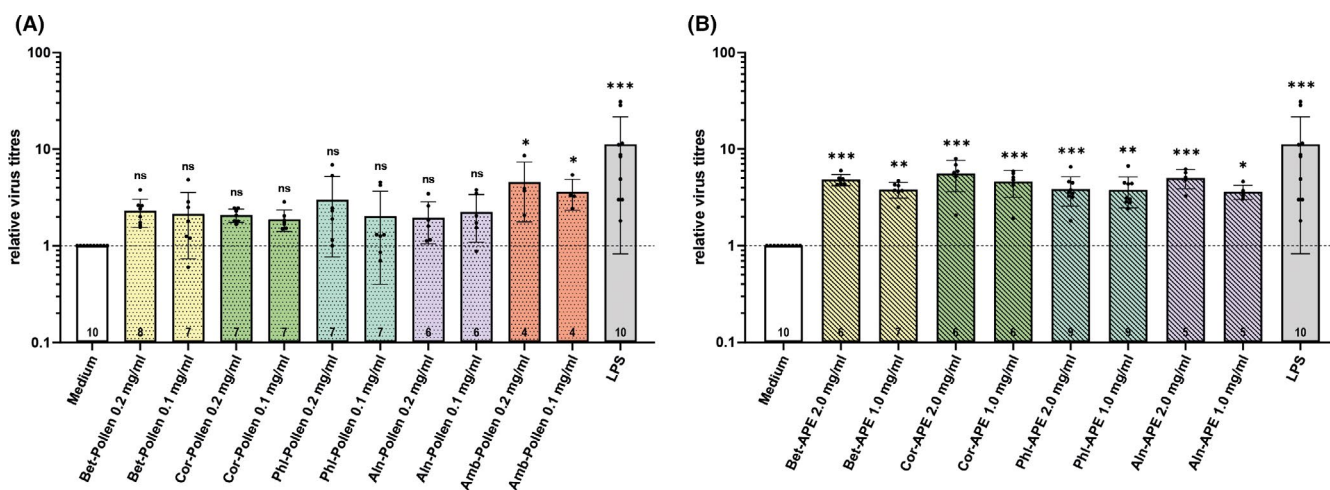


FIGURE 1 Exposure to pollen grains (A) or APE (B) induces reactivation of latent herpesvirus. ANA-1-MHV-68 were incubated with the indicated stimuli, and lytic virus was determined in supernatants by plaque assay after 72 h. Untreated cells were set as “1,” and the values for cells after stimulation were calculated relative to the control. Each black dot represents the value from an individual experiment. The columns reflect the means \pm SD from the number of independent experiments indicated at the bottom of each column. APE, aqueous pollen extract; Aln: alder (*Alnus*); Bet: birch (*Betula*); Cor: hazel (*Corylus*); Phl: grasses (*Phleum*); Amb: ragweed (*Ambrosia*)

Abbreviations: Aln-APE, aqueous extract of alder (*Alnus glomerata*) pollen; Amb-APE, aqueous extract of ragweed (*Ambrosia artemisiifolia*) pollen; ANA-1, murine macrophage cell line; APE, aqueous pollen extract; Arg 1, arginase 1 (=M2-specific marker); Bet-APE, aqueous extract of birch (*Betula pendula*) pollen; CCL, CC-motif family chemokine; Cor-APE, aqueous extract of hazel (*Corylus avellana*) pollen; CXCL, CXCL-motif family chemokine; IFN, interferon; IL, interleukin; iNOS, induced nitric oxide synthase (=M1-specific marker); LPS, lipopolysaccharide; M1, pro-inflammatory macrophage; M2, alternatively activated macrophage; MHV-68, murine gammaherpesvirus-68; MΦ, macrophage; PCA, principal component analysis; Phl-APE, aqueous extract of timothy grass (*Phleum pratense*) pollen; t-SNE, t-distributed stochastic neighbor embedding.

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Additionally, we compared the response of ANA-1-MHV-68 to that of the respective uninfected parental macrophages (ANA-1).⁷ First, we analyzed metabolic activity, apoptosis, and viability (Figure S1A-D). Overall, we did neither observe significant changes in response to stimulation nor between infected and uninfected macrophages. To evaluate whether stimulation with pollen grains or APEs leads to polarization into "M1" or "M2" macrophages, the mRNA expression of iNOS ("M1") and Arg1 ("M2") was determined. ANA-1-MHV-68 displayed an overall higher expression of iNOS (Figure 2A) and lower expression of Arg1 (Figure 2B). Within each cell line, exposure to pollen grains or APE did not significantly change the mRNA expression of the marker genes, with a few exceptions: Aln-pollen grains significantly upregulated iNOS in ANA-1-MHV-68 (Figure 2A), while Cor-APE significantly upregulated Arg1 in ANA-1 (Figure 2B). LPS increased iNOS in ANA-1 (Figure 2A). IL-4 increased Arg1 in both ANA-1 and

