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Severe Influenza Is Characterized by Prolonged Immune Activation: Results From the SHIVERS Cohort Study

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Background. The immunologic factors underlying severe influenza are poorly understood. To address this, we compared the immune responses of influenza-confirmed hospitalized individuals with severe acute respiratory illness (SARI) to those of nonhospitalized individuals with influenza-like illness (ILI).

Methods. Peripheral blood lymphocytes were collected from 27 patients with ILI and 27 with SARI, at time of enrollment and then 2 weeks later. Innate and adaptive cellular immune responses were assessed by flow cytometry, and serum cytokine levels were assessed by a bead-based assay.

Results. During the acute phase, SARI was associated with significantly reduced numbers of circulating myeloid dendritic cells, CD192^{+} monocytes, and influenza virus–specific CD8^{+} and CD4^{+} T cells as compared to ILI. By the convalescent phase, however, most SARI cases displayed continued immune activation characterized by increased numbers of CD16^{+} monocytes and proliferating, and influenza virus–specific, CD8^{+} T cells as compared to ILI cases. SARI was also associated with reduced amounts of cytokines that regulate T-cell responses (ie, interleukin 4, interleukin 13, interleukin 12, interleukin 10, and tumor necrosis factor β) and hematopoiesis (interleukin 3 and granulocyte-macrophage colony-stimulating factor) but increased amounts of a proinflammatory cytokine (tumor necrosis factor α), chemotactic cytokines (MDC, MCP-1, GRO, and fractalkine), and growth-promoting cytokines (PDGFBB/AA, VEGF, and EGF) as compared to ILI.

Conclusions. Severe influenza cases showed a delay in the peripheral immune activation that likely led prolonged inflammation, compared with mild influenza cases.

Keywords. Influenza; cellular immunity; infection; disease severity; cytokine.

While mild influenza can be subclinical or manifest as fever with unremarkable respiratory symptoms, severe influenza is characterized by respiratory complications such as pneumonia, respiratory distress, and even death. Although risk factors such as extremes of age, preexisting health conditions [1], and genetics [2] have been identified through large-scale epidemiological studies, a cohesive understanding of the underlying immunological process, particularly within cellular immunity, is still not well-defined.

Viral clearance, facilitated by neutralizing antibodies and CD8^{+} T cells [3], is an important factor for mitigating severe influenza. While neutralizing antibodies can be induced by past exposure to a similar strain, the induction and regulation of an effective CD8^{+} T-cell response are less well understood. Some studies that have compared the immune response in mild and severe influenza have identified a role for CD8^{+} T cells in mitigating severe influenza [4–6], but this finding has not been unanimous [7, 8]. Further, much less is known about the role of cellular innate immunity, particularly within the monocyte subpopulations. For example, higher proportions of CD14^{lo}CD16^{+} monocytes in the nasal airways were negatively associated with cytokines that predicted severe disease [9], while decreased functionality were reported in conventional monocytes isolated from patients with severe avian influenza virus infections [10]. How and if these changes affect downstream T-cell responses is unknown. Here, our objective is to identify immune signatures associated with severe influenza, with a particular focus on the role of monocytes and adaptive T-cell responses.

The Southern Hemisphere Influenza Vaccine Efficacy Research and Surveillance (SHIVERS) study, based in New Zealand [11], conducts epidemiologic and laboratory surveillance for respiratory pathogens among patients with mild or severe acute respiratory illness. As part of this study, we profiled

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the immune status of those with confirmed influenza during the acute and convalescent phases of disease, through a combination of serological assays and multiparameter flow cytometry.

METHODS

Study Design

Participants were enrolled upon commencement of this substudy, between August and October of 2013, which coincided with the peak influenza season in New Zealand. For mild influenza, participants were recruited from 16 sentinel general practices situated within Auckland. General practitioners and nurses assessed all consultation-seeking patients. If a patient met the World Health Organization case definition for influenza-like illness (ILI) (ie, "an acute respiratory illness [ARI] with a history of fever or measured fever of ≥38°C, and cough, and onset within the past 10 days, and requiring consultation in that general practice" [12] page 14), a respiratory specimen (nasopharyngeal swab or throat swab) was collected. For severe influenza, patients were recruited from 4 publicly funded hospitals in Auckland. Research nurses reviewed daily records of all overnight admitted inpatients with suspected ARI and interviewed them. If a patient met the World Health Organization case definition for severe ARI (SARI) (ie, "an acute respiratory illness with a history of fever or measured fever of ≥38°C, and cough, and onset within the past 10 days, and requiring inpatient hospitalization" [12] page 14), a respiratory specimen was collected. A total of 29 ILI and 39 SARI cases, which included 8 infants, were initially enrolled. We excluded the infants with SARI, to match the age groups of the ILI cases. Two patients with ILI and 4 with SARI had to be excluded because they were not registered in the central study database. A total of 27 ILI and 27 SARI cases were included for this study.

The respiratory samples were tested for influenza viral RNA by real-time reverse-transcription polymerase chain reaction analysis at the Institute for Environmental Science and Research. Patients whose respiratory sample tested positive for influenza virus were then contacted by the study nurse to undergo collection of the first whole-blood and sera specimens (ie, the acute-phase specimens). A second blood sample was collected 2 weeks later (ie, the convalescent-phase sample). Samples were processed at a central laboratory to isolate the peripheral blood mononuclear cells (PBMCs), using Ficoll-Paque (GE Lifesciences). Cells were stored in liquid nitrogen, while sera were stored at −80°C until use. Serological testing for antibody titers was done using the vaccine strains for the 2013 southern hemisphere influenza season or the relevant strain in circulation. Study data were collected and managed using Research Electronic Data Capture tools hosted at the Institute for Environmental Science and Research [13]. Additional details are provided in the Supplementary Materials.

Flow Cytometry

Antibody clones used in this study are listed in Supplementary Table 1. Intracellular cytokine (ICS) staining was performed on cells stimulated in vitro, using pooled peptides (BEI Resources) derived from the influenza virus matrix protein (M), nucleoprotein (NP), and polymerase basic 1 protein (PB1). Phytohemagglutinin or medium alone were used as controls. Samples were acquired on an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo, version 10 (FlowJo). For some assays, not all samples were analyzed, owing to sample availability (eg, poor PBMC viability). Data are expressed as absolute cell numbers or as percentages of the parent population. Medians are used to describe the average responses, and interquartile ranges (IQR) are used to describe the variability. The lower and upper limits of IQR is determined by Q1 − 1.5(IQR) and Q3 + 1.5(IQR), respectively.

