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Full details of the Zikasmell working group are available in the Supplementary Information.

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Supplementary Information

- Supplementary methods
- 1 supplementary table
- 3 supplementary figures
- Zikasmell working group

Abbreviations:

dpi = day post-infection; GBS = Guillain-Barré Syndrome; HDRS = Hamilton Depression Rating Scale; Iba1 = ionized calcium binding adaptor molecule 1; IgM=immunoglobulin M; IQR =

interquartile range; MRC = Medical Research Council; TDI = sum of threshold (T) score, discrimination (D) score, and identification (I) score; ZIKV = Zika virus.

Abstract

Background: Zika Virus (ZIKV) infection has been associated with Guillain-Barré Syndrome (GBS). Yet, little is known about the consequence of ZIKV infection on olfaction in humans.

Methods: Just right before the COVID-19 outbreak, we prospectively investigated the olfactory capacities of 19 patients with ZIKV-associated GBS from the French West Indies and compared them to 9 controls from the same population, with a GBS of similar severity but independent of Zika infection. To provide further evidence that ZIKV infection induces smell alteration, we investigated the consequences of ZIKV infection on olfactory abilities using a mouse model.

Results: Patients with GBS-Zika+ had a poorer olfactory function than GBS-non-Zika, even one to two years after the acute phase. The proportion of patients with hyposmia was significantly higher in GBS-Zika+ than in GBS-non-Zika group (68.4% *versus* 22.2%, *P*=0.042). These deficits were characterized by lower threshold and identification scores and were independent from GBS severity. Additionally, ZIKV infection was found to impair olfaction in immunodeficient mice infected with ZIKV. High viral load was observed in their olfactory system and downstream brain structures. ZIKV promoted both cellular damages in the olfactory neuroepithelium and protracted inflammation of the olfactory bulb, likely accounting for smell alteration.

Conclusions: Patients with ZIKV-related GBS had a poorer long-term olfactory function than patients with GBS-non-Zika and ZIKV-infected mice are hyposmic. These observations suggest that ZIKV belongs to the list of viruses affecting the olfactory system. Clinical evaluation of the olfactory system should be considered for ZIKV-infected patients.

INTRODUCTION

Zika virus (ZIKV) is an emergent *Flavivirus* responsible for large epidemics in the Pacific Islands and South and Central Americas. Guillain-Barré Syndrome (GBS) constitutes the major neurological complication after postnatal ZIKV infection [1-3]. The association of emerging infectious diseases, such as ZIKV infection and COVID-19, with GBS is a topic of intense debates [4]. Olfactory defects (hyposmia/anosmia) have not been reported as a common symptom in ZIKV infection. However, it has been demonstrated that ZIKV infects neurons in human brain organoids [5] and human and mouse adult brain tissue [6, 7]. In mammals, odorants are sensed by olfactory sensory neurons, of which cilia emerge within the olfactory epithelium of the posterior nasal cavity and their axons project into the brain to reach the olfactory bulb. In particular, ZIKV was found to exhibit a tropism for olfactory sensory neurons of the nasal neuroepithelium in immunodeficient mice [8]. The fact that ZIKV infects the peripheral nervous system (PNS) and olfactory tract prompt us to investigate whether olfaction defects occur in patients having experienced GBS after ZIKV infection. From the previous studies, we hypothesis that GBS might be an indicator that the patient was prone to ZIKV infection of the PNS, thus increasing probability for reporting ZIKV-infected olfactory tissues and laying the basis for olfactory deficits. We thus conducted a prospective study to investigate olfaction after a GBS associated with ZIKV infection in the French Caribbean islands of Guadeloupe and Martinique. The clinical part of this study was performed in July 2017-October 2019, just right before the COVID-19 outbreak. To unravel the pathogenesis of ZIKV infection on olfaction, a dedicated mouse model has been used.

METHODS

Study approval

The clinical study was approved by the ethical committee “Comité de Protection des Personnes Sud-Ouest et Outre-Mer 3” under reference 2010-A00282-37, MS10 and the French National Agency for Medicines and Health Product Safety under reference

2017051900102. For inclusion in the Cohort of Patients Infected by an Arbovirus (CARBO-ZIKASMELL ancillary study, NCT01099852), informed consent was obtained for all patients and controls. All animal experiments were performed after approval of protocol from the Institut Pasteur Ethics Committee (dap190107) and authorized by the French Ministry of Research (APAFIS 19469), in compliance with French and European regulations.

