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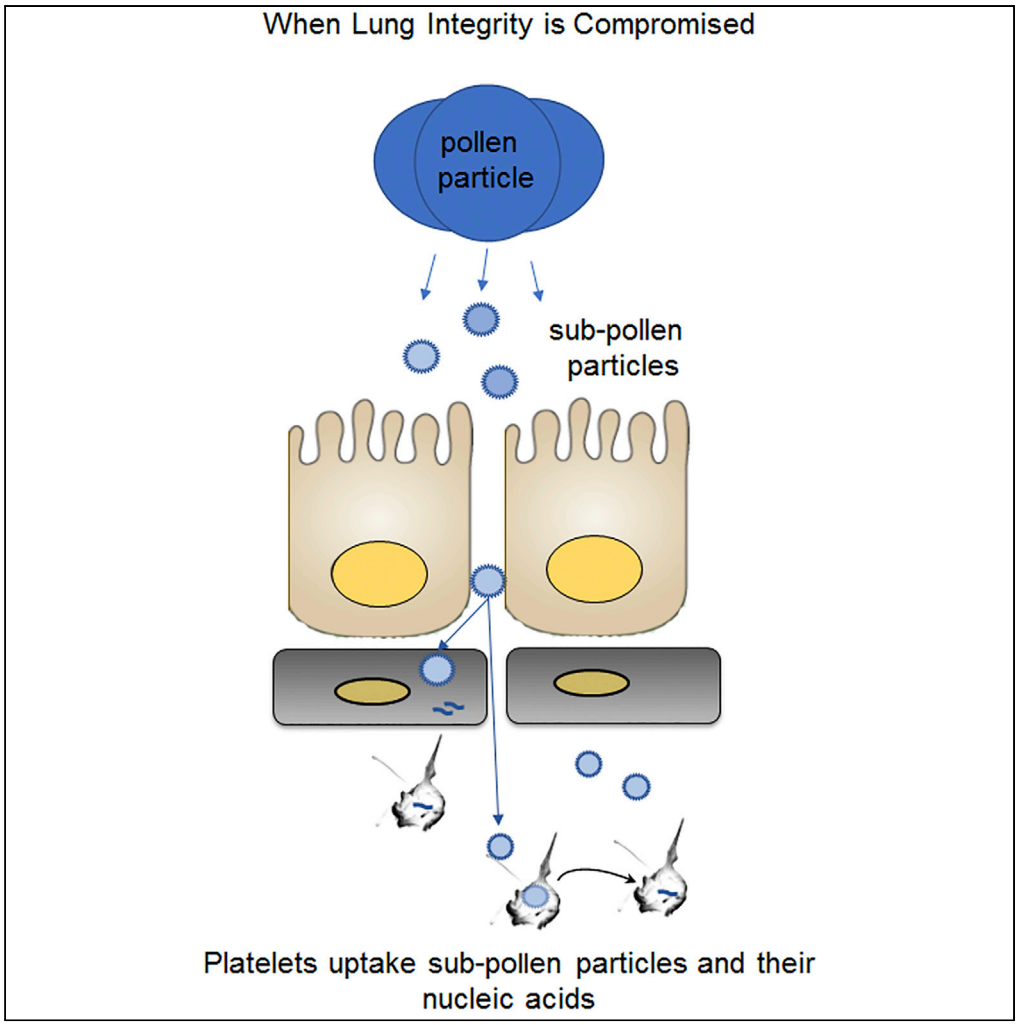

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HIGHLIGHTS

Pollen-derived miRNAs can be found in the human circulation via pulmonary transfer

This process is partially mediated by platelets' ability to uptake and digest pollen DNA

The presence of circulating pollen miRNAs has modest clinical implications

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Pollen-derived RNAs Are Found in the Human Circulation

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SUMMARY

The presence of nonhuman RNAs in man has been questioned and it is unclear if food-derived miRNAs cross into the circulation. In a large population study, we found nonhuman miRNAs in plasma by RNA sequencing and validated a small number of pine-pollen miRNAs by RT-qPCR in 2,776 people. The presence of these pine-pollen miRNAs associated with hay fever and not with overt cardiovascular or pulmonary disease. Using *in vivo* and *in vitro* models, we found that transmission of pollen-miRNAs into the circulation occurs via pulmonary transfer and this transfer was mediated by platelet-pulmonary vascular cell interactions and platelet pollen-DNA uptake. These data demonstrate that pollen-derived plant miRNAs can be horizontally transferred into the circulation via the pulmonary system in humans. Although these data suggest mechanistic plausibility for pulmonary-mediated plant-derived miRNA transfer into the human circulation, our large observational cohort data do not implicate major disease or risk factor association.

INTRODUCTION

There is a growing appreciation for the importance of non-protein coding genes in development and disease. The discovery of small RNAs, including microRNAs (miRNAs), has dramatically altered our understanding of gene expression regulation (Ambros, 2001) and their modulation of mRNA expression (Carrington and Ambros, 2003; Ambros et al., 2003). Through their regulation of many processes including transitions between pluripotency and differentiation, miRNAs help to orchestrate developmental events and play a role in cellular and tissue homeostasis that are often important for disease (Ambros, 2004, 2008, 2011). Extracellular miRNAs (exRNAs) are present in a variety of bodily fluids, including plasma, urine, and saliva, and these molecules are notably stable and resist degradation despite the presence of RNase (Mitchell et al., 2008; Argyropoulos et al., 2013). Stability of exRNA in the circulation is due to encapsulation of these small RNAs in vesicles such as exosomes or microparticles that are protected from circulating RNAses. Exosomes serve as a form of cell-to-cell communication by transferring intracellular RNA from one cell to another in close proximity or to cells in distant tissues (Maia et al., 2018). The discovery of stable RNA outside of cells has transformed our understanding of the role RNA may play in cell-to-cell communication and other complex processes. Changes in the signatures of circulating miRNAs have also been associated with the manifestation of a wide array of diseases, including myocardial infarction and chronic lymphocytic leukemia (Laterza et al., 2009; Cheng et al., 2010; Calin et al., 2005).

A very limited number of studies have shown the expression of small noncoding RNAs beyond miRNAs, but the presence of nonhuman RNAs, particularly plant-derived RNAs, in the human circulation has been questioned (Snow et al., 2013). How plant-based exRNAs enter the human circulation or if these exRNAs are genuine is not clear from the available data and the limited published information addressing this issue is contentious. Although in worms there is evidence for horizontal transfer (Timmons et al., 2001), dietary transfer of RNA in humans is not established. Functional studies suggest that rice miRNA168a could inhibit LDLRAP1 expression (Zhang et al., 2012). Several carefully conducted studies, however, cast doubt on these findings and show ineffective delivery of diet-derived miRNAs to recipient animals (Snow et al., 2013; Witwer et al., 2013; Dickinson et al., 2013; Baier et al., 2014).

We recently published the first systematic description of exRNAs, including miRNAs, from an observational cohort, currently, the largest dataset of exRNAs from human samples (Freedman et al., 2016). Characterization of these extracellular transcripts (Freedman et al., 2016) showed an association with thrombotic

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	Offspring 8 (n=2776)									
	Prevalence of exRNA Expression					exRNA Cq Expression Values				
	Positive Diagnosis		Negative Diagnosis		p Value	Positive Diagnosis		Negative Diagnosis		p Value
	N	%	N	%		Mean	SD	Mean	SD	
COPD										
pde-miR946	0	0	52	2	0.27	–	–	52	0.02	–
pta-miR1310	4	7	199	7	0.79	4	0.07	199	0.07	0.48
pta-miR948	9	15	528	20	0.32	9	0.15	528	0.20	0.92
ASTHMA										
pde-miR946	2	1	50	2	0.22	2	0.01	50	0.02	0.57
pta-miR1310	18	8	189	8	0.92	18	0.08	189	0.08	0.67
Pta-miR948	50	22	487	20	0.46	50	0.22	487	0.20	0.29
HAY FEVER										
pde-miR946	10	1	43	2	0.09	10	0.01	43	0.02	0.32
pta-miR1310	55	7	152	8	0.38	55	0.07	152	0.08	0.79
pta-miR948	177	22	361	19	**0.04**	177	0.22	361	0.19	0.99

Table 1. Association of Pollen miRNA Expression and Respiratory Diseases

Expression of pine pollen miRNAs in 2,782 people of Offspring 8 was as follows: pde-miR946 was present in 2% (n = 53), with Cq = 20.7 ± 3; pta-miR1310 was present in 8% (n = 208), with Cq = 8.3 ± 3; pta-miR948 was present in 20% (n = 543), with Cq = 20.8 ± 1.
There was no association with sex in this study.

and inflammatory disease (Mick et al., 2017; Shah et al., 2017). In this same population, we also found sequences consistent with plant-derived miRNAs. As opposed to the currently hypothesized oral horizontal transfer of non-human miRNAs, the presence of sequences consistent with pollen-derived miRNAs led us to speculate about pulmonary transfer, a mode of horizontal miRNA transfer that has not been previously proposed or investigated. It has long been known that pollen inhalation may lead to it penetrating the respiratory tract and altering disease (Michel et al., 1977). It is also known that pollen proteases compromise the airway epithelial barrier through degradation of transmembrane adhesion proteins and lung bioactive peptides (Vinhas et al., 2011). It is not currently known, however, if pollen miRNAs can be transferred into the pulmonary/systemic circulation.

