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Differential Immune Profiles in Two Pandemic Influenza A(H1N1)pdm09 Virus Waves at Pandemic Epicenter

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Background and Aims. Severe influenza A(H1N1)pdm2009 virus infection cases are characterized by sustained immune activation during influenza pandemics. Seasonal flu data suggest that immune mediators could be modified by wave-related changes. Our aim was to determine the behavior of soluble and cell-related mediators in two waves at the epicenter of the 2009 influenza pandemic.

Methods. Leukocyte surface activation markers were studied in serum from peripheral blood samples, collected from the 1st (April–May, 2009) and 2nd (October 2009–February 2010) pandemic waves. Patients with confirmed influenza A(H1N1) pdm2009 virus infection (H1N1), influenza-like illness (ILI) or healthy donors (H) were analyzed.

Results. Serum IL-6, IL-4 and IL-10 levels were elevated in H1N1 patients from the 2nd pandemic wave. Additionally, the frequency of helper and cytotoxic T cells was reduced during the 1st wave, whereas CD69 expression in helper T cells was increased in the 2nd wave for both H1N1 and ILI patients. In contrast, CD62L expression in granulocytes from the ILI group was increased in both waves but in monocytes only in the 2nd wave. Triggering Receptor Expressed on Myeloid cells (TREM)-1 expression was elevated only in H1N1 patients at the 1st wave.

Conclusions. Our results show that during the 2009 influenza pandemic a T cell activation phenotype is observed in a wave-dependent fashion, with an expanded activation in the 2nd wave, compared to the 1st wave. Conversely, granulocyte and monocyte activation is infection-dependent. This evidence collected at the pandemic epicenter in 2009 could

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help us understand the differences in the underlying cellular mechanisms that drive the wave-related immune profile behaviors that occur against influenza viruses during pandemics.

Key Words: Immune profiles, Pandemic A(H1N1)pdm2009 influenza, Pandemic waves.

Introduction

Influenza A(H1N1)pdm09 virus infection resulted in high morbidity and mortality rates in young adults during the Mexican outbreak (1,2). Some underlying conditions (3) or simultaneous contact with infectious foci (4) were related to influenza severity. Acute manifestations in young-immunocompetent subjects resembled infections by highly pathogenic pandemic influenza viruses, such as the H5N1 avian influenza and the 1918 H1N1 virus (2). The immunological profiles of previous pandemics (e.g., 1918, 1957 and 1968) showed hypercytokinemia and exacerbated leukocyte activation (5,6), making it relevant to characterize the inflammatory response to the influenza A(H1N1)pdm09 virus (7).

Multiple waves during influenza pandemics have been observed (8–10), and mathematical models show that viral mutations (11), social interventions (12–15) and ratio of immunocompromised individuals (16–18) determine the potential and magnitude of subsequent waves. These theoretical models are essential for analyzing multiple influenza outbreak waves and pandemic planning (19). In addition, seasonal influenza has shown different immune behaviors among waves (20,21). However, no evidence has been reported regarding the wave-related changes for immune soluble and cellular markers in the first influenza pandemic of the 21st century.

In addition to soluble mediators, surface cellular markers such as CD69 (an early lymphocyte activation marker), lung homing related CD62L, Triggering Receptor Expressed on Myeloid cells (TREM)-1 and HLA-DR have been useful for inflammation analysis in influenza infections (22), inflammatory systemic syndrome (23) and hypersensitivity states (24).

Furthermore, multi-wave epidemics such as in Mexico 2009 offer opportunities to test the immune nature of short-term shifts in humoral and cellular responses. We report the immunological profiles (cytokine and chemokine levels, proportion of circulating leukocytes and activation phenotype, based on surface expression markers) in RT-PCR verified patients for influenza A(H1N1)pdm2009 virus (H1N1), influenza-like illness (ILI) and healthy donors (H) during two infection waves in the epicenter of the Mexican outbreak.

