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The Malaria Metabolite HMBPP Does Not Trigger Erythrocyte Terpene Release

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Abstract

Infection with malarial parasites renders hosts more mosquito-attractive than their uninfected, healthy counterparts. One volatile organic compound, α -pinene, is associated with *Plasmodium* spp. infection in multiple studies and is a known mosquito attractant. However, how malarial infection results in elevated levels of host-associated α -pinene remains unclear. One study suggested that exposure of erythrocytes to the malarial metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) results in increased levels of α -pinene. Here we establish that endogenous levels of α -pinene are present in human erythrocytes, that these levels vary widely by erythrocyte donor, and that α -pinene levels are not altered by HMBPP treatment.

Graphical Abstract

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Keywords

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Plasmodium falciparum, the primary causative agent of lethal malaria infections, has a two-host life cycle between humans and mosquitoes. Transit between the two hosts is a critical requirement for the parasite life cycle and represents a substantial population bottleneck.¹ Mosquitoes are more attracted to humans,²⁻⁵ mice,⁶ and birds⁷ infected with malaria parasites in comparison with uninfected, healthy hosts. This observation has led to the hypothesis that *Plasmodium* species actively manipulate host odor profiles to coordinate transmission to the mosquito. Indeed, changes in the composition of host odor profiles have been observed in humans⁸⁻¹³ and mice⁶ infected with malaria; however, the molecular basis for infection-induced changes in volatile organic compound (VOC) production or release remains unknown.

Of particular interest has been the mosquito semiochemical α -pinene, which is found in higher concentrations in the breath of humans with symptomatic *Plasmodium* infection⁸ versus healthy controls. Additionally, α -pinene has been identified in the headspace above *Plasmodium falciparum*-infected erythrocytes.¹⁴ The VOC α -pinene is a member of the large and bioactive class of molecules termed terpenes. Terpenes are biosynthesized by a variety of plants, soil and environmental organisms, mammalian commensal and pathogenic microbes, and some insects.¹⁵⁻²² α -Pinene is a known component of plant-derived odorant blends that are attractive to the *Anopheles* spp. mosquitoes that transmit malaria.^{23,24} As for other terpenes, biosynthesis of α -pinene begins with the five-carbon isoprenoid precursor isopentyl pyrophosphate (IPP), which is enzymatically condensed with a second molecule of IPP by geranyl pyrophosphate synthase (GPPS) to form the 10-carbon metabolite geranyl pyrophosphate (GPP). Subsequent rearrangement and cyclization are catalyzed by a monoterpene synthase (pinene synthase) to yield α -pinene (Figure 1A).

Recently it was reported that incubation of the microbial metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) with uninfected human erythrocytes results in increased attraction and feeding behavior of anopheline mosquitoes. Concordantly, an increase in the headspace concentration of α -pinene above HMBPP-treated erythrocytes was also reported.²⁵ HMBPP is a late intermediate in the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for the synthesis of IPP and downstream isoprenoids (Figure 1A). While eubacteria and apicomplexan parasites, such as *Plasmodium* spp., utilize the MEP pathway for isoprenoid biosynthesis,²⁶ humans utilize a distinct and evolutionarily divergent biosynthetic pathway (the mevalonate pathway) to synthesize IPP.²⁷

The mechanism by which exposure of erythrocytes to HMBPP may lead to α -pinene production or release is unclear, but two possibilities may explain these findings. First, HMBPP may serve as an exogenous signal that triggers erythrocytes to release stores of α -pinene that may have accumulated via metabolic, environmental, or dietary routes. A potent activator of human V γ 9 V δ 2-T cells,²⁸ HMBPP is recognized as a pathogen-associated molecular pattern (PAMP) through its interaction with butyrophilin receptors,²⁹ suggesting that HMBPP may play a signaling role to mediate erythrocyte α -pinene release.

