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Lethal influenza in two related adults with inherited GATA-2 deficiency

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Disclosure of Conflicts of Interest

The authors declare that they have no conflict of interest.

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Abstract

The pathogenesis of life-threatening influenza A virus (IAV) disease remains elusive, as infection is benign in most individuals.

We studied two relatives who died from influenza. We Sanger sequenced GATA2 and evaluated the mutation by gene transfer, measured serum cytokine levels and analyzed circulating T- and B-cells. Both patients (father and son, P1 and P2) died in 2011 of H1N1pdm IAV infection at the ages of 54 and 31 years respectively. They had not suffered from severe or moderately severe infections in the last 17 (P1) and 15 years (P2). A daughter of P1 had died at 20 years from infectious complications. Low B-cell, NK-cell and monocyte numbers and myelodysplastic syndrome led to sequence GATA2. Patients were heterozygous for a novel, hypomorphic, R396L mutation leading to haplo-insufficiency. B- and T-cell rearrangement in peripheral blood from P1 during the influenza episode showed expansion of one major clone. No T-cell receptor excision circles were detected in P1 and P3 since they were 35 and 18 years respectively. Both patients presented an exuberant, interferon (IFN)-γ-mediated, hypercytokinemia during H1N1pdm infection. No data about patients viremia was available.

Two previously reported adult GATA-2-deficient patients died from severe H1N1 IAV infection, GATA2 deficiency may predispose to life-threatening influenza in adulthood. However, a role of other genetic variants involved in immune responses cannot be ruled out. Patients with GATA2 deficiency can reach young adulthood without severe infections, including influenza, despite long-lasting complete B-cell and natural killer (NK)-cell deficiency, as well as profoundly diminished T-cell thymic output.

Keywords

Immunodeficiency; GATA-2; influenza A virus; H1N1; immunological memory

Introduction

Influenza A virus (IAV) infection typically causes a self-limiting disease of the upper respiratory tract [1, 2]. Primary viral pneumonia (PVP) is a rare complication of seasonal influenza [2]. Severe influenza is more common in the course of pandemic than seasonal influenza. Yet, most individuals infected with the 2009 pandemic H1N1 (H1N1pdm) IAV experienced an uncomplicated flu and up to 75% of infections were even estimated to be subclinical [3–5]. However, in a small subset of patients, H1N1pdm infection rapidly progressed to PVP) with respiratory failure. A minority of patients was admitted to intensive care units (ICU), due to acute respiratory distress syndrome (ARDS) in most cases [1, 3, 5]. The case-fatality rate for symptomatic H1N1pdm illness was estimated to be, depending on the age groups, between 0.002% and 0.308% in western countries [3, 6].

Inherited and acquired variability in host immune responses may influence susceptibility and outcome of IAV infection [7–9]. However, the molecular nature of such human factors has remained largely elusive. It was recently shown that single-gene inborn errors of immunity may underlie severe influenza. Indeed, IRF7 deficiency was associated with severe influenza in one child, and two adult patients with GATA-2 deficiency died from severe H1N1 IAV infection [10–12]. It is interesting and intriguing that severe B and T cell deficiencies do not underlie severe influenza [9].

GATA-2 deficiency, due to germline heterozygous mutations in *GATA2*, was first reported in 2011 [13–16]. The initial presentation of autosomal dominant (AD) GATA-2 deficiency usually occurs in the second decade, but ranges from early childhood to late adulthood. The disease manifestations are variable, and characterized by an increased risk of infectious complications, which usually remains low until the third or fourth decade. Patients are susceptible to severe viral infections, particularly by human papillomaviruses (HPV) and herpesviruses, to nontuberculous mycobacteria, and to severe fungal infections [13–18]. Patients have an increased risk of myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), chronic myelomonocytic leukemia [13, 14, 16], chronic neutropenia [12], aplastic anemia [19], primary lymphedema, sensorineural deafness or pulmonary alveolar proteinosis [13–17].

The laboratory findings include profound monocytopenia, dendritic cells (DCs) deficiency, including in particular a deficiency of interferon (IFN)-producing plasmacytoid DC (pDCs), and B-cell and natural killer (NK)-cell lymphopenia [11–14, 17, 18]. Two previously reported adult GATA-2-deficient patients died from severe H1N1 IAV infection, although after other severe manifestations [11,12]. One patient had previously suffered from disseminated mycobacterial and HPV infections [11]. The second patient had developed AML at the age of thirteen years and, after intensive chemotherapy complicated by pulmonary aspergillosis, she suffered from numerous infections; she died at the age of eighteen from an H1N1 influenza infection in complete remission [12]. The pathogenesis of

influenza in these patients is supposed to involve the lack of pDCs, although a role of other cells, including non-hematopoietic cells, cannot be excluded.

Methods

Routine Immunologic assays

Serum IgG, IgA, IgM and IgG subclasses were measured by means of nephelometry (Siemens Nephelometer, Germany). Lymphocyte subpopulations were analyzed by using flow cytometry (BD Biosciences).

Sanger Sequencing of GATA2

Amplification and sequencing of *GATA2* in genomic DNA from blood samples was performed as previously described [11].

Plasmids, directed mutagenesis, western blot and luciferase reporter assay

Western Blots of GATA-2 were performed on HEK293T cells transfected with plasmids containing *GATA2*-WT isoform 1 (Origene RC208554), GATA2-WT isoform 2 (Origene RC208514), mutated plasmids containing the variants R396L, R396Q and R398W generated by site-directed mutagenesis (QuickChange II XL Site-Directed Mutagenesis Kit: Agilent 200521–5) or with empty vectors (EV).

Luciferase reporter assays were carried out in in HEK293T cells transfected with EV and with plasmids containing the *GATA2*-WT isoform 1 or the R396L and R398W variants. Normalization of transfection efficiency was made using Renilla luciferase gene expression modified (Kindly provided by Zhibing Zhang and Jerome Strauss) [20]. The reading of the samples was performed using Luminometer (Victor X4 model 2030, PerkinElmer).

Production of cytokines after polyclonal activation

Production of cytokines after stimulation of peripheral blood mononuclear cells (PBMCs) with 10 ng/ml of phorbol myristate acetate (PMA; Sigma Chemical Co.) plus 1 μg/ml of ionomycin (Sigma Chemical Co.) was performed as previously reported with minimal modifications [21].

Analysis of IL-17A-and IFN-γ-producing T cells

Blood diluted in culture medium (1:1) was stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin in the presence of 1 μL of Golgi Plug (BD Biosciences) for 4 hours. Then cells were washed and stained with anti-human monoclonal antibodies CD3-FITC (BD Biosciences) and CD8-PerCP (BD Biosciences) for 15 minutes at room temperature. Next, cells were washed, fixed and permeabilized following manufacturer's instructions (BD Cytofix/Cytoperm). Finally, cells were intracellularly stained with anti-human monoclonal antibodies IL-17A-PE (eBiosciences), IFN-γ-PE (BD Biosciences) or isotype-matched negative controls (BD Biosciences), washed, and analyzed on a FACSCanto II flow cytometer (BD Biosciences).

Analysis of cytokines in serum and culture supernatants

The levels of cytokines and chemokines in serum and culture supernatants were measured using flow cytometry-based bead array systems (BD Biosciences).

