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HEALTH SCIENCES

Immunological Memory to Zika Virus in a University Community in Colombia, South America

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Abstract: Zika virus appeared in South America in 2015, generating alarm worldwide as it causes microcephaly and autoimmunity. This study aims to determine the serological footprint of the incoming epidemic in a student community and to characterize the memory functional cell response during post convalescence. In a cross-sectional study, Zika-specific IgG using LIA immunoassay was found in 328 university students (CI=95%), while in the second phase, the functional cellular memory response for IFN-γ and IL-2 was quantified using post-stimulus ELISpot with inactivated virus, starting with individuals seropositive for Zika and control individuals (seropositive only for Dengue and seronegative for Zika-Dengue). Depending on the antigen used, memory humoral response (IgG) against Zika Virus was observed in >60% of the population; seropositivity for NS1 was 21.1% higher than E antigen with high intensity. The analysis of cell functionality in 22 individuals seropositive for Zika virus revealed either IFN-γ+ or IL-2+ cells in 86.3% of cases (Th1 profile), presenting multifunctionality in 50% (11 individuals), 64% of which presented> 6 SFC/10^4 PBMCs (>600 SFC/10^6 PBMC), reflecting memory circulating cells. A good agreement (Kappa= 0.754) was observed between the coexistence of both cellular and humoral responses but not in their intensity.

Key words: cytokines, flavivirus infection, immunological memory, seroepidemiologic studies, Zika virus.

INTRODUCTION

Zika Virus (ZIKV) is an arbovirus (Flaviviridae family) that comes from the African continent (Uganda, 1947), which is transmitted by the *Aedes aegypti* vector (Song et al. 2017), and that has been associated for six decades to non-relevant infections in such equatorial zone (Heinz & Stiasny 2017, Song et al. 2017). It was not after subsequent epidemics in the Pacific Islands and Australia (Kindhauser et al. 2016, Weaver & Reisen 2010), that Zika virus entered the American continent through northeastern Brazil, apparently from 2013 onwards, but first cases were reported in May 2015 (Fritzell et al. 2018, Petersen et al. 2016), being associated with altered fetal brain development and neuropathies such as Guillain-Barré Syndrome (GBS) (Broutet et al. 2016, Campos et al. 2015, Coyne & Lazear 2016, Metsky et al. 2017, Muñoz et al. 2016, Zanluca et al. 2015).

When entering the Americas, this new pathogen was reported, in Brazil alone, in 440.000-1.300.000 suspicious cases and more than 4.000 cases of possible associated microcephaly between September 2015 and

February 2016 (Victora et al. 2016). In October 2015, Zika virus was newly reported in Colombia with 91.156 suspicious cases, of which 8.221 were confirmed (9.0%), from epidemiological week 40 of 2015 to week 22 of 2016 (WHO 2016a). This is without including any subrecords since the number may be bigger in areas of co-circulation with other arboviruses such as Dengue virus (DENV) and Chikungunya virus (CHIKV), as previously reported (Roth et al. 2014).

In Colombia, 58% of suspicious cases and 53% of confirmed cases were concentrated in five out of 32 departments starting from epidemiological week 32 of 2015 to week 52 of 2016. North of Santander ranked the first position (during epidemic phase), registering an incidence rate of 765.72 cases every 100.000 inhabitants, which almost tripled the national rate (277.29 cases every 100.000 inhabitants) (SIVIGILA 2016). After this, over the course of 2018 (endemic phase), clinically confirmed cases continued to be reported in the country and the department as well (601 and 39 cases, respectively) (SIVIGILA 2018).

Zika virus infection (ZIKV) is asymptomatic in 80% of cases but associated with complications such as fetal malformation and autoimmune diseases (Cao-Lormeau et al. 2016, Coyne & Lazear 2016). During the epidemic phase in the Caribbean, the WHO declared the outbreak an international public-health emergency (WHO 2016b).

The immune response of the individual is essential for their protection. However, the interference in this response for ZIKV, attributable to the preexistence of immunoglobulins from other flaviviruses such as DENV, has demonstrated that it can either counteract the infection, even allowing its control, as observed during the ZIKV emergence (Netto et al. 2017), or strengthen the infection since its association with the disease worsening or transmission in endemic areas for various flaviviruses has been widely discussed (Heinz & Stiasny 2017, Lessler et al. 2016), in relation to the phenomenon known as "Antibody-Dependent Enhancement", ADE (Durbin 2016). This cross-reactivity appears to be higher during the convalescent phase (about 67%) compared to post-convalescence (28%), as described, compared to NS1 protein (Tsai et al. 2017).

Moreover, the role of the inhibitor of ZIKV replication by interferons is well known (Hamel et al. 2016) where specific T cells adopt functional profiles such as Th1, characterized by the production of IL-2, TNF-α and IFN-γ cytokines (Ngono et al. 2017, Pardy et al. 2017), besides showing their proliferation and cytotoxicity capacity by direct cytolysis, Granzyme B production and exposure to LAMP-1 or CD107a, as observed in the murine model (Chahal et al. 2017, Ngono et al. 2017). Thus, the reveal of such functionality in individuals with history of ZIKV infection is important for the capacity to control future infections at the expense of cellular memory (Winkler et al. 2017), which in conjunction with humoral memory, emphasizes the requirement of quantity and quality of adaptive response. This was demonstrated by experiments for murine immunization, whose generation of neutralizing antibodies was correlated with protection and survival against infection with Zika Virus-Like Particles (VLPs), both through active immunization and passive transfer (Salvo et al. 2018).

Until recently, the functionality of the cellular response to ZIKV in human individuals became known, referring to, for example, the existence of a low frequency of CD4+ IFN-γ+ T cells during acute ZIKV infection, whose cellular response focused on CD8 T lymphocytes and double-negative T lymphocytes, with significant production of IL-2 and IFN-γ, respectively, in relation to healthy donors. These parameters

were evaluated after *ex vivo* polyclonal stimulations (Cimini et al. 2017, de Sousa Barros et al. 2018).

The overexpression of 32 genes in CD8 T lymphocytes exposed to ZIKV multimer (containing previously identified immunogenic peptides), which are related to cytotoxicity, T regulation-activation, inflammation, homing and cytokine production, including TNF-α and IFN-γ in the latter group (considered by the authors to be a representative molecule of the response of ZIKV-specific CD8 T lymphocytes), has been referred to in convalescing individuals (Grifoni et al. 2018).