Alternatively, because HMBPP is itself a precursor to isoprenoids and terpenes in bacteria and plants, this metabolite may be directly incorporated into α -pinene in erythrocytes via the pathway illustrated in Figure 1A or via an as-yet-undescribed alternative enzymatic route. However, human erythrocytes do not express the known biosynthetic machinery for synthesis of α -pinene from HMBPP; mammals lack the MEP pathway and specifically do not express the final enzyme in the pathway, IspH, which converts HMBPP to the immediate α -pinene precursor IPP. Finally, no erythrocyte monoterpene synthases, nor any proteins with the terpene synthase fold, have yet been described that might mediate the final biocatalysis of GPP to α -pinene. In contrast, humans do express other prenyl diphosphate synthase orthologues, and these enzymes have been reported to moonlight as terpene synthases.²⁰⁻²² For this reason, we sought to interrogate the possibility of HMBPP-triggered, erythrocyte-produced α -pinene.

We established a working method for sampling the VOCs associated with cultured erythrocytes. Similar to Emami et al.,²⁵ we sealed donated human erythrocytes within a closed, airtight chromatography vial that had been prewarmed to 38 °C and performed headspace sampling using solid-phase microextraction (SPME) (Figure 1B). The headspace composition was determined using gas chromatography–mass spectrometry (GC–MS). Using a pure commercial α -pinene standard, we established the sensitivity and dynamic range of this assay (Figure 1C), yielding a signal-to-noise ratio of 3 and a limit of detection of 0.3 ng of α -pinene (area under the curve for ion 93, 2.36×10^3). Accommodating volumes up to 1 mL of blood, we can detect α -pinene blood concentrations as low as 2.2 nmol/L. We next sought to determine whether α -pinene was present in the headspace above untreated erythrocytes. Indeed, we confirmed that α -pinene can be detected in the headspace from donor erythrocytes, and both the retention time and mass spectra match those of the pure α -pinene standard (Figure 1D,E).

A previous study had indicated that treatment of human erythrocytes with the microbial metabolite HMBPP leads to substantial release of α -pinene. To control for batch-to-batch variability in low-level contaminants present in purified HMBPP, we acquired HMBPP from two independent chemical suppliers. Headspace sampling from both pure preparations of HMBPP confirmed that neither had contaminating levels of α -pinene above our limit of detection (Figure 2A). We next treated erythrocytes with either HMBPP or water (vehicle control) and quantified the headspace α -pinene. Because monoterpenes such as α -pinene can diffuse into the ambient air, we prealiquoted all of the blood samples into sealed individual-use aliquots. Treatment of erythrocytes with HMBPP did not result in increased levels of α -pinene (Figure 2B), and this finding was not donor-dependent. A previous study also indicated that levels of other monoterpenes (β -pinene and limonene) as well as several aldehydes (octanal, nonanal, and decanal), were increased in response to HMBPP treatment. While we searched for the presence of these additional VOCs, they were not detected in our assay. Our studies thus indicate that if erythrocytes can sense HMBPP, this signal is not accompanied by a substantial release of α -pinene within the 30 min of our experiment. Alternatively, if erythrocytes do incorporate HMBPP for the direct de novo synthesis of α -pinene, it does not occur within 30 min.

In the course of the above experiments, we noted substantial donor-to-donor variability in the endogenous levels of α -pinene present in a given erythrocyte culture. We therefore secured erythrocytes from three additional independent, unrelated donors and quantified the α -pinene levels as before. We found that the α -pinene levels were strongly dependent on the donor identity and ranged widely among our six donors (Figure 3). We found that blood α -pinene concentrations ranged from 0.37 to 2.57 ng/mL (mean $\pm$ standard deviation = 0.91 ± 0.84 ng/mL). While biosynthesis of α -pinene has not been documented in humans, α -pinene is a volatile component of several common dietary plants, suggesting that one explanation for the variability in α -pinene levels is the variability in the diets of individual donors. Alternatively, α -pinene may be synthesized by members of the human microbiome, which may contribute to endogenous α -pinene levels. Unfortunately, blood samples were provided anonymously with no dietary recall or additional sampling available, prohibiting additional analysis.

