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Longitudinal analysis of leukocyte differentials in peripheral blood of patients with acute respiratory viral infections

Micah T. McClain[a,b,*], Lawrence P. Park[a], Bradly Nicholson[a], Timothy Veldman[c], Aimee K. Zaas[b], Ron Turner[d], Robert Lambkin-Williams[e], Anthony S. Gilbert[e], Geoffrey S. Ginsburg[c], Christopher W. Woods[a,b]

[a] Durham VA Medical Center, Durham, NC, United States
[b] Division of Infectious Diseases, Duke University Medical Center, Durham, NC, United States
[c] Duke Institute for Genome Sciences & Policy, Duke University, Durham, NC, United States
[d] University of Virginia School of Medicine, Charlottesville, VA, United States
[e] Retroscreen Virology, United Kingdom

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ABSTRACT

Background: Leukocyte counts and differentials are commonly acquired in patients with suspected respiratory viral infections and may contribute diagnostic information. However, most published work is limited to a single timepoint at initial presentation to a medical provider, which may correspond to widely varying points in the course of disease.
Objectives: To examine the temporal development and time-dependent utility of routine leukocyte differentials in the diagnosis of respiratory viral infections.
Study design: We analyzed data from recent experimental human challenges with influenza A/H3N2, human rhinovirus (HRV), and respiratory syncytial virus (RSV). Routine clinical lab cell counts and differentials were measured daily from the time period immediately prior to inoculation through the eventual resolution of symptomatic disease.
Results: Approximately 50% of challenged individuals developed symptoms and viral shedding consistent with clinical disease. Subpopulations of WBC showed marked differences between symptomatic and asymptomatic individuals over time, but these changes were much more profound and consistent in influenza infection. Influenza-infected subjects develop both relative lymphopenia and relative monocytosis, both of which closely mirror symptom development in time. A lymphocyte:monocyte ratio of <2 correctly classifies 100% of influenza (but not RSV or HRV) infected subjects at the time of maximal symptoms.
Conclusions: Leukocyte differentials may suggest a viral etiology in patients with upper respiratory infection, but are not sufficient to allow differentiation between common viruses. Timing of data acquisition relative to the disease course is a key component in determining the utility of these tests.
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1. Background

Upper respiratory infections (URI) and influenza-like illnesses (ILI) are common and account for considerable morbidity and mortality each year [1–3]. However, differentiation between the many different potential viral and bacterial etiologies is often problematic. While both traditional and molecular diagnostic tests are available for many potential etiologies, there has been interest in utilization of routinely available laboratory variables to aid clinicians in pursuing the appropriate specific diagnostic pathways or empiric therapies [4–7]. In particular, simple measurements of the host immune response such as white blood cell counts and differentials have been shown to be characteristic of many infected states [7–9]. Specifically, relative lymphopenia (but not leukopenia) was found to be a prominent marker of novel H1N1 infection in 2009 [8], and in fact lymphopenia when combined with other parameters was capable of being compiled into an early 'diagnostic triad' during the influenza pandemic of 2009 [10]. Interestingly, while total WBC counts are commonly elevated in many adults with 2009 H1N1, this is not the case with seasonal H3N2 infections, but relative lymphopenia is a common finding amongst many strains of influenza [9]. Furthermore, several groups have described that at the time

* Corresponding author at: Division of Infectious Diseases, Duke University Medical Center, Durham, NC 27710, United States. Tel.: +1 919 613 5635; fax: +1 919 684 8902.
E-mail address: micah.mcclain@duke.edu (M.T. McClain).

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of clinical presentation, adults with influenza infection have both relative lymphopenia and relative monocytosis, and that the lymphocyte:monocyte ratio can be utilized in some cases to classify individuals with acute ILI as influenza-infected or not [11,12]. However, while cell counts in acute influenza infection have been fairly well described, there is a relative paucity of data describing these parameters in other common causes of viral URI like RSV and HRV [13]. Furthermore, the variability of such parameters over time in infected individuals is largely unknown, having been explored in only a few individual cases [14].

2. Objectives

The objective of the study was to examine the temporal development and time-dependent utility of routine leukocyte differentials in the diagnosis of respiratory viral infections.