ANA-1-MHV-68 (Figure 2B). Both Amb-pollen grains and LPS significantly downregulated Arg1 in ANA-1 (Figure 2B). In addition, we determined the surface expression of CD86 ("M1") and CD206 ("M2") by flow cytometry, and the cytokine and chemokine release by multiplex assays of cell culture supernatants. CD86 was significantly higher expressed on ANA-1 (Figure 2C). CD206 was expressed less on both cell lines (Figure 2D). In both cell lines, stimulation with pollen grains or APEs did not significantly change the expression of CD86 or CD206, with the exception that Aln-pollen grains upregulated, and IL-4 downregulated, CD86 expression in ANA-1 (Figure 2C). Regarding cytokines and chemokines, uninfected cells secreted higher levels of both cytokines (Figure 2E) and chemokines (Figure 2F) than infected cells. LPS stimulation of uninfected cells induced a "M1"-specific profile, while stimulation with IL-4 led to a "M2"-specific profile (Figure S2A). Uninfected cells stimulated with APEs resembled a phenotype close

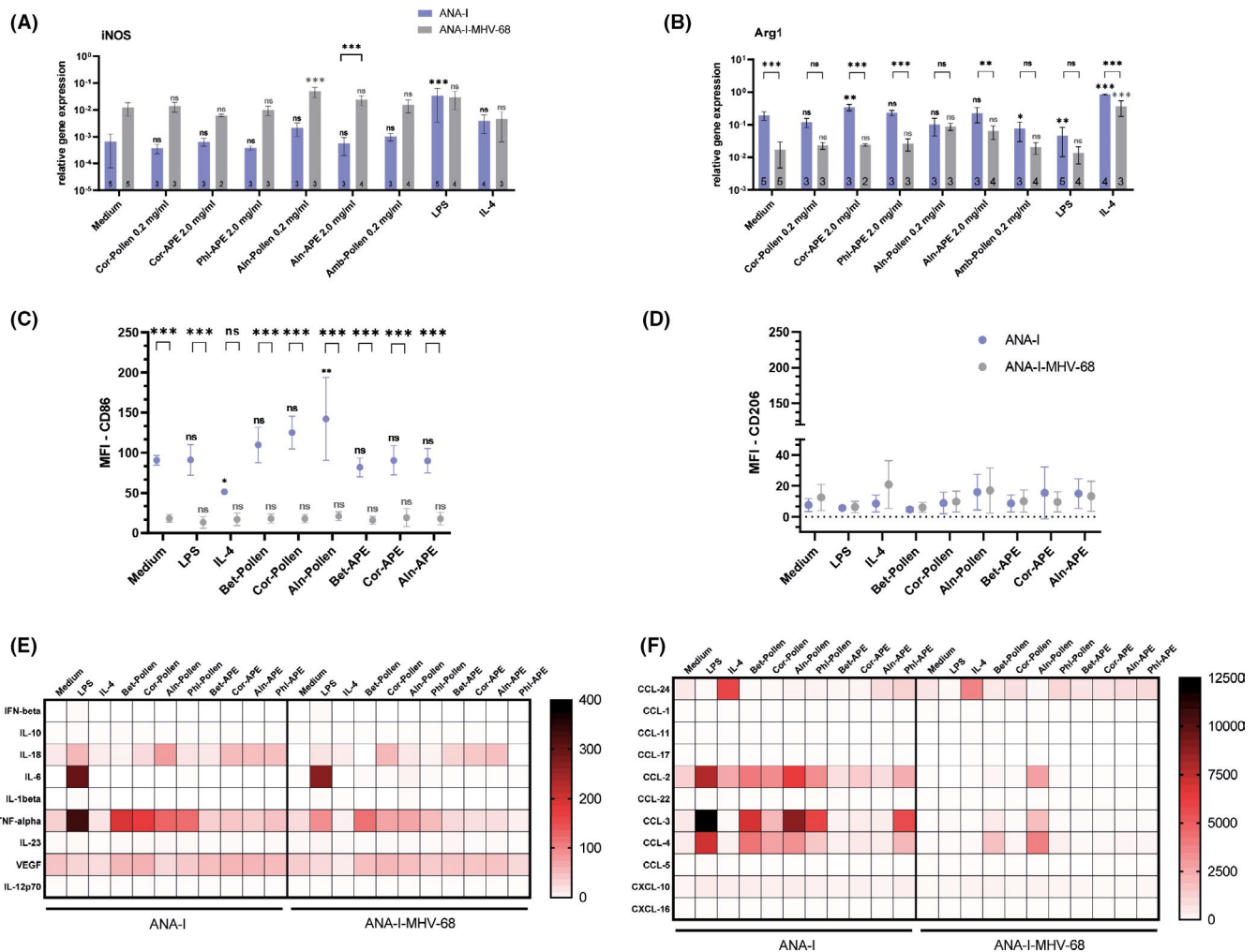


FIGURE 2 Exposure to pollen grains or aqueous pollen extract (APE) influences marker expression and cytokine and chemokine production. Cells were incubated with the indicated stimuli for 72 h, and the mRNA expression of iNOS (A) and Arg1 (B) was determined by RT-PCR. Data are expressed as relative gene expression compared to β -actin. The columns reflect the means \pm SD from the number of independent experiments indicated at the bottom of each column. The surface expression of CD86 (C) and CD206 (D) was determined by flow cytometry. Shown are mean fluorescence intensities (MFI) \pm SD from three independent experiments. The asterisks on top of the error bars indicate statistical significance between the indicated condition and the medium control and the asterisks above the brackets between the two cell lines. Cytokine (E) and chemokine production (F) was analyzed by multiplex assays. Data shown are means derived from 2 to 4 independent samples per condition

to “M2,” whereas stimulation with pollen grains induced a distinct, “M1-like” profile. Stimulation of infected cells with LPS resulted in a profile characterized by high levels of IL-6 and TNF- α , while IL-4 stimulation mainly resulted in a marked upregulation of CCL-24. Bet-pollen induced a mixed phenotype, and Aln-pollen induced a more “M1-like” phenotype (Figure S2B). To further visualize phenotypic similarities, we applied t-distributed stochastic neighbor embedding (t-SNE). In uninfected macrophages (Figure S2C), “M1” (LPS)- and “M2” (IL-4)-polarized cells appeared clearly separated. Unstimulated and cells stimulated with APEs appeared more similar to “M2,” whereas cells stimulated with pollen appeared more similar to “M1.” The phenotypic segregation of “M1” and “M2” was even more pronounced in infected macrophages (Figure