Detection of Cytokines

The Luminex MAP system was used with a Milliplex MAP 41-plex human cytokine immunoassay (Millipore) performed according to the manufacturer's protocol.

Statistical Analysis

Descriptive statistical analyses were performed for demographic and clinical data. Continuous variable data were log transformed (if the assumption of normality was not met), and differences between the ILI and SARI groups were analyzed by the Student *t* test, using GraphPad Prism (version 5.03). Probability values of <.05 are considered statistically significant and denoted by an asterisk. Cytokine data were analyzed using ordinal logistic regression in Stata 14, after adjustment for age, ethnicity, and time of sampling. Given the large number of cytokines examined, Bonferroni correction was applied for a more stringent test for significance. Univariate or multivariate associations with *P* values of ≤.001 were considered statistically significant. Data can be made available upon request.

Ethics Statement

This study received ethics approval from the New Zealand Northern (A) Health and Disability Ethics Committee under references NTX/11/11/102/AM02, AM05, AM06, and AM14. All participants provided written consent to participate in the study.

RESULTS

Study Population

A total of 27 ILI cases and 27 SARI cases were included in the study (Table 1) and were similar in terms of age, sex, and ethnicity. The time from self-reported symptoms to collection of the first blood specimen was slightly longer for the ILI group (mean [±SD], 13.7 ± 4.6 days) as compared to the SARI group (10.7 ± 4.8 days), although this difference was not statistically significant. A majority of the cases (72% in the ILI group and 83% in the SARI group) were A/H3 positive, while the

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Table 1. Demographic and Clinical Characteristics of 27 Study Participants With Influenza-Like Illness (ILI) and 27 With Severe Acute Respiratory Illness (SARI)

Variable	ILI	SARI
Interval between sampling, d, mean ± SD	15.9 ± 1.8	15.3 ± 2.4
Interval between symptom onset and initial sampling, d, mean ± SD	13.7 ± 4.6	10.7 ± 4.8
Age, y, median (range)	40 (12–77)	45 (12–78)
Sex		
Male	10/27 (37.0)	10/27 (37.0)
Female	17/27 (63.0)	17/27 (63.0)
Ethnicity		
Asian	3/27 (11)	0/27 (0)
Maori	2/27 (7)	5/27 (18)
Other	16/27 (59)	13/27 (48)
Pacific	5/27 (19)	8/27 (30)
Unknown	1/27 (4)	0/27 (0)
Infecting influenza virus (sub)type		
A(H3N2)	18/27 (72)	24/27 (83)
B	7/27 (28)	4/27 (14)
Coinfected (with IBV/other pathogen)	0/27	1/27 (3.5)
Hospitalization duration, d, median (range)	0	2 (1–10)
Reported relevant underlying conditions, no.	0	19/25 (76)
Received vaccination in past year	ND	15/26 (60)
Clinical diagnosis		
Febrile illness with respiratory symptoms	…	8/25 (66)
Suspected pneumonia	…	6/25 (25)
Exacerbation of adult chronic lung disease	…	3/25 (12)
Exacerbation of asthma	…	2/25 (8)
Exacerbation of childhood chronic lung disease	…	2/25 (8)
Other suspected acute respiratory infection	…	2/25 (8)
Respiratory failure	…	1/25 (4)
Suspected acute URTI	…	1/25 (4)
Clinical care		
Received oseltamivir	…	3/24 (12.5)
Received antibiotics	…	23/25 (92)
Received statin	…	4/24 (16)
ICU stay	…	1/27
Received assisted ventilation	…	2/27

Data are proportion (%) of patients, unless otherwise indicated.

Abbreviations: IBV, influenza B virus; ICU, intensive care unit; ND, no data; URTI, upper respiratory tract infection.

remaining cases were influenza B virus positive. Seventy-six percent of individuals with SARI reported relevant underlying conditions, the most common being asthma and chronic lung diseases, while no underlying conditions were reported in the ILI group.

Most SARI cases were relatively mild (median hospitalization duration, 2 days). Sixty-six percent received a diagnosis of uncomplicated febrile illness with respiratory symptoms. Of those who were hospitalized for >3 days, 75% (6 of 8) received a severe diagnosis, owing to exacerbation of an underlying respiratory condition or pneumonia. One patient was admitted to the intensive care unit, with a stay of 5 days. Two others received oxygen therapy. Although antibiotics were prescribed in 92% of SARI cases, we recovered detailed data on antibiotic use only for 10 cases. Only 2 patients received the immunomodulatory macrolide-class antibiotic roxithromycin. Oseltamivir was prescribed in only 12.5% of SARI cases.

We found no significant differences in antibody levels (targeting either hemagglutinin or neuraminidase) or polymerase chain reaction cycle threshold values between ILI and SARI (Supplementary Figure 1), suggesting that acute-stage antibody titers and early viral load, as detected in the upper respiratory tract swab specimens, were not strong correlates of disease severity in our cohort.

SARI Cases Have Myeloid Dendritic Cells (mDCs) and CD16+ Monocytes Increase in SARI Cases Between the Acute and Convalescent Phases of Disease

Both innate and adaptive immune cells present in the PBMCs collected during the acute and convalescent phases were evaluated. In the innate arm, the frequency and number of mDCs (HLA-DR$^+$CD14$^-$CD11c$^+$) were significantly lower during acute SARI as compared to ILI ($P < .01$; Figure 1A and 1B). By the convalescent phase, the mDC population in the SARI group had increased more significantly, compared with that in the ILI group (frequency change, 5.3% vs 4.4%; $P < .05$; Figure 1C and 1D), resulting in comparable levels between the 2 groups.