3. Study design

3.1. Human viral challenges

In collaboration with Retroscreen Virology Ltd. (London, UK) and in separate challenge trials, we intranasally inoculated 17 healthy volunteers with influenza A (A/Wisconsin/67.2005 (H3N2)), 20 healthy volunteers with respiratory syncytial virus, and 20 individuals with human rhinovirus. All volunteers provided informed consent and underwent extensive pre-enrollment health screening, including baseline antibody titers to the specific strains of virus utilized. After approximately 24 h in quarantine, we instilled the relevant virus into bilateral nares of subjects using standard methods [15]. We obtained nasal lavage samples from each subject daily for qualitative viral culture and and/or quantitative RT-PCR to assess the success and timing of infection [16]. Peripheral blood samples were drawn daily from all subjects.

3.2. Clinical case definitions

Symptoms were recorded twice daily using a modified standardized symptom score similar to the Jackson Score [17]. For all cohorts, modified Jackson scores were tabulated to determine if subjects became symptomatic from the respiratory viral challenge. A modified Jackson score of $\geq$6 over a consecutive five day period was the primary indicator of symptomatic viral infection [18] and subjects with this score and a positive qualitative viral culture or quantitative RT-PCR for at least 2 consecutive days (beginning 24 h after inoculation) were denoted as "symptomatic infection" and included in the signature performance analyses [17–19]. Subjects were classified as "asymptomatic, not infected" if the total symptom score was less than 6 and viral shedding was not documented after the first 24 h subsequent to inoculation as above.

3.3. Naturally acquired influenza and streptococcal infections

Subjects were recruited from the Duke University Medical Center Emergency Department between September 1 2011 and December 31, 2012. Subjects were considered for enrollment if they had a known or suspected influenza infection or community-acquired pneumonia on the basis of clinical data at the time of screening and if they exhibited two or more signs of systemic inflammation (SIRS) within a 24-h period. Subjects were excluded if <18 years old, if they had an imminently terminal co-morbid condition, if they had recently been treated with an antibiotic for a viral, bacterial, or fungal infection, or if they were participating in an ongoing clinical trial. In addition to residual respiratory samples collected as part of routine care, an NP swab was collected from each enrolled subject. 2009 H1N1 virus was confirmed by RT-PCR. *Streptococcus pneumoniae* CAP was defined as fever, radiographic infiltrate on chest X-ray, and culture of *S. pneumo* from blood or sputum, but without culturing of other organisms and with a negative respiratory viral battery (PCR). All patients included in these cohorts were treated as outpatients. Leukocyte differentials for these cases are from the initial blood draw CBC at the time of presentation.

3.4. Leukocyte differentials

White blood cell counts and differentials were performed on a standard Sysmex^{TM} XE-2100 automated clinical hematology system (Sysmex America Inc., Lincolnshire, IL, USA).

3.5. Statistical analysis

We generated receiver–operator characteristic curves for individual study days by fitting logistic regression models predicting symptoms as a binomial outcome and including the lymphocyte to monocyte ratio as a single independent variable. The predicted values for symptoms from the models were used to stratify the subjects as symptomatic or not at each unique value. The ROC curves plot the sensitivity and specificity comparing the observed symptoms strata across the predicted values from the models.

4. Results

4.1. Influenza challenges

We previously performed an experimental influenza virus challenge in young, healthy volunteers with influenza A/Wisconsin/67/2005 (H3N2) [15,20]. In this trial, nine (53%) of the 17 H3N2-exposed subjects developed symptoms consistent with viral upper respiratory infection with confirmed shedding of challenge virus. This infection rate is similar to previous challenge trials [21], and occurs despite similar patient profiles, vaccination history, and baseline influenza hemagglutination and neutralization titers. Subjects exhibited variability of time to initiation of symptoms as well as maximal severity of symptoms achieved, but symptom onset began at an average of 49 h after inoculation and affected individuals reached maximal symptoms at 91 h after exposure [15,20].

