



# Delayed viral clearance despite high number of activated T cells during the acute phase in Argentinean patients with hantavirus pulmonary syndrome

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## Summary

**Background** The hallmarks of HPS are increase of vascular permeability and endothelial dysfunction. Although an exacerbated immune response is thought to be implicated in pathogenesis, clear evidence is still elusive. As orthohantaviruses are not cytopathic CD8<sup>+</sup> T cells are believed to be the central players involved in pathogenesis.

**Methods** Serum and blood samples from Argentinean HPS patients were collected from 2014 to 2019. Routine white blood cell analyses, quantification and characterization of T-cell phenotypic profile, viral load, neutralizing antibody response and quantification of inflammatory mediators were performed.

**Findings** High numbers of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found in all HPS cases independently of disease severity. We found increased levels of some proinflammatory mediators during the acute phase of illness. Nonetheless, viral RNA remained high, showing a delay in clearance from blood up to late convalescence, when titers of neutralizing antibodies reached a high level.

**Interpretation** The high activated phenotypic profile of T cells seems to be unable to resolve infection during the acute and early convalescent phases, and it was not associated with the severity of the disease. Thus, at least part of the activated T cells could be induced by the dysregulated inflammatory response in an unspecific manner. Viral clearance seems to have been more related to high titers of neutralizing antibodies than to the T-cell response.

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## Introduction

Hantavirus pulmonary syndrome (HPS) is a zoonotic disease caused by distinct viruses grouped in the genus *Orthohantavirus* (subfamily *Mammantavirinae*, bunyaviral family *Hantaviridae*). Orthohantaviruses are widely

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## Research in context

### Evidence before this study

Hantavirus pulmonary syndrome (HPS) is a zoonotic disease caused by distinct orthohantaviruses. They are harboured by rodents of different species from which humans become infected through inhalation of contaminated aerosolized excreta. HPS was first described in an outbreak that occurred in the Four Corners region of United States in 1993, which led to the identification of Sin Nombre virus (SNV). In 1995, after the first HPS cases were described in Argentina, Andes virus (ANDV) was identified. This virus was soon associated with person-to-person transmission in southwestern Argentina and then in Chile. Since then, ANDV has become unique among orthohantaviruses, due to its ability to be transmitted person-to-person, increased potential to spread, and high lethality. HPS has been increasingly reported all over South America since 1995. The pathogenesis of HPS is not well understood but, as orthohantaviruses are not cytopathic, T cells, especially cytotoxic CD8<sup>+</sup> T cells, are believed to be the central players involved. However, some discrepancies still remain, probably because most results came from *in vitro* experiments and animal-model studies involving orthohantaviruses of different species. For perspective, we searched in the PubMed database for articles published up to and including December 31, 2020, using the terms hantavirus, Andes virus, and T cells. Findings indicated that studies on T-cell immune responses in acutely ill patients were only performed in North America, where a positive correlation was found between SNV-specific CD8<sup>+</sup> T-cell frequency and disease severity. However, there are no studies on T-cell responses in ANDV-like HPS acutely ill patients.

### Added value of this study

We analysed the immune response in acute ANDV-like HPS patients in Argentina from 2014 to 2019. This study cohort represents a unique collection of samples from different endemic regions in Argentina. Because HPS has low incidence, outbreaks provide unique opportunities to collect samples that avail the study of unexplored aspects of this disease. Our analysis was possible mainly due to two large outbreaks, one of which was driven by person-to-person transmission.

The phenotypic characterization of T cells in blood of acute HPS patients showed an altered CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio during the prodromal phase and high proportions of active T cells. However, the numbers of activated T cells did not associate with disease severity in our cohort. Also, we measured high viral loads in blood during the acute and early convalescent phases, offering evidence that the T-cell response was unable to resolve the infection during these phases of disease.

### Implications of all the available evidence

The accurate characterization of the immunological response to ANDV-like infections in humans is key to identifying the mechanisms leading to HPS

pathogenesis and characterizing the protective profile of T-cell responses useful for vaccine and/or therapeutic developments for South America. Our findings suggest that, although activated T cells might be implicated in immunopathogenesis, they are likely not central in the development of disease severity. This is surprising because cytotoxic CD8<sup>+</sup> T cells are known to be the primary cause of tissue damage in non-cytopathic viral infections. This apparently dysfunctional T-cell response requires further characterization.

distributed in the Americas. The species *Andes orthohantavirus* includes several human pathogens of great concern in Argentina, such as Andes virus (ANDV) and Andes-like viruses, that cause HPS with case fatality rates ranging from 22 to 40%.<sup>1,2</sup> Among the six variants of ANDV-like viruses, the most prevalent are Orán virus (ORNV) in north-western Argentina, and Buenos Aires virus (BASV or Hu39694) and Lechiguanas virus (LECV) in central-eastern Argentina.<sup>3</sup> Although HPS has been reported in most regions of the country, cases have occurred only in high-humidity areas. Human infections generally occur directly from inhalation of contaminated aerosols generated by infected rodents. However, ANDV and BASV are unique among orthohantaviruses due to their ability to be transmitted person-to-person.<sup>4–7</sup> A recent HPS outbreak reported in southwestern Argentina illuminated ANDV's pathogenic and transmission potential.<sup>7</sup>

HPS is characterized by three phases: the febrile or prodromal phase, the cardiopulmonary phase, and the convalescent phase. The prodromal phase (indistinguishable from other common viral infections) is of very short duration, after which most patients enter the cardiopulmonary phase, comprising respiratory compromise and hemodynamic instability, which can evolve to shock and death in few days. Survivors progress to the convalescent phase.<sup>8</sup> The hallmarks of HPS are an increase of vascular permeability and endothelial dysfunction, which lead to pulmonary edema in HPS patients. The origin of vascular permeability remains elusive, as orthohantaviruses are known to be non-cytopathic; thus, a dysregulated immune response is considered to be the cause. The involvement of T cells—especially cytotoxic CD8<sup>+</sup> T cells—was proposed as the central cause of HPS immunopathogenesis, but evidence is conflicting.<sup>9–13</sup> However, most results came from *in vitro* and animal-model experiments; few studies were based on naturally infected human subjects.<sup>14–16</sup> HPS studies of T-cell immune responses in acutely ill patients have only been performed in North America, where Sin Nombre virus (SNV) is the main causative agent of HPS.<sup>13</sup>

The lack of data on human immune responses to infection with ANDV-like viruses is mainly due to bio-safety concerns and logistic challenges. Besides the high virulence of all ANDV-like viruses, their potential

to cause sustained person-to-person transmission imposes biosecurity restrictions on the manipulation of samples. Logistic limitations have hindered sample collection, because HPS generally occurs in a sporadic pattern in remote areas. Outbreaks provide unique opportunities to collect samples that avail the study of unexplored aspects of this disease. We analysed the immune response in acute HPS patients whose cases were reported from 2014 to 2019 in Argentina. Our aim was to evaluate the impact of the phenotypic profile of T cells, humoral response, and inflammatory mediators on the progression of infection and disease in HPS patients.