In the 3 monocytes subpopulations that we evaluated (ie, the classical [CD14^{++}/CD16$^-$], inflammatory [CD14^{++}/CD16$^+$], and patrolling [CD14^{lo}/CD16$^+$] subpopulations) [9, 15, 16], differences between ILI and SARI were observed during the convalescent phase (Figure 2A). Expansion of classical (CD16$^-$) monocytes to CD16$^+$ monocytes is a hallmark of inflammation, and there were significantly more CD16$^+$ monocytes during the convalescent phase in the SARI group as compared to the ILI group ($P < .05$). This is due to the increase in both inflammatory monocytes {from 7.9% (IQR [upper, lower limits], 18% [−24.4, 47.6]) to 17.7% (IQR, 17% [−19.7, 48.5])} and patrolling monocytes {from 13% (IQR, 17% [−19.8, 47.1]) to 15.9% (IQR, 13% [−11.4, 41.7])} in patients with SARI. The patrolling monocytes, which are considered to have more-potent antiviral activity [16, 17], were significantly higher in the SARI group, compared with the ILI group, during the convalescent phase ($P < .05$). In the ILI group, the median frequencies remained fairly constant between the 2 time points.

CD192 Expression on Monocyte Populations Is Lower in SARI Cases During the Acute Phase, Suggesting a Reduced Mobilization Process

Aside from assessing the frequencies, we also assessed the monocytes' chemotactic potential by enumerating the proportion that expressed CD192 (otherwise known as "CCR2"). This chemokine receptor is important to mobilize monocytes from the bone marrow to the site of inflammation [18]. The

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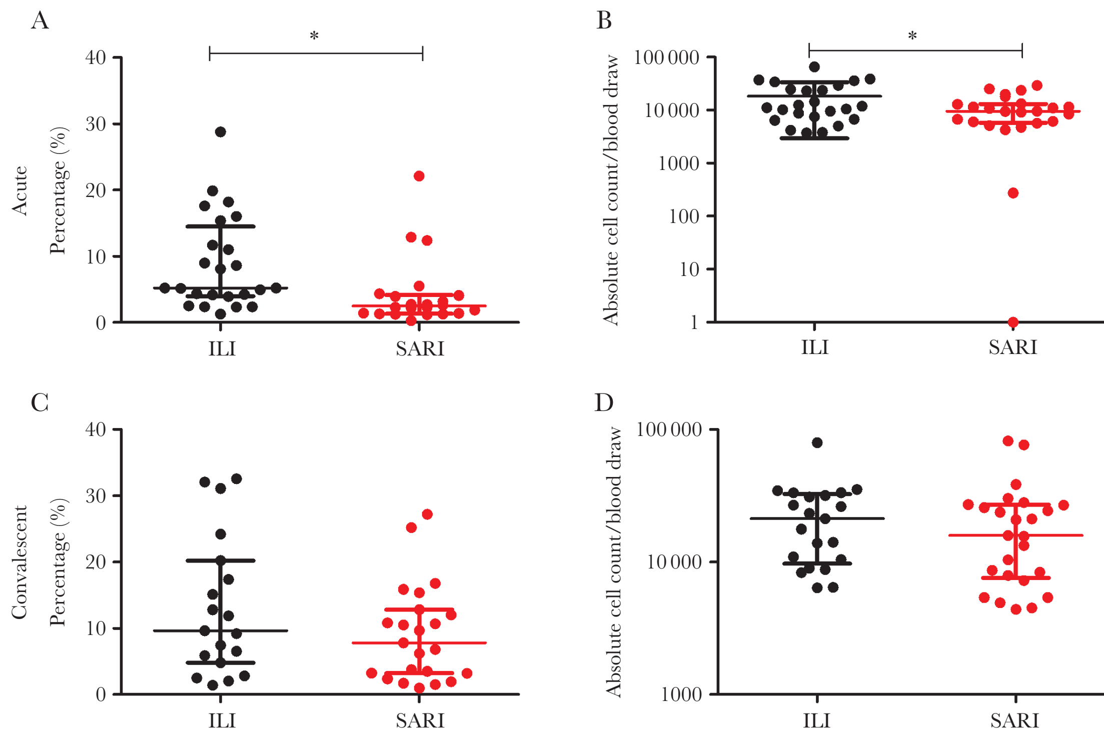

Figure 1. Differences in the myeloid dendritic cell (mDC) ($CD14^{-}CD11^{+}$) population in ILI (black) and severe acute respiratory illness (SARI; red) influenza cases during the acute and convalescent phase. Percentages and absolute cell counts of mDCs in patients with ILI and SARI during the acute (*A* and *B*) and convalescent (*C* and *D*) phases of disease. $^{*}P < .05$.

frequencies of $CD192^{+}$ monocytes were, on average, lower in the SARI group than in the ILI group {65% (IQR, 19% [29.8, 109]) vs 75% (IQR, 17% [38.9, 108]); $P = .05$} during the acute phase (Figure 2B), particularly on the patrolling monocytes {25% (IQR, 13% [−2.5, 51.1]) vs 41% (IQR, 31% [−30.4, 93.7]); Figure 2C}. By the convalescent phase, however, the frequency of $CD192^{+}$ monocytes underwent a greater increase in the SARI group than in the ILI group for both $CD16^{+}$ subpopulations (inflammatory, 19%; patrolling, 13%; Figure 2D), resulting in comparable levels between the 2 groups.

Thus, between the acute and convalescent phase, levels of mDCs, $CD16^{+}$ monocytes, and $CD192^{+}$ monocytes increased significantly in the SARI group but remained relatively stable in the ILI group, suggesting that the innate immune response in the latter group could be returning (or returned) to basal levels, while dynamic changes are still occurring in the SARI group.

SARI Cases Involved Prolonged Activation of T-Cell Responses

For the adaptive immune response, we assessed the numbers of total and influenza virus–specific $CD8^{+}$ and $CD4^{+}$ T cells. The proportions of total $CD8^{+}$ (Figure 3A) and $CD4^{+}$ (Figure 3B) T cells during the acute phase were comparable between the groups. The frequencies of these cells declined in the ILI group {from 6.0% (IQR, 4.2% [−2, 14.8]) to 4.9% (IQR, 3.7% [−2.3, 12.4]) among $CD8^{+}$ T cells and from 12.0% (IQR, 11.3% [−11.9, 33.2]) to 8.3% (IQR, 6.3% [−4.3, 21.1]) among $CD4^{+}$ T cells}, whereas they increased in the SARI group {from 5.3% (IQR, 6.8% [−6.9, 20.3]) to 8.3% (IQR, 5.0% [−1.6, 18.3]) and from 9.0% (IQR, 9.9% [−10.5, 29.1]) to 11.4% (IQR, 6.7% [−2.2, 24.6]), respectively} by the convalescent phase.