RESULTS

Pine Pollen Expression in Human Plasma

Using the Framingham Heart Study (FHS) Offspring cohort at their eighth examination visit, we recently analyzed sequencing data from plasma-derived RNA from 40 participants and identified over a thousand human extracellular RNAs and, using a targeted RT-qPCR approach in an additional 2,776 individuals, we characterized the most abundant extracellular transcripts (Freedman et al., 2016; Koupenova et al., 2018b).

The study participants had a mean age of 66.3 ± 9 years and were 54% female; 9% had asthma and 29% had hay fever (Table S1). In addition to the published extracellular RNA data (Freedman et al., 2016) from these RNA sequencing (RNA-seq) and RT-qPCR data, we found three sequences consistent with pine pollen miRNAs, specifically from *Pinus taeda* (pta) and *Pinus densata* (pde). One of these pine miRNAs, pta-miR948, was present in 20% of the participants (Table 1). Expression of these miRNAs was not associated with cardiovascular disease or heart failure, chronic obstructive pulmonary disease (COPD), or asthma (Table 1). The presence of pta-miR948 had a modest association with hay fever (Table 1), and the expression of pde-miR946 had a modest association with chronic heart failure, odds ratio = 3.04 95% confidence interval 1.34, 6.90, p-value is 0.01. Covariance adjustment removed the described significance.

Confirmation of Plant Pollen-miRNA Presence in the Circulation

To confirm the validity of our pipeline and verify the species to which the sequences were mapped, we performed additional RNA sequencing studies of plant pollens (corn, pine, and black cottonwood pollen) and analyzed the findings on the Extracellular Consortium (ERCC) pipeline (Rozowsky et al., 2019) (Figure 1A). Importantly, 94% of the black cottonwood (*Populus trichocarpa*) pollen reads align to the respective genome demonstrating the pipeline's ability to accurately map exogenous miRNA from miRBase and the genomes of sequenced species in Ensembl/NCBI. Of note, the remaining 6% of the genome of black cottonwood maps to other species, these species are only of plant origin, and do not include any mammalian genomes. Sequencing of pine-pollen (*Pinus taeda and Pinus strobus*) and alignment of the genome using the same pipeline demonstrated the presence of miRNAs for the two types of pine-pollen miRNA identified in the Offspring 8, with an additional pde-miR1314 miRNA that showed high expression. The presence of pde-miR1314 was confirmed by TaqMan RT-qPCR (Figure 1B). For our mechanistic studies, we selected this miRNA owing to its high expression in pollen, as well as pta-miR948, owing to its expression in the FHS participants. We omitted pde-miR946 present only in 2% of the population and pta-miR1310 because of homology with human exRNA. Of note, even though certain reports have proposed that column contamination may skew results toward detection of foreign miRNA (Heintz-Buschart et al., 2018), in our study, pollen miRNAs were not uniformly detected throughout the human population suggesting that pollen miRNA presence does not result from column contamination.

To evaluate the methylation status of plant miRNAs in plasma and to confirm that they are of plant origin, we oxidized plasma-derived RNA from healthy donors and screened them by RT-qPCR with miScript PCR chemistry (Zhang et al., 2012). As illustrated in Figure 1C, miRNAs of human origin were not detected in the oxidized plasma, whereas miRNAs of plant origin such as osa-mir5837.2 (currently osa-mir5837) or aly-miR774a-3p.2 (currently aly-miR774a) were present in both the oxidized and non-oxidized samples (Data S1).

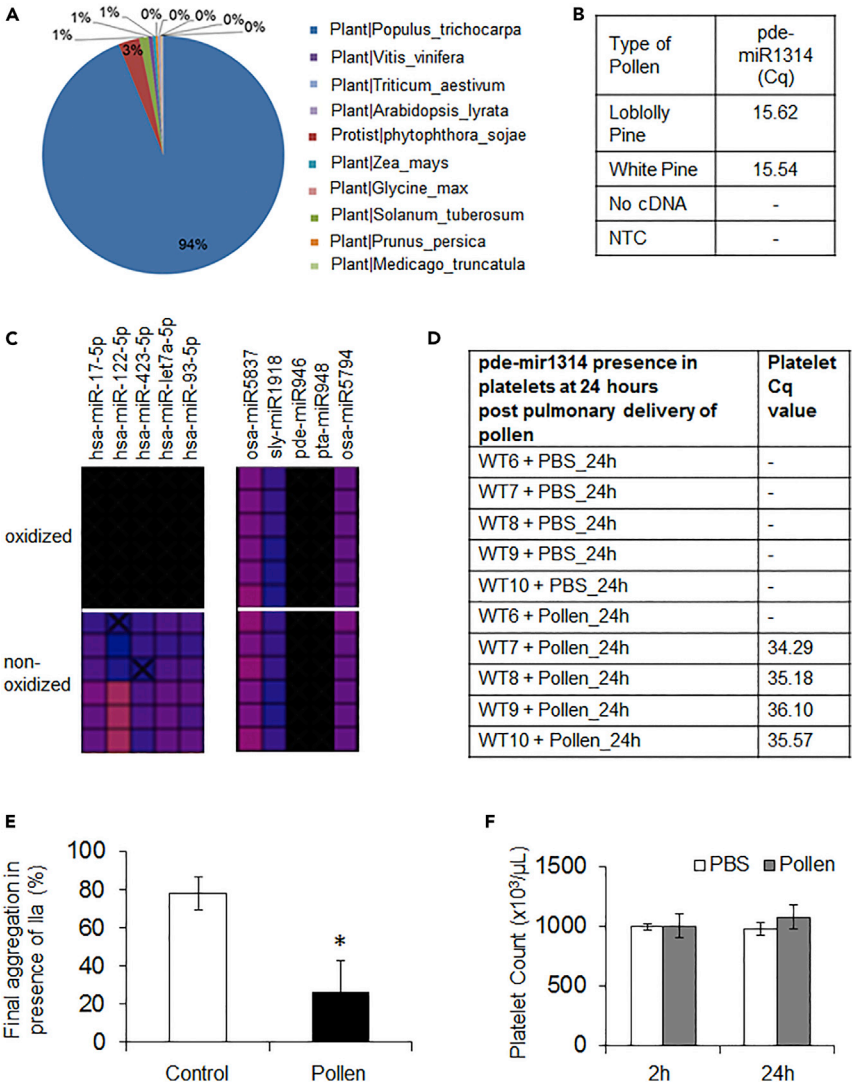
To further confirm that plant-derived miRNAs can transfer horizontally into the circulation, we utilized an *in vivo* murine model in which pollen was intranasally delivered for three consecutive days. Intranasal exposure led to the presence of pine miRNA in the platelets of mice exposed to pollen but not in the saline controls (Figure 1D; pde-mir1314); plasma presence of pollen miRNA in mice was rare (one of five mice). Interestingly, murine platelets isolated from the pollen-exposed mice at the same time point showed decreased aggregation potential in the presence of thrombin suggesting attenuated platelet function (Figure 1E). Pollen exposure did not lead to significant changes in platelet count (Figure 1F). However, similar intranasal delivery of pollen (with the additional challenge 7 days before first delivery) showed compromised lung function as evidenced by an increased bronchoconstriction, airway resistance, and dramatic leukocyte infiltration (Figure S1).

Our results suggest that the miRNA detected in the circulation of the FHS cohort is of plant origin, it can transfer horizontally into the circulation *in vivo*, and prolonged pollen exposure leads to compromised lung function even in the small airways.

Platelets Can Acquire Pine Pollen-derived miRNA from Endothelial or Epithelial Cells *In Vitro*

Platelets have the ability to take up and deliver miRNA or mRNA from cells or plasma to endothelial cells or circulating monocytes (Koupenova et al., 2018a; Clancy et al., 2017; Risitano et al., 2012; Laffont et al., 2013; Gidlof et al., 2013). Since pollen proteases can break down tight junctions between epithelial cells, we sought to evaluate if pollen particles can reach endothelial cells and if platelets can pick up pollen miRNA from endothelial cells (Runswick et al., 2007). For that purpose, we transfected primary human lung microvascular endothelial cells with synthetic pine-pollen miRNAs and circulated human platelets over the transfected cells (Clancy et al., 2017). By this model, platelets were able to take up the pine-pollen miRNA within 10 min of exposure to the transfected cells (Figures 2A and 2B).

Pollen allergens can be internalized by airway epithelial cells and are recognized by surface epithelial receptors (TLR4, PPR2) leading to chemotaxis signals and crossover of blood cells (Lambrecht and Hammad, 2014; Blume et al., 2009). To evaluate if platelets can acquire pollen miRNA directly from epithelial cells, we transfected primary airway epithelial cells from normal and asthmatic donors. Using a similar setup as described for endothelial cells, platelets also acquired pollen miRNA from flow-based exposure to epithelial cells (Figure 2C). Transfection efficiency was assessed by qPCR (Figures 2E and 2F). To assess the



Type of Pollen	pde-miR1314 (Cq)
Loblolly Pine	15.62
White Pine	15.54
No cDNA	-
NTC	-

pde-mir1314 presence in platelets at 24 hours post pulmonary delivery of pollen	Platelet Cq value
WT6 + PBS_24h	-
WT7 + PBS_24h	-
WT8 + PBS_24h	-
WT9 + PBS_24h	-
WT10 + PBS_24h	-
WT6 + Pollen_24h	-
WT7 + Pollen_24h	34.29
WT8 + Pollen_24h	35.18
WT9 + Pollen_24h	36.10
WT10 + Pollen_24h	35.57

Figure 1. Verification of Plant miRNA Origin

(A) Cottonwood pollen reads after RNA-seq and analysis on the ERCC bioinformatics pipeline maps only to plant genomes.