In this trial, asymptomatic (but exposed) individuals demonstrated no significant change from baseline in either their total WBC count or WBC subsets (lymphocytes, monocytes, neutrophils) at any time during the course of the study (Fig. 1). Individuals with symptomatic influenza infection also exhibited fairly stable total WBCs over the duration of their infection. However, symptomatic influenza-infected individuals underwent marked perturbations in WBC subsets. By day 2 post-inoculation (and around the time of symptom onset), symptomatic individuals experienced a significant drop in total lymphocyte count with a concomitant rise in both monocyte and neutrophil counts (Fig. 1). Absolute lymphopenia was common (78%, 7/9 pts), while pts exhibited only relative monocytosis and neutrophilia (Fig. 1). These alterations persisted throughout the time of maximal symptoms and then gradually returned to baseline as symptoms resolved. There were no significant changes in either eosinophil or basophil counts over time in either group (data not shown).

4.2. Respiratory syncytial virus challenge

Of the 20 subjects inoculated with RSV (Serotype A), 8 (42%) developed upper respiratory infection-like symptoms and had confirmed viral shedding [15]. Symptom onset occurred on average

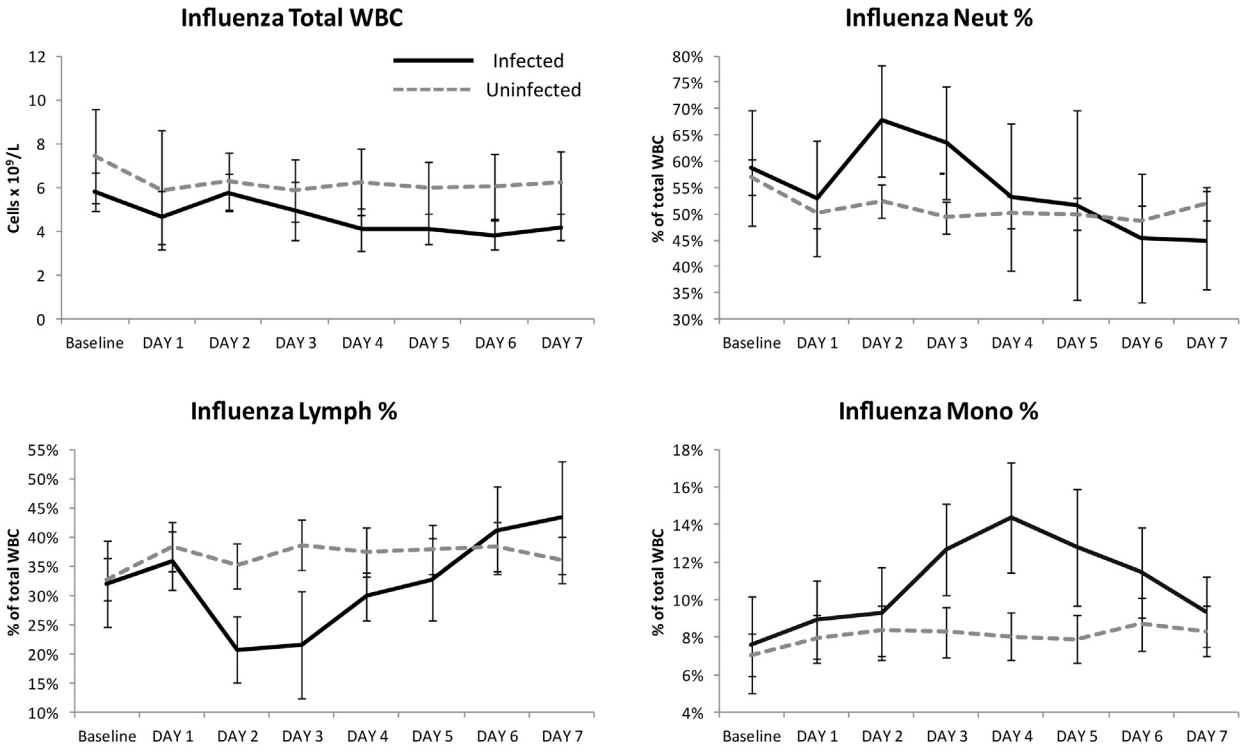

Fig. 1. White blood cell counts over time in individuals challenged with Influenza A/Wisconsin/67/2005 (H3N2). Total WBC count and percent of total WBC are presented. Day 1 represents the day of inoculation.