Materials and Methods

Patients and Sample Collection

Our study was conducted by the Medical Research Unit in Immunochemistry (UIMIQ) and approved by the Ethics and Research Committee of the National Medical Center Siglo XXI, IMSS, Mexico City (Research project: CNIC 2010-785-002). Mexico City patients from the 1st (April–May 2009) and 2nd (October 2009–February 2010) pandemic waves were enrolled.

Sixty-two patients from the Specialties Hospital of the National Medical Center Siglo XXI, IMSS or the Regional Hospital "Dr. Carlos MacGregor", and 12 healthy volunteers were analyzed. All subjects were included after informed consent had been read and signed according to guidelines established by the local ethics committee. Study groups were classified into the following: a) influenza-like illness (ILI) patients from the 1st (n = 10) and 2nd (n = 20) waves; b) confirmed influenza A(H1N1)pdm09 virus-infected (H1N1) patients from the 1st (n = 9) and 2nd (n = 23) waves; or c) healthy volunteers (H, n = 12). None of the patients from the 2nd wave was recruited in the 1st one. ILI was defined as a combination of cough, headache, and fever with one or more of the following symptoms: sore throat, rhinorrhea, arthralgia, myalgia, prostration, thoracic pain, abdominal pain, nasal congestion, diarrhea and irritability, as previously described (25). All patients were tested for influenza A(H1N1)pdm09 viral infection using specific real-time reverse transcription–polymerase chain reaction (rRT-PCR) according to the CDC protocol of real-time RT-PCR for swine influenza (H1N1) (26). The analysis was performed at the Unit for Epidemiological Surveillance, National Medical Centre "La Raza", IMSS, Mexico City.

All participants were clinically evaluated at the time of their first admission in the emergency room; the following signs or symptoms were assessed: fever (≥38°C), headache, cough, sore throat, rhinorrhea, myalgia, joint pain, dyspnea, conjunctivitis, sore back, diarrhea, asthenia, nausea, and/or vomiting. Systemic Inflammatory Response Syndrome (SIRS) was diagnosed according to the definition of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (27). None of the patients had bacterial co-infections or previous influenza vaccination. Afterwards, blood specimens from each subject were collected in silicone and EDTA-coated tubes (BD Vacutainer, Franklin Lakes, NJ). Serum samples were obtained and stored at −70°C and EDTA samples were processed immediately. Due to logistic problems, in some cases we were unable to obtain serum and blood cells from the same subject for all immune evaluations.

Determination of Anti-Influenza Titers

The titer of anti-hemagglutinin antibodies was determined by hemagglutination inhibition test. Serum samples were

heated at 56°C for 30 min and serially diluted in PBS-BSA, pH = 6.9. Fifty µL of serum dilutions were incubated for 30 min at 37oC with an equal volume of pre-diluted virus (influenza A/Mexico/4482/2009, provided by InDRE) containing 8 HA units. After incubation, titers were measured by inverse dilution where 100% of erythrocytes agglutinated. Titers of 1:40 or above were considered positive (28,29).

Cytokine and Chemokine Quantification

Serum cytokine (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A) and chemokine (CXCL8, CXCL9, CCL2 and CXCL10) concentrations were determined using a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA), according to the manufacturer's instructions. Log-transformed data were used to construct standard curves that were fitted to 10 discrete points using a 4-parameter logistic model. The concentrations in the test samples were calculated interpolating from their corresponding standard curves. Data analysis was performed using the GraphPad Prism software (GraphPad Software, San Diego, CA).