## Methods

### Ethics

Procedures for sampling and analysis were approved by the Ethics Committee of the Administración Nacional de Laboratorios e Institutos de Salud Dr. Carlos G. Malbrán. Written informed consent was obtained from all patients and healthy volunteers prior to analysis.

### Study population and samples

The enrolment process included patients according to the confirmation criteria used by the National Reference Laboratory for Hantaviruses (NRLH).<sup>2</sup> This study included 154 out of 441 patients with confirmed HPS from 2014 to 2019 in Argentina (Table 1). Standardized clinical and epidemiological information was obtained for each case through clinical/epidemiological forms. Additionally, daily information about disease progression was obtained from 33 patients during the Epuén outbreak. The classification of samples according to the clinical phase of disease was based on laboratory and clinical data. Disease severity was classified according to the clinical presentation in four grades (I = prodromal symptoms without respiratory compromise, II = prodromal symptoms with only oxygen delivery, III = cardiopulmonary phase that responded to treatment, and IV = cardiopulmonary phase with fatal outcome), as previously described.<sup>17</sup> Further details are provided in the supplementary appendix. Most of the samples were obtained from two large outbreaks. The first outbreak occurred from October 2017 to February 2018 in Buenos Aires Province, in the central-eastern part of the country. The second outbreak, driven by person-to-person transmission, took place from November 2018 to February 2019 in Epuén, in the southwestern part of the country.<sup>7</sup> Healthy volunteers were included as controls. Blood samples were collected from each patient in ethylenediaminetetraacetic acid (EDTA) blood-collection tubes for viral load quantification and purification of peripheral blood mononuclear cells (PBMCs) and in serum-separator tubes for serum collection. Samples were aliquoted, immediately transported to NRLH, and stored at -80 °C until used. For

the analysis of viral load, we classified samples with immunoglobulin M (IgM) titers greater than immunoglobulin G (IgG) titers (hospitalized symptomatic patients) and those with IgM titers less than IgG titers or with negativized IgM but high IgG titers (discharged asymptomatic patients). From the HPS patients shown in Table 1, 30 were included for hematologic analysis, 39 for phenotypic characterization of T cells, 154 in viral load quantification and IgM-to-IgG ratio determination, 48 patients for neutralization assays, and 38 for inflammatory mediators quantification.

### Viral RNA quantification

RNA was obtained from 200 µL of whole blood using TRIzol LS (Invitrogen, Waltham, MA, USA) and then purified with the RNAid kit (QBioGene, Solon, OH, USA) according to the manufacturers' recommendations. Viral RNA quantification and genotype characterization were performed as described previously.<sup>7</sup>

### Analysis of cellular response to the infection

Routine white blood cell analyses were performed with an automatic hematological counter (Sysmex XN-550; Sysmex, Hamburg, Germany), and the presence of immunoblasts and other immune cells, such as, neutrophils, were confirmed microscopically (Axio Scope A1; ZEISS, Jena, Germany) by May Grünwald-Giemsa staining. Lactate dehydrogenase (LDH) activity was measured by an automated dry chemistry analyser.

PBMCs were purified from fresh blood using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) solution for density gradient centrifugation according to the manufacturer's instructions; phenotyping surface markers were used to identify specific subpopulations and detected with the appropriate antibody conjugate (BD Biosciences, Franklin Lakes, NJ, USA). To study the phenotypic profile of T cells, PBMCs were incubated for 30 min at 4 °C with anti-HLA-DR-FITC (Clone: G46-6), anti-CD38-PE (Clone: HIT2), anti-CD8-PerCP Cy5.5 (Clone: SK1), and anti-CD3-APC (Clone: BW264/56), or with anti-HLA-DR-FITC (Clone: G46-6), anti-CD38-PE (Clone: HIT2), CD4-PE-Cy5 (Clone: RPA-T4), and anti-CD3-APC (Clone: BW264/56). Cells were washed and resuspended in phosphate-buffered saline (PBS) containing 1% heat-inactivated fetal calf serum (FCS) with 1% paraformaldehyde (PFA). Then, 100,000 events were analysed. Samples from healthy volunteers were included as controls. Data acquisition was performed using an Accuri C6 flow cytometer (BD Biosciences). Flow cytometry data were analysed using FCS Express 6 software (De Novo Software, Glendale, CA, USA).

### Focus reduction neutralization test

Humoral responses were evaluated by focus reduction neutralization test against ANDV, as described in the

Characteristic	HPS	Healthy
Geographic origin	<i>n</i> (case-fatality rate)	<i>n</i>
Central/East	70 (17.1)	6
North/West	33 (24.2)	
South/West	51 (29.4)	18
Age		
Median (range)	33 (2-90)	45.8(25-75)
Distribution in age-groups	<i>n</i> (%)	
0–10	4 (2.6)	
11–20	20 (13)	
21–30	41 (26.6)	3
31–40	43 (27.9)	8
41–50	25 (16.2)	6
51–60	9 (5.8)	2
61–70	7 (4.5)	4
71–80	3 (1.9)	1
81–90	2 (1.3)	
Sex	<i>n</i> (%)	
Male	109 (70.8)	10
Female	45 (29.2)	14
Clinical findings	<i>n</i> (%)	NA
Fever (> 38 °C)	154 (100)	
Gastrointestinal manifestations	67 (43.5)	
Hemorrhagic manifestations	21 (13.6)	
Respiratory compromise	135 (87.6)	
Hepatic compromise	96 (89.7)	
Renal compromise	33 (21.4)	
Neurological manifestations	10 (6.5)	
Hemodynamic engagement	51 (33.1)	
Exposure information	<i>n</i> (%)	NA
Person-to-person transmission clusters	5	
Clustered members	43 (27.4)	
Contact with an HPS Contact with other suspected case case	38 (24.2)	
Rodent source infection	116 (75.8)	

**Table 1: Epidemiological and clinical description of the cohort of HPS patients from 2014 to 2019 in Argentina.**

supplementary appendix. Focus-forming units (FFU) were counted and neutralizing antibody titers were defined as the reciprocal of the highest serum dilution that resulted in an 80% reduction in the number of FFU compared to a non-neutralized virus control.