at 84 h post-inoculation, with peak symptoms occurring on average at 141 h. Similar to the influenza challenge, asymptomatic (but exposed) individuals demonstrated no significant changes in either total WBC or WBC subsets (Fig. 2). Those with symptomatic RSV infection also exhibited minimal change in their total WBC over time, but underwent a marked increase in their monocyte counts starting on day 3–4 post-inoculation, along with a milder reduction in lymphocyte and neutrophil counts. Again, counts had normalized almost to baseline by the end of the observation period. Similar to Influenza infection, there were no significant

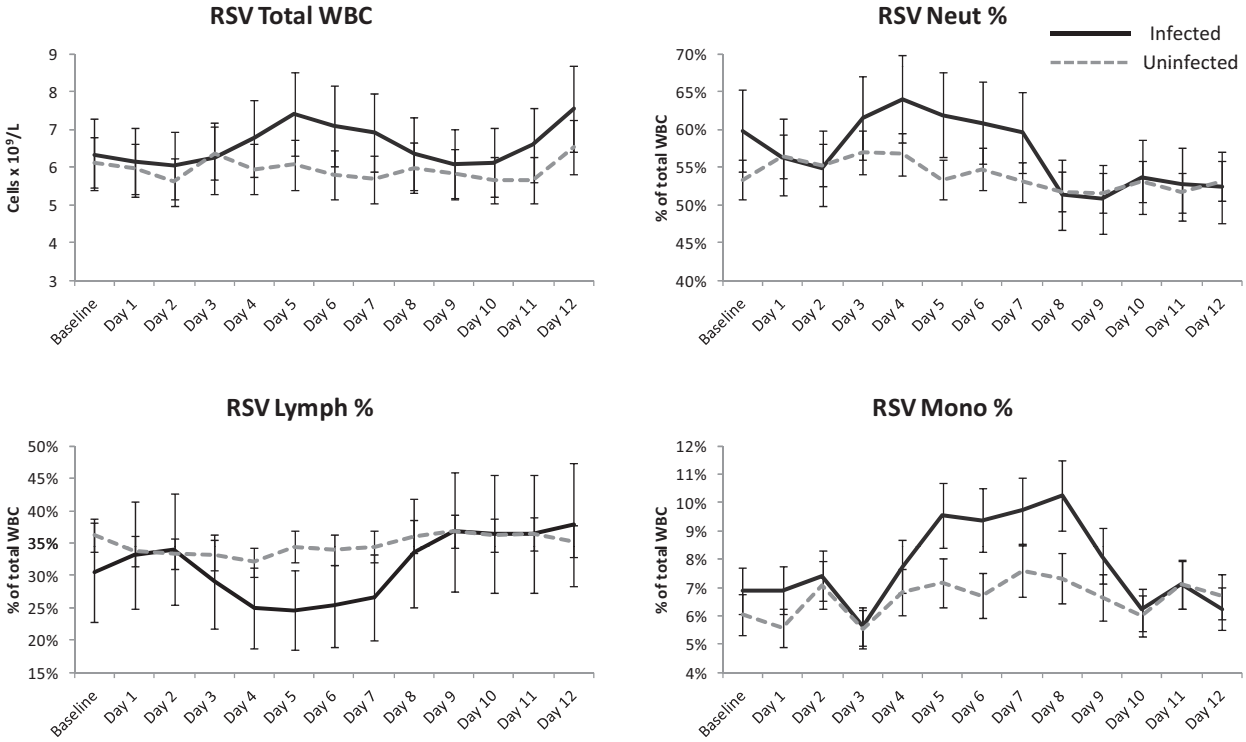

Fig. 2. White blood cell counts over time in individuals challenged with respiratory syncytial virus. Total WBC count and percent of total WBC are presented. Day 1 represents the day of inoculation.