Peripheral Leukocyte Surface Marker Assessments

Whole blood samples were evaluated for: a) autofluorescence; b) six-color antibody-conjugated cocktail, which included anti-CD19/FITC (Clone: HIB19, BD Bioscience), anti-TREM-1/PE (Clone: 193015, R&D Systems), anti-CD86/PE-Cy5 (Clone: FUN-1, BD Bioscience), anti-CD14/PE-Cy7 (Clone: M5E2, BD Bioscience), anti-CD62L/APC (Clone: Dreg-56, Invitrogen) and anti-HLA-DR/APC-Cy7 (Clone: M5E2, BD Bioscience); or c) five-color cocktail, which included anti-CD3/FITC (Clone: HIT3, BD Bioscience), anti-CD69/PE (Clone: HIB19, BD Bioscience), anti-CD4/PE-Cy5 (Clone: RPA-T4, BD Bioscience), anti-CD8/APC (Clone: 3B5, Invitrogen) and anti-CD19/PerCP (Clone: SJ25-C1, Caltag). Appropriate isotype controls were also used. After 15 min in the dark at room temperature (RT), 500 µL of FACS Lysing Solution (Becton-Dickinson, CA) were added, and incubated for 10 min at RT. The cell suspensions were washed once with a 1 mL fraction of phosphate-buffered saline by centrifugation at 900 × g for 5 min at RT. Ten thousand single leukocytes were acquired using FACSDiva 6.1.3 software in a FACSAria I flow cytometer (BD Biosciences). Final analysis was performed using the Infinicyt Analytical Software (Cytognos). The analysis algorithms to identify and characterize leukocytes are presented in Supplementary Figure 1. Percentages of CD69, CD62L, TREM-1 and HLA-DR positive cells were determined in: $CD3^+CD4^+$ (helper T lymphocytes), $CD3^+CD8^+$ (cytotoxic T lymphocytes), $CD19^+$ (B-lymphocytes), $CD14^+$ (monocytes) or granulocyte-gated cells (defined as $FSC^{high}SSC^{high}$ as routinely analyzed in clinical hematocytometers) (30). Additionally, granulocytes were $CD3^-CD19^-CD14^-$ (31). For each population, the relative expression of each marker was determined by Mean Fluorescence Intensity (MFI). Representative histograms are presented in Supplementary Figure 2.

Statistical Analysis

To know if medians were different among groups, Kruskal-Wallis test and Dunn's multiple comparison post-test were calculated; $p < 0.05$ was considered statistically significant. All statistical analyses and graphics were performed with Prism 5 software (Graphpad Software, La Jolla, CA).

Results

Demographic and Clinical Characteristics

Demographic and clinical characteristics of the patients for the 1st and 2nd influenza waves are summarized in Tables 1 and 2. Based on gender distribution, no significant differences were detected among the groups. However, we found that ILI patients were significantly older than H1N1 patients in the 2nd wave ($p < 0.05$). Major influenza signs and symptoms, underlying conditions, and fatal outcomes were similar among groups. In contrast, the SIRS rate was higher in the 1st wave (60% for ILI and 90% for H1N1) compared to the 2nd wave (15% for ILI and 17% for H1N1). Both ILI and H1N1 patients from the 1st wave were negative to anti-A(H1N1)pdm2009 antibodies, showing titers <40; on the other hand, seven patients (five ILI and two H1N1) in the 2nd wave were positive for anti-A(H1N1)pdm2009 with titers >40 (Table 2).

No hypercytokinemia but differential cytokine/chemokine concentrations between waves are described. Table 3 summarizes the cytokine and chemokine determinations. No significant differences were found for IL-2, TNF-α,

Table 1. Epidemiological data

	1st wave ($n = 19$)		2nd wave ($n = 43$)		
	ILI ($n = 10$)	H1N1 ($n = 9$)	ILI ($n = 20$)	H1N1 ($n = 23$)	H ($n = 12$)
Gender					
Female	4	4	12	10	7
Male	6	5	8	13	5
Age (years)					
Media	41	38.2	48[a]**/[b]*	30.3[b]*	30.8[a]**
Max	49	78	76	59	64
Min	15	15	18	16	22

1st wave: April–May 2009; 2nd wave: October 2009–February 2010.
*$p < 0.05$.
**$p < 0.01$.
[a]ILI or H1N1 (2nd wave) vs. H.
[b]ILI vs. H1N1 at the same wave; Kruskal-Wallis test with Dunn's multiple comparison post-test.