#### Cytokine, chemokines, and growth factor assays

Serum inflammatory mediator profiles were established using a 48-Plex Bio-Plex Pro Human Cytokine Screening Panel (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. A total of 72 serum samples from 38 HPS patients and six healthy volunteers were included. After assay completion, plates were read on the LuminexFlexmap 3D (MiraiBio, San Bruno, CA, USA). Data were exported to Bio-Results Generator 3.0 and Bio-Plex Manager (Bio-Rad Laboratories). Sample replicates were run on separate plates. The first set of replicates was processed immediately

after thawing; the second set was stored at 4 °C after thawing and processed 24 h later. All samples were clarified at 10,000 *xg* for 10 min at 4 °C immediately prior to processing. Replicate values from different plates were manually combined, and percent coefficient of variation (%CV) was calculated. Due to low quality in the second read after 24 h at 4 °C for proinflammatory cytokines interleukin 2 (IL2) and IL3, concentrations were calculated from the samples processed immediately after thawing.

#### Statistics

To analyse the empirical distribution of the incubation period of person-to-person transmission cases we employed the quantile-quantile (Q-Q) plot method by using the qqplotr package implemented in R software, version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria). Fitting to normal and log-normal

distribution models was applied. A *p*-value of less than or equal to 0.01 of fitting of the observed data set to this theoretical curve was considered significant. Student's *t*-test or Mann–Whitney–Wilcoxon test was used for comparison of two groups of data. Comparisons of HPS groups and healthy volunteers were done by a nonparametric analysis of variance (ANOVA), Kruskal–Wallis *H* test, and Dunn's multiple comparisons test. Spearman's ranked correlation test was used for correlation analysis. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego CA, USA), and statistical significance was assumed when the *p*-value was less than 0.05. Multivariate analysis of evolution of serum biomarkers profile was undertaken in a subset of patients by principal component analysis (PCA) by using the FactoMineR package implemented in R software.

#### Role of funders

The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

## Results

### Clinical course of hantavirus pulmonary syndrome by Andes virus-like infection

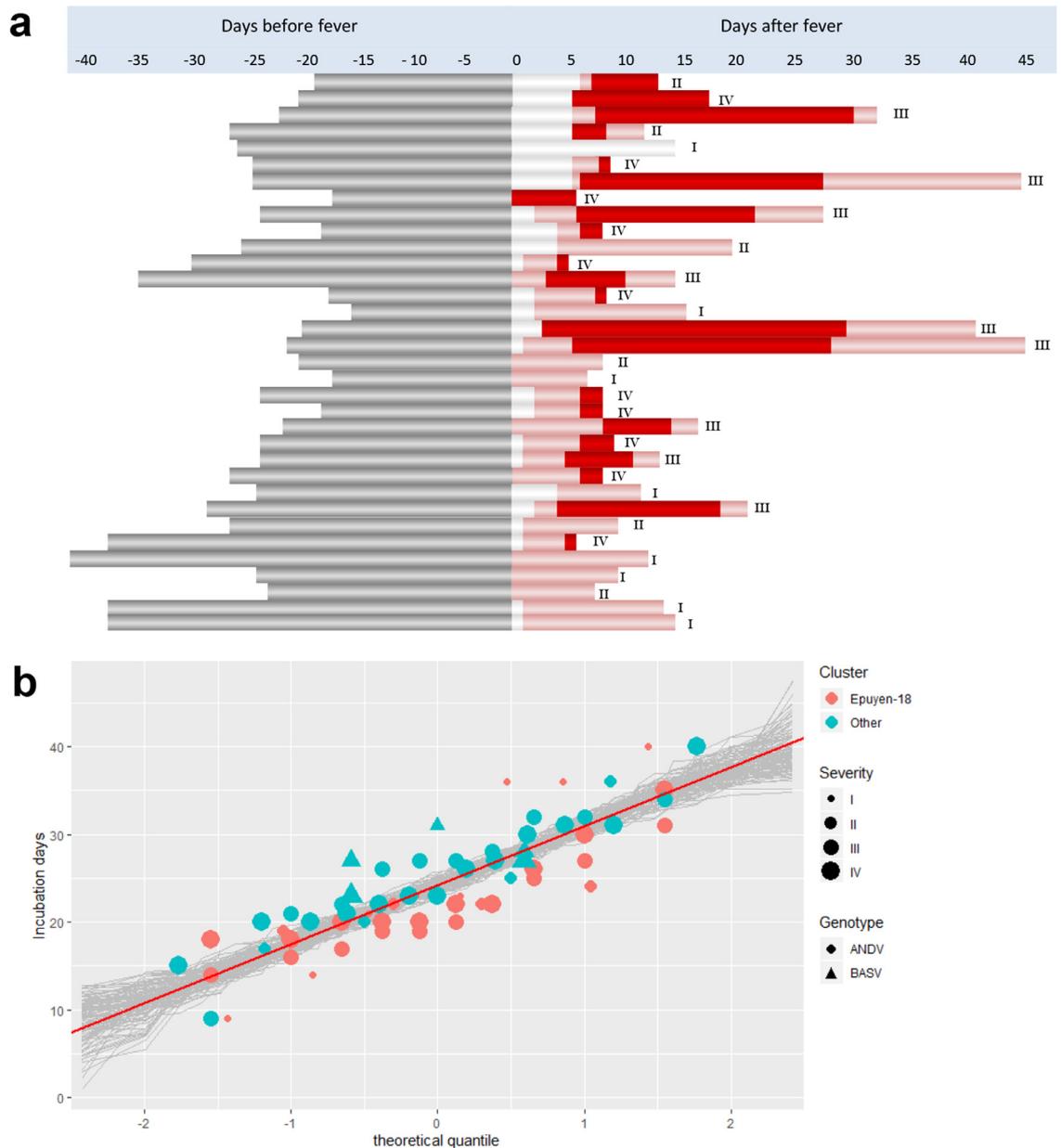
The most relevant clinical parameters during hospitalization are summarized in Table 1. The ANDV Epuyén person-to-person transmission outbreak was an opportunity to identify HPS cases earlier than usual and, among the 34 cases, disparate clinical pictures were observed (Fig. 1-a). Most of the patients (56%) progressed to severe disease (grades III and IV), requiring supportive treatment in the intensive care unit (ICU). Patients with severe disease entered the cardiopulmonary phase approximately 4 d after illness onset (median 4 d; range 0–6 d). Survivors required an average of 14 d in the ICU (median 14 d; range 3–25 d), whereas those with fatal outcomes remained 1.5 d before death (median 1.5 d; range 1–11 d). Of the patients who entered the ICU, 57.8% died, resulting in a case fatality rate of 32.3%.<sup>(7)</sup> The incubation periods were accurately determined for almost all patients (Fig. 1-b). The analysis of incubation days of these and other person-to-person events occurring during the same period were in agreement with a normal distribution model, the median being 26 d (range 9–40 d)<sup>7</sup>. Interestingly, the Epuyén person-to-person transmission cluster showed greater deviation from normality than person-to-person transmission clusters previously reported in Argentina, especially with regard to mildly symptomatic cases, which also tended to locate at the extremes of the distribution range values (Fig. 1b).