(B) RT-qPCR (TaqMan) for one of the most abundant miRNAs in pine pollen identified by RNA-seq confirming that the human plasma-pollen miRNA reads are correctly derived from two types of pine pollen.

(C) Heat plots of miScript RT-qPCR after oxidation of plasma-derived RNA with $NaIO_4$ confirming that the pollen miRNA reads are of plant origin. Plant miRNAs have a methyl group at the 3′ end that cannot be oxidized and the miRNA can be transcribed and detected by miScript chemistry. (D–F) Pollen was delivered intranasally in mice over 3 consecutive days.

(D) Pulmonary transfer of pine pollen-derived miRNA *in vivo*. Platelets from 12-week-old male C57BL/6J mice were isolated 24 h post the third dose and tested for pollen miRNA presence by RT-qPCR. Mice were challenged intranasally with pollen for 3 consecutive days.

(E) Platelet aggregation was measured in isolated (washed) murine platelets 24 h after the last challenge in the presence of low concentration of thrombin (factor IIa, 0.5 U/mL) (n = 3/group, platelets of two mice were pooled to form one n, p = 0.008).

(F) Platelet number measured in blood at the indicated time points post last delivery of pollen by blood counter (n = 5 mice/group, p = n.s.).

Data in graphs are represented as average ± SEM of *n* indicated for each panel; significance was assessed by unpaired t test (two-tailed value) and is indicated as a star symbol (*).

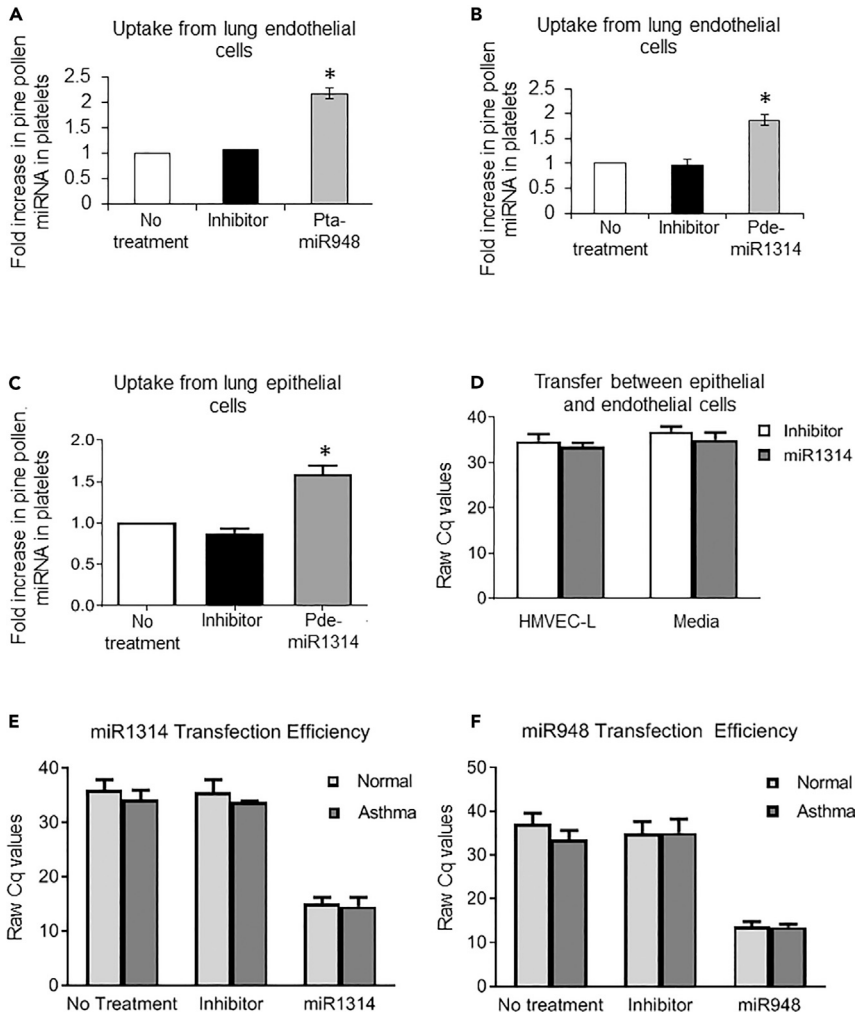


Figure 2. Transfer of Pollen miRNA between Platelets and Relevant Pulmonary and Vascular Cells

(A–D) Endothelial or epithelial cells were transfected with the respective synthetic pollen miRNA, and platelets were circulated over the cells for 10 min. miRNA presence after incubation was analyzed by RT-qPCR. Uptake of (A) pta-miR948 (raw Cq values of no treatment 32.9 ± 7; inhibitor 31.9 ± 5; miR1314 16.5 ± 1) and (B) pde-miR1314 (raw Cq values of no treatment 30.5 ± 4; inhibitor 30.4 ± 5; miR1314 16.3 ± 2) by platelets from endothelial cells (HMVEC-L) and of (C) pde-miR1314 (raw Cq values of no treatment 31.6 ± 1; inhibitor 33.2 ± 2; miR1314 18.3 ± 1) from epithelial cells (NHBE). Platelets in (A) and (B) were isolated from three donors (F, age 23 years; M, age 49 years; F, age 22 years) and in (C) also from three donors (F age 35 years; F age 43 years; M age 42 years). (D) Transfer of pde-miR1314 from transfected epithelial cells to endothelial cells in co-culture transwell system was not significant. Each condition was normalized to the baseline pollen pde-miR1314 content in each person's platelets without treatment.

(E and F) Transfection efficiency for synthetic pollen miRNAs used in this study. Epithelial cells were transfected with pollen miRNA and tested for mRNA expression by qPCR. Transfection efficiency was established for (E) pde-miR1314 and (F) pta-miR948 pollen miRNAs using primary human cells derived from normal (NHBE) and asthmatic (D-HBE-As) sources. Similar results were observed for HMVEC-L cells.

Data in each graph are representative of means ±SEM for each condition; significance in (A), (B), and (C) was assessed by one-way ANOVA, followed by Bonferroni post-test; significance in (D), (E), and (F) was assesed by two-way ANOVA and star symbol (*) indicates $p < 0.05$.

possibility of direct transfer of pollen miRNA between epithelial and endothelial cells we utilized transwell co-culture of transfected epithelial cells in the presence of endothelial cells. There was no transfer of pollen miRNA to the endothelial cells nor was pollen miRNA present in the media (Figure 2D). Our results indicate that platelets can directly acquire pollen miRNA from either of the cells that compose the epi-endothelial barrier in lungs.

Platelets Are Found in BAL but Uptake Pollen DNA under Physiological Conditions

In the alveolar capillaries, platelets are instrumental in keeping the endothelial barrier intact. Thrombocytopenic patients have a destabilized pulmonary vascular endothelial barrier, asthmatic patients have dysfunctional platelets (Weyrich and Zimmerman, 2013; Gresele et al., 1987), and our results show decreased aggregation potential of platelets in pollen-exposed mice. Since platelets can be found in the broncho-alveolar fluid after lavage (BAL) in asthmatic patients (Metzger et al., 1987), we sought to evaluate if pollen increased platelet presence in BAL in mice.

Utilizing the Hemavet HV950 system, designed specifically to measure the blood differential for mice, we were able to detect a modest rise of platelet number in BAL over the 24 h following the last exposure (Figure 3A). Platelet influx in BAL, however, was not as great as the influx of lymphocytes or basophils (Figure S2), and immunofluorescence of BAL showed singular platelets with barely identifiable CD41 expression and resting morphology (Figure 3B). Reduced CD41 expression in the alveolar fluid was consistent with the BAL pH which is around 6.92. At this pH platelets have a reduced aggregation ability and velocity (Scharbert et al., 2011). Interestingly, pine pollen was identified, clearly surrounded by numerous amounts of macrophages some of which contained platelet-CD41 (Figure 3C). Occasionally, platelet CD41 was found on the pollen particle outside of macrophages (Figure 3C). Pollen particles were detectable 24 h post the last exposure, although the DNA no longer was concentrated as in the first 15 min (Figure 3C). Lack of activation of platelets in BAL, significant interaction of platelets with pollen, and overwhelming pulmonary macrophage presence suggest that platelets are not likely to pick up genetic material directly from pollen in BAL.

Since pollen particles (70 μm) in the presence of water can spontaneously form sub-pollen particles (Bacsi et al., 2006) of much smaller size (1–10 μm, Figure S2) and these particles can possibly enter the capillary circulation owing to the compromised endothelial barrier because of impaired platelet function, we assessed whether platelets can acquire pollen nucleic acids under physiological conditions (re-calcified HEPES Buffered Tyrode Solution, pH 7.4). Incubating platelets *in vitro* with pollen (10 ng/μL) led to uptake of pollen DNA starting at 5 min post initial mixing (Figure 3D). Platelets are anucleate, and the bright DAPI stain inside of them indicates DNA uptake. Platelets began digestion of DNA at 15 min post initial mixing, and the DNA was completely digested by 60 min. Overall our results suggest that, as a function of pollen exposure, there is a compromised epithelial-endothelial barrier, consistent with impaired platelet function. The compromised epithelial-endothelial barrier may result in pollen sub-particles leaking into the circulation with their genetic material subsequently taken up by platelets and digested to RNA.