Patients and clinical study design

Consecutive patients with ZIKV-related GBS were seen at the University Hospitals of Guadeloupe and Martinique between one and two years after ZIKV infection during the French West Indies 2016 outbreak ("GBS-Zika+" group). The control group was formed by patients with non-ZIKV GBS patients seen at the same place in January 2018-October 2019, after the Zika epidemics ("GBS-non-Zika" group).

Diagnosis of ZIKV infection

As previously defined by the Pan American Health Organization and the World Health Organization [9], patients were considered to have confirmed ZIKV recent infection in the presence of: i) detection of viral genome in urine, plasma, or CSF samples; or ii) detection of immunoglobulin M (IgM) for ZIKV and plaque reduction neutralization test positive for ZIKV; or probable recent ZIKV infection in the presence of ZIKV IgM and no dengue IgM, or suspected recent ZIKV infection in the presence within the previous month of a clinical picture consistent with ZIKV (rash with two or more of the following signs or symptoms: fever, arthralgia, myalgia, conjunctivitis or edema). The acute phase of patients of the GBS-Zika+ group has been described in previous publications for nine patients from Guadeloupe [2] and nine patients from Martinique [2,10].

GBS diagnosis and severity assessment

Diagnosis of GBS was made by a neurologist based on clinical profile, CSF analysis and neurophysiological examination [2,11]. The level of GBS diagnostic certainty was classified ac-

cording to the Brighton criteria [12]. Motor deficit severity was rated according to the MRC score [13].

Olfactory assessment

The T, D and I score

The Sniffin' Sticks test was used to assess olfactory functions [14]. It provides a validated quantification of threshold (T) score, discrimination (D) score, identification (I) score, and a total score (T+D+I=TDI total score) [15].

Olfactory Pleasantness

During the Sniffin' Sticks "I" subtest, patients were asked for the *Pleasantness* of the 16-odor smelling [16]. The sum of the number of odors rated as pleasant, unpleasant or neutral was 16.

Memory assessment

Since memory impairments are associated with olfactory dysfunctions [17], the memory immediate recall task from the Wechsler Memory Scale – Revised [18] has been rated.

Depression intensity

Since olfactory dysfunctions are associated with depression [16], the Hamilton Depression Rating Scale 17 items (HDRS) [19] was rated.

The mouse model

Mice deficient for the receptors to interferon a/b receptor and interferon g (homozygous for the *Ifnar1*^{tm1Agt} and *Ifngr1*^{tm1Agt} alleles on the 129S2/SvPas background, named AG129) were bred under specific-pathogen-free conditions.

Asian ZIKV strain FG15 was isolated from a patient during a ZIKV outbreak in French Guiana in December 2015 and obtained from the Virology Laboratory of the Institut Pasteur of French Guiana, as previously described [20]. Twenty-four 6-7-week-old AG129 mice were

housed by groups of 3-6 animals in isolators in a Biosafety level-3 facility. Animals were anesthetized with an intraperitoneal injection of 70 mg/kg ketamine (Imalgène 1000, Merial) and 2 mg/kg xylazine (Rompun, Bayer), and 20 µL of 0.9% sodium chloride containing 10^6 focus-forming units of ZIKV was administered intranasally to six male and six female mice (10 µL/nostril) as previously described [21]. Mock-infected mice (six males, six females) received 20 µl of 0.9% sodium chloride intranasally.

At predefined days post-infection (dpi), animals were submitted to behavioral tests or blood sampling for plasma viral load assessment. All mice were killed by cervical dislocation at 11 dpi. For half of the mice, whole heads (after removal of skin, upper cranium, and lower jaw) were collected and formalin-fixed (formalin solution 10% neutral buffered (HT-5011-1CS, Sigma). For the other half, samples of nasal turbinates and the brain (separated in olfactory bulbs, cerebellum, cortex and brainstem) were collected and immediately frozen at -80°C.

Buried food-finding test

To assess olfaction, we used the buried food-finding test as previously described [22]. Twenty hours before testing, mice were fasted and individually placed into a clean cage (37 x 29 x 18 cm) with autoclaved bedding (pine shaving) for 20 minutes. Mice were then placed in another, identical cage for 2 minutes with a pellet of familiar food hidden in 1.5 cm bedding at the corner of the test cage. Then, mice were placed in the opposite corner and the latency to find the food (defined as the time to locate cereals and start digging) was quantified using a chronometer. The test was carried out during a 15 min period. Mice were removed from the cage as soon as they found the food. One minute later, mice performed the same test but with a visible pellet positioned upon the bedding. Then mice performed the same test but with about 5-10 pieces of chocolate cereals (Coco pops, Kellogg's) hidden under the bedding.