### Hematological markers for early detection of Andes virus infection

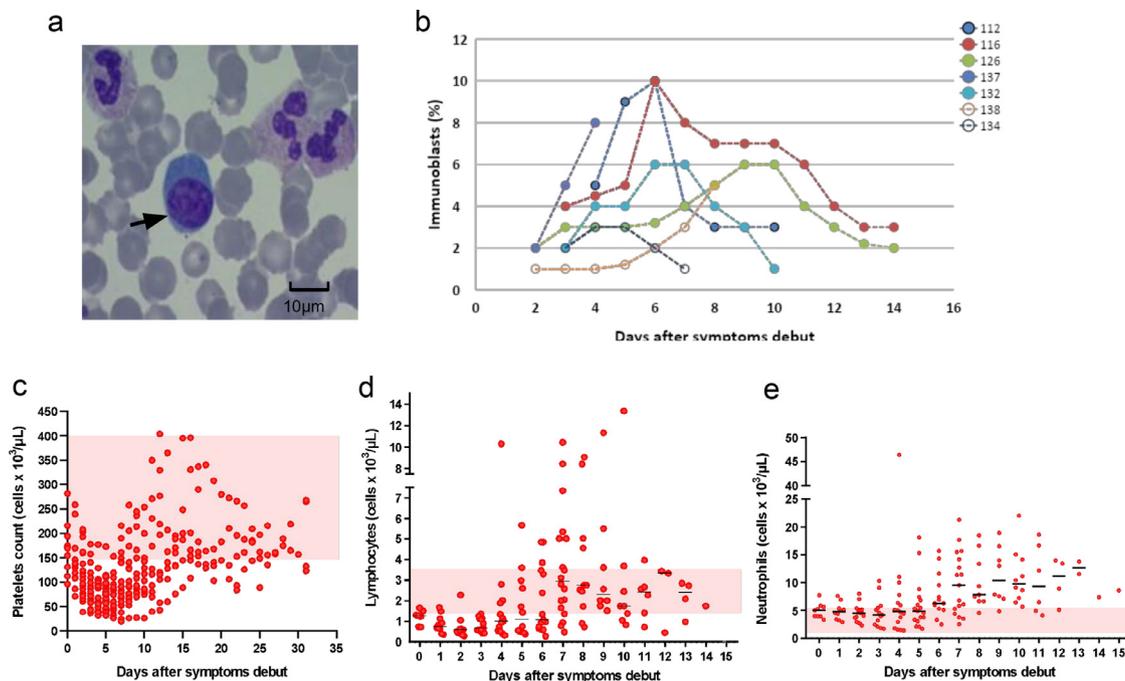
We analysed the kinetics of different blood cell subpopulations during the acute phase of illness in patients during the Epuyén HPS outbreak (Fig. 2). The presence of immunoblasts in the prodromal phase and the decrease in platelet counts were the earliest findings in HPS patients. Lymphopenia was frequent immediately after fever onset and throughout the prodromal phase and, in general, was followed by neutrophilia. Thrombocytopenia and increasing LDH activity marked the beginning of the cardiopulmonary phase. The analysis of the proportion of leukocyte subpopulations showed that most patients with severe disease had a lower proportion of lymphocytes than neutrophils in the cardiopulmonary phase, (Fig. 3-a), showing higher neutrophil-to-lymphocyte ratios (NLR)—1.6:1 for mild cases, grade II; 3.7:1 for severe that responded to treatment, grade III; 8:1 for severe with fatal outcome, grade IV. As markers of tissue injury, LDH activity and NLR were higher in severe cases during the cardiopulmonary phase (Fig. 3-b and c). In absolute numbers, neutrophil numbers were more strongly positively correlated with LDH activities than lymphocyte numbers (ratio: 0.69, *p* < 0.0001 and ratio: 0.44, *p* < 0.0001, respectively; Spearman's ranked correlation) (Fig. 3-d and e), suggesting a more preponderant role for neutrophils than for lymphocytes in tissue injury.

### Highly activated T-cell subpopulations did not achieve viral clearance during the acute phase

For the phenotypic characterization of T-cell subpopulations from PBMCs by flow cytometry, the lymphocyte gate, defined by forward and side scatter, was followed by CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> gating (Fig. S1). An altered CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio was marked in the prodromal phase, and CD8<sup>+</sup> tended to recover faster than CD4<sup>+</sup> T cells (Figs. 4-a and S2-a). We then analysed the kinetics of activation of T cells during the acute phase by monitoring the co-expression of CD38 and HLA-DR on the cell surface. The frequency of activated T cells was increased in HPS cases during the acute phase compared to healthy volunteers (CD8<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>: range 15.71–75.95%, median 51.7%; CD4<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>: range 4.7–31.82%, median 11.4%). The highest frequency of active CD8<sup>+</sup> T cells was detected during the second and third weeks of illness (cardiopulmonary phase, Figs. 4-b and S2-b), whereas active CD4<sup>+</sup> T cells were more abundant in the second week (Fig. 4-c). However, there were no significant differences in the frequencies of activated CD8<sup>+</sup> and CD4<sup>+</sup> T cells between mild and severe cases (Fig. 4-d and data not shown). Furthermore, longitudinal analysis showed that viral RNA load in blood remained high throughout the entire hospitalization period (up to the fourth week) (Figs. 4-e and S2), and several patients still had



**Figure 1.** Incubation period and the clinical course of hantavirus pulmonary syndrome patients. (a) Patients reported during the Andes virus-caused person-to-person transmission outbreak in southwestern Argentina. Each patient is represented as a horizontal bar, stacked in order according to the date of fever onset. The first case was reported in November 2018 and the last in February 2019 ( $n = 34$ ). The most probable incubation periods are represented in dark grey; the symptomatic period between fever onset (Day 0) and hospital admission is shown in light grey, the stay at low-complexity rooms in pink, and the period required in intensive care unit in red. Severity grade is indicated after each bar (Roman numerals). (b) Distribution analysis of the incubation period of person-to-person infected patients. Southwestern Argentina person-to-person events (salmon dots,  $n = 33$ ) were plotted with other person-to-person events during the period and historical person-to-person data (turquoise symbols,  $n = 33$ ) in a quantile–quantile plot, indicating that together they fit to a normal distribution model ( $p < 0.001$ ) with a mean of 22 d. Comparing both groups, the 2018–2019 outbreak data showed greater deviations from normality than clusters previously reported. Grey lines show 100 simulated normal data sets with equal sample size, mean, and standard deviation (using the Visual Studio bootstrap method) and compares the theoretical curve of perfectly normal data with a slope equal to standard deviation and an intercept equal to mean.