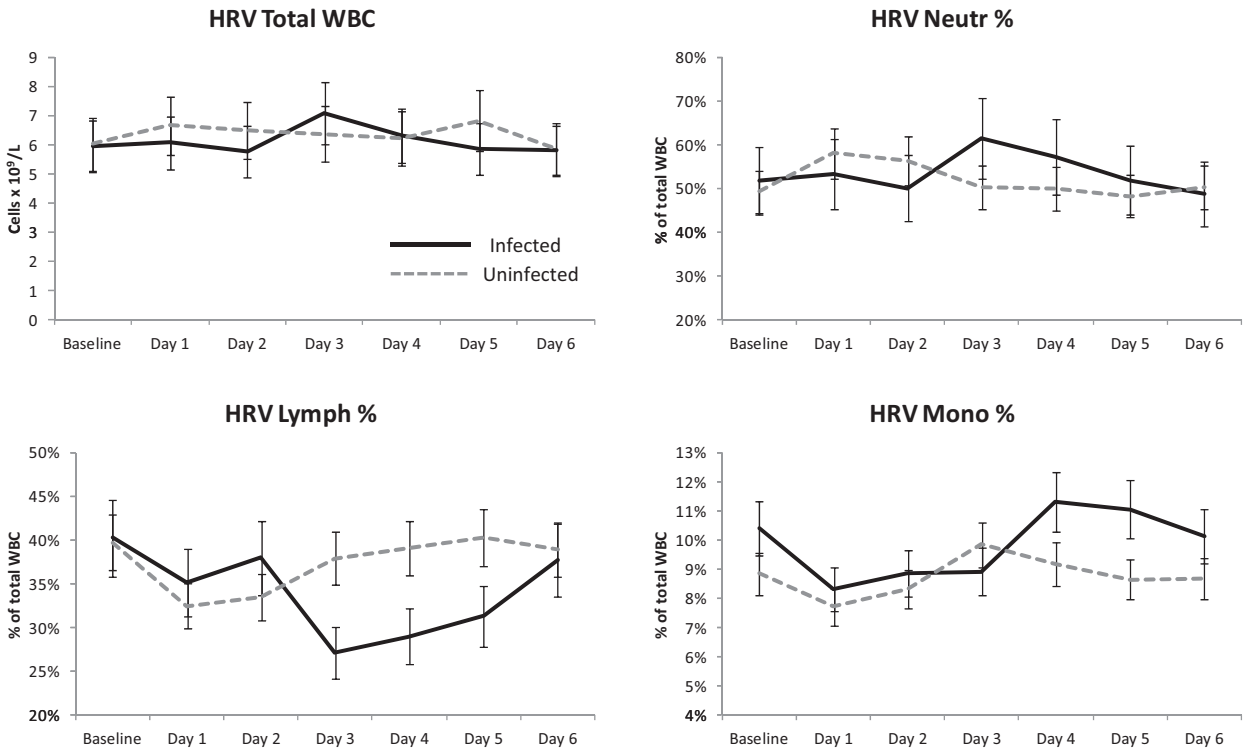

Fig. 3. White blood cell counts over time in individuals challenged with human rhinovirus. Total WBC count and percent of total WBC are presented. Day 1 represents the day of inoculation.

changes in either eosinophil or basophil counts over time in either group.

4.3. Human rhinovirus challenge

In the HRV challenge, ten of 20 HRV-inoculated subjects (50%) developed upper respiratory infection symptoms and had confirmed viral shedding [15]. Symptom onset occurred on average 40 h after inoculation, with peak symptoms occurring on average at around 72 h. Similar to the previous viral challenges, asymptomatic individuals underwent no significant alteration in their WBC counts while enrolled in the study (Fig. 3). Those with symptomatic HRV infection demonstrated relative lymphopenia along with monocytosis similar in character to the influenza challenges, although absolute lymphopenia and monocytosis were rare and no significant changes in neutrophil counts were noted in HRV-infected individuals (Fig. 3). Again, as above there were no significant changes in either eosinophil or basophil counts over time in either group.