Table 2. Clinical data

	1st wave		2nd wave	
	ILI ($n = 10$)	H1N1 ($n = 9$)	ILI ($n = 20$)	H1N1 ($n = 23$)
Major signs and symptoms				
Fever (>38 °C)	10/10	8/9	20/20	23/23
Headache	8/10	9/9	19/20	21/23
Cough	8/10	9/9	20/20	23/23
Throat pain	4/10	6/9	4/20	6/23
Diarrhea	3/10	0/9	1/20	1/23
Days of evolution at diagnosis	3.7 ± 2.8	5.7 ± 3.6	4.2 ± 4.5	4.5 ± 5.9
SIRS	6/10[a*]	8/9[b*]	3/20[a*]	4/23[b*]
Underlying conditions				
Tobacco consumption	3/10	6/9	8/20	5/23
Diabetes	0/10	1/9	5/20	2/23
COPD	0/10	4/9	2/20	1/23
Dyslipidemia	1/10	2/9	3/20	1/23
Hypertension	0/10	4/9	7/20	2/23
Asthma	1/10	2/9	4/20	2/23
H1N1pdm2009 antibody titers (>40)	0/10	0/9	5/20	2/23
Fatal outcome	0/10	0/9	2/20	0/23

SIRS, systemic inflammatory response syndrome; COPD, chronic obstructive pulmonary disease; 1st wave: April–May 2009; 2nd wave: October 2009–February 2010; [a]ILI (1st wave) vs. ILI (2nd wave); [b]H1N1 (1st wave) vs. H1N1 (2nd wave); Kruskal-Wallis test with Dunn's multiple comparison post-test.
*$p < 0.05$.

IFN-γ, IL-17A, CXCL10 and CXCL9 levels among groups. Additionally, outlier values had no correlation with any ILI or H1N1 group, disease severity or fatal outcome. IL-6 concentration in ILI and H1N1 groups was higher than in H group for the 1st and 2nd waves ($p < 0.05$). In contrast, CXCL8 was significantly lower in ILI and H1N1 compared to the H group in the 1st wave ($p < 0.05$), and still lower in the 2nd wave; however, they did not reach statistical significance. Finally, IL-4 concentration in the H1N1 group was higher than in the ILI group ($p < 0.05$); IL-10 concentration in the H1N1 was elevated in contrast with the H group ($p < 0.05$), and IL-4 and IL-10 concentrations in the H1N1 group were higher in the 2nd wave compared to the 1st wave.

Leucocyte Activation Is More Evident During the 2nd Pandemic Wave

No significant differences in the peripheral percentages of granulocytes, monocytes or lymphocytes were observed among groups (Supplementary Table 1). In contrast, percentage of helper T cells in the ILI and H1N1 groups was significantly lower in the 1st wave than in the 2nd wave ($p < 0.01$); helper T cells in the ILI and H1N1groups were also significantly lower than in the H group (Supplementary Table 1 and Figure 1A, $p < 0.05$ and $p < 0.01$, respectively). Similar results were observed for cytotoxic T cells in ILI and H1N1 groups where the percentage was lower in the 1st wave than in the 2nd wave (Supplementary Table 1 and Figure 1B, $p < 0.01$ and $p < 0.05$).

Finally, we observed that CD69 expression on helper T cells in the ILI and H1N1 groups was lower in the 1st wave compared to the 2nd wave ($p < 0.01$ and $p < 0.05$, respectively); however, for the 2nd wave, CD69 expression on helper T cells was higher in the ILI and H1N1 groups in comparison with the H group (Figure 1C; $p < 0.05$ and $p < 0.01$, respectively). We did not observe statistical differences for the expression of CD69 in cytotoxic T cells