**Figure 2.** Hematological findings during the person-to-person transmission outbreak in southwestern Argentina. (a) Representative smear from a hantavirus pulmonary syndrome patient (cells were subjected to May Grünwald-Giemsa staining and analysed by microscopy, Axio Scope A1); the black arrow indicates an immunoblast. (b) Frequency (%) of immunoblasts along the acute phase ( $n = 7$ , 55 samples). (c) Platelet count during the acute phase ( $n = 30$ ; 180 samples). (d–e) Lymphocyte and neutrophil absolute numbers during the acute phase ( $n = 30$ ; 180 samples). Pink areas in each graph show the reference range of normality.

detectable viral RNA during the early (IgM<IgG) and the late (only IgG) convalescence phases (Fig. 4-f). Interestingly, no significant differences were observed in viral RNA load in blood among cases showing different severity grades (Fig. S3-d).

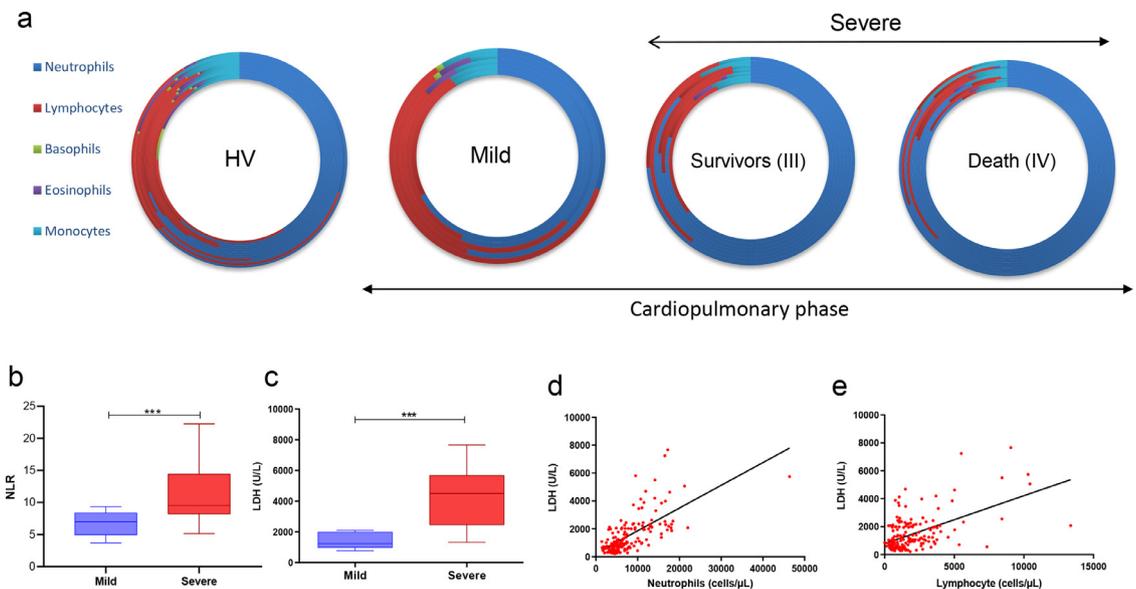
#### Viremia completely cleared after the development of high titers of neutralizing antibodies

Increasing titers of neutralizing antibodies began to be detectable approximately 10 d after fever onset and reached the highest titers after 18 weeks. Although patients infected with different ANDV-like viruses had neutralizing antibodies against ANDV, the highest titers were found in ANDV patients (Fig. 5 and Table S1). Longitudinal analysis showed that viral RNA clearance from blood was achieved as titers reached their maximum levels (Fig. S2-c). Only one patient still had detectable viral RNA ( $4.1 \times 10^3$  copies per mL) 202 d after fever onset (the last sample obtained) despite having high neutralizing antibody titers.

#### Broad dysregulated cytokine and chemokines patterns with a predominance of Th1 profile

We used Luminex technology to define the cytokine profile in the HPS patients. We found that ANDV

infections promoted increased secretion of numerous proinflammatory cytokines (interleukin 1 subunit beta [IL1B], IL1 receptor antagonist [IL1RN], IL2, IL2 receptor subunit alpha [IL2RA], IL6, IL10, interferon gamma [IFNG], and IFN alpha [IFNA]), chemokines (chemokine C-C motif ligand 2 [CCL2], CCL3, CCL7, C-X-C motif chemokine 9 [CXCL9], CXCL10, and leukaemia inhibitory factor [LIF]), and growth factors associated with the activation of granulocytes (colony-stimulating factor [CSF2] and CSF3) (Fig. 6 and Table S1). Among these biomarkers, significant differences between mild and severe disease were only observed for CCL7 and CXCL10 in the prodromal phase (1–4 d;  $p = 0.04$  and  $p = 0.03$ , respectively, Mann–Whitney–Wilcoxon test) (Fig. S4). Concentrations of some cytokines produced by T cells were marginally increased (IL4), whereas others were expressed at very low levels (IL9 and tumour necrosis factor beta [TNFB]), similar to or lower than in healthy volunteers (Figs. 6, S4, and Table S1). Table S1 shows concentrations of some circulating immune biomarkers in five severe HPS patients, from whom sequential blood samples could be obtained. The kinetics showed that they were differentially expressed, suggesting a tendency towards a Th1 profile. In these patients IL10 was greatly increased (up to 50-fold) in the first days of illness and showed a general tendency to normalization during early convalescence. Finally, in



**Figure 3.** Quantification of leukocyte subpopulations in peripheral blood in hantavirus pulmonary syndrome (HPS) patients during the Epuyén person-to-person transmission outbreak in southwestern Argentina. (a) The percentage of different cell types are represented in each ring graph in different colors in the cardiopulmonary phase. From left to right, healthy volunteers (HV,  $n = 12$ ) and HPS cases grouped as mild or severe according to disease severity grade: mild (II,  $n = 5$ ), severe that survived (III,  $n = 7$ ), and severe that were fatal (IV,  $n = 10$ ). Cell quantification was performed by an automatic hematological counter. (b) Maximum neutrophil-to-lymphocyte (NLR) ratios in mild (I and II,  $n = 12$ ) and severe patients (III and IV,  $n = 18$ ), Mann–Whitney–Wilcoxon test ( $p = 0.0006$ ). (c) Maximum lactate dehydrogenase (LDH) activities (unit per Litre [U/L]) recorded in survivors with mild ( $n = 12$ ) and severe ( $n = 18$ ) disease. (d) Correlation of LDH activity (U/L) in serum with neutrophil absolute numbers (cells per  $\mu\text{L}$ ) in acute patients; Spearman's ranked correlation (ratio: 0.69,  $p < 0.0001$ ). (e) Correlation of LDH activity (U/L) in acute serum samples with lymphocyte absolute numbers (cells per  $\mu\text{L}$ ); Spearman's ranked correlation (ratio: 0.44,  $p < 0.0001$ ).