To reconcile our findings with previous studies that had reported HMBPP-induced α -pinene release, we hypothesized that loss of volatile α -pinene through diffusion following repeated sampling of the same sample over time may be a possible explanation for the results observed by the previous study. To test this hypothesis, we filled a single airtight sample tube with erythrocytes from a single donor. At $t = 0$ we removed 1 mL of erythrocytes from the conical tube and measured the headspace concentration of α -pinene according to our previous assay. We left the remainder of the sample sealed (Figure 4A). We repeated this process for a total of 10 iterations, allowing the tube of erythrocytes to re-equilibrate for 1 h between sampling. We found that α -pinene levels decreased by 25–60% ($100 \times$ first run/final run) as a result of repeated sampling (Figure 4B). As expected on the basis of its vapor pressure (4.75 mmHg at 25 °C), α -pinene is in a vapor–liquid equilibrium.³⁰ Each time our pooled erythrocytes are uncapped and sampled, vaporous α -pinene diffuses away, and a new vapor–liquid equilibrium is established. The total concentration of liquid α -pinene is thus depleted over time, thereby resulting in a reduced pool of α -pinene in each subsequent sampling. To confirm that this is not unique to α -pinene naturally absorbed within erythrocytes, we supplemented erythrocytes with 10 ng/mL (73.4 nM) α -pinene and found that the α -pinene levels dropped by 66–80% over the course of repeated sampling (Figure 4C).

While run-to-run variability and the run-order effect are commonly observed problems for mass spectrometry, our results highlight an additional precaution that needs to be taken when biologically generated volatiles are sampled. All samples should be aliquoted and sealed in an airtight container prior to the start of the experiment, as repeated sampling from the same container will result in artificially decreased concentrations of volatiles over time. Investigators should continue to control for run-order effects by randomizing the order in which samples are run.

While mounting evidence suggests that *Plasmodium* infection alters host odor profiles and results in increased mosquito attraction, the mechanism by which this occurs remains unclear. One class of molecules, terpenes, notably α -pinene, has been repeatedly highlighted for being both mosquito-attractive and enriched during *Plasmodium* infection. The metabolic origin of terpenes during *Plasmodium* infection remains unclear, as mammals

do not express orthologues of the terpene synthases required for terpene production. Here we have established that endogenous levels of α -pinene are present in human erythrocytes. While α -pinene levels from erythrocytes from a single donor sample are highly reproducible, α -pinene levels vary widely by erythrocyte donor. While the source of erythrocyte α -pinene remains enigmatic, it is possible that α -pinene may be dietary in origin, explaining the donor-to-donor variability that we observe.

While HMBPP-mediated α -pinene release has been previously reported,²⁵ we do not find evidence that the headspace of HMBPP-treated erythrocytes contains increased levels of α -pinene. HMBPP-treated erythrocytes also appear to be more mosquito-attractive than untreated erythrocytes.²⁵ Human erythrocytes bind several chemokines,^{31,32} and human V γ 9 V δ 2-T cells actively respond to HMBPP,²⁸ raising the possibility that HMBPP exposure of erythrocytes may result in other properties that increase mosquito attraction independent of α -pinene release. CO₂ emission from erythrocytes has also been reported to be elevated upon HMBPP exposure. As CO₂ is also a mosquito semiochemical,^{33,34} elevated CO₂ levels could be responsible for mosquito attraction to HMBPP-treated erythrocytes. However, supplementation of 5 ppm CO₂ to untreated erythrocytes was not sufficient to sway mosquitoes from HMBPP-treated erythrocytes.

Subsequent experiments are needed to identify the origin of *Plasmodium*-infection-associated volatiles. Infection of germ-free animal models may be valuable in discerning volatiles that arise from microbiome versus host or *Plasmodium* parasite metabolism. Identification of either human or malarial terpene synthases or metabolic labeling studies are required to understand the origin of *Plasmodium*-infection-associated terpenes. Carefully controlled dietary recall studies are necessary to understand whether erythrocyte endogenous α -pinene is biosynthesized by humans or human microbiome members.