Table 3. Serum cytokine and chemokine levels

Cytokine/ Chemokine (pg/mL)	1st wave		2nd wave		
	ILI ($n = 7$)	H1N1 ($n = 9$)	ILI ($n = 18$)	H1N1 ($n = 23$)	H ($n = 12$)
IL-2	2.9 ± 0.9	LOD	2.5 ± 0.3	2.7 ± 0.8	LOD
IL-4	6.0 ± 3.2	5.4 ± 4.6	3.9 ± 59.1[b]*	14.3 ± 40.4[b]*	3.9 ± 0.00
IL-6	16.3 ± 21.1	47.7 ± 85.1	58.9 ± 257.4[a]*	173.4 ± 795.3[a]*	2.3 ± 1.2[a]*
IL-10	LOD	4.9 ± 2.5	5.5 ± 27.2	11.4 ± 20.5[a]*	3.9 ± 1.3[a]*
TNF-α	LOD	LOD	3.4 ± 2.6	3.3 ± 2.30	LOD
IFN-γ	LOD	LOD	5.6 ± 8.8	7.8 ± 16.2	LOD
IL-17A	LOD	LOD	LOD	19.3 ± 3.1	22.8 ± 12
CXCL10 (μg/mL)	2 ± 1.2	3.6 ± 1.4	2.4 ± 1.9	3.5 ± 1.8	2.3 ± 1.4
CCL2	111.2 ± 124.8	156.3 ± 216.0	147.3 ± 148	665.7 ± 1044.3	192.1 ± 37.2
CXCL9 (μg/mL)	0.9 ± 0.5	1.6 ± 1.6	2.5 ± 1.9	2.9 ± 1.8	1.2 ± 0.9
CXCL8	26.3 ± 31[a]*/[c]*	35.8 ± 14.87[a]*/[d]*	236.4 ± 349[c]*	301.7 ± 529.5[d]*	580.7 ± 766.4[a]*

Limits of detection (pg/mL): 2.6 (IL-2); 4.9 (IL-4); 2.4 (IL-6); 4.5 (IL-10); 3.8 (TNF-α); 3.7 (IFN-γ); 18.9 (IL-17A); 2.8 (CXCL10); 2.7 (CCL2); 2.5 (CXCL9); 0.2 (CXCL8); 1st wave: April–May 2009; 2nd wave: October 2009–February 2010; LOD: lower limit of detection. Mean ± SD.
Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$.
[a]ILI or H1N1 (1st wave) vs. H.
[b]ILI vs. H1N1 at the same wave.
[c]ILI (1st wave) vs. ILI (2nd wave).
[d]H1N1 (1st wave) vs. H1N1 (2nd wave).