Scanning electron microscopy

Fixed samples were dissected and post-fixed by incubation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. As previously described, they were processed by alternating incubations in

1% osmium tetroxide and 0.1 M thiocarbohydrazide [23]. After dehydration, samples were dried, mounted on a stub, and analyzed by field emission scanning electron microscopy with a Jeol JSM6700F operating at 3 kV (see the Supplemental material for details).

Immunofluorescence

Forty-micrometer coronal sections of fixed brains were obtained using a vibrating microtome (VT1000S, Leica). After epitope retrieval and blocking, sections were incubated with rabbit anti-ionized calcium-binding adaptor molecule (Iba1; 1/500, Wako Chemicals, 016-20001) antibodies and then with the appropriate secondary antibodies (Alexa Fluor 568-labeled anti-rabbit, A11036; Invitrogen). Sections were counterstained with Hoechst (H3570, Invitrogen) before observation with a Zeiss LM 710 inverted confocal microscope (see the Supplementary information for details). Quantitation of cells was performed using the Icy open source platform [24].

ZIKV RNA levels from mouse plasma and tissues

Viral RNA was extracted, reversed transcripted, and quantified by PCR as previously described (forward primer 5′-CCG CTG CCC AAC ACA AG- 3′; reverse primer 5′-CCA CTA ACG TTC TTT TGC AGA CAT-3′; probe: 5′-6-carboxyfluorescein-AGC CTA CCT TGA CAA GCA ATC AGA CAC TCA A-MGB-3′) [20]. Details are provided in the Supplementary Information.

Statistics

Quantitative variables were summarized as median with interquartile range (IQR) and compared across groups using Mann-Whitney non-parametric test. Categorical data were expressed as percentages and compared using Fisher exact test between groups. To study the associations between demographical and clinical characteristics, and olfactory function, Spearman non-parametric test was used. In animal experiences, time to event was analyzed using Kaplan-Meier estimates and compared across groups using the Logrank test. Statistical

analyses were performed using Prism software (GraphPad, version 9, San Diego, USA), significance was considered at the level 5%.

RESULTS

Olfactory dysfunction in ZIKV-associated GBS

One to two years after GBS, olfactory performances were evaluated in 19 patients with ZIKV-associated GBS ("GBS-Zika+" group) at the University Hospitals of Guadeloupe and Martinique and compared to nine controls with a GBS of similar severity but not related to ZIKV ("GBS-non-Zika" group) (Table 1). The diagnosis of recent ZIKV infection was biologically supported at the disease onset in all GBS-Zika+ cases: 12/19 by RT-PCR, 1/19 by immunoglobulin (Ig) M ZIKV serology, 6/19 by IgG ZIKV serology, and Zika typical symptoms within the previous month (Supplemental Table 1). The control group was formed by patients with non-ZIKV GBS seen at the same place in January 2018-October 2019, after the Zika 2016 epidemics.

While general characteristics of the GBS-Zika+ and GBS-non-Zika groups were similar (Supplemental Table 1), ZIKV-associated GBS had lower olfactory scores (TDI, median [IQR]: 24.3 [18.5-32.3] and 34.3 [28.4-36.5], *P* =0.005, Figure 1A). The proportion of patients with hyposmia (a TDI<31) was significantly higher in GBS-Zika+ than in GBS-non-Zika group (68.4% and 22.2%, *P*=0.042, Table 1). The proportion with major hyposmia (a TDI<24) was significantly higher in GBS-Zika+ than in GBS-non-Zika group (47.4% and 0.0%, *P*=0.026, Table 1). ZIKV-associated GBS had lower olfactory threshold scores (T, median (IQR): 3.3 [2.0-6.3] and 8.0 [6.3-10.1], *P*=0.002, Figure 1B), and the proportion of patients with olfactory perception deficit (T<6) was significantly higher in GBS-Zika+ than in GBS-non-Zika group (73.7% and 11.1%, *P*=0.004, Table 1). The proportion of patients with olfactory identification deficit (I<11, Figure 1D) was significantly higher in GBS-Zika+ than in GBS-non-Zika group (47.4% and 0.0%, *P*=0.026, Table 1). There was no difference between the two groups for the Medical Research Council (MRC) score, the olfactory discrimination score, the pleasantness score of odorants, the memory task scores, and the depression severity (Table 1). Finally, there was a correlation between the olfactory score and age in the two groups (GBS-Zika+: *r*=-

0.459, *P*=0.049; GBS-non-Zika: Spearman *r*=-0.745, *P*=0.026). There was no relation between olfactory score and delay between Zika and the olfactory test (GBS-Zika+: *r*=0.013, *P*=0.959; GBS-non-Zika: *r*=0.008, *P* =0.987).