Pine Pollen miRNA Targets Mammalian Gene Expression

Although plant miRNAs have a methyl group on the ribose of the last nucleotide instead of a hydroxyl group as in mammalian miRNAs, they still have the potential to target mammalian mRNA levels by shared 7-mer seed sequences. Using the miRNA nucleotide sequence for the pine miRNAs (mirbase.org) and the "custom prediction" function in miRDB database (mirdb.org) we found many possible human mRNA targets. Pde-miR946 had 329 human mRNAs predicted, including prostaglandin E receptor 4 and fibronectin type III domain. Pta-miR948 had 763 predicted human gene targets, including insulin-like growth factor binding protein and coagulation factor II (thrombin) receptor. Pde-miR1314 had only 36 human mRNA targets, one of which was matrix metallopeptidase (MMP24). Epithelial or endothelial cells transfected with pine pollen miRNA and gene expression from transcripts with the highest predicted targets was measured using epithelial cells derived from normal and asthmatic sources. Interestingly, epithelial cells from normal and asthmatic patients, or endothelial cells, showed differences in regulation of gene expression according to cell type or pathology (Table 2). Overall pollen miRNA can affect the expression of certain human mRNAs, although regulation differs according to cell type and asthmatic origin.

DISCUSSION

Limitations in bioinformatic analyses of RNA sequencing have precluded broad assessment of non-human RNAs from large numbers of samples. Using a specialized pipeline developed by the NIH Common Fund Extracellular RNA Consortium, we re-analyzed the sequencing data and identified many small RNAs mapped to non-human origins. Using these data, we found that plant RNAs appear to be present in a subgroup of people in variable amounts. We re-analyzed each plant miRNA individually using stringency analysis to eliminate sequence homology to human miRNAs. It is highly relevant that even when examining pollen miRNA expression in a large human cohort, no clear association with disease was observed.

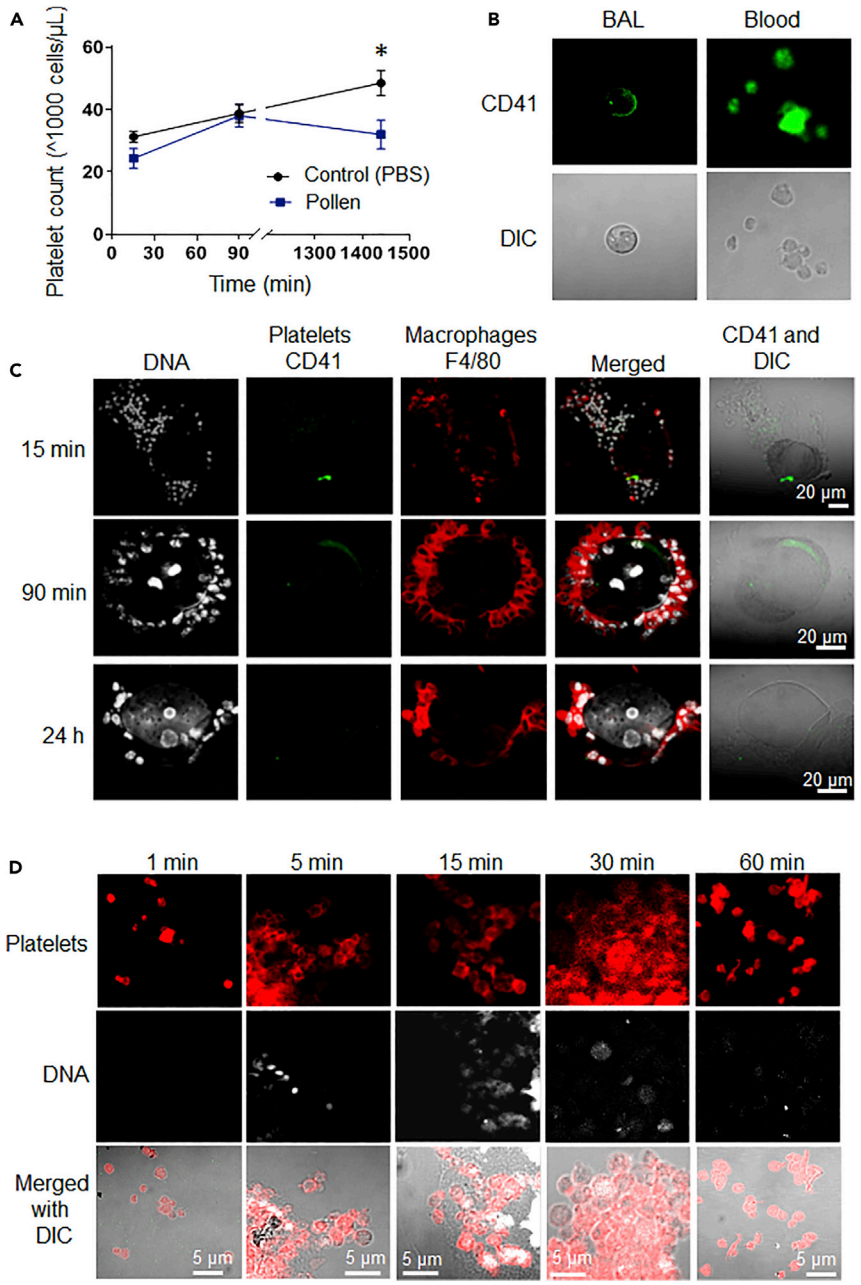


Figure 3. Platelet Function in Plasma and Interaction with Pollen in BAL and under Physiological Conditions

(A and B) Twelve-week-old male C57BL/6J mice were challenged intranasally with pollen for 3 consecutive days. Platelet presence in murine BAL was assessed by (A) Blood counter (n = 4 mice/group/time point) and by (B) Immunofluorescence (in PBS challenged mice) and compared with the immunofluorescence in blood. Platelet number measured in BAL at the indicated time points post last delivery of pollen by blood counter (Hemavet 950HV).

(C) Immunofluorescence of BAL collected at the indicated time points shows overwhelming macrophage presence and platelets rarely found to interact with pollen particles; scale bar is 20 µm.

(D) Washed human platelets were incubated with 10 ng/µL of pine pollen in HEPES-buffered Tyrode solution for the indicated times (representative of n = 5 mice per group is shown). (D) Platelets, anucleated cells, are able to take up pollen DNA (DAPI, bright and concentrated 5 min) and digest it to RNA (DAPI low fluorescence and spotty, 30 min to 1 h). Images are representative of n = 3 human donors (2 F and 1 M); scale bar is 5 µm. In all cases, data are represented as mean ± SEM for each condition; significance was assessed by two-way ANOVA, followed by Bonferroni post-test, and star symbol (*) indicates $p < 0.05$.

Cells	pta-miR948			pta-miR1314		
	Normal Epithelial	Asthmatic Epithelial	Endothelial (HMLEC)	Normal Epithelial	Asthmatic Epithelial	Endothelial (HMLEC)
Gene Target						
HIVEP3	1.32 ± 0.2[a]	0.92 ± 0.3[a]	0.59 ± 0.001[b]	1.66 ± 0.4[b]	1.09 ± 0.2[b]	0.85 ± 0.1[b]
	p=0.03	p = n.s.	p=0.00005	p = n.s.	p = n.s.	p = n.s.
PIK3AP1	0.80 ± 0.4[a]	0.54 ± 0.3[a]	2.24 ± 3.6[b]	0.44 ± 0.03[b]	0.53 ± 0.07[b]	2.03 ± 4.9[b]
	p = n.s.	p=0.02	p = n.s.	p = n.s.	p = n.s.	p = n.s.
PRR11	1.07 ± 0.6[b]	1.39 ± 0.7[b]	1.84 ± 0.8[b]	0.72 ± 0.7[b]	1.46 ± 0.7[b]	1.75 ± 1.0[b]
	p = n.s.	p = n.s.	p = n.s.	p = n.s.	p = n.s.	p = n.s.
MMP24	1.29 ± 0.3[b]	0.64 ± 0.05[b]	0.75 ± 0.1[b]	0.89 ± 0.3[a]	1.40 ± 0.6[a]	1.08 ± 0.01[b]
	p = n.s.	p = n.s.	p=0.02	p = n.s.	p = n.s.	p = n.s.
PAD5	0.70 ± 0.2[b]	0.64 ± 0.1[b]	1.28 ± 0.1[b]	0.46 ± 0.3[b]	1.11 ± 0.4[b]	1.19 ± 1.2[b]
	p = n.s.	p = n.s.	p = n.s.	p= 0.02	p = n.s.	p = n.s.

Table 2. Fold Change of Gene Targets in Primary Human Lung Cells Transfected with Synthetic Pollen miRNAs

Gene targets were chosen based on the highest scores predicted using http://mirdb.org/and the sequence of each pollen miRNA listed in http://mirbase.org. HIVEP3-Human Immunodeficiency Virus Type 1 Enhancer Binding Protein; PIK3AP1-Phospho-inositide 3-Kinase Adaptor Protein 1; PRR11-poly(A) polymerase D5; MMP24-Matrix Metallopeptidase 24; PAD5-Proline Rich 11.

[a]Expression at 6 h post transfection

[b]Expression at 24 h post transfection.

Once thought to be static, platelets are now known to interact and communicate in broader ways. The platelet, a cell central in hemostasis and thrombosis, has seen its biological roles expanded exponentially over the last decade to include immunity, inflammation, and mediation of oncogenesis (Clancy and Freedman, 2015). Platelets, although anucleate, contain a wealth of transcriptomic information with distinct expression profiles as compared with white cells (Freedman et al., 2010). Platelets' ability to participate in diverse systemic responses is noted by our growing understanding of their contents and the revelation of their capacity to share these contents (Risitano et al., 2012; Clancy et al., 2017).