Moreover, the existence of a possible modulating capacity of ZIKV for the production or function of type I interferons from mononuclear cells has been known (Bowen et al. 2017).

Similarly, an increase in the profile of both pro-inflammatory and plasma regulatory mediators has been previously described. In a study conducted in 36 individuals with acute ZIKV infection in Brazil, IL-2, IFN-γ and ZIKV-induced IP-10 chemokine were identified, among others, in relation to the presence of symptoms in individuals (de Sousa Barros et al. 2018). The pro-inflammatory profile in response to the infection produced by this flavivirus also appears to characterize the innate immune response (evidence of strong IL-1β induction) (Wang et al. 2018).

It is now required to identify the quantitative and qualitative characteristics of the cellular memory response against ZIKV based on a model that involves the specific viral stimulation of circulating mononuclear cells by using markers associated with both central memory and effector memory T cells, which reveals the panorama of the long-term immune status in a primoinfected individual.

The University of Santander (Universidad de Santander) campus located in the city of Cucuta, Colombia constitutes an environment with conducive geographical and environmental (typical) conditions for the existence of the *Aedes* sp., whose presence has been constantly reported.

This study aimed to assess the effect (in terms of seroprevalence) of the incoming ZIKV epidemic in a community in the northeastern region of Colombia, South America, as well as to determine the functional characteristics of the adaptive cellular response against the virus and its relationship with the humoral response, given the relevance of the event in our region since the American continent has been considered a potential endemic zone for flavivirus, due to the permanent circulation of the vector (Holbrook 2017, Song et al. 2017).

MATERIALS AND METHODS

It is a cross-sectional descriptive correlational study.

Study population

Based on a population of 2.300 students enrolled in the University of Santander, 328 students were included (CI = 95%) by probabilistic (random) sampling, including other departments of the School of Health Sciences (n = 1.380, 60% of the students enrolled) and other different schools (n = 920, 40% of the students enrolled). Participation was voluntary, subject to previously signed informed consent in compliance with ethical standards.

This research was conducted following the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects, which was amended by the 64th WMA General Assembly in Fortaleza, Brazil in October 2013 on issues such as Risks, Privacy and Confidentiality, Research Protocols

and Research Ethics Committees. In this regard, this study represented a minimal risk for the participants since venous puncture was performed by qualified health personnel with a permanent observation of the participants during and after the procedure. The protection of health, dignity, integrity, privacy and personal information confidentiality of the participants was ensured by assigning an internal numerical code to each participant, also ensuring the bioanalysis blind control. Additionally, both the informed consent and a complete protocol containing the methodological description of the research were presented and approved by the Institutional Bioethics Committee (created as per Agreement 08 of the University's Superior Council in October 2012). It is necessary to underline that this committee is an independent body from researchers, as well as from the sponsor of this study, which ensures its transparent operation.

Serological analysis

Once consent for participation in the research was given, a sample of 6 mL whole blood was collected from each individual, of which serum was obtained, being later preserved at -80° C until its processing. The humoral response was measured by detecting ZIKV-specific IgG using the Line Immuno Assay (LIA) analytical method for two viral antigens: non-structural protein (NS1) and envelope structural E protein. The recomLine Tropical Fever IgG commercial kit (Mikrogen Diagnostik, Germany) was used following manufacturing instructions.

In addition, two control sera were traced (not included in the population sample), in support of sensitivity and specificity of the analytical technique used as follows: a first serum was taken from an individual with a history of past ZIKV infection (clinical and laboratory diagnosis), whose result was obtained by means of the technique used in the study (LIA). The result was ZIKV+DENV- (reactivity for ZIKV anti-NS1 IgG). A second serum was taken from an individual with a diagnosis of DENV infection (positive IgG, previously traced by ELISA Capture technique in External Laboratory, without reactivity for ZIKV), whose result was obtained by means of the technique used in the study (LIA). The result was ZIKV-DENV+ (reactivity for DENV anti-NS1 and anti-E IgG).

Stimulation of human PBMC with ZIKV

Peripheral Blood Mononuclear Cells (PBMC) were obtained from heparinized whole blood from 36 individuals seropositive for ZIKV and DENV by density gradient centrifugation using Histopaque®-1077 (Sigma® Life Science, United Kingdom). 4 mL of whole blood was diluted with the same quantity of PBS pH 7.4 (Thermo Fischer Scientific, USA) under sterile conditions and then with 4 mL of Histopaque®-1077 solution and centrifuged at 400Xg for 30 minutes ideally at 4°C. The interface containing the mononuclear cells was recovered and three washes were done using RPMI 1640 medium, supplemented with HEPES and L-Gln (LonzaTM BioWhittakerTM, USA) and centrifuged at 400Xg for 10 minutes each wash. The pellet was resuspended in the same medium. Equal parts of the cell suspension were mixed with trypan blue (Merck KGaA, Germany) for cell viability/counting. Then, 10^4 viable PBMCs in RPMI 1640 medium (100uL) were stimulated with 10^5 ZIKV PFU, PRVABC59 strain, Asian/American Lineage inactivated with Ultraviolet light at intensity of 100mJ per cm^2 (Lum et al. 2018), for 20 minutes, at a 1:10 ratio (PBMC:PFU) (Lum et al. 2018), on a plate presensitized with monoclonal antibody against each INF-γ and IL-2 cytokine (as described below), overnight at 37° C, CO_2 at 5%. A duplicate assembly was performed in conjunction with negative controls (no viral stimulus, overnight

at 37°C, CO_2 at 5%) and positive controls (cells stimulated with PhorbolMyristate Acetate, PMA-Merck KGaA, Germany-, 25ng/mL for 4 hours at 37°C) (Cárdenas et al. 2015a).

Cell functionality analysis

Cell functionality analysis was performed by quantifying the secretion of IL-2 and IFN-γcytokines using the ELISpotmethod. The number of cells producing IL-2 and IFN-γcytokine was determined using the ELISpotmethod (R&D Systems®, USA), based on sandwich-type immunoassay for antigen detection, where the secreted cytokines were captured by specific antibodies impregnated in a polyvinylidene fluoride membrane that contains monoclonal antibody (for capturing). Following the manufacturer's indications. The number of ZIKV-specific cells producing each type of cytokine, called Spot Forming Cells (SFC), was established by subtracting the number obtained from the same individual in the assembly without stimulus, based on the determination of the functional capacity using positive control (polyclonal stimulus) in each case.