Identification of influenza A virus (IAV) and measurement of neutralizing antibodies against influenza A viruses

Influenza A H1N1pdm virus was detected in nasopharyngeal swabs using Real-Time Polymerase chain reaction (PCR) AH1N1 assay (F. Hoffmann-La Roche Ltd, Basel, Switzerland) [22]. Titers of neutralizing Ab against H1N1pdm (strain A/California/07/09) and a seasonal IAV strain (A/Brisbane/59/07) were measured in serum samples from P1 and P2 after H1N1pdm infection according to standard protocols [23].

PCR analysis of the rearranged T-cell receptor and immunoglobulin genes

PCR analysis of the immunoglobulin heavy chain (*IGH)* and T-cell receptor gamma (*TCRG*) genes rearrangement was performed with commercial kits (Master Diagnóstica SL, Granada, Spain).

Signal joint T cell receptor excision circles (sjTRECs) quantification

sjTRECs levels were measured in gDNA from peripheral blood using a quantitative real-time PCR adaptation of the original technique [24].

This research has been performed in accordance with the Declaration of Helsinki. The protocols were approved by Clinical Research Ethics Committees of hospitals involved. Written informed consent for the study was obtained from their legal representative. For a detailed description of materials and methods see the Supplementary Material.

Results

Case reports

We studied three related Caucasian patients from a non-consanguineous family from and living in Spain (Figure 1A and Table S1). The proband (P1) was born in 1953. He had a history of chronic neutropenia. A diagnosis of refractory anemia secondary to MDS with dyserythropoiesis, dysgranulopoiesis and dysmegakariopoiesis with abundant macrophages and no reticulin fibrosis in bone marrow (BM) aspirates was made when he was 30 years old. Splenectomy was performed one year after. He suffered one episode of pneumonia at the age of 37 years, and mild recurrent upper and lower respiratory tract infections between ages 37 to 44. He was doing well without medication, when in January 2011, at the age of 54, he was hospitalized for PVP by H1N1pdm IAV. He was treated with oseltamivir, but the disease evolved in the next three days to ARDS. Viral pneumonia was complicated by secondary infection by *Klebsiella pneumoniae* and *Acinetobacter baumannii* and he died 28 days after admission due to refractory septic shock. No necropsy or further BM analyses were performed. One month earlier, the son of P1 (P2) had died due to severe H1N1pdm infection. P2 also had a history of MDS with dyserythropoiesis, dysgranulopoiesis and dysmegakariopoiesis, and a perianal abscess, diagnosed when he was 15 years old. At the age of sixteen years, he was hospitalized for one episode of pneumonia. At the age of 31 he

was admitted to the hospital due to flu-like symptoms lasting for three days. He presented with acute respiratory insufficiency. An X-Ray showed an infiltrate in the left lower lobe. The patient was treated with levofloxacin and ceftriaxone. In few hours his condition evolved to severe respiratory failure with progressive pulmonary infiltrates. Empirical treatment with oseltamivir was started and pharyngeal swabs were later positive for H1N1pdm IAV. Three days later he was admitted to the ICU because of ARDS. Oseltamivir was withdrawn and intravenous zanamivir was instituted. The infection led to death three days later due to refractory hypoxemia, despite the use of prone positioning ventilation and recruitment maneuvers. Unfortunately, extracorporeal membrane oxygenation was not available. No necropsy or further BM analyses were performed.

The daughter of P1 (P3) had developed flu-like symptoms with pulmonary interstitial infiltrates in October 2005, at the age of 17. Lung biopsy showed interstitial fibrosis and focal alveolar proteinosis with presence of abundant foamy macrophages. BM biopsy showed no abnormalities, except a high percentage of macrophages (84%). She died at the age of 20 from complications of SLE-like syndrome management (Table S1). No GATA-2 deficiency-related diseases were observed in the other relatives.

GATA-2 deficiency in three patients

Blood samples from P1, obtained when he was 54 years, six days after hospital admission for H1N1 infection, were recruited to be included in a survey aimed to study the role of genetic variability in the severity of IAV [25, 26]. Routine immunological analysis showed neutropenia, monocytopenia and a nearly complete absence of peripheral NK and $CD20^+$ B-cells. No immunological analysis had been performed on P2 during the flu episode. Historical immunological analysis from P1, P2 and P3 at the ages of 43, 21 and 13 years respectively showed severely reduced numbers of B-cells and monocytes; P3 also had severely reduced numbers of NK-cells (Table 1). On the basis of these data, familial GATA-2 deficiency was suspected. By Sanger method, we found a novel missense heterozygous R396L mutation in *GATA2* in the three patients. The mutation was not observed in their healthy relatives (Figure 1A and B). We did not find the R396L mutation in public data-base (dbSNP, 1000 genome), in 55 healthy Caucasian individuals and in 1022 individuals from 52 ethnic groups from the HGDP-CEPH panel. Residue 396 is highly conserved across species (Figure 1C). In silico analyses performed by means of PolyPhen-2 and PROVEAN/SIFT showed that the damaging effect of the R396L mutation is highly probable. Mutations in the zinc finger-2 domain, particularly R398W (one of most frequent mutations causing GATA-2 deficiency), R398Q, R396W and R396Q have been reported in several independent studies [12, 13, 16, 17, 19], underscoring the key role of these residues on GATA-2 function. The novel R396L mutation suggests that the residue R396 at *GATA2* may be a mutational hotspot.

Expression and function of R396L GATA2 allele in HEK 293T cells

The protein expression of isoforms 1 and 2 of GATA-2 in HEK293T transfected cells was shown to be slightly reduced in the mutant *GATA2* R396L, R396Q and R398W alleles compared to the WT (Figure 2A and Figure S1A-B).

Only GATA-2 isoform 1 has transcriptional activity. Promyelocytic leukemia protein (*PML*) gene is a member of the tripartite motif (TRIM) family and potentiates transactivation activity of GATA-2. HEK293T cells transfected with *GATA2*-WT showed a functional activity that was enhanced when the cells were co-transfected with PML. However, the function of GATA2-R396L, GATA2-R396Q and GATA2-R398W mutants were severely impaired and did not increase with co-expression of PML (Figure 2B), suggesting that R396L is dysfunctional and hypomorphic.

We then performed luciferase assays in transient cotransfections with PML and combined vectors of *GATA2*-WT and *GATA2*-R396L, as well as vectors of *GATA2*-R398W and *GATA2*-R396Q as negative controls, in different amounts mimicking heterozygosity (Figure 3A). We observed a non-significant decrease in GATA2-mediated relative luciferase activity with increasing levels of the *GATA2*-R396L, *GATA2*-R398W and *GATA2*-R396Q mutants. Similar results were obtained with constant amounts of *GATA2*-WT vector and different amounts of *GATA2* R396L, R396Q and R398W mutants (Figure 3B). Interestingly, GATA-2-mediated decrease of relative luciferase activity was observed to decrease at higher concentrations of R396L vector, which could suggest promoter competition. However, when statistical analysis was performed, the difference of GATA2-mediated relative luciferase activity between this point and the activity of the WT alone was not found to be significant; indicating that the observed decrease in relative luciferase activity does not fall into the loss of function category, which could be expected in a dominant-negative model. These results suggest that haplo-insufficiency could be the mechanism of GATA-2 deficiency caused by the R396L, R398W and R396Q mutations.