Platelets are now known to horizontally transfer ribonucleic acids (RNAs), traffic pathogens, and regulate physiological and pathophysiological processes far beyond hemostasis (Koupenova et al., 2014; Risitano et al., 2012). Platelets can transfer transcripts, including messenger RNA (mRNA) and small noncoding microRNA, to recipient cells or extracellular vesicles (including exosomes and microparticles) with functional implications (Risitano et al., 2012; Gidlof et al., 2013). The observation that platelets possess the capacity to transfer cytosolic RNA suggested a new function for platelets in the regulation of vascular homeostasis (Risitano et al., 2012). The processes involved in this horizontal cellular transfer of RNA are not fully understood; however, it is believed to be multifactorial with some contribution of platelet exosome release and uptake by other platelets or vascular cells. Platelet-specific exosomes have been investigated and found to be functional, mediating vascular processes. For example, platelets from patients with myocardial infarction exhibit loss of specific miRNAs, and activated platelets shed miRNAs that can regulate endothelial cell gene expression (Gidlof et al., 2013).

Recently, the field of plasma-derived RNA or extracellular RNAs (exRNAs) has rapidly and greatly expanded, and it is thought that the most abundant microparticle subtype in circulation is platelet derived. Previous investigations have demonstrated an association of plasma miRNA with cardiovascular disease and studied the relevance of platelet miRNA to platelet function (Nagalla et al., 2011; Ward et al., 2013). It has been previously shown that platelet miRNAs can repress expression of platelet proteins, and miRNA profiles are associated with platelet reactivity (Nagalla et al., 2011). Platelets and their response to antiplatelet therapy may be important to the circulating miRNA pool (Willeit et al., 2013). It is known that platelet microparticles, rich in miRNAs, can modify the transcriptome of macrophages and reprogram their function toward a phagocytic phenotype (Laffont et al., 2015).

There are several possible mechanisms of transfer within the pulmonary vasculature. Grass pollen exposure of lung epithelial cells affects immunological barrier properties by modulating release of mediators such as CXC chemokine ligand (CXCL) 8/interleukin 8 (IL-8) (Leino et al., 2013) and MMPs that can degrade the basement membrane and destroy the intraepithelial/interendothelial junctions, disrupting the alveolar-capillary barrier (Aschner et al., 2014). Once the epithelial cells are "activated" by the pollen, the epithelial cell may (1) release MMPs, which can degrade the collagen in the basal membrane, and (2) simultaneously the epithelial barrier can become leaky because of a reduction in adhesion proteins at the junctions. Pollen particles may then reach the endothelium and affect the endothelial gap junctions to transfer to platelets. Another possibility is that epithelial cells release vesicles/vacuoles after engaging the pollen, and this could lead to the formation of vesicles with processed pollen miRNA. Additionally, the epithelial cells may release IL-8 as a chemo-attractant for neutrophils. Platelets bind neutrophils during inflammatory processes, and this interaction could bring them closer to the epithelium. Also, direct treatment of endothelial cells with pollen extract causes an increase of E-selectin and VCAM-1 protein levels as well as an increase of IL-8 production (Taverna et al., 2008). Here we report that platelets are able to uptake pollen miRNA from both endothelial and epithelial cells *in vitro*. This process can be of particular importance in the capillary beds of the alveoli where the epithelial and endothelial cells are very close in proximity. Evidence of crossover of platelets is seen in the BAL of pollen-treated mice described in our study and in human asthmatic patients described previously (Metzger et al., 1987).

Our study, together with previous work, outlines another plausible mechanism by which pollen can affect the epithelial-endothelial pulmonary barrier and lead to the presence of pollen nucleic acids in the circulation. As pollen enters the lung, certain pollen particles can spontaneously form smaller pollen sub-particles. Pollen destabilizes the epithelial-endothelial barrier leading to crossover of pollen sub-particles in the circulation. Platelets encounter these sub-particles full of pollen DNA and quickly take them up. Platelets completely digest the pollen DNA within an hour. Chronic exposure to pollen (in a process similar to allergens or asthmatic patients) leads to dysfunctional platelets as evident by the reduced aggregation potential in our murine model and in asthmatic patients (Gresele et al., 1987). Reduced aggregation potential of platelets in asthmatic patients has been explained as platelets becoming exhausted and losing their contents, which can be found in plasma (Gresele et al., 1987). Dysfunctional platelets in turn can no longer stabilize the epithelial-endothelial barrier leading to the possibility of a larger influx of pollen sub-particles in the circulation. As platelets become exhausted, their ability to get rid of pollen nucleic acids may be reduced and certain pollen RNA material can become detected in the circulation. There is a possibility that, since mice have approximately four-times more platelets per microliter than humans, we were only able to detect pollen miRNA in platelets.

In summary, we demonstrate that, with the assistance of platelet uptake, pollen miRNA can transfer into the circulation and platelets appear to play a previously undescribed role in pollen nucleic acid clearance. Although these data suggest mechanistic plausibility for pulmonary-mediated plant-derived miRNA transfer into the human circulation, our large observational cohort data do not implicate disease or risk factor association.

Limitations of the Study

We have utilized various databases to analyze our results and confirmed that the miRNAs found in the human circulation are of plant origin. Although none of the miRNAs that we have used throughout this study aligned with any human miRNA that can be detected by sequencing, we are relying on what is currently available in the mirbase.org and we cannot account for any future discoveries. By utilizing various methods in addition to sequencing and our animal studies our data suggest that pollen miRNA indeed can be transferred in the circulation.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.08.035.

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AUTHOR CONTRIBUTIONS

All authors assisted with the preparation and editing of the manuscript. M.K., K.T., and J.E.F. designed and M.K., K.T., H.A.C., A.S., S.E.T., O.V., A.M.K., M.E.M., and M.K.E. executed all experiments. E.M. performed the statistical analysis for the FHS participants. J.E.F. is the lead author and oversaw the entire study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Pollen-derived RNAs

Are Found in the Human Circulation

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TRANSPARENT METHODS

Study Population

The Framingham Heart Study (FHS) is a long-term, ongoing population study (Kannel et al., 1979, Wilson et al., 1998). The FHS Offspring Study is a community-based, prospective study of general disease as well as cardiovascular disease (CVD) and its risk factors. Previous reports have detailed the sample composition and study design (Wilson et al., 1998). The study began in 1948 with 5,209 adult subjects and is now on its third generation of participants. Cohorts undergo an examination at the FHS once every ~4-8 years and have been densely phenotyped over multiple prior examinations with a wide variety of noninvasive tests. Participants in the Offspring Study have been examined every 4-8 years since the 1970s, for 8 prior exams. At exam 8, FHS participants had a mean age of 66 years, 55% were women (Table S1). Studies outlined by the FHS protocol were approved by and carried out in accordance with Boston University Medical Center and by UMass Medical School Institutional Review Board Committees. We postulated that the expression of specific genes is influenced by environmental factors and is related to subclinical phenotypes.

Cohort Blood Sample Processing and RNA Isolation

Blood samples were collected at the Framingham Heart Study (Framingham, MA, USA) on Offspring exam 8 (March 2005 - Jan. 2008) as previously described (Freedman et al., 2016). Blood collection tubes were centrifuged and plasma was separated from the cells and frozen at -80°C within 90 minutes of draw. RNA was isolated from citrated plasma samples using a miRCURY RNA Isolation Kit (Freedman et al., 2016).

FHS RNA Sequencing and Real-Time PCR Analyses

The exact protocols have been previously published in full (Freedman et al., 2016). Briefly, the Ion Total RNAseq Kit v2 (Cat. No: 4479789, Life Technologies, USA) was used for creating libraries for sequencing. RNA Sequencing was performed on the Ion Proton System with Ion PI Chip Kit v2 BC and Ion Proton System (Life Technologies, USA) using the Ion PI Sequencing 200 Kit v2 (Life Technologies, USA). Sequencing reads were at maximum 200 nucleotides.

Sequencing Data Analysis Using the Genboree Sequencing Pipeline

Small RNA-seq reads were processed and quantified using the exceRpt tool (Rozowsky et al., 2019) available on the Genboree Workbench [http://www.genboree.org/] (Freedman et al., 2016). ExceRpt (Rozowsky et al., 2019) incorporates several modifications to existing analysis methods used to assess cytosolic micro-RNAs (miRNAs) that specifically address experimental issues pertinent to exRNA profiling, such as variable contamination of ribosomal RNAs, the presence of endogenous non-miRNA small-RNAs, and the presence of exogenous small-RNA molecules derived from a variety of plant, bacteria, and viral species. Briefly, the software processes each sample independently through a cascade of read-alignment steps designed to remove likely contaminants and endogenous sequences before aligning to exogenous miRNAs as we have previously shown (Freedman et al., 2016). Exogenous alignment of the miRNAs and the genome was done via Genboree exceRpt small-RNA seq pipeline (v3.3.0) (Rozowsky et al., 2019).

Reverse Transcription for Pollen miRNAs (FHS and oxidized miRNAs)

Specific pollen miRNAs were chosen directly from the list generated by RNAseq. The most abundant and most commonly expressed pine pollen miRNAs were selected for measurement by RT-qPCR. RNA samples (2,782) were reverse transcribed and subjected to real time PCR (Freedman et al., 2016). For pine miRNAs, miScript Plant RT Kit (Cat. No: 218762, Qiagen, Frederick, MD, USA) was used due to 3' modifications. Real-Time PCR analysis was performed on the BioMark Real Time PCR System.