In the in vitro stimulation assay to assess influenza virus–specific T-cell responses, there were relatively high numbers of interferon γ (IFN-γ)–expressing cells in the unstimulated samples (considered the baseline response), potentially due to the recent infection. Thus, in the interest of transparency, we analyzed our peptide-stimulated response both without and with subtraction of baseline values. Although the latter represents data from only a fraction of individuals within each group, it nonetheless represents a bona fide influenza virus–specific response.

Without baseline subtraction, although there was no statistically significant difference, acute SARI cases tended to have fewer influenza virus–specific IFN-γ–expressing $CD8^{+}$ T cells than acute ILI cases under all stimulation conditions (Figure 3C). By the convalescent phase, these numbers and percentage had increased for the SARI group but declined for the ILI group. Similarly, there were also significantly fewer IFN-γ–expressing $CD4^{+}$ T cells during acute SARI as compared to acute ILI, but by the convalescent phase these numbers had increased to comparable levels between the 2 (Figure 3D). After subtracting the baseline response, the SARI group had fewer IFN-γ–expressing

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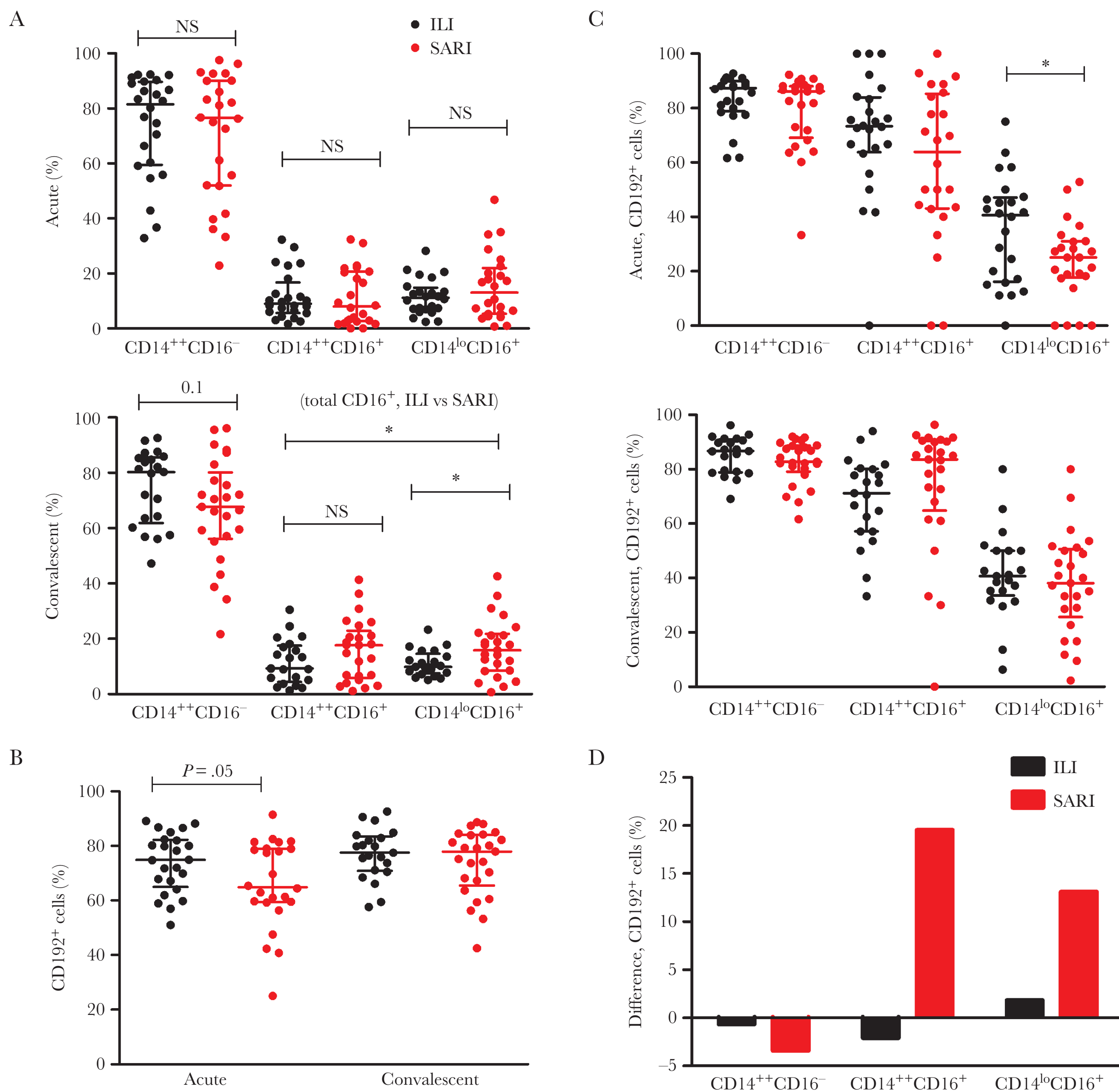

Figure 2. Differences in the monocyte subpopulations in influenza-like illness (ILI; black) and severe acute respiratory illness (SARI; red) influenza cases during the acute and convalescent phases of disease. Monocyte populations are identified on the basis of the relative expression level of the markers CD14 and CD16 as $CD14^{++}/CD16^{-}$ (classical), $CD14^{++}/CD16^{+}$ (inflammatory), and $CD14^{lo}/CD16^{+}$ (patrolling) monocytes. *A*, Percentages of each monocyte subpopulation (per the total monocyte population) during the acute and convalescent phases. *B*, Percentages of $CD192^{+}$ cells in the total monocyte population during the acute and convalescent phases. *C*, Percentages of $CD192^{+}$ cells per monocyte subpopulation in ILI and SARI during the acute and convalescent phases. Lines represent medians, and error bars represent interquartile ranges. *D*, Change in percentages of $CD192^{+}$ monocytes in each subpopulation between acute and convalescent samples. Values were obtained by subtracting acute values from the convalescent value. $^{*}P < .05$; NS, not statistically significant.