Analysis of B and T lymphocytes

A nearly normal proportion, (5.4% of lymphocytes), although at reduced absolute numbers, of peripheral $CD19^+$ B-cells (49 $CD19^+$ B-cells/μL; P10-P90 normal values in our healthy adult controls are 135–500 $CD19^+$ B cells/μL) was observed in P1 during the H1N1pdm infection (Table 1, at age 54 years). However, all B-cells were found to be $CD19^{low}CD20^-CD27^{+++}CD24^-CD38^{+++}IgD^-IgM^-$ plasmacytoid B-cells (Figure 4A). CD4 T-cell lymphopenia with an inverted CD4:CD8 ratio, and a severe deficiency of regulatory T cells (Treg) were also observed (Table 1 and Figure S2). The ratio of naïve to memory ($CD45RA^+/CD45RA^-$) CD4 T-cells was found to be within normal values. However, very high numbers of $CD3^+CD56^+$ and $CD3^+CD57^+$ T-cells were found, probably by accumulation of terminal effector $CD8^+$ T-cells. Historical analysis from P2 and P3 showed severely reduced numbers of peripheral B-cells, a normal ratio of $CD45RA^+/CD45RA^-$ $CD4^+$ T-cells and high numbers of $CD3^+CD56^+$ cells. Unfortunately, none biological material was available to analyze peripheral DCs.

Analysis of antibodies

Immunoglobulin levels (IgG, IgA, IgM, and IgG subclasses) were normal in the three patients. IgG antibodies (Ab) against Epstein-Barr virus, cytomegalovirus, herpes simplex virus, varicella-zoster virus, and rubella virus were detected in serum obtained during the influenza episodes from P1 (aged 54 years) and P2 (31 years), although these viral infections had been unremarkable or silent. Surprisingly, neutralizing Ab titers against H1N1pdm

increased from <1:8 to 1:37 and 1:55 in serum samples from P1 taken at days 4, 12 and 19 respectively after admission. Interestingly, similar titers of neutralizing antibodies against the previous annual H1N1 strain were also detected (Figure 4B). Neutralizing Ab against H1N1pdm were not detected in an acute serum sample from P2 taken three days after hospital admission, although he had serum antibodies against a seasonal strain. No later samples from P2 were available.

Analysis of B-cell clonality

High numbers of peripheral plasmacytoid B cells were observed in P1 in spite that he had no peripheral B-cells for at least 11 years. However, a high number of plasma cells were observed in BM from patient P1 and from previously reported patients, which would account for maintenance of antibody production [19, 27]. Spectratyping analysis of *IGH* repertoire in DNA from peripheral blood from P1 obtained during the flu episode showed a restricted, pauciclonal, pattern with the presence of one unusually abundant fragment length. These results suggest that the observed peripheral, plasmacytoid, B cells result from the expansion of one major clone (Figure 4C). Unfortunately, no later samples were available for characterizing the antibody produced by this major clone.

Functional analysis of T lymphocytes

Roughly half of GATA-2-deficient patients show $CD4^+$ T-cell lymphopenia, and accumulation of $CD56^+$ terminal effector $CD8^+$ T-cells, usually with an effector memory RA (EMRA) phenotype [18, 27, 28]. Only a few studies have analyzed T-cell function in GATA-2-deficient patients [11, 27, 29]. T-cell proliferative responses in PBMC from P3 were severely impaired, but they normalized upon addition of a co-stimulus, suggesting that the defect was due to the absence of peripheral monocytes/macrophages (Figure S3) [27]. Analysis of IFN-γ- and IL-17A-producing T-cells in response to polyclonal activation, performed during the flu episode, showed that P1 had a strongly increased expansion of IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells (Figure 5A). PBMC from P1 also showed a strong IFN-γ production after polyclonal activation (Figure 5B). These results contrast with those from Bigley et al [11], who reported low IFN-γ production by GATA-2-deficient cells in response to LPS.

Analysis of T-cell clonality

Less than half of GATA-2-deficient patients show abnormal T-cell receptor rearrangement patterns [19]. Spectratyping analysis of the *TCRG* gene rearrangement in DNA obtained from whole blood from P1 during the flu episode showed a pauciclonal pattern with expansion of a major clone. A severely reduced *TCRG* pattern was already detected in P1 at the ages of 35, and 44 years (Figure 5C). The same analysis in DNA from P3 at the ages of 13 and 18 years showed a slightly reduced pattern of rearrangement (Figure 5C).

sjTRECs quantification

TRECs were only previously analyzed in eight pediatric GATA-2-deficient patients under 18 years of age, showing normal values in most patients [30]. No TRECs were showed in P1 at the age of 35, 44 and 54 years (Table 2). A low number of TRECs was observed in blood

samples from P3 at the age of 13 years, whereas no TRECs were observed in samples obtained when he was 18 year-old (Table S2). Overall, our data would suggest that T-cell thymic output is more impaired in GATA2-deficient patients than previously thought. Memory T-cells against conserved epitopes of IAV, as those previously described [31, 32], could have expanded in patient P1. Unfortunately, no samples to test IAV specific responses were available.

Serum cytokine and chemokine levels during H1N1pdn infection

Hypercytokinemia has been reported to play a direct role in the development of severe ARDS secondary to IAV infection [1, 8]. Serum levels of interferon gamma-induced protein 10 (IP-10, CXCL10) and monokine induced by gamma interferon (MIG, CXCL9) as well as IFN-γ, monocyte chemotactic protein-1 (MCP-1, CCL2) or IL-8 (CXCL8), were strongly elevated in P1 and P2 in the course of H1N1pdm infection. This increase was particularly evident when cytokine/chemokine levels were compared with those observed in other patients with severe H1N1pdm infection from our (Figure 6) or other previously reported studies (Table S3). The pattern of serum cytokines/chemokines was also similar to that observed in patients with severe H5N1 or H7N9 IAV infection (Table S3). High levels of IL-6 and IL-10 were also observed in P1 and P2.

Discussion

We report here three relatives with AD GATA-2 deficiency, two of whom, with no recent history of severe infections, died of influenza during the 2009 H1N1pdm at one month interval. Patients carried a novel, R396L, germline heterozygous mutation of the *GATA2* gene. This mutant protein was expressed normally yet was functionally hypomorphic, underlying haplo-insufficiency in heterozygotes. The severity of the deficiency is illustrated by the death of the three patients from infectious complications, including two deaths from influenza.