METHODS

Materials and Reagents.

HMBPP was purchased from both Sigma-Aldrich and Echelon Biosciences Incorporated (Salt Lake City, UT, USA), resuspended at 4 mM in highly purified water, and stored at -80°C . Human erythrocytes (types A, B, and O, leukocyte-reduced and irradiated) were obtained from the St. Louis Children's Hospital Blood Bank (St. Louis, MO, USA).

Volatile Collection and GC–MS Analysis.

Erythrocytes were washed three times with equal volumes of RPMI-1640 medium (Sigma-Aldrich, SKU R4130) supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 0.01 mM thymidine, 10 $\mu\text{g}/\text{mL}$ gentamycin, and 0.5% albumax (Thermo Fisher Scientific, 11020039) and stored at 50% hematocrit at 4°C . When testing responses of erythrocytes to HMBPP and water, erythrocytes were stored as 1.4 mL aliquots in individual 1.5 mL microfuge tubes (Thermo Fisher Scientific, 05-408-129), wrapped in Parafilm (Sigma-Aldrich, SKU P7793) and stored at 4°C . Prior to sampling, 1 mL aliquots of 50% erythrocytes were transferred to 4 mL glass autosampler tubes (Thermo Fisher Scientific, 03-391-19), closed with screw

caps with septa (Thermo Fisher Scientific 03-391-21), and equilibrated at 38 °C for 15 min. Following equilibration, 2.5 μL of 4 mM HMBPP (final concentration 10 μM) or purified water was added to the erythrocytes, caps were closed, and Parafilm was used to seal the vials. Volatiles were immediately collected from the headspace using solid-phase microextraction ($n = 5$, randomized order for each sample). Directly before sampling, the divinylbenzene/Carboxen/polydimethylsiloxane fiber (Sigma-Aldrich, SKU 57348) was conditioned for 30 min at 225 °C in the inlet of an Agilent 7890A gas chromatograph. Headspace sampling occurred over 30 min with the temperature maintained at 38 °C. Following sampling, the collected volatiles were desorbed onto the injector of the Agilent 7890A gas chromatograph equipped with an Agilent HP-5MS column (30 m, 0.25 mm inner diameter, 0.25 μm film thickness) and interfaced with an Agilent 5975C mass spectrometer. Throughout the run, the inlet temperature was held constant at 225 °C. Injection was performed in the splitless mode for 1 min before split vent opening. The oven program followed a linear temperature gradient with an initial temperature of 60 °C (held for 2 min), a ramp of 10 °C/min until 225 °C, and a final hold for 5 min at 225 °C. Helium was used as the carrier gas with a constant flow rate of 1 mL/min (25.6 cm/s). The transfer line temperature was held constant at 300 °C. Ionization was performed using electron ionization, with an ion source temperature, electron energy, and emission current set at 230 °C, 70 eV, and 300 μA , respectively. Mass spectra were acquired in scan mode between m/z 40.0 and 170.0. α -Pinene was identified on the basis of the retention time of an analytical standard (Sigma-Aldrich, SKU 80605), and its abundance was quantified using the area under the curve of extracted ion 93. Integration was performed in Agilent MassHunter (version B.05.00, build 5.0.519.0) using the Agile integrator. To measure the background contamination of HMBPP with α -pinene, 2.5 μL of 4 mM HMBPP (final concentration 10 μM) was added to 1 mL of erythrocyte storage medium.

An α -pinene standard curve was generated through the addition of 2.5 μL of commercial α -pinene diluted in hexanes to autosampler tubes containing 1 mL of pure water and sampled as with erythrocyte treatments. Tested amounts of α -pinene were 500, 250, 100, 75, 50, 25, 10, and 0 ng (hexanes spiked into water). The standard curve was generated by measuring samples in three independent experiments. The limit of detection was defined as 3 times the area under the curve (ion 93) at the retention time for commercial α -pinene in negative controls containing only water and sampled as with erythrocytes.