$CD8^{+}$ T cells than the ILI group only during the acute phase ($P < .05$; Figure 3E). No significant differences in the IFN-γ–expressing $CD4^{+}$ T-cell response between ILI and SARI cases were detected at any time point (Figure 3F). Collectively, these data demonstrate that there is a reduced influenza virus–specific IFN-γ^{+} T-cell response during SARI, particularly for the $CD8^{+}$ T-cell compartment, compared with ILI, during the acute phase. No significant difference between the ILI and SARI groups were observed for T cells expressing tumor necrosis factor α (TNF-α) or both IFN-γ and TNF-α (data not shown).

We also assessed the proliferation ($Ki67^{+}Bcl\text{-}2^{-}$) and activation ($HLA\text{-}DR^{+}CD38^{+}$) status [19, 20] of these T cells (Figure 4A). The differences in these T-cell populations between the ILI and SARI groups were again more marked during the convalescent

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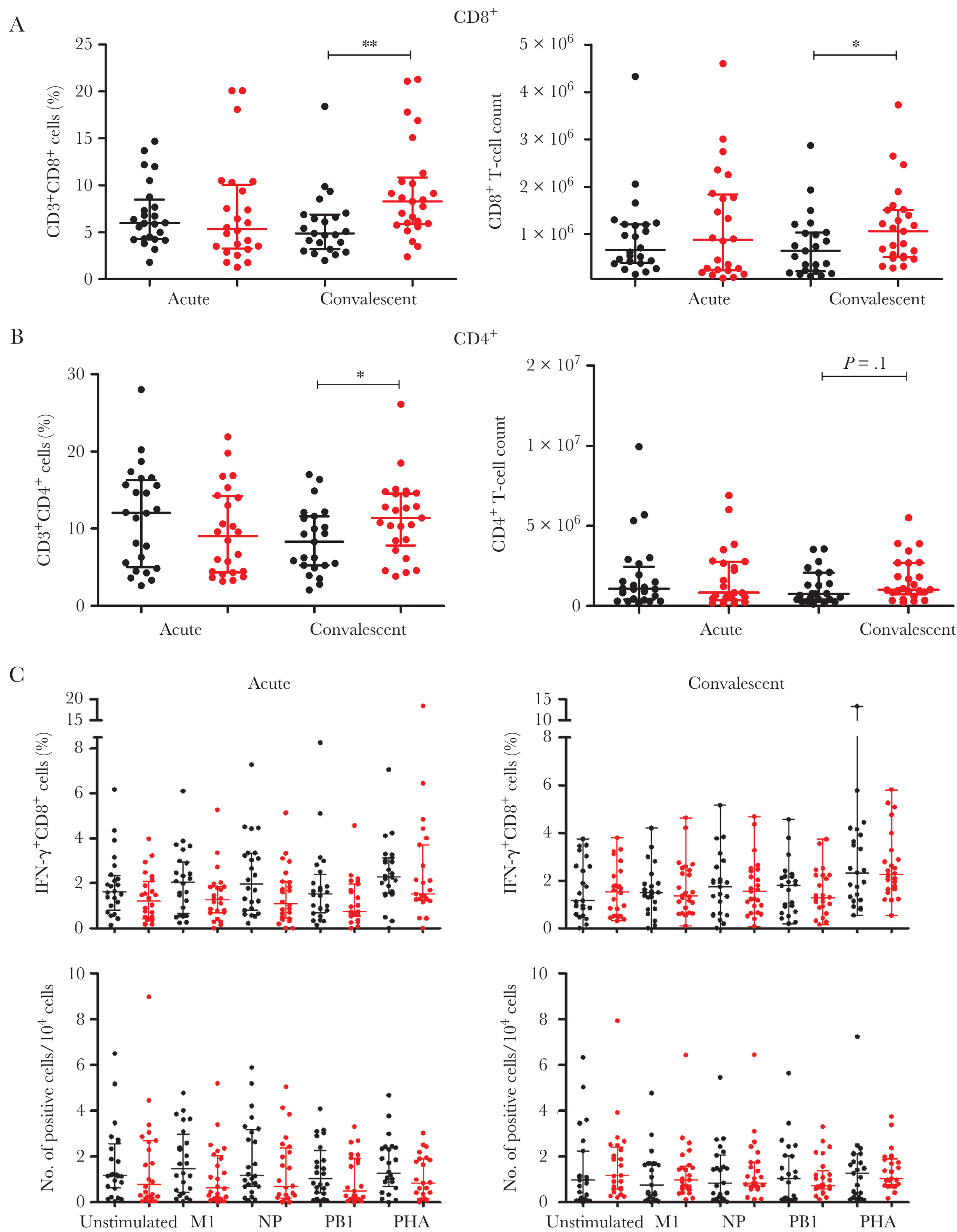

Figure 3. Differences in the adaptive immunity in influenza-like illness (ILI; black) and severe acute respiratory illness (SARI; red) influenza cases. *A* and *B*, Percentages and absolute cell counts of CD3+CD8+ (*A*) and CD3+CD4+ (*B*) T cells during the acute and convalescent phases. *C* and *D*, Interferon γ (IFN-γ)–expressing CD8+ (*C*) and CD4+ (*D*) T cells, expressed as percentage of gated lymphocytes, and absolute cell counts detected in each stimulation condition (unstimulated, matrix 1 protein [M1], nucleoprotein [NP], polymerase basic 1 protein [PB1], and phytohemagglutinin [PHA]; without baseline subtracted). *E* and *F*, Total influenza virus–specific IFN-γ–expressing CD8+ (*E*) and CD4+ (*F*) T-cell responses after peptide (M1, NP, and PB1) stimulation (with the baseline value subtracted). Data are expressed as medians, and error bars represent interquartile ranges. *$P < .05$, **$P < .01$.