Statistical analysis

The statistical analysis of the data was based on the preparation of simple frequency distributions and contingency tables in categorical variables with their respective relative frequencies (percentages). Descriptive measures such as average, median, range and standard deviation in numerical variables were calculated. To establish the relationship between the results for the laboratory tests and the sociodemographic variables, the Chi-square test of association and the Fisher's exact test were carried out. The Kappa coefficient was also used to measure the degree of agreement between the humoral response and the cellular response.

The hypothesis contrast for the results of the humoral and cellular response among analyzed groups (seronegative, seropositive for dengue, seropositive for Zika) was carried out using the Mann-Whitney U test with paired data. The established significance level was 0.05 using the Windows SPSS statistics pack version 24.

Qualitative variables of nominal (yes, no) or ordinal (scales or ranges) type, survey question items, serological analysis and sociodemographic variables were considered, as well as quantitative variables: age and frequency of IFN-γ and IL-2 cytokine-producing cells.

RESULTS

Description of the sample

328 individuals (95% CI, universe = 2.300 students of the University of Santander Campus in Cucuta, Colombia) between 16 and 47 years old were counted in the representative sample, with an average of 20.6 ± 4.1 years. 73.5% of the participants were men and 26.5% were women.

79.3% of the students were from academic programs in the School of Health Sciences and 20.7% from other school programs, of which 36.3% were in their third or fourth year in the program (fifth to tenth academic term).

Serological footprint of the ZIKV epidemic

The humoral memory response (IgG) against Zika Virus was determined in the entire population sample, obtaining seroprevalences above 60% depending on the antigenic specificity for the proteins: non-structural NS1 and structural E (viral envelope) proteins. Thus, the seropositivity for NS1 antigen was 21.1% higher than the one found for the E antigen and at the expense of a high-intensity level, which was 1.5 times higher frequency than the intermediate and low levels of response. In total, the seroprevalence for

ZIKV in this study was 86.3% (283 individuals seropositive for either or both antigens).

In general terms, 89% of the seropositive population for NS1 showed an intermediate or high response (2 or 3+), while less than a third (28.6%) part did so with such intensity against the E antigen (Table I).

It is important to point out that in a large majority of students positive for specific IgG against E antigen, 93.8% (200/213) simultaneously presented a response against ZIKV NS1 and 13 showed an exclusive response against the first (by adding the 270 students seropositive against NS1 antigenbrings a total of 283 individuals seropositive for ZIKV).

When comparing the serological findings in terms of age, sex, type of academic program and start date of the program, it was observed that the prevalence of seropositivity increased with age, being higher in students of professional degrees in the School of Health Sciences (83.1%) who were in the fifth semester (academic level) or above (84.9%) at the time of carrying out this study. However, no relevant association was found between the results (seropositivity) and these variables (Table SI - Supplementary Material).

According to the observation, there was higher participation of the population from the Health Sciences programs than from other schools. The above considering that the population from the previous school (School of Health Sciences) is usually the majority in our Institution, clarifying that such participation was simply subject to voluntary participation, as previously specified.

When students were asked about symptoms such as fever, headache, myalgia, eye pain, conjunctivitis, arthralgias, rash and vomiting in any of the periods concurrent with the epidemic phase for ZIKV (October 2015-June 2016), it was found that 10.4% (34 individuals) reported: fever (85.3%), rash (61.8%), headache (56%), arthralgia (53%), myalgia and eye pain (23.5% each), vomiting (20.6%) and only a minority, conjunctivitis (2.9%).

When crossing this information with the evidence of humoral immunological memory (IgG), it was observed that despite there was consistency in most cases (87.5%, 49/56 individuals with medical history), 81.3% of the individuals (221/272) who did not show any symptoms during this period, were seropositive. Therefore, no statistically significant association was found ($p > 0.05$) between the appearance of symptoms in epidemic phase and evidence of past ZIKV infection as it did occur when relating the antecedent diagnostic variable (reference of medical diagnosis) in that period with the evidence of humoral response of specific IgG memory (97% concordance, 92/95 cases), taking as a model the NS1 antigen ($p < 0.01$) (Table SI).

Similarly, the analysis of the association between the antecedent diagnostic variables and the presence of anti-protein E IgG also revealed agreement in 77.9% of the cases (74/95 individuals) with a very significant association ($p = 0.002$).

It is surprising the high percentage of the population (76.4%, 178/233 individuals) that did not refer to a diagnosis (essentially clinical diagnosis) but showed an immunological footprint of infection by the virus. This suggests that the population does not have a reliable reminder of the clinical picture associated with ZIKV infection, but it appears that there was consistency between the medical opinion (based on clinical findings over the epidemic phase, given the limited access to laboratory confirmations for most cases in the region due to the emergence of the virus) and the infection event.

Table I. Zika flavivirus seroprevalence (n = 328).

ZIKV humoral response	Result n (%)				
	Negative	Positive[a]			
		+ (Low)	++ (Medium)	+++ (High)	Total
IgG anti-NS1	58 (17.7)	30 (9.1)	94 (28.7)	146 (44.5)	270 (82.3)
IgG anti-E	115 (35.1)	152 (46.3)	59 (18.0)	2 (0.6)	213 (64.9)

[a] Results on a semi-quantitative scale (recomScan v01 software-Mikrogen Diagnostik, Germany).

Functional cellular response to ZIKV in human population

Based on the recognition of the serological status for ZIKV in the analyzed population and having the results of the additional evaluation carried out for specific IgG against NS1 and E proteins of Dengue virus as well (which showed simultaneous seropositivity for the last one in 268/283 cases ZIKV+, considering that North of Santander is a strongly endemic region for DENV-unpublished data from a parallel study), two subgroups of control individuals were established: one seronegative for ZIKV and DENV viruses (ZIKV-DENV-), another seropositive only for Dengue virus and seronegative for ZIKV (ZIKV-DENV+), and a third group (test group: ZIKV+DENV+) made up of individuals seropositive for ZIKV (and simultaneously positive for DENV mostly), for specific stimulation with suspension of inactive Zika virus (see materials and methods). It should be noted that given the high seroprevalence found against both flaviviruses in the evaluated population, the creation of the latter group was essentially at the expense of individuals with a history of coinfection.