P1 and P2 presented an exuberant IFN-γ-mediated, hypercytokinemia during H1N1pdm infection. Accumulating evidence suggest that hypercytokinemia, particularly of IFN-γ, IL-6, IL-8, CCL2, CXCL9 and/or CXCL10 (a IFN-γ-mediated signature), plays a direct role in ARDS development and in IAV-mediated lung pathology and mortality, and that this effect is independent of viral titers [1, 33–35]. No data about viremia or inflammatory response in the other GATA-2 deficient patients with fatal influenza were reported [11, 12]. Plasma levels of Th1 cytokines were also found to be high in GATA-2-deficient patients and severe Epstein-Barr virus (EBV) disease with a marked EBV viremia [36]. Hemophagocytic lymphohistiocytosis (HLH) secondary to an EBV-positive lymphoma [36] severe herpes simplex virus infection [37] and disseminated *Mycobacterium kansasii* infection (C Rodríguez-Gallego et al unpublished results) developed in GATA-2-deficient patients. HLH is a deadly systemic hyperinflammatory condition described as a cytokine storm, particularly of Th1 cytokines [38]. Taken together, our and previous data suggest that, in spite of the profound defects in mononuclear cells, hyperinflammatory responses (particularly Th1-mediated), may develop in GATA-2-deficient patients. Abundant tissue-resident macrophages in lung, BM and healthy skin can be observed in GATA-2-deficient patients

lacking circulating monocytes [11,17,39]. In fact, abundant macrophages had been previously observed in BM biopsies (P1 and P3) and lung biopsies (P3) from our patients. Although alveolar macrophages were found to be, at least partially, dysfunctional, these cells might offer an alternative route of antigen presentation in the absence of DC [17,39]. Treg deficiency could underlie the exuberant IFN-γ-mediated inflammatory response and immunopathology in these patients [40, 41]. Alternatively, a role of genetic variants involved in pathogen sensing and/or inflammatory responses to infection cannot be ruled out [42]. However, in the absence of data about the patient's viremia, we cannot exclude that overwhelming IAV infection is due to the observed deficiencies in mononuclear cells and underlies such an inflammatory response and ARDS in our patients.

In spite that P1 lacked peripheral B-cells for at least eleven years, he was able to produce neutralizing antibodies against the novel H1N1pdm IAV strain and a previous annual H1N1 strain. Broadly neutralizing antibodies directed against conserved regions of the hemagglutinin stalk and receptor binding domain were recently described [43–45]. Since this patient had not been vaccinated against H1N1pdm, our results suggest that one or a few clones of long-living memory B-cells against a previous IAV expanded in patient P1; and that these cells produced antibodies able to cross-react and to neutralize H1N1pdm. Specific Ab are recognized as the main factor to prevent IAV infection, whereas cell-mediated immunity is thought to be crucial for the control of an established infection [1, 31, 32, 46]. This is the rationale for influenza vaccination. However, patients lacking T- and/or B-cells are not prone to severe influenza, although vaccination is considered not to be effective [9]. In any event, Abs able to neutralize H1N1pdm were unable to fight the ongoing infection in P1. In contrast to B-cells, NK-cells, DCs and monocytes, T-cells are thought to be relatively well-preserved in patients with GATA-2 deficiency [27, 30, 39]. Our results suggest that T-cells are more affected in GATA-2-deficient patients than previously suspected and, like in other leukocyte subsets, the effect of GATA-2 deficiency on T-cells is progressive. P1 did not suffer from severe viral, mycobacterial or fungal infections despite complete B-cell deficiency for at least 11 years and the absence of thymic output of T-cells for at least 19 years. Overall, our data emphasize that GATA2-deficient patients may live for a long time off one's immunological memories. Our data underscores the role that long-living memory T- and B-cells can play in the resistance to infection in adulthood, particularly in patients with late-onset primary or acquired immunodeficiencies.

Several PID predisposing to viral infections were previously reported [47–49]. However, severe PID affecting T- and B-cells, such as severe combined immunodeficiency or agammaglobulinemia do not predispose to severe influenza [9]. This contrasts with parainfluenza and other viruses, which often kill children with inborn errors of T-cells [9]. No predisposition to severe influenza was either reported in patients with isolated NK-cell deficiencies or DC deficiency [9, 43–50]. PIDs affecting IFNs type I and II have not been reported to have severe influenza [9]. To our best knowledge, IRF7 deficiency and GATA-2 deficiency are the only described single-gene inborn errors of immunity associated with lethal IAV infection so far. The only known patient with complete IRF7 deficiency developed life-threatening influenza at the age of 2.5 years [10]. Her leukocytes, including pDCs, as well as fibroblasts and iPSC-derived pulmonary epithelial cells, produced diminished amounts of type I IFNs. The child is now 7 years old and well with annual

vaccination against influenza as her sole prophylaxis, suggesting that IRF7 deficiency might not impair immunity against secondary IAV infection. IRF7 is ubiquitously expressed and so susceptibility to severe influenza in IRF7 deficiency cannot be ascribed only to a hematopoietic impairment. Patients with AD GATA-2 deficiency have a large clinical phenotype including viral infectious disease. Due to the profound impairment in so many leukocyte subpopulations, including loss or diminished pDCs and NK, it is difficult to propose the mechanism underlying susceptibility to severe influenza in GATA-2-deficient patients. Besides its role in hematopoiesis, GATA-2 is expressed in endothelium and lymphatic valves, and it is also involved in adipogenesis [17, 28, 39]. However, the clinical infectious phenotype of GATA-2 deficiency is reversed by allogenic hematopoietic stem cell transplantation [39], suggesting that hematopoietic anomalies are by themselves responsible for susceptibility to severe infection. Undoubtedly, the lack of pDCs and NK-cells may contribute. However, unlike the IRF7-deficient patient, who survived life-threatening influenza in childhood, our and the two previously reported patients with GATA-2 deficiency who died by IAV infection, with no hematologic malignancy at the moment of the IAV infection, died in adulthood [10–12]. Such deficiencies should be considered in selected patients with life-threatening flu, even in the absence of any personal or familial history. It is not known why some GATA-2-deficient patients present with specific complications, particularly severe viral infections, while others (even family members with the same mutation) do not [17,39]. A role of other genetic variants involved in innate immune responses cannot be ruled out. Our description of two adult relatives with AD GATA-2 deficiency died of flu, with no recent history of other severe infections, suggests that lethal flu may result from and actually reveal single-gene inborn errors of immunity, at least in some patients [51].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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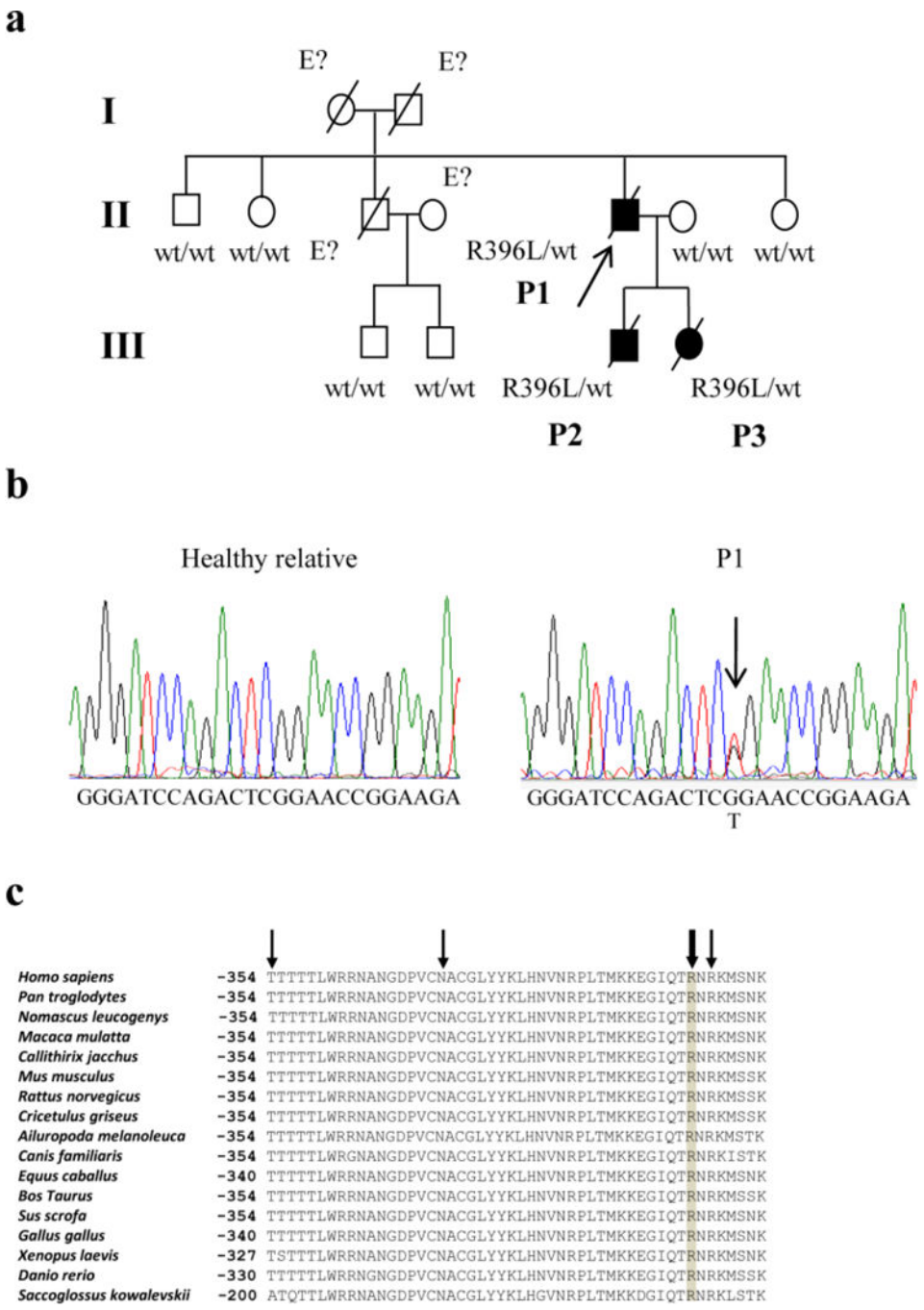