Confirmation of plant miRNA origin utilizing $NaIO_4$ oxidation

Plasma RNA was isolated from healthy donors as described below in 14 µL elution volume. RNA was split evenly and one of the parts was subjected to oxidation with a final concentration of 10 mM sodium periodate ($NaIO_4$) for 40 min, in the dark, on ice. After oxidation the volume of both parts (oxidized and non-oxidized) was adjusted to 200 µL with H_2O. 1 mL of Qiazol was added and RNA was re-isolated using the plasma protocol of the Qiagen RNeasy Micro Kit according to the manufacturer's protocol. RNA was then subjected to RT-qPCR utilizing miScript chemistry. Plant miRNAs have a 3' modification (2'-O-methylation), instead of a hydroxyl group as in mammalian miRNAs, which makes them resistant to oxidation with sodium periodate. Since miScript technology utilizes polyadenylation of mature miRNAs in the first step of reverse transcription and the poly(A) polymerase requires a hydroxyl group at the 3' end, human miRNA cannot be amplified and detected when oxidation has occurred. TaqMan RT-qPCR is not appropriate for this verification since it does not involve polyadenylation in the first step of the reaction and does not account for the lack of oxidation at 3'-end of plant miRNAs.

Platelet pollen-miRNA transfer from lung Endothelial and Bronchial-epithelial Cells using Flow adhesion model

Primary lung endothelial cells (HMVEC-L, Lonza, cat. no. CC-2527, isolated from F age 52 y) or bronchial epithelial cells (normal, NHBE, Lonza cat. no. CC-2540, isolated from M age 42 y, or asthmatic, D-HBE-As, Lonza cat. no. 194911, isolated from F age 54 y) were plated on glass slides at 60,000 cells/slide overnight and then transfected with 5 nM of 2 synthetically designed pollen miRNAs (pta-miR948 and pde-miR1314, Integrated DNA Technologies, USA) or with miScript Inhibitor Negative Control (Qiagen, cat. no. 1027271) for 24h. Washed human platelets isolated from healthy human donors ($2x10^8$ platelets/slide) were run over the endothelial cells for 10 min at 3 mL/min (Clancy et al., 2017). Platelets were collected and assessed for pollen miRNA presence by RT-qPCR.(Clancy et al., 2017)

Co-culture of pollen-miRNA-transfected Primary Bronchial Epithelial Cells and HMVEC-L

Normal and asthma bronchial epithelial cells were cultured to Passage 4 and plated at $1.2 x10^5$ cells per well in a 12-well plate in 1.1 mL of BronchiaLife Complete Medium and were grown for two days prior to transfection. On the day prior to transfection, Human Microvascular Endothelial Cells – Lung (HMVEC-L, Lonza, cat. no. CC-2527) grown in VascuLife VEGF-Mv Endothelial Cell Culture Medium supplemented with VascuLife VEGF-Mv LifeFactors Kit (Lifeline Cell Technology, cat. no. LL-0005) and phenol red (Lifeline) to passage 6, were plated at $0.6 x 10^5$ cells per well in a 24-well plate in 0.550 mL of VascuLife Complete Medium. On the day prior to transfection, ThinCert Cell Culture Inserts, 0.4 µm membrane (Greiner Bio-One, Austria, cat. no. 662641) were coated with 200 µL of 50 µg/mL collagen (Chrono-Log Corp, PA, USA, cat. no. 385) diluted from stock with 60% ethanol made with sterile water. The coated inserts were dried in a closed biosafety cabinet under UV light overnight with the cabinet sash closed and fan off.

On the day of transfection, the HMVEC-L cells were washed with 1 mL pre-warmed PBS and 700 µL of media consisting of BronchiaLife Complete Media and VascuLife VEGF-Mv Complete Media at 1:1 proportion. Normal and asthma bronchial epithelial cells were transfected with 5 nM pde-miR1314 miRNA or miScript Inhibitor Negative Control, or in some cases cells were left untreated as described above for 6 hours. After transfection, epithelial cells were washed three times with pre-warmed Hepes-Buffered Saline Solution (Lonza, cat. no. CC-5022). Each well was trypsinized with 0.500 mL Trypsin-EDTA (Lonza, cat. no. CC-5012) until cells detached. The trypsin was neutralized with 1 mL of BronchiaLife Complete Media containing 2% FBS. Cells were transferred to a 15 mL tube and resuspended with a pipette. The wells were rinsed with 0.500 mL Hepes-BSS. The cells were centrifuged at 220 x g for 5 minutes. The cells were washed a second time with Hepes-BSS and

centrifuged at 220 x g for 5 minutes. Cells were re-suspended in media consisting of BronchiaLife Complete Media and VascuLife VEGF-Mv Complete Media at 1:1 proportion, counted on a hemacytometer, and plated onto the collagenized inserts (which had been washed with 300 µL of mixed media just prior) at 0.275 x 10^5 cells per insert in 300 µL of mixed media. The inserts were transferred to the wells containing the HMVEC-L cells and then co-cultured at 37°C for 24 hours. After 24 hours the inserts were removed. 500 µL of media was removed from the HMVEC-L cell wells and 700 µL of QIAzol Lysis Reagent (Qiagen, cat. no. 79306) was added. The HMVEC-L cells were washed with pre-warmed PBS. 700 µL of QIAzol was added to the wells and after 10 minutes the cells were collected and frozen at -80°C.

RT-qPCR for human genes targeted by pollen miRNA

Genes for measurement were selected based on target score calculated as a function of complementarity between the seed sequence and targeted sequence in the 5'-UTR region of human mRNA using mirdb.org. Transfected endothelial or bronchial epithelial cells were collected at the appropriate time points, 700 µL of Qiazol reagent was added and after 10 minutes, the samples were frozen at -80°C until processing. RNA was isolated from these samples using the miRNeasy Mini Kit (Qiagen, cat. no. 217004) with on-column DNA digestion using the RNase-free DNase kit (Qiagen, cat. no. 79254) and eluted in 30 µL. RNA was normalized to total RNA concentration and cDNA was synthesized in a 10 µL reaction using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, cat. no. 4368813). cDNA was preamplified in a 5 µL reaction using the TaqMan PreAmp Master Mix (Life Technologies, cat. no. 4391128). Preamplification was performed for 10 human genes targeted by pollen miRNA as well as ACTB. Preamplification product was diluted with 40 µL of DNA Suspension Buffer (Teknova, cat. no. T0223) prior to qPCR. qPCR was performed in a 10 µL reaction using TaqMan Gene Expression Master Mix (Life Technologies, cat. no 4369016), on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, MA, USA). The following genes were screened for using TaqMan assays (Life Technologies, cat. no. 4331182): PAPD5 (Hs00900790_m1), MMP24 (Hs00198580_m1), PRR11 (Hs00383634_m1), FRS2 (Hs00183614_m1), MAFG (Hs00361648_g1), CSDE1 (Hs00918650_m1), CREB3L3 (Hs00962115_m1), PIK3AP1 (Hs00381030_m1), ARF4 (Hs01070798_g1), HIVEP3 (Hs00962949_m1), ACTB (Hs99999903_m1).

***In vivo* Pollen Inhalation Model and TaqMan RT-qPCR**

Plant pollens were delivered to mice via the intranasal route and pollen miRNA presence was assessed in platelets and plasma after 24 hours. To ensure horizontal transfer, PBS dissolved pine pollen was intranasally delivered to C57BL/6J mice for 3 consecutive days. Pollen from *Pinus taeda* and *Pinus*

strobus (Greer, cat# RM132 and RM130) was resuspended at 50 µg/µL in sterile saline and 30 µL of the suspension were delivered intranasally. At the times indicated, after the final delivery, blood was collected by heart puncture and plasma and platelets were immediately isolated. RNA was isolated from 100 µL of plasma or from washed murine platelets using miRNeasy Micro Kit (Qiagen cat no. 217084) according to the manufacturer's protocols (Freedman et al., 2016). RT-qPCR for pine pollen miRNA analysis used TaqMan gene expression assays and the TaqMan® MicroRNA Reverse Transcription Kit (ThermoFisher Scientific, MA, USA, cat. no. 4366597).

Cell Count in blood or BAL

Blood (500 µl) from mice was collected by heart puncture in 200 µl of CPD after CO_2-asphyxiation. Blood cell count was determined using an AcT8 hematology analyzer (Beckman Coulter, USA). Broncho-alveolar lavage (BAL) was collected in sterile 1 mL PBS supplemented with CPD buffer (9:1 ratio). Immediately after euthanasia, murine lungs were gently flushed and BAL was fixed and stained as described below. 50 µL of BAL was run for blood cell differential on a Hemavet HV950 (Drew Scientific Group, FL, USA). Of note, the numbers measured using this blood hematology analyzer (Figure 3a and Figure S2) represent only cells that are singular; any aggregates that are formed between white blood cells (Figure 3c) cannot be accounted for by this method or by flow cytometry.

Confocal Microscopy and Antibodies

The volume of isolated BAL, or human platelets treated with pollen, was adjusted to 1 mL with PBS and fixed with 333 µL of 16% PFA for 10 min at constant rotation of 1000 rpm using magnetic stir bars (Bio/Data Corporation, cat. no. 105990) in glass tubes (Bio/Data Corporation, cat. no. 101521). Cells were spun down for 7 min at 1200 x g and washed once with ice-cold PBS. Cells were then resuspended in 100 µL of HEPES-modified Tyrode's buffer supplemented with 2% FBS and blocked for 1h at room temperature, then antibodies were added for an additional hour. At the end of incubation, samples were washed with 1 mL of 1X PBS and mounted on slides. The following antibodies were used throughout this study: anti-human: CD41-FITC or CD41-APC (clone HIP8, eBioscience, CA, USA, cat. no. 11-0419 and cat. no. 17-0419); anti-mouse: CD41-FITC (clone MWReg30, eBioscience, cat. no. 11-0411), Ly6G-APC (clone RB6-8C5, eBioscience, cat. no. 17-5931), F4/80-APC (Biolegend, cat. no. 123116).