Out of the total number of individuals identified, 14 individuals from control groups and 22 from the test group participated in a second round. Table II illustrates the description of the population groups analyzed and the value of the median functional cells obtained post-stimulus for each of the IFN-γ and IL-2 cytokines evaluated. Individual data per group are shown in Table SII.

The intrinsic cellular functionality parameter for the immunological markers of interest in each individual was established using polyclonal stimulus with PhorbolMyristate Acetate (PMA) as a positive control (see Materials and Methods section), from which the result was considered after specific stimulation.

Similarly, the frequency of functional cells specific to Zika Virus per individual was determined by subtracting the value obtained after stimulation with the Zika virus from that obtained without viral stimulation (concomitant assembly for each case; see material and methods section).

With the aim of establishing similarities between the groups of ZIKV+DENV+ individuals and ZIKV-DENV+ individuals in relation to the DENV IgG seropositivity (to make them comparable), the median of this response was compared for the non-structural and envelope type antigens (Mann-Whitney U Test), finding that there was no statistically difference ($p = 0.823$ and 0.381 for DENV anti-NS1 and anti-E IgG, respectively). The above allowed considering the response of human PBMCs to ZIKV between

Table II. Descriptive variables for cell and humoral response between groups.

Population Group	Descriptive variable	Cell functionality (# SPC[a]/10^4 PBMC[b])		IgG anti-NS1[c] (Humoral response)	IgG anti-E[c] (Humoral response)
		IFN-γ	IL-2		
ZIKV-DENV-	n	7.0	7.0	7.0	7.0
	Media	0.0	0.0	0.0	0.0
	SD[d]	0.0	0.0	0.0	0.0
	Median	0.0	0.0	0.0	0.0
	Range	0.0	0.0	0.0	0.0
ZIKV-DENV+	n	7.0	7.0	7.0	7.0
	Media	0.6	1.9	2.0	0.1
	SD	1.0	3.0	0.6	0.4
	Median	0.0	0.0	2.0	0.0
	Range	0.0 – 2.0	0.0 – 8.0	1.0 – 3.0	0.0 – 1.0
ZIKV+DENV+[e]	n	22.0	22.0	22.0	22.0
	Media	9.4	6.1	2.8	1.3
	SD	21.7	9.3	0.5	0.6
	Median	2.0	3.0	3.0	1.0
	Range	0.0 – 101.0	0.0 – 40.0	1.0 – 3.0	0.0 – 3.0

[a] SFC: Spot Forming Cell; [b] PBMC: Peripheral Blood Mononuclear Cells; [c] Value based on the initial parameters for measuring humoral response intensity from 0 to 3 crosses (0-3+) through semi-quantitative scale: to DENV (middle row) or to ZIKV (last row); [d] Standard Deviation (SPSS v24); [e] correspond to the test group (interest group).

the ZIKV+ vs ZIKV- individuals groups (data per case provided in Table SII).

The cellular response analysis showed immunological memory in 64% (14/22), 73% (16/22) and 50% (11/22) of individuals seropositive for ZIKV (evaluated for this parameter), regarding the production of IFN-γ, IL-2 and both cytokines (multifunctionality) post-viral stimulus, respectively. Therefore, in general, 86.3% (19/22 individuals) of this population group showed some response (Figure 1a and Table SII). Interestingly, there was no evidence of multifunctionality in the ZIKV-DENV+group.

Regarding the response at the expense of IFN-γ only (monofunctional), this cytokine was between 6-20 SFC/10^4 PBMC (moderate intensity) in 9% of this population subgroup (2 individuals) and was higher than 20 SFC/10^4 PBMC (high intensity) in 4.5% (1 individual). 36.4% (8 individuals) did not show any production of this cytokine.

On the other hand, the monofunctional response for IL-2 (exclusively) was 1-5 cells/10^4 PBMC (low intensity) in 18.2% (4 individuals) and intermediate in only 4.5% (1 individual). 27.3% (6 individuals) showed no IL-2 production.

As observed, the response was exclusively monofunctional in 36.2% of individuals, prevailing a low-intensity response; while at the multifunctional level, the response was not only more representative (50% of ZIKV-positive individuals), but an intermediate intensity prevailed (22.7% of the individuals) and, definitely, a minority of the population (13.6%, n = 3) did not reveal any cellular response for these biomarkers (Figure 1a).

When comparing the median functional response for each of the markers, a statistically significant increase ($p< 0.001$) was observed in the number of cells producing IFN-γ and IL-2 in the group of individuals seropositive for ZIKV regardingthe ZIKV-DENV- group (seronegative population), which tends to have a higher level of cells producing IFN-γ compared to the ZIKV-DENV+ group ($p = 0.061$), although not in relation to IL-2 (Figure 1b and Table SIII).

Finally, to assess the relationship between the humoral and cellular immune response to the Zika virus in individuals, in terms of existence and intensity, three subgroups of response according to cellular functionality were also established for the evaluated population, as follows: 1-5 SFC/10^4 PBMC (Low); 6-20 SFC/10^4 PBMC (Moderate or medium); and > 20 SFC/10^4 PBMC (High). This classification has been already established for the humoral response parameter to favor the comparison of the two types of response (Table I).

By dichotomizing the results of the cellular and humoral responses (presence or absence), it was possible to determine an overall concordance of 90%, an expected random concordance of 58.0%, and a concordance coefficient of 75.4%, which defines a good concordance according to the Landis and Koch classification.

On the other hand, a weak or null concordance was found when contrasting low, moderate or high categories for each type of specific response (cellular and humoral), suggesting a connection between the possibility of joint generation but independence between both magnitudes (Table III).