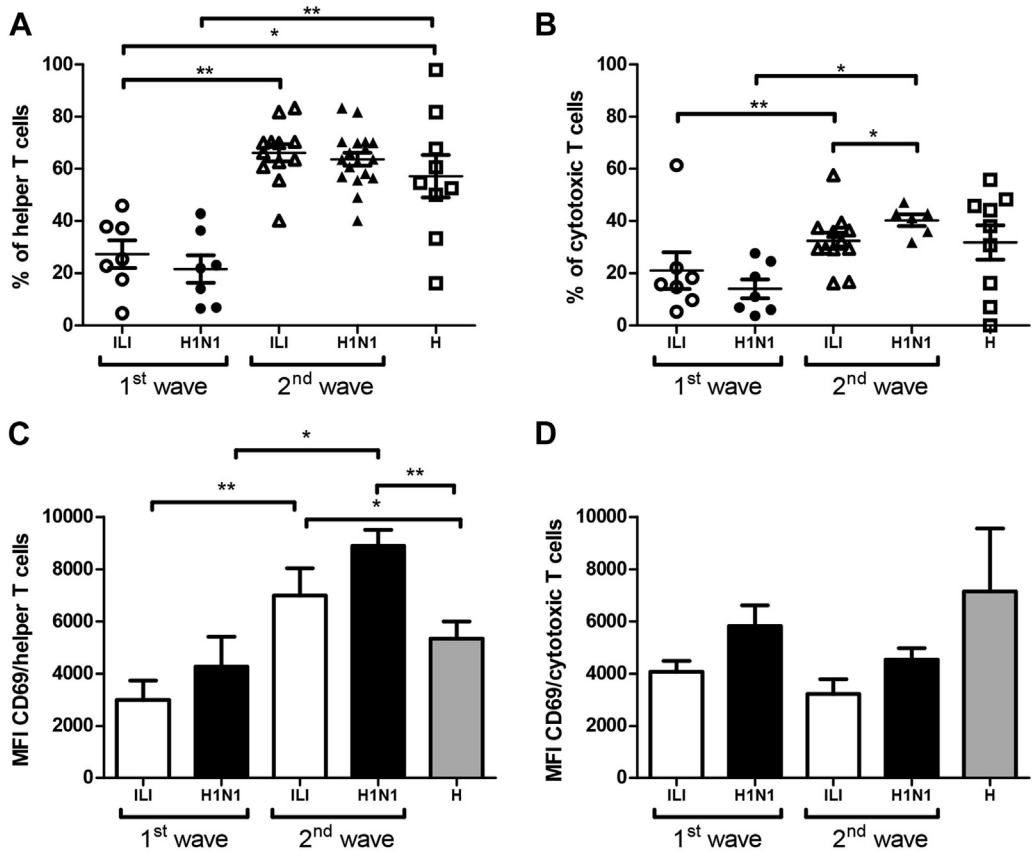

Figure 1. T cell phenotype in the ILI, H1N1 and H groups from the 1st and 2nd pandemic waves. Peripheral blood leukocytes were immunostained with CD3-, CD4-, CD8- and CD69-specific antibodies and analyzed with flow cytometry. The CD69 percentages and relative expression levels in helper (A,C) and cytotoxic (B,D) T cells are shown as mean and standard deviation. A Kruskal-Wallis test, which was followed by a Dunn's multiple comparison test, was performed. *$p < 0.05$, **$p < 0.01$.

(Figure 1D). CD62L expression in granulocytes and monocytes in the ILI group was higher than in the H group at the 2nd wave (Figure 2A−B granulocytes: $p < 0.01$; monocytes: $p < 0.05$). Unlike CD62L expression, TREM-1 expression in monocytes in the 1st wave was higher in the H1N1 group than in the H group (Figure 2C, $p < 0.01$). Additionally, HLA-DR expression in monocytes in both groups was higher in the 1st wave than in the 2nd wave, and both waves displayed higher expression in comparison with the H group (Figure 2D, $p < 0.05$).

Discussion

Influenza A(H1N1)pdm09 virus has shown different rates of morbidity and mortality among different countries, seasons and waves (26,32,33), reaching less mortality than the 1918 pandemic virus (0.01−0.06 vs. 2−3%) (34). However, the influenza A(H1N1)pdm09 virus had a higher lethality rate in young adults compared to neonates and elderly persons, but similar lethality in patients immunocompromised or affected by comorbidities during the 2009 outbreak (35). We did not find any age-associated or clinical differences between the waves that could explain a different immunological response against the pandemic virus (Tables 1 and 2). Moreover, we observed that the comorbidity and clinical diagnosis was not useful to differentiate between the ILI and the H1N1 groups as reported previously (36). However, we observed a lack of antibody titers >40 in the 1st wave patients, supporting the hypothesis for a faint immunological response against influenza A(H1N1)pdm09 virus in the first wave. In contrast, seven patients (five ILI and two H1N1) expressed titers >40 during the 2nd wave, suggesting an elevated immune response during the 2nd wave. Seroprevalence analysis in Mexico showed that 39% of the population was positive after the 2nd wave, with significantly higher titers in subjects <20 years of age (49.5%); interestingly one third of the seropositive subjects were asymptomatic (36). This could result from vaccination programs leading to homologous reinfections (37), suggesting a wide spread of wild type pandemic virus resulting in inter-pandemic influenza exposure, thus providing short-lived protection (38). Mathematical models have noted that early vaccination programs lead to limitation for a 2nd wave; however, despite an early vaccination program in Mexico a 2nd