phase. Patients with SARI had higher frequencies of proliferating CD8+ T cells {1.3% (IQR, 0.8% [−0.4, 2.9]) specific for M1 epitopes, 1.2% (IQR, 0.7% [−0.1, 2.5]) specific for NP epitopes, and 1.2% (IQR, 0.7%[−0.3, 2.7]) specific for PB1 epitopes} than patients with ILI {1.0% (IQR, 0.8% [−0.72, 2.5]), 0.8% (IQR, 0.6% [−0.2, 2.1]), and 0.8% (IQR, 0.7% [−0.62, 2.3]), respectively;

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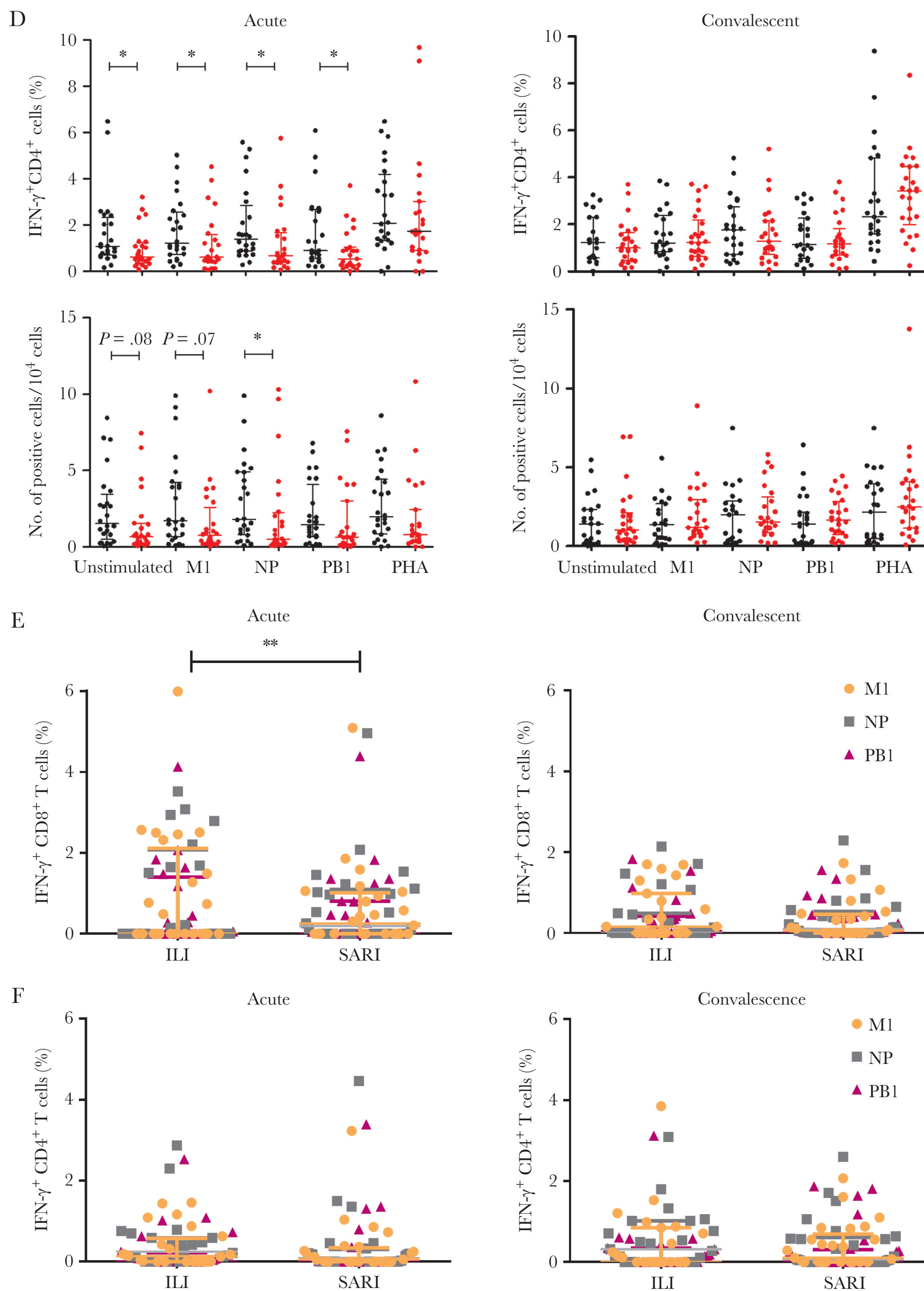

Figure 3. Continued.

$P < .05$; Figure 4B}. The SARI group also had greater percentages of activated CD38⁺CD8⁺ T cells, particularly those specific for NP and PB1 epitopes {4.2% (IQR, 3.3% [−3.2, 10.2]) and 3.1% (IQR, 1.6% [−0.15, 6.3]), respectively}, than the ILI group {2.6% (IQR, 2.7% [−2.9, 7.8]) and 2.3% (IQR, 2.2% [−2.4, 6.4]), respectively; $P < .05$; Figure 4C}. These differences were not observed in the CD4⁺ T-cell population. We also found no significant correlation between the CD4⁺ T-cell responses and the