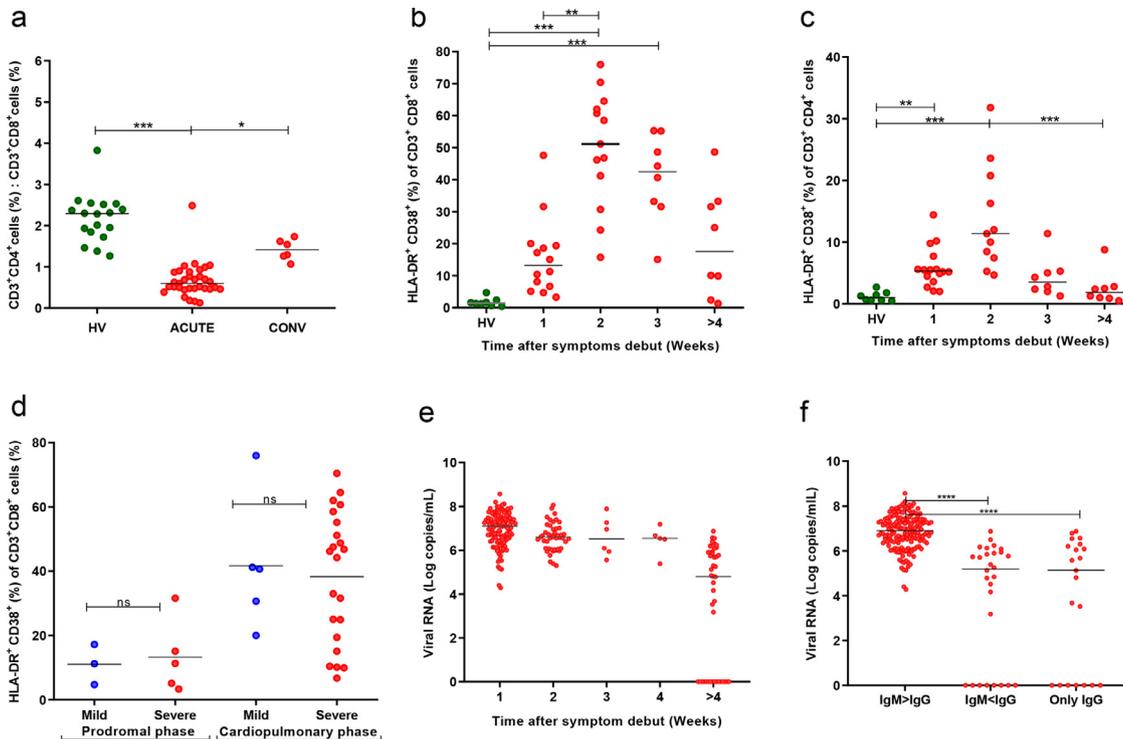
order to follow the evolution of cytokine profile in a subset of patients in sequential blood samples, we performed a dimensional reduction analysis with PCA to identify new meaningful underlying variables. The first two dimensions account for more than 65% of variability of this data set (Fig. S5-a). Considering the period after symptom onset, most acute samples ( $\leq 2$  weeks) grouped together in the lower-right quadrant, showing a relationship with a higher level of specific proinflammatory cytokines, such as the IL6 axis and type I and type II interferons (Fig. S5-b). At later time points, sample values tended to fall in the upper-right quadrant. Interestingly, the exceptions are the samples from the only patient infected by LECV, for which the PC2 remained in the range of healthy volunteers (Fig. S5-c).

## Discussion

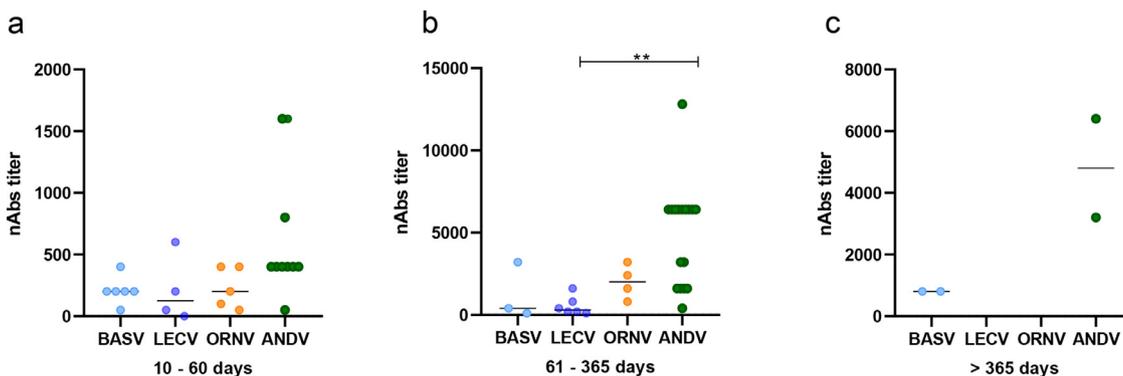
The accurate description of the immunological response to American orthohantavirus infections in humans is key to identifying the mechanisms leading to HPS pathogenesis and characterizing the protective profile useful for vaccine and/or therapeutic developments. The wide geographic extension of the country and the low incidence of disease usually hinder the collection of fresh anticoagulated blood for immunological studies, due

mainly to logistical issues. However, we have carefully included samples from patients, allowing us to evaluate variables that could be representative. Consequently, this study, which involved around 35% of HPS cases reported in the period, included samples that were selected based on (1) geographic origin (different viral variants), (2) age, (3) sex, and (4) severity of disease. Additionally, strict inclusion criteria in terms of information and quality were imposed on the samples in order for the conclusions to be strong. The outbreak in Epuyén provided an opportunity to collect samples from ANDV-infected patients for immunological purposes. Instances of person-to-person transmission provided accurate evidence to precisely define the incubation period for HPS, which is relevant for diagnosis, disease surveillance and control, and treatment with medical countermeasures—many of which are most effective when administered before or immediately after symptom onset.