Fig. 1. Novel missense mutation in *GATA2*.

(a) Pedigree of the family of the GATA-2-deficient patients. Patients (P1, P2, and P3) and their relatives are indicated by a black square or circle. *GATA2* genotypes at residue 396 (R396L, mutants; wt, wild-type; E?, unknown) are indicated. The index patient is indicated by an arrow. (b) Electropherograms showing a heterozygous G>T substitution at nucleotide 16913 (exon 7) of *GATA2* in P1. (c) Alignment of the portion of the human GATA-2 molecule containing residue 396 and the corresponding regions in other species. Residue 396 is indicated in gray and by a thick arrow. Other residues in this region, found to be

mutated (T354M, N371K, R396Q, R396W, R398W and R398Q) in previously reported patients with autosomal dominant GATA-2 deficiency are indicated by a thin arrow.

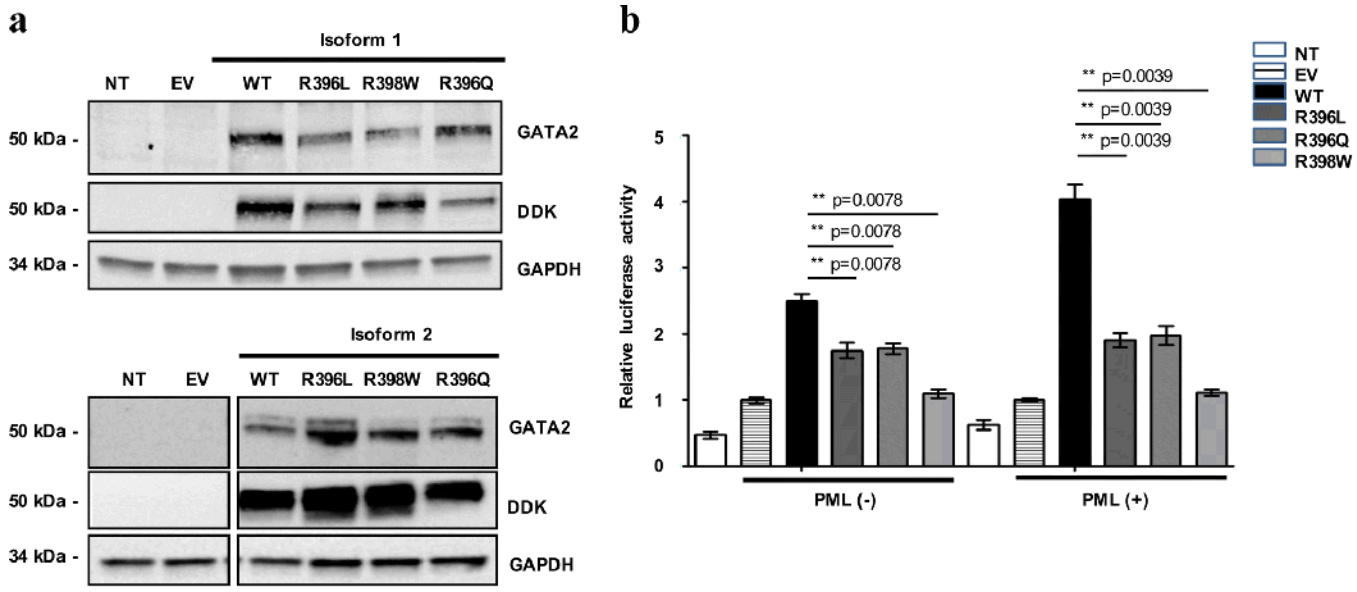

Fig. 2. Expression and transcriptional activity of the R396L allele in overexpression system.

(a) Expression of GATA2 protein. HEK293T cells were transfected with an empty vector (EV), a construct encoding a GATA2 WT (wild type) DDK-tagged or mutated *GATA2* variants (R396L, R396Q, R398W) for 48 hours. Protein expression of GATA2 isoforms 1 (480 aminoacids - aa) and 2 (466 aa) was evaluated by western blotting with an anti-GATA2 antibody and an anti-DDK tag. GAPDH was included for normalization. NT corresponds to no transfected cells. The results shown are representative of three independent assays. (b) Functional characterization of R396L allele. GATA2-mediated luciferase activity was measured in HEK293T cells. The GATA2-dependent transactivation potential was evaluated with GATA2 luciferase reporter vector. Cells were transiently transfected with (+) or without (−) promyelocytic leukemia protein (PML), empty vector (EV) and different *GATA2*-vectors (WT, R396L, R396Q or R398W). The results shown are representative of three independent assays, each performed in triplicate.