Washed Human Platelets and Murine Platelet/Plasma Isolation

Human platelets were isolated from venous citrated blood of healthy human donors by a two-step centrifugation method (Koupenova et al., 2014). Platelets were re-suspended in 37°C HEPES-

Tyrode Buffer (140 nM NaCl, 6.1 KCl, 2.4 mM $MgSO_4.7H_2O$, 1.7 mM Na_2HPO_4, 5.8 mM Na-HEPES, pH 7.4, supplemented with 0.35% BSA and 0.1% Dextrose) and used in the transfer experiments.
Murine plasma/platelets were isolated from citrated blood collected by heart puncture. To control for variations in volume from each mouse, 500 µL of blood was collected in 200 µL of CPD buffer throughout the entire study. Blood counts were measured immediately after draw, using a Coulter AcT 8 hematology analyzer. Blood was immediately spun down at 500 x g for 10 min at room temperature. The top plasma layer was removed and centrifuged for an additional 10 min at 2000 x g at room temperature and 200 µL of plasma was removed and immediately frozen on dry ice. Isolation of platelets for RNA: the remaining blood fraction was gently transferred to Corning Falcon Round Bottom Polystyrene Tubes (Corning Incorporated - Life Sciences, Oneonta, NY, USA, cat. no. 352058) containing 4 mL of CPD Buffer (16 mM Citric acid, 102 mM Sodium citrate, 18.5 mM NaH_2PO_4, 142 mM Dextrose, pH 7.4) and centrifuged at room temperature for 5 min at 300 x g with no brake. The top layer (~1 cm left above the RBC layer) was transferred to a new polystyrene tube and centrifuged at room temperature for 7 min at 3500 x g with no brake. Any remaining red blood cells in the platelet pellet were manually removed with a pipette; platelets were immediately lysed in 700 µL of QIAzol Lysis Reagent.
Isolation of platelets for platelet aggregation: The bottom cell layer was re-suspended in 4 mL of platelet wash buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Dextrose, pH 7.4) supplemented with 100 ng/mL PGE_2 (Koupenova et al., 2014) in 5 mL Corning Falcon Round Bottom Polystyrene Tubes. The rest of the procedure was carried out similarly as for RNA, with the exception that the platelet pellet was re-suspended in 300-500 µL of HEPES-Tyrode Buffer.

Human Platelet and Pollen Interaction

Human platelets were isolated as described above. $2x10^5$ platelets/µL, in 200 µl of HEPES-Tyrode Buffer supplemented with Ca^{2+}/Mg^{2+}, (Koupenova et al., 2014) were incubated with 10 ng/µL of *Pinus strobus* pollen dissolved in PBS. Samples were incubated for the times indicated in the figure legends, at 37°C in a PAP-8E aggregometer (Bio/Data Corporation), at constant rotation of 1000 rpm. At the end of each time point, the volume was adjusted and platelets were fixed and stained as described in the “Confocal Microscopy and Antibodies” section.

Mice

All plasma and platelet isolation, and in vivo models were carried out in male C57BL/6J mice (The Jackson Laboratory, cat.no. 000664), age 12-14 weeks. All procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee and conducted accordingly.

Murine Platelet Function

Washed murine platelets at $2x10^5$ platelets/µl in 200 µl of HEPES-Tyrode Buffer (supplemented with 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.3 µM fibrinogen) were tested for aggregation potential in the presence of murine thrombin for 10 minutes at 37°C (Koupenova et al., 2014) using a PAP-8 aggregometer.

Murine Pulmonary Function

Whole body plethysmography (PenH): Pulmonary resistance quantifying breathing in awake spontaneously breathing mice was measured non-invasively. Unanesthetized, unrestrained mice were placed in a clear Plexiglas chamber, and both ambient and chamber pressure and temperature were used to calculate mouse ventilation.[8,14,15] Baseline measurements were taken under conditions of normoxia (21% oxygen; nitrogen balance) followed by a 10-minute exposure to hypercapnia (7% carbon dioxide; 21% oxygen; nitrogen balance). This method assesses the ability of mice to respond to a respiratory challenge. During baseline measurements, PenH was used to assess airway responsiveness. Penh is an empirical and dimensionless parameter and increased PenH signifies increased bronchoconstriction (Verheijden et al., 2014). In order to confirm this increased airway responsiveness, we directly measured airway resistance and pulmonary mechanics. *Pulmonary Mechanics:* Pulmonary mechanics at baseline and in response to incremental doses of methacholine were performed using forced oscillometry (FlexiVent system, SCIREQ, Montreal, Canada) in tracheotomized and anesthetized mice. Mice were anesthetized and placed on a computer controlled piston-ventilator FlexiVent system (SCIREQ, Montreal, Canada) (Keeler et al., 2017). Measurements were obtained by analyzing pressure and volume signals acquired in reaction to predefined, small amplitude, oscillatory airflow waveforms (perturbations) applied to the subject's airways. After an initial mechanical scan protocol, animals were subjected to incremental doses of nebulized methacholine. Respiratory system resistance (Rrs) was obtained by assessing the mouse's response to a single frequency forced oscillation maneuver (McGovern et al., 2013). Resistance of central airways (Rn) and small airway and tissue resistance (G) was measured (McGovern et al., 2013). To ensure that the animal was alive during these *in vivo* pulmonary mechanical scans, the heart beat was visualized between each dose during the deep inflation and at the end of the study.

H&E staining of Murine Lungs

After the Murine Pulmonary Function Experiment, lungs were inflated with 1 mL of 4% formaldehyde and fixed overnight. After 24h, lungs were transferred to 1X PBS for 24h, paraffin embedded, sectioned, and stained by the UMass DERC Morphology core. Images were taken with an Amscope IN300T-FL microscope and ToupViewX software (ToupTek, China).

Statistical Analyses of the Pollen miRNAs from the FHS

As previously described (Freedman et al., 2016), we studied plasma samples from the FHS offspring 8 cohort. There were 2,782 subjects for analysis. All statistical analyses related to the FHS were performed using STATA 13.0. Descriptive statistics are displayed as mean ± SD for continuous variables and count (percentage) for categorical variables. The RT-qPCR Cq values were not normalized because a precise and homogeneous plasma volume was used for each included sample. Ordinary least squares linear regression models were used to test for association with median age and sex and the Cq value for each pine pollen miRNA was assessed. In the mechanistic *in vitro* or *in vivo* studies, all values were expressed as the average ± SD of at least 3 different experiments. Fold changes in gene expression (RT-qPCR) were calculated based on beta-actin normalization and standard calculation methods. Statistical analyses were performed by either student-t test or ANOVA single factor test followed by Bonferroni test post analysis or unpaired t-test using Prism software (GraphPad Prism v7; La Jolla, CA, USA) using α=0.05.

Data and Software Availability

All RNA sequencing data in this manuscript has been previously deposited in dbGaP, accession number phs000007.v27.p10; the RNA-seq data can be accessed under Jane Freedman at http://genboree.org/exRNA-atlas/exRNA-Grids.rhtml?grid=analysisTable.

SUPPLEMENTAL EXCEL FILE

Raw Cq values from RT-qPCR that generated the plots in Figure 1c

Cq after miScript RT-qPCR after oxidation of plasma-derived RNA with $NaIO_4$ confirming that the pollen miRNA reads are of plant origin. Plant miRNAs have a methyl group at the 3' end that cannot be oxidized and the miRNA can be transcribed and detected by miScript chemistry.