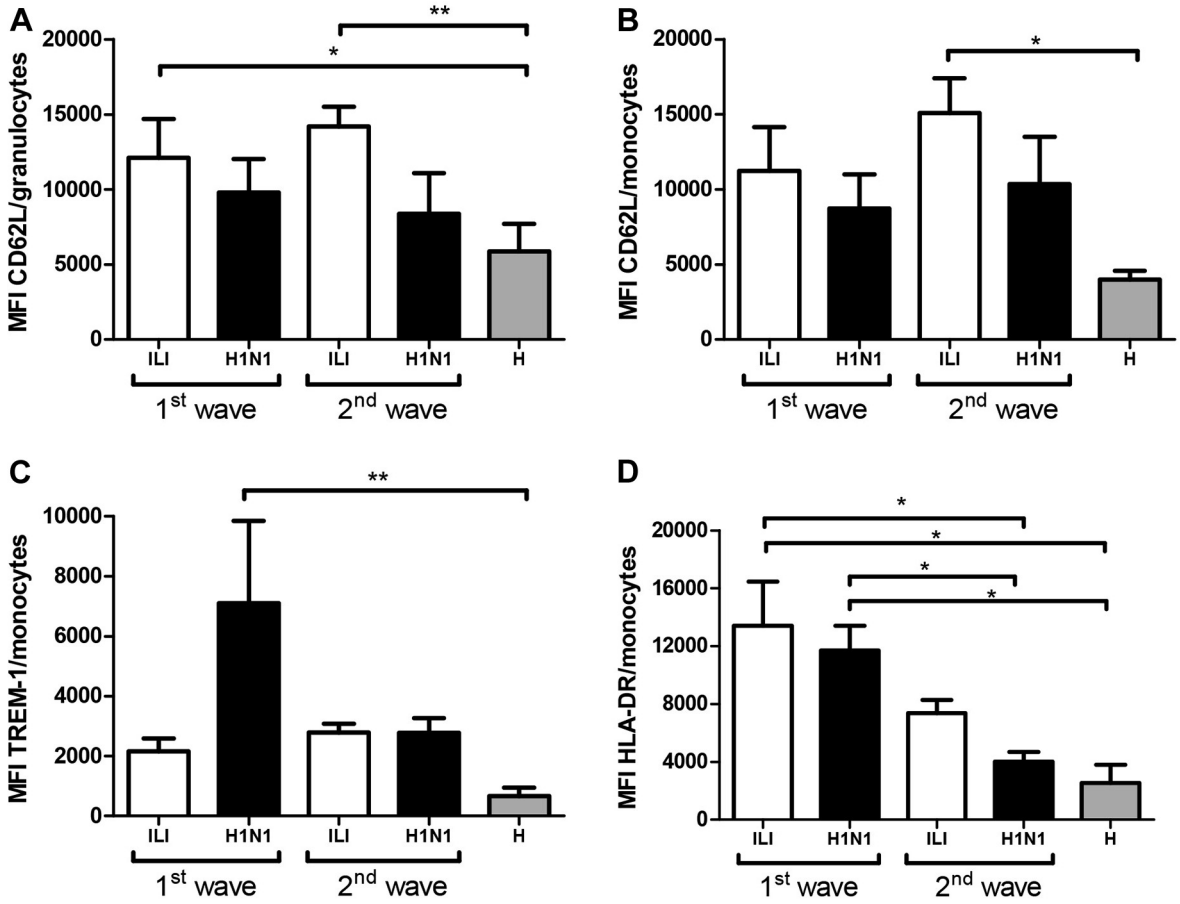

Figure 2. Monocyte and granulocyte phenotypes in the 1st and 2nd pandemic waves for the ILI, H1N1 and H groups. Peripheral blood leukocytes were immunostained with CD14-, CD62L-, TREM-1- and HLA-DR-specific antibodies and analyzed with flow cytometry. The relative neutrophil CD62L (A), monocyte CD62L (B), monocyte TREM-1 (C) and monocyte HLA-DR (D) expression levels are shown as mean and standard deviation. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed. $*p < 0.05$, $**p < 0.01$.

wave developed, suggesting a greater virulence of the influenza A(H1N1)pdm09 virus.