4.4. Lymphocyte:monocyte ratio

Based upon previous work suggesting the utility of the lymphocyte-to-monocyte ratio in classifying acute influenza infection [11,12], we analyzed this parameter in each challenge trial depicted above (Fig. 4). In all three viral challenges, infected subjects develop, on average, a decrease in the L:M ratio compared to asymptomatic individuals. Interestingly, in all three trials the decrease in this ratio, as well as its recovery back to baseline, closely parallels the rise and fall of clinical symptoms. An L:M ratio of less than 2 (similar to previously published work with Influenza) correctly classifies 100% of influenza-infected individuals at the time of maximal symptoms (ROC curves, Fig. 4). However, at times 24 h earlier or later, a cutoff of <2 misclassifies one sick individual as 'asymptomatic', and at times more than 24 h from maximal disease only two symptomatic individuals show a ratio <2. An L:M threshold of <2 does not, however, perform well classifying cases of HRV or RSV at any time points, despite the relative changes over time in these cell subsets. Thus, changes in the lymphocyte and monocyte counts in experimental influenza infection were much more consistent and profound than was seen with the other two viruses.

4.5. Naturally acquired influenza infection vs. Streptococcus pneumoniae pneumonia

As a comparator to our viral infection cohorts, we looked at the lymphocyte differentials from naturally occurring cases of influenza (PCR-proven novel H1N1) as well as cases of community-acquired pneumonia due to *S. pneumoniae* which presented to the Duke University Emergency Department in the years 2011–2012. Given the location of enrollment, these groups were somewhat older on average than in our challenge trials, but patients with severe disease (requiring admission) were excluded in order to focus on cases with broadly similar degrees of clinical severity. Overall, naturally acquired influenza infection resulted in lower lymphocyte counts at the time of sampling (average of 14% lymphocytes in H1N1 cases vs. 20% for H3N2, Table 1) but less monocytosis (average of 7% monocytes for H1N1 and 15% for H3N2). Cases of *S. pneumo* infection did have a higher proportion of neutrophils (81% vs. 71%) as well as total WBC (13.4 vs. 8.5, Table 1). Despite these apparent differences, however, using these values as classifiers offers only moderate sensitivity and specificity (67% and 72% for a WBC cutoff of 10, 67% and 67% for a neutrophil cutoff of 82) given the wide range of values in these subsets, and combinations of these variables do not improve this discriminative ability (data not shown). Absolute lymphopenia was common in both types of natural infection (8/18 H1N1 and 12/18 *S. pneumo*), whereas monocytosis was uncommon (2/18 H1N1 and 1/18 *S. pneumo*). Furthermore, we observed a decrease in the lymphocyte:monocyte ratio below 2 in significantly more H1N1 (12/18, 67%) than *S.*