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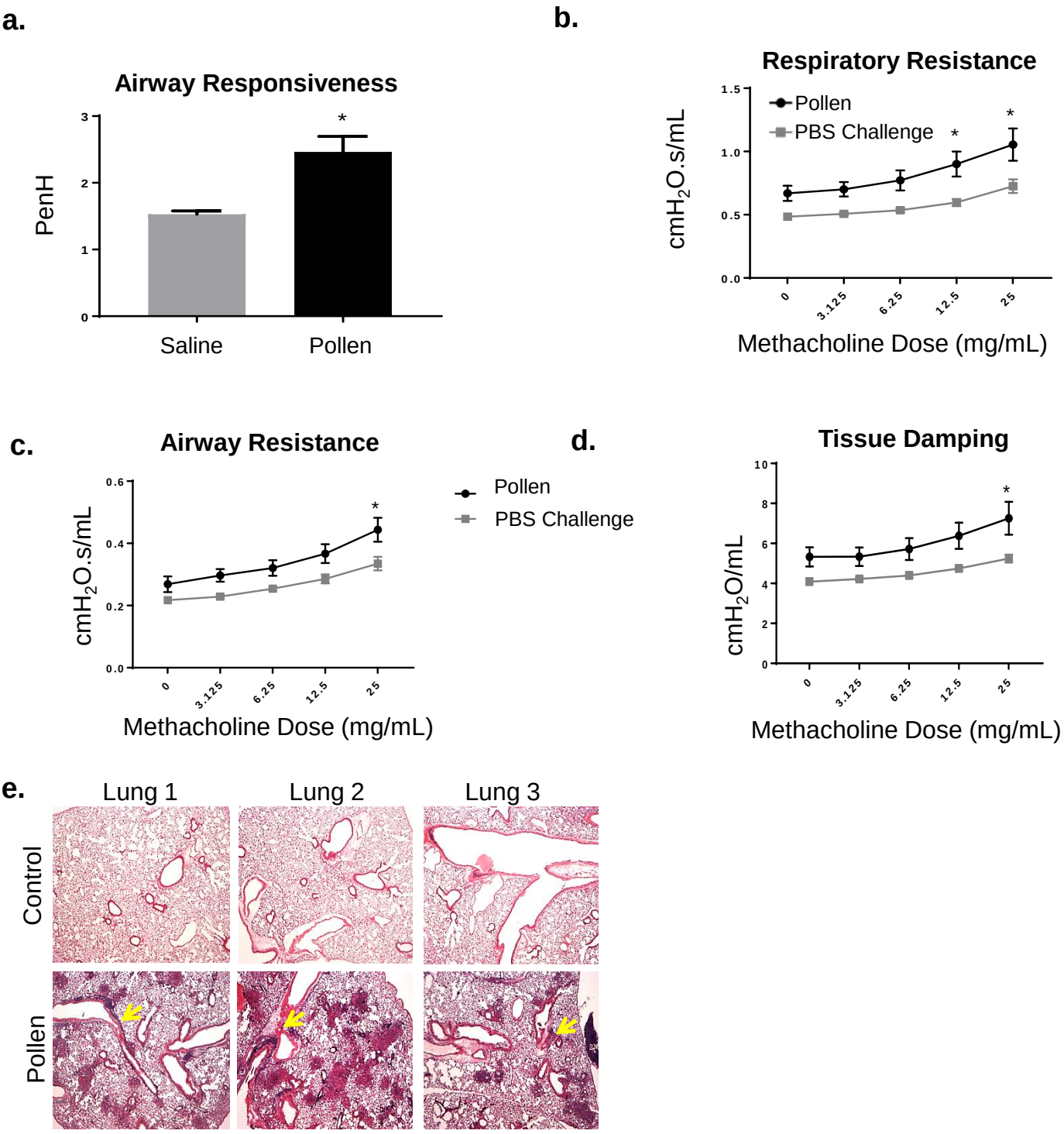

Figure S1 (Related to Figure 1). Effect of pine pollen on pulmonary mechanics. Pollen was delivered intranasally in mice over 3 consecutive days. **a.** Platelets were isolated 24h post the 3rd dose and tested for pollen miRNA presence by RT-qPCR. Effect of pollen on pulmonary mechanics: **a.** Airway responsiveness measured non-invasively in unanesthetized mice by unrestrained whole body plethysmography, as a function of enhanced pause (PenH). PenH is an empirical parameter assessing increases in bronchoconstriction. b-d. Pulmonary mechanics assessed in the same mice as in a. (after PenH) by the ventilated lung resistance method in anesthetized animals by increased exposure to methacholine. **b.** Total respiratory system resistance; **c.** Central airway resistance; **d.** Tissue damping, a measurement of the function of the smallest airways in tissue resistance. **e.** H&E Histology of the lungs of 3 of 4 different mice after the highest dose of methacholine exposure. Pictures of the lobes of each mouse are compared to the same lobe position as in control. Blue staining indicates a dramatic increase in leukocyte presence in the lungs of pollen mice. Yellow arrows point to constricted airway structures. a-d. Each test involved n=8 mice/group (male). Data is represented as the average ± SEM. Significance is established using unpaired t-test in b. and 2-way repeated-measures ANOVA followed by Bonferoni post hoc analysis in c-e. Significance in all cased is considered at $p<0.05$.

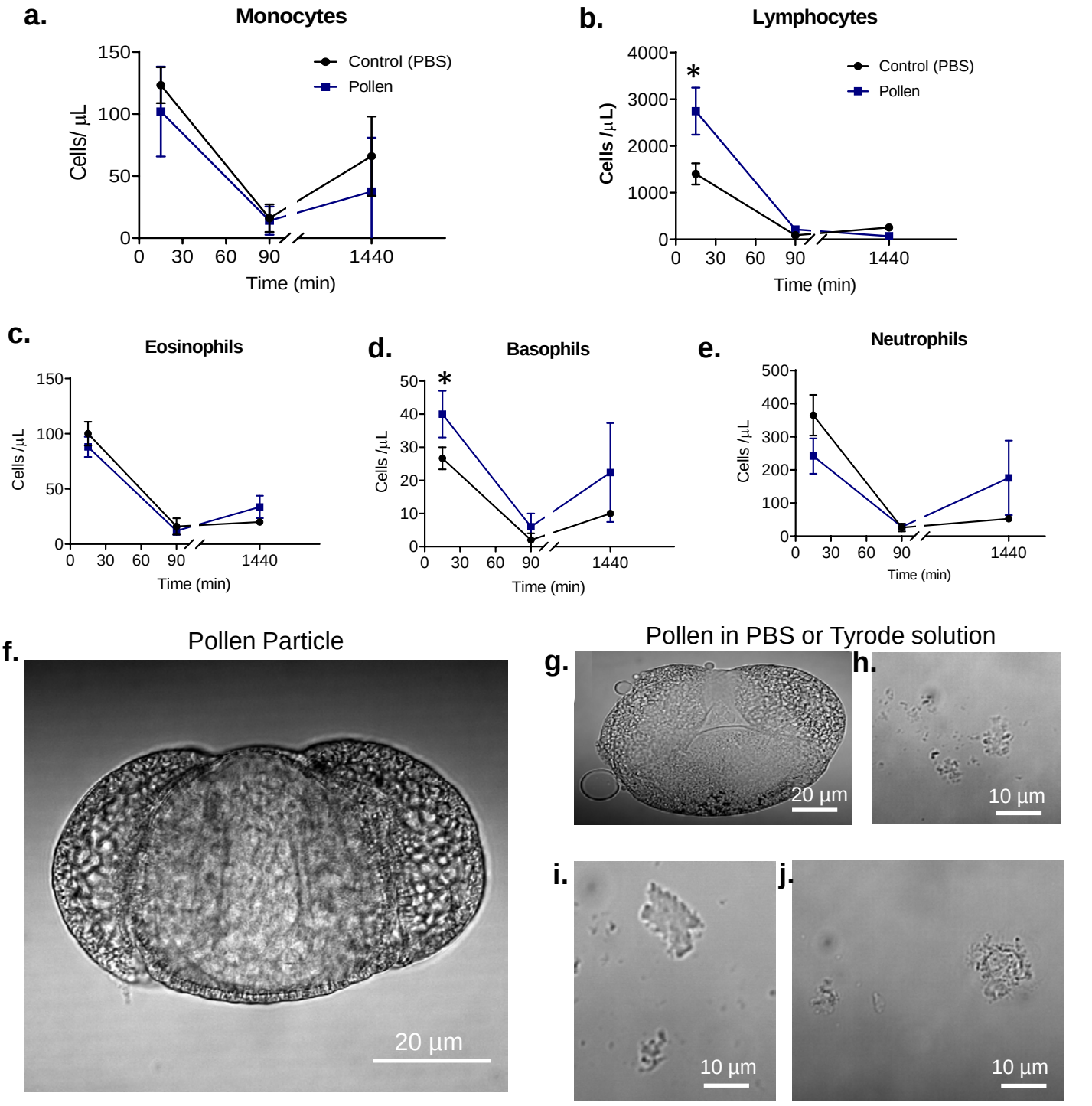

Figure S2 (Related to Figure 3). Blood cell numbers in BAL post pollen delivery and spontaneous formation of sub-pollen particles from pollen under hydrophilic conditions. Pollen was delivered intranasally in mice over 3 consecutive days. BAL was collected at the indicated time points and 50 µl of blood was tested for blood differential with a Hemavet HV950 blood counter. **a.-e.** Counts of different blood cells as indicated in each graph. Graphs represent mean ± SD. Significance was assessed by two-way ANOVA with $p<0.05$, followed by Bonferroni post test. Of note, this method only tests singular cells and does not account for cell aggregates as seen in Figure 3c. f-j: Sub-pollen particle formation from pine pollen particles under hydrophilic conditions assessed by differential interference contrast (DIC) microscopy of: **f.** Intact pine-pollen particle visible after immediate fixation of dissolved pollen in PBS. **g.** Pollen in BAL (PBS flush) 15 min after 3 days of sequential delivery of intranasal pollen in mice. **h-j:** Sub-pollen particles of various sizes formed in PBS or HEPES buffered Tyrode solution after 5 min of mixing. Scale bars are indicated on the images.

Table S1 (Related to Table 1): **FHS Sample Characteristics**

	Offspring 8 (n=2,776)	
Characteristics	**mean / n**	**SD / %**
Age (years)	66.3	9.0
Sex (female)	1499	54%
Systolic BP	128.5	17.2
Diastolic BP	73.5	10.1
BMI (mg/kg2)	28.3	5.4
Total Cholesterol to HDL Ratio	3.5	1.1
Triglycerides	118.4	69.7
Fasting Blood Glucose	106.7	23.7
Diabetes	378	14%
Blood Sugar Treatment	252	10%
Anti-hypertensive Treatment	1330	48%
Lipid Lowering Treatment	1179	43%
COPD	61	2%
Asthma	232	9%
Hay Fever	798	29%

BP-blood pressure; BMI- body mass index; HDL-high density lipoprotein; COPD- Chronic obstructive pulmonary disease