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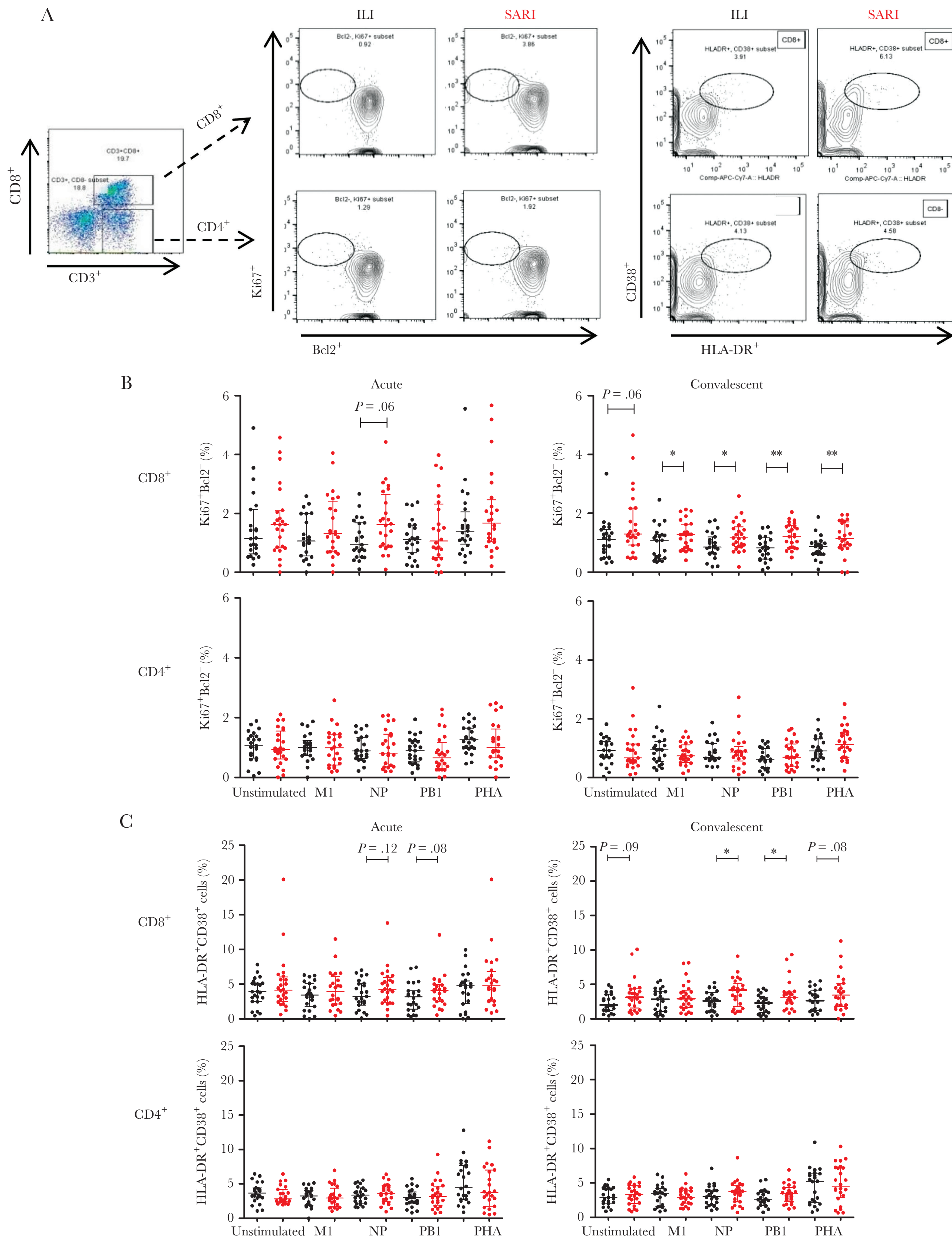

Figure 4. Differences in the proliferating and activated, effector T cells and in influenza-like illness (ILI; black) and severe acute respiratory illness (SARI; red) influenza cases. *A*, Representative flow plots depicting the gating strategy for identification of Ki67⁺Bcl2⁻ proliferating (top left) and CD38⁺ activated, effector (top right) populations. *B* and *C*, Percentages of proliferating (Ki67⁺Bcl2⁻) CD4⁺ or CD8⁺ T cells (*B*) and percentages of effector (CD38⁺) CD4⁺ or CD8⁺ T cells (*C*) during the acute and convalescent phases of disease. Lines represent medians, and error bars represent interquartile ranges. *$P < .05$.

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Table 2. Cytokines That Were Negatively or Positively Associated With Severe Acute Respiratory Illness (SARI)

	Regression Coefficient		*P*	
Association, Cytokine	Univariate	Adjusted[a]	Univariate	Adjusted[a]
Negative				
IL-3	−3.3	−3.29	<.001	.001
IL-12p40	−2.55	−2.44	<.001	<.001
IL-12p70	−2.37	−2.09	<.001	.002
TNF-β	−1.85	−2.03	.003	.001
IL-13	−2.01	−1.81	<.001	<.001
IL-4	−1.53	−1.46	<.001	.004
GM-CSF	−1.67	−1.32	<.001	.003
IFN-α2	−1.32	−1.26	.001	.008
Positive				
sCD40L	1.52	1.54	<.001	.001
EGF	1.66	1.72	<.001	.002
G-CSF	2.32	1.87	.001	.015
PDGFBB	1.76	1.9	<.001	.001
TNF-α	2.47	2	<.001	<.001
MCP-1	2.47	2.14	<.001	<.001
IL-8	2.67	2.25	<.001	.001
IL-5	3.79	3.71	<.001	<.001
GRO	4.18	3.83	<.001	.002
Fractalkine	16.88	16.52	<.001	<.001
VEGF	17.33	17.34	<.001	<.001
PDGFAA	18.22	19.24	<.001	<.001

Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-α2, interferon α2; IL-3, interleukin 3; IL-4, interleukin 4; IL-5, interleukin 5; IL-8, interleukin 8; IL-12, interleukin 12; IL-13, interleukin 13; sCD40L, soluble CD40L; TNF-α, tumor necrosis factor α; TNF-β, tumor necrosis factor β.

[a]Adjusted for age, ethnicity, and time to sample collection. Only cytokines with *P* values of ≤ .001 in either univariate or adjusted models were listed.

convalescent-phase antibody titers (data not shown), despite evidence suggesting otherwise [5, 21]. Of note, there were also no statistically significant differences in the convalescent HAI, NAI, and influenza virus–specific IgG antibody titers between the ILI and SARI groups (data not shown).

Differences in Cellular Immunity Are Reflected in Cytokine Profiles

To determine whether the cellular immune responses were reflected in cytokine profiles, we analyzed the levels of 41 cytokines in patients' sera. By adjusting for age, ethnicity and sampling time, we were able to identify key cytokines that were negatively or positively associated with SARI (Table 2). Descriptive statistics for the cytokine concentrations are shown in Supplementary Table 2 and Supplementary Figure 2.