Influenza H1N1 1918 and H5N1 avian viruses are known for eliciting a hyper-immune response that included high circulating cytokine and chemokine concentrations (39). In addition, studies in patients with severe illness during the 2006–2007 seasonal influenza, showed a high concentration of IL-6 (20) and IL-8 (21); in contrast, *in vitro* studies showed the influenza virus as a poor inducer of pro-inflammatory cytokines in macrophages and dendritic cells (40). Our study reveals that IL-6 was significantly elevated (>10-fold above controls) in both pandemic waves (Table 3), suggesting a better infection control because high concentrations of IL-6 have proven to confer protective effects against influenza (41). In contrast with other reports associating IL-6 augmentation with a poor outcome (42) or SIRS (43), recent reports show that IL-6 is not a definitive biomarker to determine systemic inflammation (42). Moreover, we observed that IL-10 concentration during the 2nd wave was higher in the H1N1 group than in the H group. High concentrations of IL-10 could resolve influenza infection by promoting virus clearance and autoregulation (44,45). Probably in the 1st wave IL-10 was diminished in young patients and increased in elderly patients (46).

T cell lymphopenia was associated with influenza A(H1N1)pdm09 infections (47,48), probably as a result of differential lymphocyte migration into the lungs (49,50). We observed T cell lymphopenia in patients from the 1st but not the 2nd wave. Helper T cells showed an increase in early activation markers such as CD69 only during the 2nd wave (Figure 1). This could be related to differential migration patterns to lymph nodes and lungs from naive and, predominantly, memory T lymphocytes (51,52). The higher proportion of circulating $CD69^+$ CD4 T cells during the 2nd, but not the 1st wave, could be explained by memory induced by group immunizations.

Neutrophils, the major component of granulocytes, could contribute to lung damage; however, reduced numbers or impaired function of these cells may lead to severe influenza disease (53) with a high mortality rate (54,55). Because granulocytes could enter the lungs by

using CD62L (56), we tested its expression in peripheral blood. An augmented CD62L expression in granulocytes was observed for both waves in all patients compared with the H group. Although CD62L is diminished during some infections and influenza virus *in vitro* induces CD62L shedding on neutrophils (57), it has also been reported that CD62L is upregulated in circulating leukocytes early after injury (58). Accordingly, CD62L is overexpressed in human proinflammatory neutrophils exposed to IFN-γ (59,60), which is elevated during acute stages of illness in influenza infection (61). In addition, expression of TREM-1 and HLA-DR was also evaluated in monocytes. Interestingly, we observed increased levels in TREM-1 only during the 1st wave, suggesting a wave-dependent differential migration in H1N1 patients; therefore, we propose that the expression of both CD62L and TREM-1 could be useful in the study of waves in influenza infection.

Considering all statistical differences observed in the ILI and H1N1 groups compared with the H group, our results showed greater immunological activity in the 2nd wave compared with the 1st wave of influenza during the 2009 outbreak; accordingly the mortality rates were greater during the 2nd wave (62). Our study suggests that some differential leukocyte phenotypes such as CD69 for helper T cells, and CD62L, TREM-1 and HLA-DR for myeloid cells could explain this epidemiological observation. Our study has some limitations associated with: a) a relatively small number of observations and sizeable SD for most parameters; b) serial samples were not collected; therefore, a peak time point could have been missed; and c) signs and symptoms from patients were not stratified, just registered as present or absent.

In conclusion, to our knowledge this is the first report that describes differences in the immune response during pandemic periods, with prominent activation during the 2nd pandemic wave, which was more likely explained by the immune response than by underlying diseases or age. Further investigation is needed to fully understand the underlying mechanisms driving the wave-like behavior of immune responses against pandemic influenza virus so that more focused therapeutic strategies can be designed.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.arcmed.2015.12.003.

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