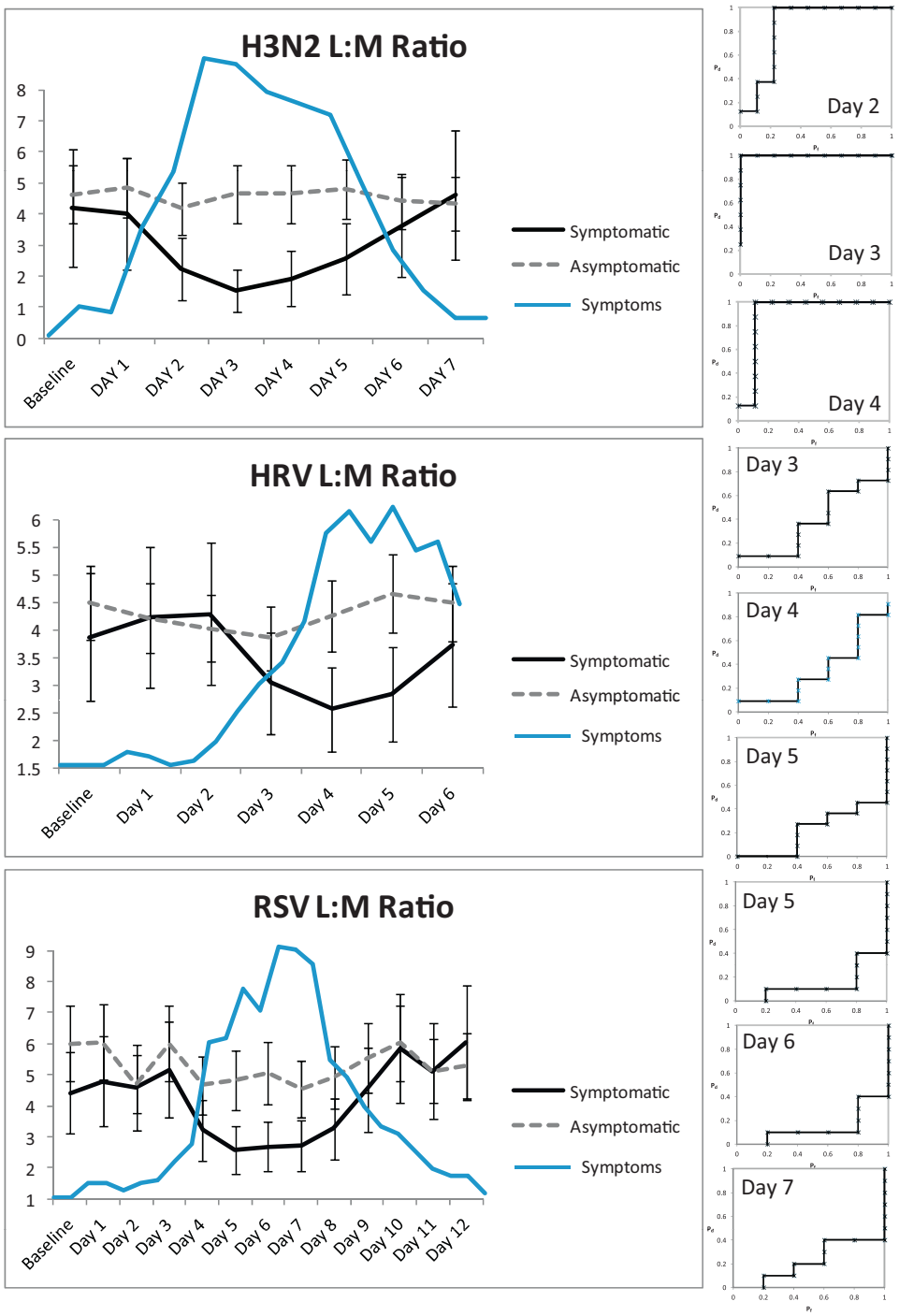

Fig. 4. Lymphocyte:monocyte ratios in peripheral blood from each viral challenge trial, superimposed on a graph of total symptom scores over time for each group. At right, ROC curves for performance of a lymphocyte:monocyte ratio of ≤2 at classifying symptomatic and asymptomatic individuals.

pneumo (7/18, 38%, $p = 0.05$) cases. Thus, while a decreased L:M ratio was much more common in naturally acquired respiratory viral infection than in bacterial CAP, such a ratio alone correctly classifies individuals much less consistently than in our viral challenge model.

5. Discussion

The host response to infection varies widely by pathogen type as well as by individual variability in the hosts themselves, as determined by myriad factors including genetics and a lifetime of environmental and infectious exposures. One of the simplest and most common clinically assayed aspects of the immune response is the white blood cell count and differential. Perturbations in these variables can lend critical (if often non-specific) information to the treating clinician and help to guide more directed testing and treatment decisions, as well as aiding in the monitoring of response to therapy. For the first time we have undertaken to demonstrate the time-dependent variability in makeup of peripheral white blood cells (as detectable by routine clinical laboratory methods) throughout the entire clinical course of human respiratory viral infections.

Table 1
Characteristics of individuals with symptomatic respiratory infection.

	Influenza H3N2[a] (*n* = 17)	RSV (*n* = 20)	HRV (*n* = 20)	Influenza H1N1[b] (*n* = 18)	*S. pneumo*[b] (*n* = 18)
Age	27.4	26.7	20.1	33.1	41.4
Male sex (%)	53	55	60	33	44
Cell counts					
Avg. lymph (%)	20 (0.98)[c]	25 (1.85)	29 (1.83)	14 (1.19)	9 (1.21)
Avg. mono (%)	15 (0.74)[c]	9 (0.66)	11 (0.69)	7 (0.59)	5 (0.67)
Avg. neut (%)	64 (3.14)[c]	62 (4.58)	57 (3.59)	71 (6.03)	81 (10.8)
Avg. WBC	4.9 (3.4–6.6)	7.4 (4.9–8.7)	6.3 (4.7–7.9)	8.5 (3.4–14.7)	13.4 (3.0–26.7)
L:M ratio <2	100% (9/9)	60% (6/10)	18% (2/11)	67% (12/18)	38% (7/18)

[a] Averages for challenge subjects represent values recorded at time of maximal symptoms.
[b] Naturally occurring cases of infection.
[c] Percent of total WBC followed by absolute cell counts (K/cmm) in parentheses.