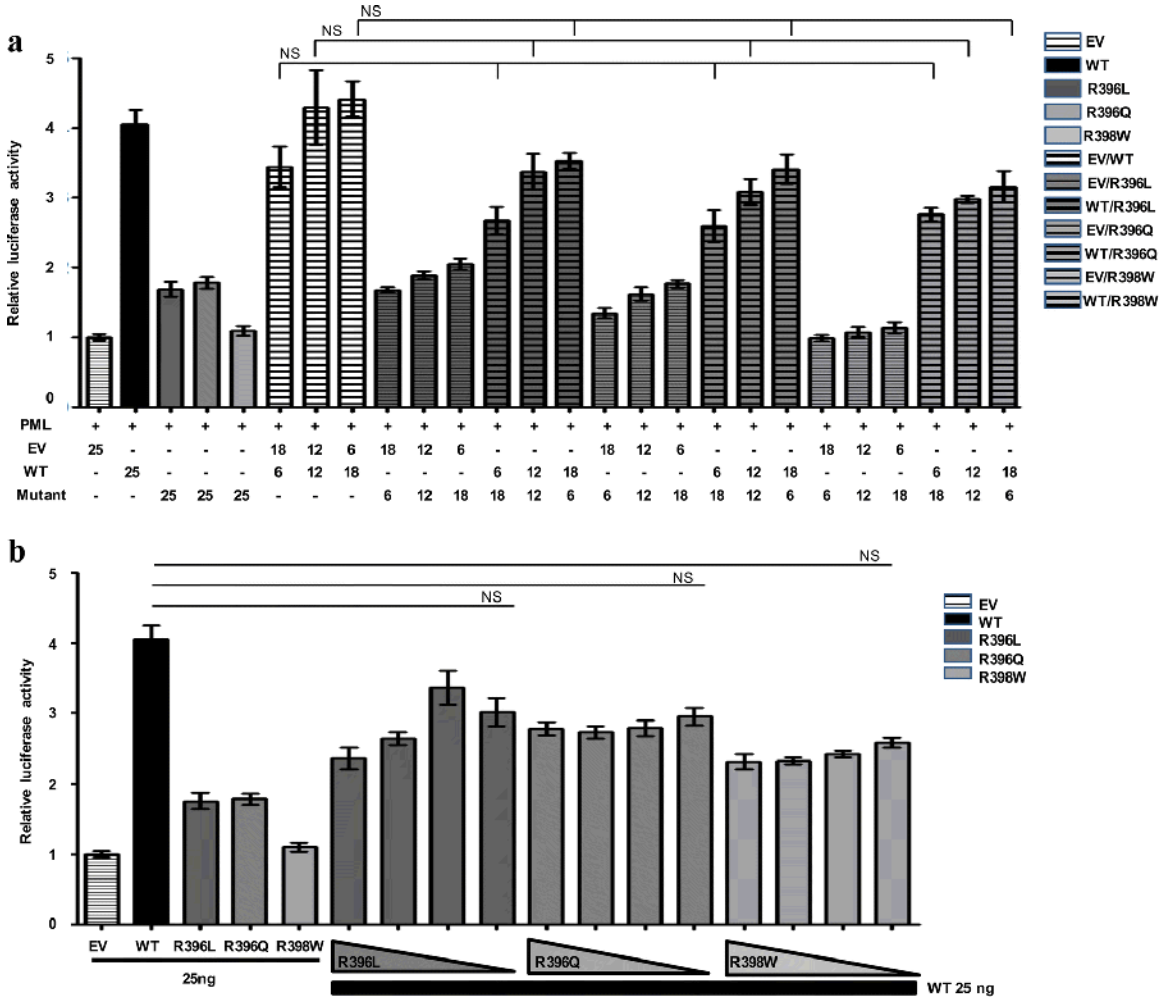

Fig. 3. Analysis of the impact of heterozygosity for the R396L allele in overexpression system.

GATA2-mediated luciferase activity was measured in HEK293T cells. (a) Transient cotransfection of cells with promyelocytic leukemia protein (PML) (+) and different amounts of vectors (empty vector –EV-, wild type – WT-, R396L, EV/WT, EV/R396L, WT/R396L, EV/R396Q, WT/R396Q, EV/R398W or WT/R398W) (b) Transient cotransfection of cells with PML and constant amount of *GATA2*-WT vector with decreasing amounts of *GATA2* mutant (R396L, R396Q or R398W) vectors were used in the experiment. The results shown are representative of three independent assays, each performed in triplicate. Statistical analysis was performed using GraphPad Prism version 5.03 by means of an unpaired t-test (Wilcoxon matched-pairs signed rank test). Data were considered significant when $p<0.01$.