Olfactory dysfunction in ZIKV-infected mice

To assess whether ZIKV infection leads to the olfactory deficit, we intranasally inoculated AG129 mice of both sexes with 10^6 plaque forming units of ZIKV and followed them up between 24 hours and 11 dpi. These mice were chosen since they cannot respond to type-I and type-II interferons, making them available for viral infections and thus clinically relevant to study Zika infection [6]. All infected mice looked healthy until 7 dpi. The first symptoms appeared on day 8 with decreased body weight (Figure 2A) and neurological signs such as toe walking, tremors, loss of balance and partial or total paralysis of hindlimbs. Then, disease severity increased rapidly. On day 11, all mice were moribund. They were euthanized and necropsied. High viral loads were detected in the plasma of infected mice at 2, 7, and 11 dpi (Figure 2B), consistent with previous findings after intranasal inoculation [21]. High viral loads were detected at 11 dpi in the olfactory mucosa and in various brain parts, including the olfactory bulb, cerebral cortex, brainstem and cerebellum (Figure 2C).

Olfactory performances were assessed in ZIKV-inoculated mice at 1, 3, 8 and 10 dpi (Figure 2 D-G). We found that infected mice of both sexes exhibited signs of hyposmia/anosmia at 8 and 10 dpi during food findings experiments. Indeed, they needed more time to find buried food than uninfected mice, and a substantial proportion of them (22% at 8 dpi and 33% at 10 dpi) failed to find the food during the test (Figure 2 F, G and Supplemental Figure 1). Nevertheless, all infected mice succeeded in finding visible food (Figure 2 F, G) despite sickness behavior or locomotor deficit.

Imaging by scanning electron microscopy revealed cellular damages in the olfactory epithelium of infected mice (Figure 3 A-C), which might contribute to the observed hyposmia. Cellular damages and viral particles were also observed in the eyes of the same animals, but neither in the respiratory epithelium nor in the ear (Supplemental Figure 2). Finally, we investi-

gated the neuroinflammation in brain tissues, which exhibited high viral loads (Figure 2C). Robust neuroinflammation, with numerous Iba1 positive microglial cells, were found in the olfactory bulb (Figure 3 D-I). A robust neuroinflammation was also observed in the dentate gyrus of the hippocampus and the cerebellum of infected mice (Supplemental Figure 3).

DISCUSSION

By combining investigations of mice experimentally infected with ZIKV and data from GBS-Zika+ patients, we found that ZIKV infection was responsible for hyposmia, with GBS-Zika+ patients exhibiting long-term deficits. These sequels are characterized by lower threshold and identification scores but are independent of GBS severity. This pioneering study indicates that olfactory disorders can be part of the neurological spectrum of neuroZika. The strengths of this study are: i) its sample size, taking into account the absolute rarity of GBS-Zika+, ii) the comparison to a control group from the same population, with GBS of similar severity, but not linked to Zika, and iii) its translational nature allowing the generation of hypothesis about the pathogenesis.

Limitations of our study include the olfactory assessment at distance of the GBS onset. Extending these investigations to a group of patients with *de novo* and early ZIKV-associated GBS would clarify the implications of the current findings. Moreover, this study could be underpowered to detect some olfactory differences, such as olfactory discrimination and pleasantness. Another limitation of our study is the use of AG129 mice for modeling ZIKV-induced hyposmia. These mice, dramatically susceptible to ZIKV attack, do not survive beyond 10-12 dpi, limiting studies of long-term infection consequences [6]. Less severe models such as Collaborative Cross strains may provide relevant mouse resources for investigating delayed ZIKV-induced smell impairment mechanisms [20].

It is generally believed that olfactory threshold impairment corresponds to a PNS disorder whereas impairment of olfactory identification, when tested with suprathreshold concentra-

tions of odors, rather reflects dysfunction of the central nervous system (CNS). This CNS involvement is unexpected in a PNS disorder such as a Guillain-Barré, although a combination of CNS and PNS disorders has been already reported for neuroZika [2].

The mechanisms underlying olfactory dysfunctions are unknown in this setting. To ensure proper olfactory system homeostasis, new neurons are continuously added to the olfactory epithelium and the olfactory bulb. We found sustained ZIKV infection in the olfactory neuroepithelium and the olfactory bulb of infected mice with protracted inflammation. We thus hypothesized that smell loss in GBS-Zika+ patients could result from a post viral sensorineural dysfunction, which might involve a chronic inflammatory process, interfering with the turnover of olfactory sensory neurons [25]. Although we focus on olfactory dysfunctions in GBS-Zika+ patients, animal studies may suggest that olfactory abilities are impaired even in Zika+ patients without GBS. Extending these investigations to ZIKV-infected patients, with and without neurological symptoms, should characterize these initial findings.