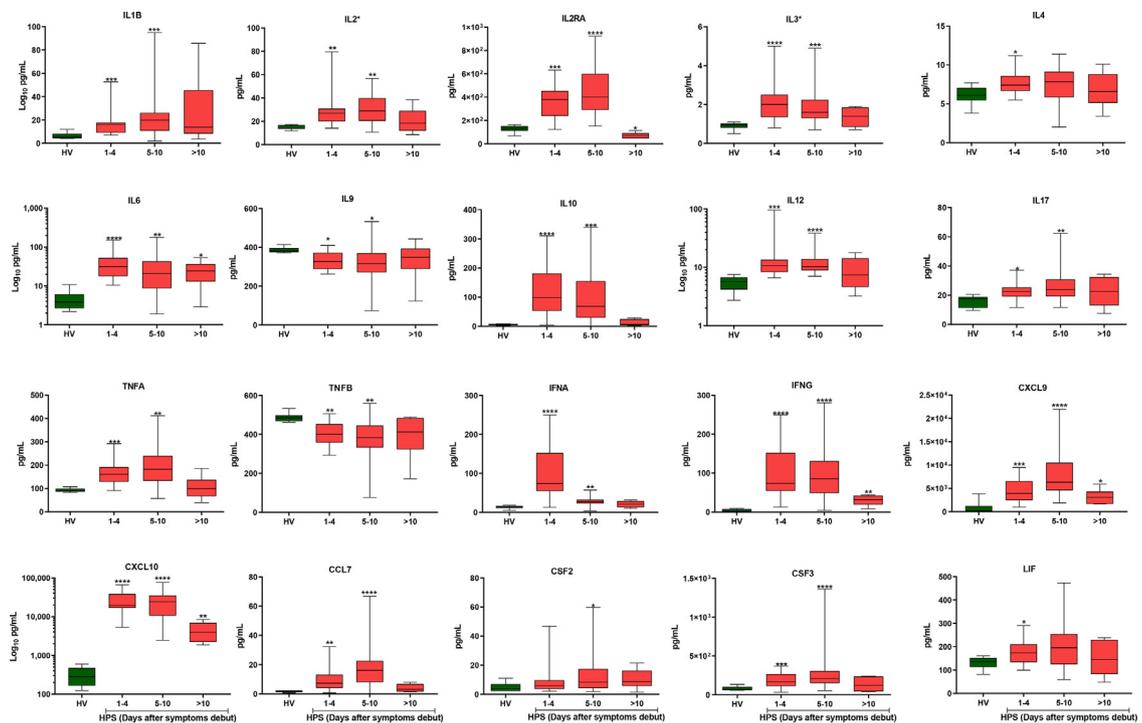
The presence of immunoblasts in the early prodromal phase would be helpful in identifying ANDV infections among high-risk contacts. These cells were previously characterized as activated B and T cells in orthohantavirus-infected patients.<sup>17,18</sup> Also, high numbers of neutrophils were found, and positive correlations of LDH activity (a tissue injury marker) with



**Figure 4.** T lymphocyte subpopulations and viral load in acute hantavirus pulmonary syndrome (HPS) patients. (a)  $CD4^+$  to  $CD8^+$  ratio in acute and convalescent HPS patients (red dots) and healthy volunteers (green dots). (b–c) Comparison of the frequency (%) of activated  $CD8^+$  T cells (week 1,  $n = 14$ ; week 2,  $n = 13$ ; week 3,  $n = 8$ ; > 4 weeks,  $n = 8$ ) or  $CD4^+$  T cells (week 1,  $n = 16$ ; week 2,  $n = 11$ ; week 3,  $n = 8$ ; > 4 weeks,  $n = 8$ ) between HPS patients (red dots); and healthy volunteers (green dots,  $n = 8$ ) according to time after symptoms debut. (d) Comparison of the frequency of active cytotoxic  $CD8^+$  T cells in mild and severe patients during the prodromal and cardiopulmonary phases, Mann–Whitney–Wilcoxon test (ns: not significant). Viral RNA in blood was quantified in patients (e) in different weeks after fever onset: week 1,  $n = 117$ ; week 2,  $n = 46$ , week 3,  $n = 6$ , week 4  $n = 5$ , and weeks > 4 (up to 233 days).  $n = 44$  and (f) during different phases of disease: acute, in patients with  $IgM > IgG$  ( $n = 169$ ); early convalescence,  $IgM < IgG$  ( $n = 28$ ); and late convalescence, no  $IgM$  and high  $IgG$  ( $n = 21$ ). Kruskal–Wallis H test followed by Dunn’s Multiple Comparison test (a, b, c, and f,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ).



**Figure 5.** Neutralizing antibody (nAb) responses in hantavirus pulmonary syndrome (HPS) patients infected by different Andes-virus-like viruses. Neutralizing antibody titers against Andes virus (ANDV) in cases grouped by viral variant: Buenos Aires virus (BASV; light blue), Lechiguanas virus (LECV; purple), Orán virus (ORNV; orange) and ANDV (green). Samples from survivors were divided in three different group of periods, from left to right: (a) 10–60 d (BASV:  $n = 6$ , mean: 29 d; LECV:  $n = 4$ , mean: 30.8 d; ORNV:  $n = 5$ , mean: 35.5 d; ANDV:  $n = 10$  mean: 33.7 d), (b) 61–365 d (BASV:  $n = 3$ , mean: 108.3; LECV:  $n = 6$ , mean: 121 d; ORNV:  $n = 4$ , mean: 176 d; ANDV:  $n = 17$ , mean: 170 d) and (c) more than 365 d after onset of disease (BASV:  $n = 2$ , mean: 1,904 d; ANDV:  $n = 2$ , mean: 1,495 d). Kruskal–Wallis H test ( $p = 0.0018$ ); Dunn’s multiple comparison test ( $**p = 0.0023$ ).