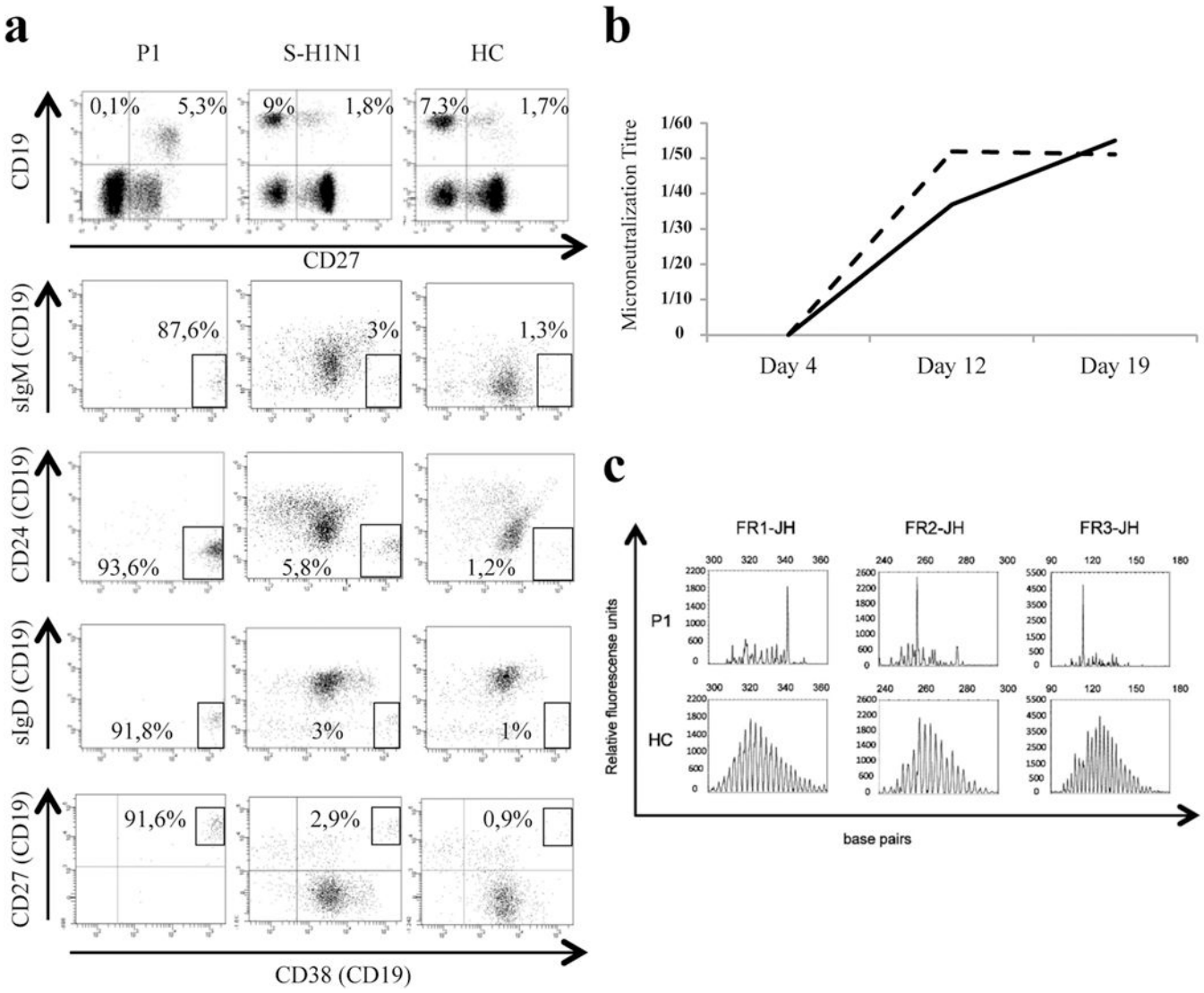

Fig. 4. Peripheral B lymphocytes and production of antibody against influenza A virus in patient 1 during H1N1pdm infection.

(a) B cell phenotype from P1, from one patient with severe acute respiratory failure due to H1N1pdm infection (S-H1N1) and from one healthy control (HC). Plasmacytoid B cells are indicated by rectangles. Acquisition was stopped when 20.000 lymphocytes were gated based on CD45 expression and side scattering. (b) Measurement of neutralizing antibodies against H1N1pdm (thin line) and a seasonal IAV strain (dashed line) in P1. Influenza human antibody standard 09/194 to A/California/07/09-like viruses with a neutralization titer of 1/516 (The National Institute for Biological Standards and Control , UK) was used for titer normalization. Seroconversion is considered to occur when the neutralization titer increase is higher than 4-fold. Microneutralization titers at day 4 were lower than 1/8 (the limit od detection of the analysis). (c) Spectratyping analysis of the B cell receptor heavy chain (*IGH*) repertoire in P1 and in an adult healthy control (HC).

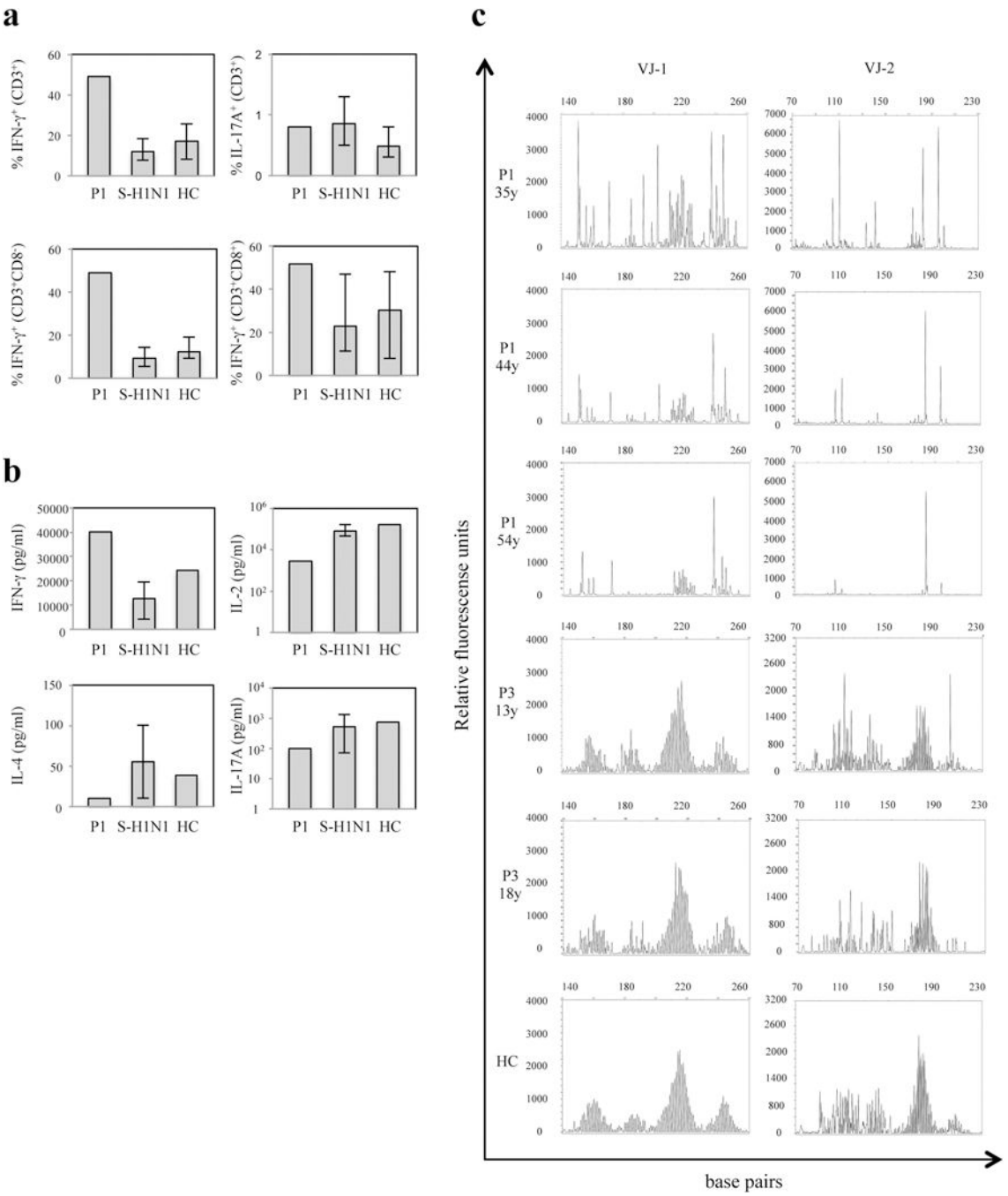

Fig. 5. Analysis of peripheral T lymphocytes.

(a) Analysis of IFN-γ- and IL-17A-producing T cells in response to polyclonal activation in P1, in patients suffering from severe acute respiratory failure due to H1N1pdm infection (S-H1N1pdm; N=5) and in healthy controls (HC, N=5). (b) Production of IFN-γ, IL-2, IL-4 and IL-17A by PBMC after polyclonal activation. The experiments were performed when the patient was attended at Intensive Care Unit during the severe H1N1pdm infection. (c) Spectratyping analysis of the T-cell receptor gamma gene (*TCRG*) repertoire. Analysis

performed in DNA from whole blood from P1 and P3 at different ages and from one representative healthy control (HC).