Relative lymphopenia is well-described as being common at the time of presentation with acute influenza infection [7,14,22]. More recently, a few authors have also demonstrated that monocytosis may be a marker of acute influenza infection as well, and that when combined together the ratio of lymphocytes to monocytes (in particular a ratio of <2) might be useful in directing clinicians to consider influenza as the etiology in cases of ILI [11,12]. In the current study, we have demonstrated that relative lymphopenia and monocytosis (and to a lesser degree neutrophilia) are found in experimental influenza infections and that their development closely corresponds with the rise and fall of clinical symptoms (Fig. 4). However, we also note that while not diagnostic, lymphopenia and monocytosis can also be present in RSV and HRV infections, and that when they occur they track with clinical symptoms in those disease states as well. Thus, changes in the lymphocyte-to-monocyte ratio seem to be a common factor in experimental viral upper respiratory infections in humans, although the degree and consistency of these changes varies from virus to virus. At the time of maximal symptoms, an L:M ratio of <2 correctly classifies 100% of influenza-exposed subjects. However, with HRV and RSV-infected individuals the response is much more variable and the L:M ratio does not perform well as a classifier at any cutoff value.

Perhaps the most striking facets of the changes in WBC subsets in experimental viral challenges are the time-dependency and relative rapidity with which these effects occur and then resolve. The greatest perturbation in a given value tends to only last for a day or two, highlighting the importance of understanding where in the course of disease sampling is occurring. Furthermore, these values tend to trend tightly with overall symptom score over time, and therefore are likely subject to (or part of) underlying forces which drive symptomatology. This is also biologically consistent with previously published changes in PBMC gene expression over time in these viral challenges, where interferon-response genes and markers of monocyte/macrophage activation are key drivers of the host response, and also track closely with symptom development over time [15,20,23].

In naturally acquired influenza infection (albeit with a different virus, the 2009 H1N1 strain), a similar general trend toward lower lymphocyte and higher monocyte counts is seen with 67% having a L:M ratio <2, but this finding is much less profound and consistent than in the influenza challenge model. Patients with acute bacterial CAP due to *S. pneumo* exhibit more pronounced neutrophilia and lymphopenia but significantly less monocytosis, but still roughly 1/3 of them exhibit a L:M ratio <2. Interpretation of cell differentials is complicated and likely influenced by the experimentally demonstrated importance of timing in interpreting WBC changes, as with naturally occurring cases our knowledge of where in the course of disease a given patient resides is incomplete. Thus, while a low L:M ratio in the setting of normal total WBC and upper respiratory symptoms may in general be suggestive of a viral etiology, and may therefore have some utility in directing clinical evaluation down the appropriate pathway, these changes are clearly not specific enough to support focusing solely on a narrow clinical entity.

Despite the benefits, utilization of experimental human challenge models does carry limitations, as the clinical symptoms seen in human viral challenge trials, while similar in character to natural infection, are often less severe than those which bring a given patient to seek medical evaluation [21]. Challenges also utilize primarily young, healthy volunteers, which may limit the broad applicability of results to more varied, high-risk populations. Nonetheless, these data suggest that patterns in leukocyte differentials as defined through routine clinical analysis may suggest a viral etiology in patients with suspected upper respiratory viral infection, but are not sufficient to allow reliable differentiation between common viral causes of URI. Timing of data acquisition relative to the disease course remains a key component in determining the utility of these tests. While clearly remaining a highly nonspecific part of the workup of patients with clinical URI, interpretation of the leukocyte differential may prove able to play a role in aiding the clinician to pursue appropriate workup and management of such a common clinical presentation.

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Competing interests

Dr. Gilbert and Dr. Lambkin-Williams are employed at Retroscreen Virology.

Ethical approval

All protocols were reviewed by the relevant institutional ethical review boards for human studies and were conducted in accordance with the Declaration of Helsinki.

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