Most of the 8 cytokines that were negatively associated with SARI were involved in priming and regulating T-cell responses. Interleukin 4 (IL-4) and interleukin 13 (IL-13) are important regulators of the T-helper type 2 (Th2) response, while interleukin 12p40 (IL-12p40) and IL-12p70 (as part of the IL-12 complex) are important for priming Th1 responses. The proinflammatory cytokines, TNF-β and IFN-α2 and the growth factors, interleukin 3 and granulocyte-macrophage colony-stimulating factor were also negatively associated with SARI cases. Conversely, cytokines that were positively associated with SARI cases were growth factors and proinflammatory, chemotactic cytokines that regulate innate immunity. Cytokines such as VEGF, EGF, PDFGBB, and PDFGAA are important in tissue regeneration and wound repair, while granulocyte colony-stimulating factor stimulates the proliferation of leukocytes, particularly neutrophils. MCP1, fractalkine, interleukin 8 (IL-8), and GRO are chemoattractants for monocytes, neutrophils, natural killer cells, and T cells. Other cytokines that were associated with SARI cases were the proinflammatory cytokine TNF-α and the Th2-promoting (but not cell-derived) cytokines interleukin 5 (IL-5) and soluble CD40L. Collectively, the cytokine profile in SARI individuals suggested an overall condition that was suboptimal for the induction of adaptive T-cell responses but enriched in cytokines that promote cellular infiltration of innate cells and tissue repair.

DISCUSSION

Differences in study design and definitions of study groups have proved to be a major challenge in identifying a clear immune signature for severe influenza [4, 5, 7, 9]. Here, by comparing the response in ILI and SARI cases, we found a discernible immunophenotype, suggesting a difference in response kinetics that does not seem to be strain-dependent.

The cytokine profile in SARI cases suggested a potential link between the innate immune response within the macrophage and DC populations and downstream T-cell responses. Interleukin 3 and granulocyte-macrophage colony-stimulating factor are potent hematopoietic stimulators secreted by macrophages and epithelial cells and, in the presence of IL-4, promote the maturation of dendritic cells [22]. Suboptimal levels of these cytokines could explain the lower numbers of mature DCs in SARI. Since activated DCs and macrophages are in turn the major sources of IL-12, a potent activator of the downstream Th1 response [23], this could lead to the reduced $CD8^+$ T-cell responses seen in SARI. Lower serum IL-12 levels have been observed in severely ill patients with influenza or respiratory syncytial virus infection, compared with patients with mild cases [10, 22, 24–26], confirming that IL-12 is important in modulating the outcome of respiratory infections. The SARI cytokine profile also revealed a strong association with chemotactic cytokines regulating the innate immune cells. However, this enhanced chemotactic potential may not be beneficial, as extensive immune cell infiltration could lead to excessive tissue damage. GRO and IL-8, which are potent neutrophil chemoattractants, are the key mediators of acute lung injury during inflammation [27] and have been associated with acute respiratory distress syndrome [27, 28]. The positive association of platelet factors that are an integral part to the wound healing process [29] also suggests that individuals with severe influenza

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may have experienced more tissue damage than individuals with mild influenza.

Unexpectedly, the canonical Th2 cytokines IL-4 and IL-13 were negatively associated with SARI, although another Th2 cytokine, IL-5, was not. IL-4 and IL-13 signal through a shared receptor, IL-4Rα, but not IL-5, suggesting that different Th2-responses might underlie severe and mild disease processes. It is possible that stimulation of Th2 responses through IL-4 and IL-13 contributed to the rapid tissue repair, as has been proposed recently [30, 31], in mild influenza. In contrast, IL-5 and some IL-13 cytokines are produced mainly by innate lymphoid type 2 cells and invariant natural killer T cells and are key mediators during allergic airway inflammation [32, 33]. Given that asthma and chronic lung diseases were the most commonly reported underlying condition for our SARI cases, it is possible that the positive association of IL-5 with SARI reflected the exacerbation of underlying conditions in these patients.

Numerous studies have studied the cytokine responses of patients with influenza in the attempt to identify markers of disease progression or severity. Our finding that severe influenza is associated with cytokines that regulate the innate immune cells, particularly IL-8, MCP-1, and TNF-α, are in agreement with findings by Lee et al [34] and Davey et al [35], whose studies involved patients infected with 2009 pandemic influenza A(H1N1) virus. However, in contrast to these and other studies [26, 36], our model did not find any statistically significant association between interleukin 6 and interleukin 10 and disease severity. This is likely related to the relatively late collection of the acute-phase sample, since serum levels of interleukin 6 could return to baseline within a week after symptom onset [36]. In addition, direct comparison between our findings and those of these studies is complicated by the differences in study designs and virus strains.

There are 2 possible interpretations of the findings in our study. The first is that severe influenza is caused by a delayed activation of critical immune cells, as was shown for human cases of avian influenza A(H7N9) virus [5] and 2009 pandemic influenza A(H1N1) virus [4] infection. These studies showed that severity was associated with a delayed induction of natural killer cells and influenza virus–specific $CD8^+$ T-cell responses [5]. However, the lower numbers of peripheral immune cells during the acute phase in severe influenza could also be a consequence of aggressive trafficking into the airways and not a truly deficient immune response. Certainly, the few studies that compared peripheral versus site-of-infection profiles have reported much more robust local immune responses in the airways and lungs [7, 9] as compared to the peripheral blood [37]. Nonetheless, regardless of either scenario, the higher numbers of activated immune cells in the circulation during the convalescent phase suggests that individuals with severe influenza continue to experience a prolonged state of immune activation.

There are several limitations in the present study. First, the majority of the SARI cases were relatively mild, with very few cases requiring intensive care unit admission. Thus, our data does not truly capture the immune profile of those on the far end of the severity spectrum. In addition, there could also be an enrollment overlap between the patients with ILI and the briefly hospitalized patients with SARI, owing to health-seeking behavior. This could explain the low statistical support for some of our data. Second, a majority of the SARI cases have underlying conditions. As alluded to previously, it is possible that the immune response profile differs in those with specific underlying conditions. This study is not powered to examine this or other potentially confounding factors and thus could only generalize the immune features underlying mild and severe influenza. Third, owing to logistical challenges, our first sampling time point was relatively late after symptom onset, limiting our ability to capture early events after infection. Nevertheless, our data shows that, cases of mild and severe influenza exhibit differing kinetics of peripheral immune response, consequently resulting in prolonged immune activation in the severe influenza cases.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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