Patients with ZIKV-related GBS had a poorer long-term olfactory function than patients with GBS-non-Zika and ZIKV-infected mice are hyposmic. These observations suggest that ZIKV belongs to the list of viruses affecting the olfactory system such as Epstein-Barr virus, cytomegalovirus [22, 26], and several other respiratory viruses including rhinovirus, influenza, and SARS-CoV-2 [23]. Clinical evaluation of the olfactory system should be considered for ZIKV-infected patients. It is important for clinical practice as these patients may benefit from dedicated olfactory rehabilitation programs through smell training [27, 28].

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CONFLICTS OF INTEREST

Authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: FL; Methodology: FL, YM, AL, XM, ER; Investigation: FL, AL, AC, VM, HC, IC, MF, XM, ER, the Zikasmell Working group; Funding acquisition: AC, FL, PML; Project administration: FL; Supervision: FL, AL, AC, XM, PML; Writing – original draft: FL; Writing – review & editing: FL, AL, ER, XM, PML

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request. The data are not publicly available because they contain information that could compromise the privacy of our patients.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplementary methods.

Supplementary Table 1: Characteristics of the GBS-Zika+ and GBS-non-Zika Groups at the Disease Onset.

Supplementary Figure 2: Scanning electron microscope imaging of the respiratory epithelium, cochlea and eye following ZIKV infection.

Supplementary Figure 3: ZIKV promotes brain neuroinflammation in AG129 mice.

ZikaSmell Working Group.

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Table and figure legends.

Table 1. Demographics, Clinical Characteristics, olfactory screen, psychometric and memory tests.

Figure 1. Threshold, Discrimination, Identification and TDI Olfactory Scores in ZIKV-associated GBS Patients and GBS-non-Zika controls.

Panels A-D show the TDI (A), the olfactory threshold (B), the discrimination (C) and identification (D) scores in ZIKV-associated GBS patients and non-ZIKV-associated GBS controls. TDI, total score= T (olfactory threshold score) + D (olfactory discrimination score) + I (olfactory identification score). Box showing median, 25 percentile and 75 percentile, and whiskers showing minimum/maximum in bar graphs. $P<0.05$ are shown. In gray is the area under the 10 percentile for healthy controls that defines the criteria for hyposmia (normative values from Oleszkiewicz et al., 2019) [14]. Red points represent patients with declared hyposmia.

Figure 2. Clinical, molecular and behavioral characteristics of ZIKV-infected AG129 mice.

(**A**) Variation in body weight of mock- and ZIKV-infected AG129 mice at 11 days post-infection (dpi). (**B, C**) Quantification of ZIKV RNA in plasma (**B**) and in different brain areas (**C**) of mock- and infected-animals at 2, 7 and 11 dpi. (**D-G**) Fraction of mock or infected mice successfully finding hidden or visible food in 15 minutes. Food-finding assays were performed at 1, 3, 8- and 10-dpi. Results in (A) are expressed as the mean ± standard deviation. Mann-Whitney test comparing infected animals to mock (A), Mann-Whitney test (B, C), and Log-rank (Matel-Cox) test (D-G). n=12/ timepoint in (A, B, D-G); n=6/timepoint in (C); P value is indicated in bold when significant. ***$P<0.001$.

Figure 3. ZIKV promotes cellular damages in the olfactory epithelium and induces neuroinflammation of the olfactory bulb in infected AG129 mice.

(**A**) Dissected mouse head, sagitally cut in half, showing the close relationship between the olfactory epithelium, the olfactory bulb and the cortex. (**B-C**) Scanning electron microscope imaging showing changes in olfactory epithelium following ZIKV infection at 11dpi. (**D-E**) Coronal sections showing Iba1+ cells in the olfactory bulb of mock and ZIKV infected mice at 11 dpi. Images are maximum intensity projection over a 26-µm depth. (**F**) Iba + cell density in the granule cell layer, the external plexiform layer and the glomerular layer of the olfactory bulb. Horizontal red lines indicate the medians. N=4 mock (2 males, 2 females) and n=4 ZIKV (2 males, 2 females) at 11 dpi in (F). Mann-Whitney test (F); the P value is indicated in bold when significant. Scale bars: 100 µm (D-E), 5 µm (D1-E1), 2 µm (B, C), 500 nm (C1).