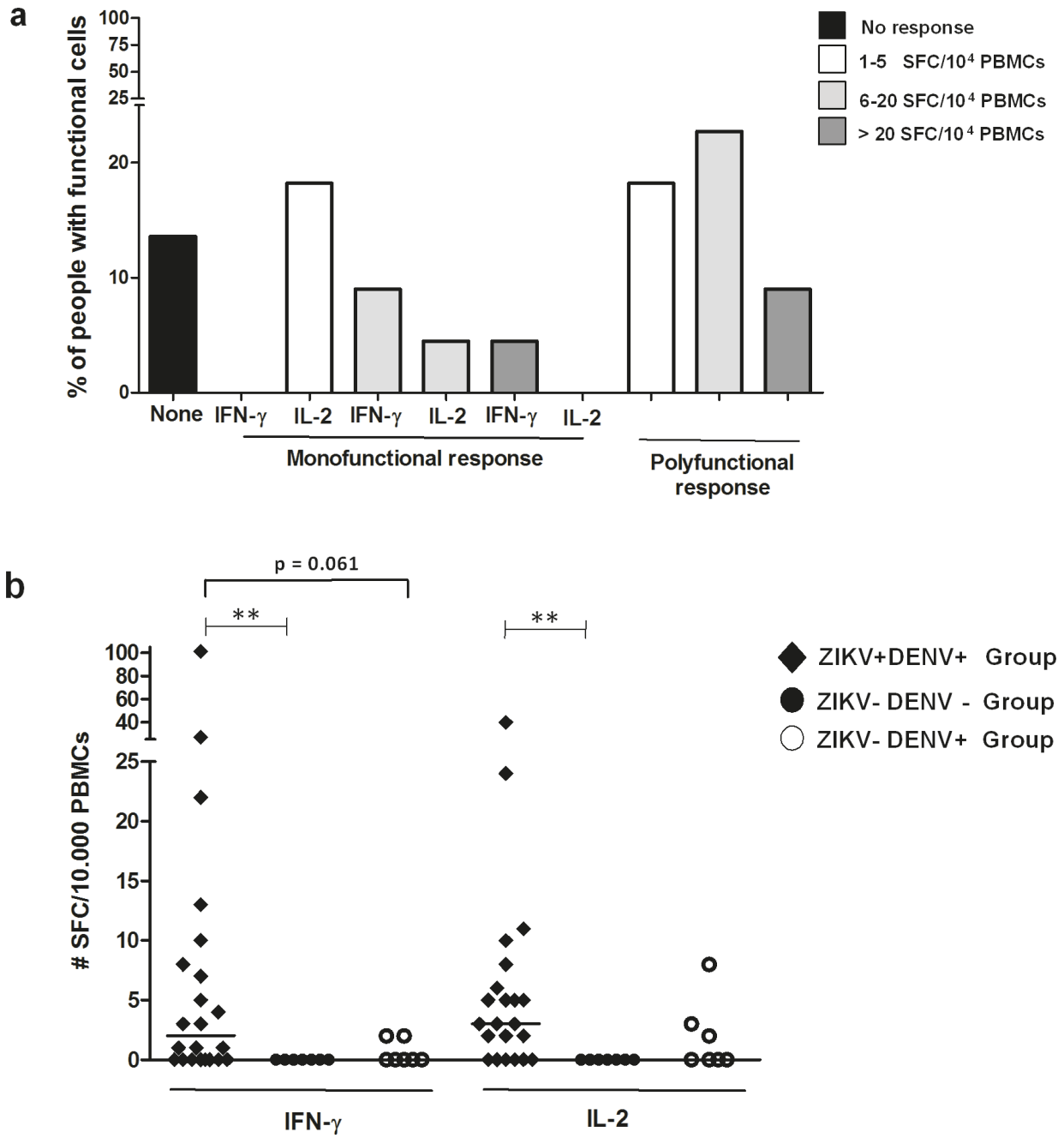

Figure 1. Cell functionality against ZIKV. (**a**) Bars represent the relative frequency of population with monofunctional (single cytokine) or multifunctional (two cytokines) response following *ex vivo* stimulation with Zika virus. At multifunctional level for the lower scale (low intensity) it was taken into account that for each individual both cytokines, a range of 1-5 functional cells/10^4 PBMCs were identified; the number of cells producing one of the two cytokines was in the range of 6-20 functional cells /10^4 PBMCs for the intermediate scale, and for the higher scale (high intensity), the number of cells producing one of the two cytokines was greater than 20 functional cells/10^4 PBMCs. (**b**) the dots represent the number of Spot Forming Cells for each individual evaluated within the ZIKV+DENV+, ZIKV-DENV- and ZIKV-DENV+ groups after the viral stimulus. The horizontal line corresponds to the median value. *p> 0.05 and ** p> 0.01 using Mann-Whitney U test.

Table III. Humoral and cellular response magnitude concordance analysis.

Contrast Low[a]		Cellular response (IFN-γ)			Total	Concordance index Kappa)
		Moderate[b] (medium)	High[c]			
Humoral response IgG (NS1)	Low	1	0	0	1	0.127
	Medium	1	2	0	3	
	High	13	2	3	18	
Total		15	4	3	22	
Low		Cellular response (IL-2)				
		Medium	High			
Humoral response IgG (NS1)	Low	1	0	0	1	0.057
	Medium	2	1	0	3	
	High	13	3	2	18	
Total		16	4	2	22	
Low		Cellular response (IFN-γ)				
		Medium	High			
Humoral response IgG (E)	Low	7	3	3	13	0
	Medium	7	1	0	8	
Total		14	4	3	21	
Low		Cellular response (IL-2)				
		Medium	High			
Humoral response IgG (E)	Low	9	2	2	13	0.019
	Medium	6	2	0	8	
Total		15	4	2	21	

[a.b.c] **Cellular response categories: [a] 1-5 SFC (Spot Forming Cells)/10^4 PBMC (Peripheral Blood Mononuclear Cells); [b] 6-20 SFC/10^4 PBMC; [c]> 20 SFC/10^4 PBMC.**

DISCUSSION

The emergence of the Zika virus in the American Continent and Colombia (2015-2016) was strikingas the clinical behavior of the infection changed, implying alterations in fetal brain development (Brady et al. 2019, Driggers et al. 2016, Mlakar et al. 2016, Song et al. 2017), apart from being proposed as a possible TORCH agent (Coyne & Lazear 2016) causing Guillain-Barré syndrome (Cao-Lormeau et al. 2016, Simon et al. 2016) and some registered cases of acute myelitis (Mécharles et al. 2016) and meningoencephalitis (Carteaux et al. 2016) during the epidemic phase. Its importance is mainly focusing on pregnant women or women of childbearing age (the case of university student population who could become pregnant), as well as the young population in general since it is known that one of the transmission routes is sexual through semen (Gornet et al. 2016, Musso et al. 2015, Song et al. 2017, Wilder-Smith et al. 2018), with an infecting load even greater than that of other body fluids like blood and urine (Mansuy et al. 2016).