Measuring α -Pinene Time-Dependent Concentration.

To measure α -pinene loss over time, 14 mL of washed erythrocytes was placed in a 15 mL conical vial (Sigma-Aldrich, SKU CLS430791) and stored at 4 °C. For some experiments, analytical α -pinene, diluted in water, was added to a final concentration of 10 ng/mL in erythrocytes at the time of aliquoting. During the experiment, erythrocytes were stored, capped, and placed on ice, and samples were iteratively taken. Volatile collection and GC–MS analysis proceeded as above.

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REFERENCES

- (1). Alavi Y, Arai M, Mendoza J, Tufet-Bayona M, Sinha R, Fowler K, Billker O, Franke-Fayard B, Janse CJ, Waters A, and Sinden RE (2003) The dynamics of interactions between *Plasmodium* and the mosquito: a study of the infectivity of *Plasmodium berghei* and *Plasmodium gallinaceum*, and their transmission by *Anopheles stephensi*, *Anopheles gambiae* and *Aedes aegypti*. *Int. J. Parasitol* 33, 933–943. [PubMed: 12906877]
- (2). Koella JC, Sørensen FL, and Anderson RA (1998) The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proc. R. Soc. London, Ser. B* 265, 763–768.
- (3). Lacroix R, Mukabana WR, Gouagna LC, and Koella JC (2005) Malaria infection increases attractiveness of humans to mosquitoes. *PLoS Biol.* 3, e298. [PubMed: 16076240]
- (4). Busula AO, Bousema T, Mweresa CK, Masiga D, Logan JG, Sauerwein RW, Verhulst NO, Takken W, and de Boer JG (2017) Gametocytemia and attractiveness of *Plasmodium falciparum*-infected Kenyan children to *Anopheles gambiae* mosquitoes. *J. Infect. Dis* 216, 291–295. [PubMed: 28859429]
- (5). Batista EP, Costa EF, and Silva AA (2014) *Anopheles darlingi* (Diptera: Culicidae) displays increased attractiveness to infected individuals with *Plasmodium vivax* gametocytes. *Parasites Vectors* 7, 251. [PubMed: 24885914]
- (6). De Moraes CM, Stanczyk NM, Betz HS, Pulido H, Sim DG, Read AF, and Mescher MC (2014) Malaria-induced changes in host odors enhance mosquito attraction. *Proc. Natl. Acad. Sci. U. S. A* 111, 11079–11084. [PubMed: 24982164]
- (7). Cornet S, Nicot A, Rivero A, and Gandon S (2013) Malaria infection increases bird attractiveness to uninfected mosquitoes. *Ecol. Lett* 16, 323–329. [PubMed: 23205903]
- (8). Schaber CL, Katta N, Bollinger LB, Mwale M, Mlotha-Mitole R, Trehan I, Raman B, and Odom John AR (2018) Breathprinting reveals malaria-associated biomarkers and mosquito attractants. *J. Infect. Dis* 217, 1553–1560. [PubMed: 29415208]
- (9). Berna AZ, McCarthy JS, Wang XR, Michie M, Bravo FG, Cassells J, and Trowell SC (2018) Diurnal variation in expired breath volatiles in malaria-infected and healthy volunteers. *J. Breath Res* 12, 046014. [PubMed: 30129561]
- (10). De Moraes CM, Wanjiku C, Stanczyk NM, Pulido H, Sims JW, Betz HS, Read AF, Torto B, and Mescher MC (2018) Volatile biomarkers of symptomatic and asymptomatic malaria infection in humans. *Proc. Natl. Acad. Sci. U. S. A* 115, 5780–5785. [PubMed: 29760095]
- (11). Berna AZ, McCarthy JS, Wang RX, Saliba KJ, Bravo FG, Cassells J, Padovan B, and Trowell SC (2015) Analysis of breath specimens for biomarkers of *Plasmodium falciparum* infection. *J. Infect. Dis* 212, 1120–1128. [PubMed: 25810441]
- (12). Robinson A, et al. (2018) *Plasmodium*-associated changes in human odor attract mosquitoes. *Proc. Natl. Acad. Sci. U. S. A* 115, E4209–E4218. [PubMed: 29666273]
- (13). de Boer JG, Robinson A, Powers SJ, Burgers SLGE, Caulfield JC, Birkett MA, Smallegange RC, van Genderen PJJ, Bousema T, Sauerwein RW, Pickett JA, Takken W, and Logan JG (2017) Odours of *Plasmodium falciparum*-infected participants influence mosquito-host interactions. *Sci. Rep* 7, 9283. [PubMed: 28839251]
- (14). Kelly M, Su C-Y, Schaber C, Crowley JR, Hsu F-F, Carlson JR, and Odom AR (2015) Malaria parasites produce volatile mosquito attractants. *mBio* 6, e00235–15. [PubMed: 25805727]
- (15). Stotzky G, Schenck S, and Papavizas GC (1976) Volatile organic compounds and microorganisms. *CRC Crit. Rev. Microbiol* 4, 333–382. [PubMed: 780055]
- (16). Cane DE, and Ikeda H (2012) Exploration and mining of the bacterial terpenome. *Acc. Chem. Res* 45, 463–472. [PubMed: 22039990]