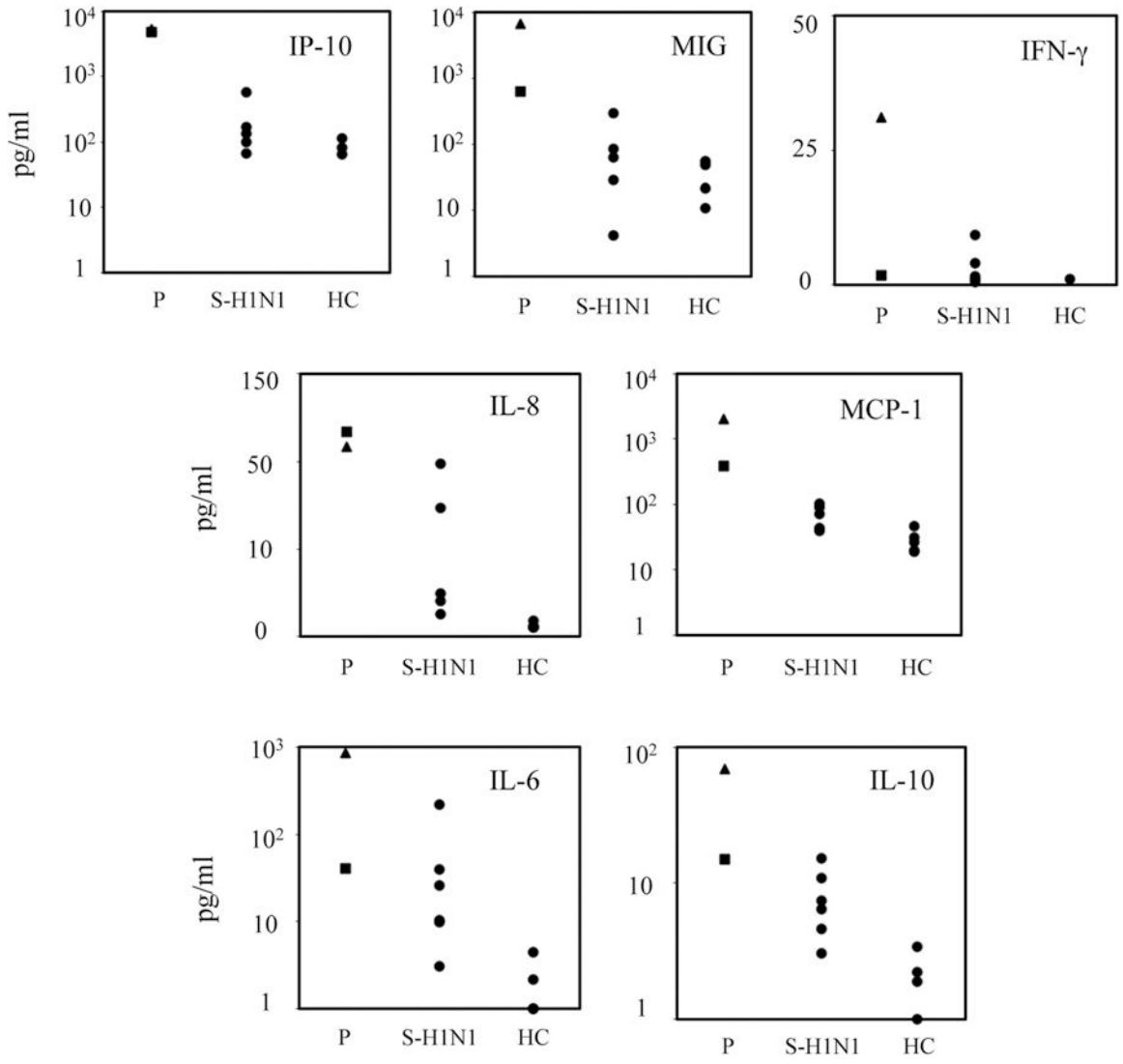

Fig. 6. Serum cytokine and Chemokine levels.

Serum from P1 (filled square) and P2 (filled triangle) were obtained 6 and 4 days after admission to Intensive Care Unit respectively. Samples from patients with severe acute respiratory failure due to H1N1pdm infection (S-H1N1pdm; N=6) obtained 5.4 (range 3–7) days after hospital admission and seven healthy controls (HC) are also included. No differences were observed when serum levels of IL-1-β, IL-2, IL-4, IL-12p70, IL-17A, TNF-α and CCL5 (RANTES) were compared.

Table 1.

Leukocyte count and lymphocyte subpopulations.

	P1 54 years	P1 43 years	P2 21 years	P3 13 years	S-H1N1pdm (N=6)	HC (N=29)
Leukocyte count (cells/μl)						
Lymphocytes	900	4730	1530	2060	1355 (955–1880)[a]	2666 (1640–3410)[a]
Monocytes	290	0–183	10–70	0–100	645 (371–1042)[a]	601 (445–777)[a]
Neutrophils	420	870	660	4660	5208 (3070–7610)[a]	4531 (2790–6810)[a]
Lymphocyte subpopulations (%)[b]						
T cells						
$CD3^+$	95	92	84	96	66 (46–77)	72 (65–81)
$CD3^+CD4^+$	49	42	41	29	46 (30–60)	45 (35–56)
$CD45RA^-$ ($CD3^+CD4^+$)[c]	34		73	39	49 (34–62)	48 (30–76)
$CD3^+CD8^+$	64	63	45	68	19 (11–29)	25 (18–32)
$CD4^+CD8^+$ (CD3)	20.3				1.40 (0.4–2.2)	2.0 (0.7–2.4)
TCR γδ	7	10	6	19	2 (1–8)	4 (1–9)
Treg[d]	1.4				6.4 (4.7–8.7)	6.5 (4.9–8.2)
$CD3^+CD56^+$	62		33	24	4 (2–11)	6 (2–11)
$CD3^+CD57^+$	66	50	29	19	7 (2–17)	12 (4–26)
B cells						
$CD19^+$	5.4	0	0,8	1,7	23 (10–48)	12 (8.0–18)
$CD20^+$	0.2	0	0,7	1,6	21 (8–46)	12 (8–17)
$CD19^+CD27^+$	5.4				8.3 (1.7–23)	3.2 (1.6–4.7)
NK cells						
$CD3^-CD16^+$	0.3	0.6			11 (8–15)	14 (8–22)
$CD3^-CD56^+$	0		12	0.8	9 (6–13)	13 (7–19)
$CD3^-CD57^+$	0.2		11	0.6	4.2 (1.5–7.5)	7 (2–13)

S-H1N1pdm: patients with primary viral pneumonia and severe acute respiratory failure due to H1N1pdm infection; HC: adult healthy controls. Treg: regulatory T cells.

[a] Values are mean (percentiles 10–90).

[b] In the group S-H1N1pdm values are mean (range); in the group HC values are mean (percentiles 10–90).

[c] $CD45RA^-$ ($CD3^+CD4^+$) cells were $CD45R0^+$.

[d] Treg were estimated as the percentage of $CD4^+$ T-cells expressing $CD25^{high}CD127^{-/low}$ (see also Figure S2). P1 had 6,2 Treg cell/μL, a 92% reduction compared with the median values observed in healthy controls (78 Treg/μl; percentile 10–90, 38–136 Treg/μl).

Table 2.

Quantification of sjTRECs.

Patient	Age (years)	sjTRECs[a]
P1	35	**0**
	44	**0**
	54	**0**
P3	13	**844**
	18	**0**
Healthy donors (N=15)	10–15	**>1500**
Healthy donors (N=30)	18–65	**> 100**

[a]The results are expressed as sjTRECs per 100ng of gDNA from whole blood.