In geographical regions such as the department of North of Santander, and especially the city of Cucuta, where there are optimal conditions for the proliferation of the *Aedes aegypti* vector (Ortiz & Argote 2016), it is very important to observe the humoral and cellular immune footprint for ZIKV, considering that the region is also a DENV endemic zone (as confirmed by the finding of the parallel study above-mentioned). Therefore, an analytical tool was used for differential serological diagnosis among flaviviruses (as is the case here using several viral antigens), from which it was possible to establish groups of seropositive individuals for some/both ZIKV antigens, as well as totally seronegative for ZIKV, in the presence of both positive and negative serological response to DENV (in support of the specificity of the analysis).

The findings of this study showed that the prevalence of antibodies against Zika virus in the academic community analyzed was considerably high (86.3%), regardless of variables such as gender, age and even aspects such as the circulation in areas of greater presence of the vector in the academic institution (which is especially observed in Health Sciences Degrees laboratories), which make us infer that the population studied could constitute a reflection of the community in general in the region. This bearing in mind that the department of North of Santander registered an incidence rate three times higher than the national rate for Colombia during the epidemic phase (ZIKV emergency) (SIVIGILA 2016). In addition, this finding contrasts with the seroprevalence data determined for other infectious events in our region (Cárdenas et al. 2015b).

These data also contrast with that referred to in the population of northeastern Brazil, which is the most affected country by the emergence of ZIKV in the Americas, where 63.3% of seroprevalence was reported in a sample of 633 individuals (75% between 20-49 years old) among pregnant women, patients with HIV infection or tuberculosis and university population (staff), by detecting IgG against ZIKV NS1 using ELISA (Netto et al. 2017). Similarly, global seroprevalences of 73% and 66% have been reported in the Yap Islands of Micronesia and French Polynesia, respectively, (Aubry et al. 2017, Duffy et al. 2009), with reports for the Americas of 19-30% in pregnant women (n = 3.050), blood donors (n = 814) and even in individuals with acute febrile syndrome in the Colombian-Venezuelan border (18.5%, n = 157) (Carrillo-Hernández et al. 2018, Flamand et al. 2017, Villarroel et al. 2018), all of which contrast with the high seroprevalence determined in the population studied.

13 individuals were found to be seropositive only for E protein and 57 were exclusively positive for NS1, as well as 4 times more of probability for detection using NS1 as antigen. In addition to this, the fact that the humoral memory response to E antigen found in the population analyzed was not only lower in frequency but also in intensity (71.4% of the cases in low level), allows inferring a greater potential of the use of NS1 antigen for screening purposes, in qualitative and quantitative terms. These results are consistent with studies previously referred to as the inclusion of non-structural antigens such as NS1 or NS5 for optimizing the diagnosis of ZIKV infection in terms of precision and lower cross-reactivity when searching for antibodies in relation to the use of E protein only (Stettler et al. 2016, Wong et al. 2017).

The population analyzed showed a greater and stronger humoral memory response at the expense of ZIKV NS1 antigen. This is consistent with recent reports, even at the cellular level (using peptides as a stimulus) (Delgado et al. 2018), related to E antigen, although the latter

differs only in 45% of its homologous amino acid sequence in relation to DENV 1-4, for which its serological cross-reactivity has been referred to the same marker among flaviviruses (Heinz & Stiasny 2017), likewise, NS1 is the center of our attention in relation to the differential diagnosis between flaviviruses since this one has the lowest sequence homology within other non-structural proteins such as NS3 and NS5 (Wen & Shresta 2017), being necessary to underline the referred specificity in the serological test used in this study, not only for NS1 but for also for E protein (mutated antigenic variation) of ZIKV, in relation to other flaviviruses and specially DENV, but acknowledging that given the existing discussion on cross-reactivity in serological tests among flaviviruses, the only fully accepted analysis is the Plaque Reduction Neutralization Test (PRNT) (Sharp et al. 2019).

This study suggests the importance of using this biomolecule as a marker of the epidemiological footprint when seeking to demonstrate a specific response for Zika virus (based on the nature of the immunoassay technique used to differentiate the reactivity to ZIKV among other flaviviruses such as DENV even in endemic areas). However, it does not rule out the usefulness of the concomitant detection of the humoral response to E protein, taking into account that the serological footprint was determined only using NS1 antigen in 21.1% of cases (57/270 individuals), but such evidence of immunological memory was generated only at the expense of the use of ZIKV-specific E protein as antigen in 6.1% of cases (13/213 individuals). These types of findings make these proteins a key element in the development of diagnostic test and therapeutic vaccines, as previously mentioned. Current mRNA and DNA subunit vaccines for Zika infection are based on E protein, but due to the potential of NS1, some vaccines have been designed based on attenuated vesicular stomatitis viruses that co-express NS1, E protein and prM protein (Li et al. 2018), or models using Zika VLPs (Salvo et al. 2018), conferring protection against the virus through cellular and humoral components.

On the other hand, if the diagnosis of Zika infection is frequently based on the presentation of clinical symptoms, as occurs in this environment, the findings of contrasting information between the antecedent of infection symptoms and the serological footprint of Zika virus presented in a high percentage in the evaluated population will imply (or have an effect on) a lower probability of access to diagnosis in the face of clinical warning signs, possibly related to the mild symptomatology attributed to flavivirus in at least 80% of cases (symptomatic infection is reported in only 20-25% of the affected population), as a self-limiting mild clinical presentation of 4-10 days of duration (Duffy et al. 2009, Song et al. 2017), where mild fever, rash and arthralgia are present in most cases, as previously referred to (Bozza et al. 2019). The above is consistent with the findings of this paper, apart from cephalea in 19 of the 34 students who reported symptoms during the epidemic phase of Zika (56% of the population subgroup).