S2D). To identify the factors that contribute most to these differences, we performed factorial analysis of mixed data. For uninfected macrophages, dimensions 1 and 8 explained $\approx 35\%$ of the overall variance (Figure S3A). The main contributing factor within dimension 1 was “treatment” (Figure S3B). For infected macrophages, dimensions 2 and 10 accounted for $\approx 16\%$ of the overall variance (Figure S3C). Within dimension 2, the main contributing factor was again “treatment” (Figure S3D). Finally, to investigate whether stimulation with pollen grains or APEs influences macrophage functions, we analyzed cAMP production and phagocytic capacity. In ANA-1-MHV-68, baseline cAMP production was higher than in ANA-1, and there was a tendency toward decreased production after stimulation with IL-4 and pollen grains (Figure S4A). In ANA-1, stimulation with pollen grains decreased cAMP production to levels below detection, while LPS and IL-4 increased cAMP production. In both cell lines, APE did not change cAMP production. Treatment with pollen grains and APEs (except Aln-APE) decreased the phagocytic capacity of ANA-1-MHV-68 (Figure S4B). However, a different response was observed in ANA-1. Here, APEs (again except Aln-APE) increased the phagocytic capacity, whereas it was not changed by stimulation with pollen grains (Figure S4B).

In conclusion, we show that pollen grains and APEs can induce reactivation of a latent gammaherpesvirus. This is in line with our recent findings⁷ and identifies pollen as a hitherto unrecognized “novel” trigger of herpesvirus reactivation.⁸ Constantly recurring reactivation of herpesviruses by pollen grains and their associated compounds may result in chronic inflammatory processes that may lead to immunopathology and disease. Notably, seasonal variation in reactivation and shedding of Epstein-Barr virus (EBV) in healthy adults has been described earlier, with an increase of EBV shedding in saliva during spring and autumn months.⁹ The authors hypothesized that one possible explanation might be that these months represent the peak allergy seasons in the area where the study group was recruited. By causing an influx of infected immune cells, the increased presence of allergens might contribute to the increased shedding.⁹ Our data provide now the first experimental evidence for this hypothesis.

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CONFLICT OF INTEREST

Dr Traidl-Hoffmann reports grants and personal fees from Töpfer GmbH, personal fees from Lancome, grants and personal fees from Sebapharma, personal fees from Sanofi Genzyme, personal fees from Novartis, personal fees from Lilly Pharma, personal fees from Danone Nutricia, personal fees from Bencard, and personal fees from La Roche Posay, outside the submitted work. All other authors have nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.