- (17). Yamada Y, Kuzuyama T, Komatsu M, Shin-Ya K, Omura S, Cane DE, and Ikeda H (2015) Terpene synthases are widely distributed in bacteria. *Proc. Natl. Acad. Sci. U. S. A* 112, 857–862. [PubMed: 25535391]
- (18). Ditengou FA, Müller A, Rosenkranz M, Felten J, Lasok H, van Doorn MM, Legué V, Palme K, Schnitzler JP, and Polle A (2015) Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat. Commun* 6, 6279. [PubMed: 25703994]
- (19). Mann FM, and Peters RJ (2012) Isotuberculosinol: the unusual case of an immunomodulatory diterpenoid from *Mycobacterium tuberculosis*. *MedChemComm* 3, 899–904. [PubMed: 23926455]
- (20). Beran F, Rahfeld P, Luck K, Nagel R, Vogel H, Wielsch N, Irmisch S, Ramasamy S, Gershenzon J, Heckel DG, and Kollner TG (2016) Novel family of terpene synthases evolved from trans-isoprenyl diphosphate synthases in a flea beetle. *Proc. Natl. Acad. Sci. U. S. A* 113, 2922–2927. [PubMed: 26936952]
- (21). Gilg AB, Tittiger C, and Blomquist GJ (2009) Unique animal prenyltransferase with monoterpene synthase activity. *Naturwissenschaften* 96, 731–735. [PubMed: 19277597]
- (22). Lancaster J, Khrimian A, Young S, Lehner B, Luck K, Wallingford A, Ghosh SKB, Zerbe P, Muchlinski A, Marek PE, Sparks ME, Tokuhisa JG, Tittiger C, Köllner TG, Weber DC, Gundersen-Rindal DE, Kuhar TP, and Tholl D (2018) De novo formation of an aggregation pheromone precursor by an isoprenyl diphosphate synthase-related terpene synthase in the harlequin bug. *Proc. Natl. Acad. Sci. U. S. A* 115, E8634–E8641. [PubMed: 30139915]
- (23). Wondwosen B, Birgersson G, Tekie H, Torto B, Ignell R, and Hill SR (2018) Sweet attraction: sugarcane pollen-associated volatiles attract gravid *Anopheles arabiensis*. *Malar. J* 17, 90. [PubMed: 29466989]
- (24). Wondwosen B, Birgersson G, Seyoum E, Tekie H, Torto B, Fillinger U, Hill SR, and Ignell R (2016) Rice volatiles lure gravid malaria mosquitoes, *Anopheles arabiensis*. *Sci. Rep* 6, 37930. [PubMed: 27901056]
- (25). Emami SN, Lindberg BG, Hua S, Hill SR, Mozuraitis R, Lehmann P, Birgersson G, Borg-Karlson AK, Ignell R, and Faye I (2017) A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection. *Science* 355, 1076–1080. [PubMed: 28183997]
- (26). Lange BM, Rujan T, Martin W, and Croteau R (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. U. S. A* 97, 13172–13177. [PubMed: 11078528]
- (27). Goldstein JL, and Brown MS (1990) Regulation of the mevalonate pathway. *Nature* 343, 425–30. [PubMed: 1967820]
- (28). Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, and Jomaa H (2003) Microbial isoprenoid biosynthesis and human $\gamma\delta$ T cell activation. *FEBS Lett.* 544, 4–10. [PubMed: 12782281]
- (29). Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, McWilliam HEG, Hudson C, Tutuka C, Wheatley AK, Kent SJ, Villadangos JA, Pal B, Kurts C, Simmonds J, Pelzing M, Nash AD, Hammet A, Verhagen AM, Vairo G, Maraskovsky E, Panousis C, Gherardin NA, Cebon J, Godfrey DI, Behren A, and Uldrich AP (2020) Butyrophilin 2A1 is essential for phosphoantigen reactivity by $\gamma\delta$ T cells. *Science* 367, No. eaay5516. [PubMed: 31919129]
- (30). Daubert T, and Danner R (1989) *Physical and Thermodynamic Properties of Pure Chemicals: Data Compilation*, Taylor & Francis, Washington, DC.
- (31). Darbonne WC, Rice GC, Mohler MA, Apple T, Hébert CA, Valente AJ, and Baker JB (1991) Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J. Clin. Invest* 88, 1362–1369. [PubMed: 1918386]
- (32). Neote K, Darbonne W, Ogez J, Horuk R, and Schall TJ (1993) Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. *J. Biol. Chem* 268, 12247–12249. [PubMed: 8389755]
- (33). Omondi BA, Majeed S, and Ignell R (2015) Functional development of carbon dioxide detection in the maxillary palp of *Anopheles gambiae*. *J. Exp. Biol* 218, 2482–2488. [PubMed: 26056246]
- (34). Dekker T, Geier M, and Cardé RT (2005) Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. *J. Exp. Biol* 208, 2963–2972. [PubMed: 16043601]