This suggests two important aspects: a possible underestimation of the epidemiological reality for ZIKV (aspect to be researched), especially if the evidence of exporting cases in the absence of local transmission records is taken into account as it has happened in countries such as the Philippines, Thailand, Cameroon, Vietnam and Indonesia (Wilder-Smith et al. 2018); the second aspect is the need to implement permanent and proactive surveillance strategies for this virus in the region since it is located in the tropical zone. This importance has been fully recognized in other areas that have been strongly hit by

the incoming epidemic of ZIKV, such as the Northeastern of Brazil (Brady et al. 2019), bearing in mind that due to its antecedent, these areas could be the focus of new epidemics.

It is globally recognized the tendency to underestimate the actual number of cases because of variables such as accessibility to health services, among others, based on statistics especially on medical, hospital or laboratory reports (Fritzell et al. 2018). In this context, the lack of perception of symptoms by the individual would only worsen this subrecord, as shown in our results. This aspect becomes even more important once the case of infection is transferred to a vulnerable population such as pregnant women (indirectly included in our study as women of childbearing age) or with a predisposition to the development of an immunological disorder, as it has been widely exemplified (Brady et al. 2019, Broutet et al. 2016, Coyne & Lazear 2016, Muñoz et al. 2016), hindering the possibility of its timely and less consequent detection.

Previous dengue virus infections can explain the existence of this subrecord. An event that has been discussed in recent years is whether pre-existing immunity to dengue virus has any association regarding clinical presentation and transmission of Zika infection or not. Rodriguez-Barraquer et al. (2019) demonstrated in a Brazilian cohort where the Zika epidemic began in 2015 that the presence of high antibody titers against Dengue virus reduced the risk of infection. Even more interesting, the clinical picture associated with Zika virus infection (Rodriguez-Barraquer et al. 2019), which could be extrapolated to the findings in this research since in a parallel study of our group (unpublished results), it was determined that more than 80% of the individuals evaluated had a joint serological footprint for the two flaviviruses.

Regarding our findings of cellular functionality against ZIKV, although no significant difference was observed between the median functional cells for IFN-γ and IL-2 cytokines between individuals ZIKV+DENV+ (test group), with respect ZIKV-DENV+ (with serological evidence), this is possibly subject to the variability of response between individuals due to aspects such as the infectious load received (at the time of natural infection), the time elapsed post-contact, the immunological (intrinsic) competition to generate and maintain memory response after primoinfection, which started from the stimulus of 10.000 circulating mononuclear cells. The observed statistical trend (to IFN-γ) may be subject to the limited number of individuals evaluated.

In natural conditions, the similarity among flaviviruses leads to the generation of immune cross-reactivity, which in turn can be translated into cross-protection (Heinz & Stiasny 2017, Ngono & Shresta 2019, Oliveira et al. 2019), to the point of including cross memory B cells for ZIKV and DENV flaviviruses (Adam et al. 2018). However, it is also stated that previous infection with DENV could exacerbate the infection and effects related to ZIKV in pregnant women (Bhaumik et al. 2019).

Therefore, findings on the response to ZIKV in our population of seropositive individuals simultaneously for other flaviviruses could be a close mirror of the adaptive cellular immunity to the first in our region, given the impact of the co-circulation of ZIKV and DENV viruses in endemic areas such as the city of Cucuta in the northeastern region of Colombia, which even recently ranked first in Colombia in frequency of Dengue cases (SIVIGILA 2018).

These results show consistency with the recently referred cross-reactivity at T cell level in the functional response (IFN-γ) to both non-structural NS1, NS3 and NS5 antigens and

structural (envelope protein) antigens between ZIKV and other flaviviruses (Delgado et al. 2018, Lim et al. 2018, Reynolds et al. 2018). This may favor the protection of individuals in areas of co-circulation of agents since such antigenic-based cross-reactivity is considered key for the maintenance of human memory CD4+ T subpopulations. These constitute a large part of the responding cells, even during primoinfection (as shown in other viral models) (Jameson & Masopust 2018, Su et al. 2013). However, the effect of immunization prior to ZIKV is still unknown concerning the protection-potency balance of the infection and disease by Dengue Virus (Roth et al. 2018).

At the same time, in populations of other endemic areas of dengue such as India, cross-reactivity has been identified, where CD8+ T cell proliferation was observed in response to Japanese Encephalitis Virus in individuals seronegative for it but seropositive for DENV (Turtle et al. 2017). Likewise, it was found in Nicaragua and Sri Lanka that CD8+ T lymphocytes of individuals previously exposed to DENV responded to ZIKV by overexpressing granzyme B and PD-1, with respect to those seronegative for DENV (Grifoni et al. 2017) or in Singapore, with expansion and cytotoxic response of CD4+ and CD8+ T lymphocytes (Lim et al. 2018), although using in most cases ZIKV antigens as a stimulus.

It is important to point out that although cell response to ZIKV was observed in the ZIKV-DENV+ population, this in turn differs from the typically multifunctional response known for DENV (from the cytokines studied) and associated with protection against secondary infection (de Alwis et al. 2016, Tian et al. 2019, Weiskopf et al. 2013), which suggests that although crossed cellular immunity is generated between Zika and Dengue viruses, the characteristics and quality of this response could be different to the one observed specifically for each one, which shows the need to evaluate the characteristics of the cellular memory response to these two flaviviruses in a comparative way between ZIKV+/-DENV+/- individuals in order to understand what would happen in each type of population, especially in endemic areas of Dengue.

However, the aspect of possible cellular cross-reactivity is still controversial considering that Stettler et al. (2016) also researched the role of memory CD4+ T lymphocytes in terms of specificity and cross-reactivity in Zika infection, finding high specificity of Th subpopulations to Zika or Dengue viruses, which show low cross-reactivity even in individuals that have been previously exposed to Dengue (Stettler et al. 2016). These authors stated that while E protein is responsible for the cross responses between Zika and Dengue flaviviruses, NS1 protein promotes a strong cellular response translated into the elimination of infection in a specific-virus way. All this evidence encourages us to conduct our studies focusing on studying in more depth the role of NS1 in cellular immunity to Zika infection in the same way that our study has been initially carried out by evaluating the Th1 immune profile.