**Figure 6.** Concentrations (pg/mL) of over-expressed and under-expressed cytokines, chemokines, and growth factors in hantavirus pulmonary syndrome (HPS) patients (red boxes), compared to healthy volunteers (green boxes,  $n = 6$ ). HPS patients were divided into three groups according to the days after symptoms debut. HPS 1–4 d  $n = 23$ ; 5–10 d  $n = 28$ ; >10 d  $n = 6$  (range 16–28 d). Mann–Whitney–Wilcoxon test; (\* $p < 0.05$ ; \*\* $p < 0.01$  \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ). Abbreviations: IL, interleukin; IL1B, interleukin 1 subunit beta; IL2RA, interleukin 2 receptor subunit alpha; TNFA, tumour necrosis factor alpha; TNFB, tumour necrosis factor beta; IFNG, interferon gamma; IFNA, interferon alpha; CXCL, Chemokine C-X-C motif ligand; CCL, Chemokine C-C motif ligand; CSF, colony-stimulating factor; LIF, leukaemia inhibitory factor; HPS: hantavirus pulmonary syndrome; HS: healthy volunteers. \* indicates cytokine concentrations calculated from only one read (see Methods).

lymphocyte and neutrophil numbers suggests their possible involvement in endothelial barrier dysfunction in the most affected tissues. Furthermore, high NLRs were observed in severe cases in the cardiopulmonary phase. Previous studies have related increased NLR with lethality in various diseases, including inflammatory disorders.<sup>19,20</sup> The activation and functionality of neutrophils in HPS patients need to be addressed in future studies. The low concentration of IL8 in serum, the major chemotactic factor for neutrophils, is intriguing, since high numbers of these cells were observed in the most severely ill HPS patients. In contrast, high concentrations of IL8 were measured in patients with another disease caused by orthohantaviruses, haemorrhagic fever with renal syndrome (HFRS), and positively correlated with kidney dysfunction, the hallmark clinical finding of HFRS.<sup>21</sup>

Because of contradictory evidence defining the role of T cells in orthohantavirus infections as detrimental or beneficial, we performed a phenotypic characterization of T cells in acute HPS patients, resulting in important findings. Besides the lymphopenia observed in the prodromal phase, there was no decrease in T-cell counts. Thus, the cause of lymphopenia could be

attributed to the migration of T cells or other lymphocyte subpopulations to the most affected tissues, such as lung and liver. The altered CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio could be due to a faster proliferation of CD8<sup>+</sup> compared to CD4<sup>+</sup> T cells. T-cell characterization showed a highly activated phenotype in all patients regardless of clinical condition, suggesting that this phenotype is not related to the progression of disease. This finding was surprising because cytotoxic CD8<sup>+</sup> T cells are known to be the primary cause of tissue damage in non-cytopathic viral infections.<sup>22</sup> Also, the T-cell response was unable to resolve the infection during the acute and early convalescent phases. The persistence of ANDV RNA in blood for long periods of time was described in previous studies on HPS patients.<sup>17–23</sup> More studies are needed to determine if the persistence of ANDV-like viruses in the blood of patients is related to infectiousness and the risk of person-to-person transmission beyond the prodromal phase. Another finding was that viral load was not associated with disease severity. Previous studies in SNV-infected HPS patients showed contradictory evidence related to correlation between viral titers and disease severity.<sup>24</sup> Robust and highly effective antiviral T-

cell responses usually contribute to the clearance of acute infections, while persistent viruses are associated with deficient T-cell responses. In the present work, viral clearance seems to be related to rising titers of neutralizing antibodies during the convalescent phase of ANDV-infected patients. This delay in the neutralizing-antibody response, which was also observed in Lassa fever patients,<sup>25</sup> is intriguing and requires further study. Interestingly, some levels of cross-neutralization were detected in patients with ANDV-like viruses (LECV, BASV, and ORNV) in the same phase.

Additionally, we found increased levels of inflammatory mediators with a predominant Th1 cytokine profile. Anergy or exhaustion of T cells states, characterized by the expression of multiple co-inhibitory receptors, could be the cause of the inefficacy of T cells due to the immune system's attempt to avoid a harmful response to the host. The loss of IL2 is one of the earliest signs of exhaustion.<sup>26</sup> Though, in our cohort of patients, the expression of inhibitory receptors on T cells could not be addressed, the elevated levels of circulating IL2 and IL2R indicate that T cells were probably functional in these patients. Indeed, we observed increased levels of some proinflammatory cytokines and interleukins secreted by T cells in acute patients. These findings indicate that T cells were perhaps neither exhausted nor anergic. In addition, in a previous work, we measured high activities of granzymes A and B acutely ill HPS patients in Argentina.<sup>27</sup> However, functional studies are needed to confirm or rule out this possibility. A previous study in patients with HFRS (nephropathia epidemica), caused by Puumala virus, found no evidence of upregulation of the programmed cell death protein 1 inhibitory receptor in responding CD8<sup>+</sup> T cells, while another study detected cytotoxic CD8<sup>+</sup> T cells highly expressing NKG2D, a TCR-independent activating receptor.<sup>28,29</sup> Bystander activation can be triggered by several viral infections, resulting in the rapid activation of non-specific T cells upon hyperinflammatory stimuli.<sup>30</sup> This is in line with the irrelevance of T cells in the disease pathogenesis subsequent to ANDV infection in golden hamsters, the gold-standard animal model for HPS.<sup>15</sup>

One limitation of this study was the impossibility of performing analysis directly in the lungs, the most-affected organ in HPS patients, since lung dissection is discouraged on ANDV-contaminated corpses due to high infection risk. However, as the primary targets for orthohantavirus replication are endothelial cells and orthohantaviruses are able to establish systemic infections, our analysis in peripheral blood is an important contribution.

In summary, our findings show that the high numbers and phenotypic profile of T cells found in peripheral blood during the acute phase did not correlate with a decrease in viral load or disease severity. Functional studies on samples from HPS patients are needed to explore the highly complex immunological responses to

ANDV-like infections and to address the roles of T cells in pathogenesis.

Conversely, the increment of the neutralizing antibody response was related to viral clearance during convalescence. This finding may have important implications for understanding immune responses to HPS and also for the design of novel immunotherapeutic and preventive vaccines approaches.

### Contributors

All authors read and approved the final version of the manuscript. AAI: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing; NP: Data curation, Investigation, Methodology; CMB: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization; LL: Methodology, Resources; CO: Methodology, Resources; CA: Methodology, Resources; MG: Data curation, Investigation, Methodology; RC: Data curation, Investigation, Methodology; DOA: Data curation, Investigation, Methodology; BD-K: Methodology, Validation; HS: Methodology, Validation; NDP: Data curation, Investigation, Methodology; JHK: Formal Analysis, Funding acquisition, Writing-review & editing; MSL: Formal Analysis, Writing-review & editing; GP: Formal Analysis, Writing-review & editing; PS: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing-review & editing; VPM: Conceptualization, Formal Analysis, Investigation, Visualization, Writing-original draft, Supervision; Writing-review & editing

### Declaration of Competing Interest

All authors declare no competing interests.

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**Data sharing statement**

All raw data, deidentified patient data and statistical analysis will be available after the approval of Ayelen Iglesias Ph.D. thesis, which will be published according to policies of the Universidad de Buenos Aires. The informed consent form is available as requested by e-mail to the corresponding author.

**Supplementary materials**

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103765.

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