Figure 1.

(A) Metabolic pathways leading to α -pinene. Enzymes are highlighted with salmon boxes. Abbreviations: HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; MPP, mevalonate pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate. (B) Schematic of the α -pinene detection assay. (C) α -Pinene standard curve generated using commercial α -pinene over a range of 500 to 0 ng/mL. Displayed are the means of standards measured in triplicate, and error bars denote the standard deviation. (D) GC-MS traces of commercial α -pinene (bottom) and the erythrocyte headspace (top) for α -pinene parent ion, 93. (E) Mass spectra from retention time 4.77 min, the elution time for commercial α -pinene, for the erythrocyte headspace (top) and commercial α -pinene (bottom).

Figure 2.

(A) Abundances of α -pinene in HMBPP from Echelon Biosciences or Sigma-Aldrich or in vehicle control (water). (B) Erythrocyte α -pinene abundance following treatment with HMBPP or vehicle control (water). The values are not significantly different by the Mann–Whitney U test (Donor 1, $p = 0.841$; Donor 2, $p = 0.548$; Donor 3, $p = 0.420$). All of the assays were performed with $n = 5$. Lines indicate the means of the samples, and error bars indicate the standard error of the mean.

Figure 3.
Abundance of α -pinene in the headspace of untreated human erythrocytes ($n = 5$).

Figure 4.

(A) Schematic of the repeated sampling mechanism. The time between each sampling was 1 h. At each sampling, 1 mL of erythrocytes was removed, and the headspace composition of the removed cells was assessed. (B, C) Headspace concentrations of α -pinene over (B) untreated human erythrocytes and (C) erythrocytes supplemented with 10 ng/mL commercial α -pinene as a function of the number of GC-MS runs (and accordingly the number of tube openings). Displayed are the values of experiments performed in duplicate, with connecting lines indicating values from an individual replicate.