NS1 stands out as one of the antigens associated with protection against flavivirus infection in experimental animal models, in the absence of neutralizing antibodies (Costa et al. 2006). This Zika-specific protection mediated by T cells was evidenced by the presence of CD4+ cells with Th1 (T helper) profile (IFN-γ+ and TNF-α+) (Li et al. 2018) in mice immunized with the vaccine, suggesting that the presence of NS1 modulates the Th1 response. These findings are related to those obtained in this study regarding the evidence of cellular production of Th1 profile cytokines (also including IL-2 in this case) in the evaluated individuals, taking into account the presence of NS1 protein in the complete virus.

Interestingly, Li et al. (2018) did not just revealed the participation of Th1 subpopulations in the response to ZIKV, but also a balanced activity due to the presence of Th2 and Th17 profile lymphocytes (Li et al. 2018), an aspect that would be interesting to evaluate in the future in our cohort as it is consistent with the strong humoral response observed in the individuals.

Our findings reveal the characteristics of a cellular response to the ZIKV, sustained over time, where cases with levels > 20 functional cells/10^4 PBMC (referring to circulating memory cells) for the two markers evaluated (cytokines) and associated with Th1 profile stand out, which is something that needs to be confirmed in a larger population group. However, this is consistent with the study referred to by Delgado et al. (2018) in nine individuals seropositive for ZIKV (approximately one year post-infection) without any antecedent of DENV infection.

About 200 functional cells or SFC were found for IFN-γ/10^6 PBMCs (post-stimulus with pooled peptides of structural and non-structural ZIKV proteins) in this population, which in most cases is translated into 2 SFC/10^4 PBMCs, according to the scale used in this study. However, the magnitude of the response was concentrated between 200-600 functional cells/10^6 PBMC or more (which for our scale corresponds to 2-6 SFC/10^4 PBMC) in a group of 11 individuals that had previously been seropositive or in acute phase together for ZIKV and DENV flaviviruses (Delgado et al. 2018).

Our stimulus model was based on the use of inactivated whole virus, simulating a scenario close to the reality of infection, which allows revealing medians of 2 and 3 functional cells/10^4 PBMC (200 and 300/10^6 PBMC) for IFN-γ and IL-2, respectively, with individual responses of up to 101 IFN-γ+/10^4 PBMC (10.100 functional cells/10^6 PBMC) and 40 IL-2+/10^4 PBMC (4.000 functional cells/10^6 PBMC) in the group of 22 individuals seropositive for ZIKV (in conjunction with DENV). However, a median of 0 was observed for IFN-γ+ or IL-2+/10^4 PBMC in individuals seronegative for ZIKV and seropositive for DENV (Control group 2) (Table III). At the same time, this magnitude of response is similar to that referred to in the human population against ZIKV African lineage, after stimulation with peptides of the NS3 protease and helicase enzymes within a range of 57-466 IFN-γ+cells/10^6 PBMC (Herrera et al. 2018).

On the other hand, our results regarding multifunctionality for IFN-γ and IL-2 after 16 to 24 months of ZIKV infection (according to the epidemic phase in Colombia and our region) constitute a relevant parameter strongly associated with the quality of the protective response, especially of subpopulations of CD8+ T lymphocytes facing viruses in humans (Cárdenas et al. 2015a, Ning et al. 2011), which are in contrast with that referred to by Grifoni et al. (2018) in individuals infected by ZIKV in convalescent phase, 1-2 years post-infection, in which the response based on IFN-γ was especially evident (Grifoni et al. 2018), whose role in the onset of inflammatory response and rapid control of viral replication is widely known (Hobbs et al. 2018). However, it is relevant that the findings of IL-2 cytokine involved in the proliferation of activated T lymphocytes, which are associated with central memory subpopulations and tissue-resident memory (TRM), are responsible for maintaining the protective status of individuals in the long term (Schreiner & King 2018).

Although the two cytokines evaluated in this study are recognized for their immunological relevance, the low or no detection of producing cells for some of them does not exclude cell functionality to other markers, making possible to expand the range of biomarkers in a future research. Then this study can be considered a model or pilot study of immunological evaluation against ZIKV in humans. In this

regard, it is worth mentioning the antecedent on the low frequency of CD4 T functional effectors lymphocytes for IFN-γ in response to ZIKV, found in infected individuals in the acute phase (Cimini et al. 2017). This is an aspect that could affect in general the functionality observed for this cytokine, in consistency with what was observed in this study. The evidence indicates a possible modulating capacity of ZIKV exactly for the production or function of interferons by mononuclear cells, as it has also been referred to for type II (Bowen et al. 2017, Tappe et al. 2016).

Taking it all, this study shows that the result of serological screening for ZIKV in our population is different depending on the viral antigen used for IgG detection, with a considerably high seroprevalence (> 85% of the population evaluated) for NS1accompanied by a lack of knowledge of previous infections or antecedents of diagnosis or clinical manifestations in 76.4% of cases, suggesting the convenience of a serologic surveillance, especially in endemic areas with co-circulation of arboviruses (Fritzell et al. 2018).

We have offered evidence of cellular response against ZIKV after the use of a specific stimulus with the whole virus (close to natural conditions) in population seropositive to this flavivirus, characterized by a wide biological variability between individuals at a moderate or low level in most cases of functional cells for IFN-γ and IL-2 cytokines associated to Th1 memory profile (despite the only two immunological markers that were initially measured), which is relevant in antiviral control, apparently without difference between them. This does not allow giving a conclusion on the central or peripheral memory profiles that have dominated the cellular response against this infectious agent.

Further research is required to expand the sample evaluated in relation to the analysis of cell-mediated response in seropositive individuals only for ZIKV (elucidating findings regarding concomitantly seropositive population for other flaviviruses such as Dengue), in order to determine their response profile associated with T lymphocytes, as well as to confirm any possible cellular cross-reactivity between Zika and Dengue flaviviruses.

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SUPPLEMENTARY MATERIAL

Tables SI, SII and SIII.

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DMC was responsible for the design and implementation of this work, the monitoring of the experiments, the samples collection and the data analysis, definition of intellectual content, literature search, manuscript preparation and further edition. MAJ, LDV and NLO collected samples, performed the experiments and analysed data. JAS supported the data analysis, definition of intellectual content, literature search, manuscript preparation and review. CRC performed the population selection, sampling process and conduction of the experiments. JEO advised on the design and implementation of the work, supported the definition of intellectual content and critically reviewed the manuscript. KAC advised and supported the performance of the cellular experiments, and critically reviewed the manuscript.