T-Cell Responses in Adults During Natural Respiratory Syncytial Virus Infection

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Background. The pathogenesis of respiratory syncytial virus (RSV) in older adults may be due to age-related T-cell immunosenescence. Thus, we evaluated CD4 and CD8 T-cell responses during RSV infection in adults across the age spectrum.

Methods. Peripheral blood mononuclear cells collected during RSV infection in adults, age 26–96 years, were stimulated with live RSV and peptide pools representing F, M, NP, and G proteins and analyzed by flow cytometry.

Results. There were no significant age-related differences in frequency of CD4⁺ T cells synthesizing interferon (IFN)γ, interleukin (IL)2, IL4, IL10, or tumor necrosis factor (TNF)α or in CD8⁺IFNγ⁺ T cells. IL4⁺CD4⁺ T-cell numbers were low, as were IL13 and IL17 responses. However, in univariate analysis, CD4 T-cell IFNγ, IL2, IL4, IL10, and TNFα responses and CD8⁺IFNγ⁺ T cells were significantly increased with more severe illness requiring hospitalization. In multivariate analysis, viral load was also associated with increased T-cell responses.

Conclusions. We found no evidence of diminished RSV-specific CD4 or CD8 T-cell responses in adults infected with RSV. However, adults with severe disease seemed to have more robust CD4 and CD8 T-cell responses during infection, suggesting that disease severity may have a greater association with T-cell responses than age.

Keywords. elderly adults; respiratory syncytial virus; T-cell response.

Respiratory syncytial virus (RSV) is best known as a cause of bronchiolitis in young infants and children [1]. Immunity to RSV is incomplete and reinfections occur throughout life. Although reinfections are generally mild in young adults, RSV can cause serious illness in older adults, especially those with cardiopulmonary conditions [2–8]. Studies indicate that RSV is second only to influenza as an important viral respiratory pathogen in adults [9–11].

Although the role of underlying cardiopulmonary conditions seems clear, viral and host factors involved in adult RSV pathogenesis have not been fully elucidated [12, 13]. Respiratory syncytial virus is not a highly cytopathic virus, and adults shed ~1000-fold lower viral titers than infants [14, 15]. Innate immune factors may contribute to severity, because hospitalized adults have higher mucosal levels of inflammatory mediators interleukin (IL)6 and macrophage inflammatory protein 1α [16]. Although serum and mucosal antibodies to RSV

contribute to protection from infection and illness severity, an absolute protective level is undefined [16–18].

T cells have been implicated in protection and disease pathogenesis of RSV [13, 19, 20]. Animal models show memory T cells to RSV protect from disease and accelerate virus clearance, although they also contribute to immunopathology [21–24]. CD4 and CD8 T cells are critical for recovery from primary infection in infants, but they may also be mediators of excessive inflammation under certain conditions [13, 24–26]. A balance of T cell-derived inflammatory (interferon [IFN]γ) and immunomodulatory (interleukin [IL]10) cytokines seems to be important for immune-mediated control of infection while minimizing tissue pathology [27]. In experimental animal models and in infants, a Th2 biased CD4 response may be associated with severe disease [20, 28]. Of note, aging is associated with changes in T-cell function, specifically declines in CD8 cytotoxic T-cell function and a shift from Th1 dominant to a more Th2 response [29]. Collectively, these age-related changes have been termed “immunosenescence”, and their effects have been termed “inflammaging” [30–32]. Several investigators reported age-related decline in resting RSV-specific memory T cells, both CD4 and CD8, after in vitro exposure of peripheral blood mononuclear cells (PBMCs) to RSV [33–35]. In contrast, we previously did not find age-related changes in baseline T-cell function in older persons, although we did note increases in activated proliferating CD8 T cells in more severely ill older adults [16, 36].

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Thus, in a study of disease pathogenesis in adults, we evaluated CD4 and CD8 T-cell responses by flow cytometry during natural RSV infection. The approaches were intended to determine magnitude, specificity, and effector phenotypes of T-cell subsets in the acute and convalescent phases of infection, specifically seeking to differentiate Th1 and Th2 cytokine production, as well as age, in relation to disease severity.

METHODS

Patient Populations

Two subject cohorts were recruited during 3 winters in Rochester, New York from 2005 to 2008, as described in Table 1 [16]. The first included community living adults ≥ 21 years, some with underlying chronic medical conditions. Most were enrolled before the RSV season and followed by passive and active surveillance for RSV infection. The second cohort included adult winter admissions to Rochester General Hospital (RGH) with diagnoses consistent with respiratory infection. Exclusion criteria included residence in long-term care or presence of immunocompromising conditions or medications. Subjects were enrolled after obtaining written informed consent, and they had a nasopharyngeal swab (NPS) tested for RSV by reverse transcriptase-polymerase chain

reaction. Clinical and demographic data were collected from subjects who tested positive for RSV, along with collection of respiratory secretions (NPS, sputum) every 1–2 days until viral clearance was confirmed. Fifty milliliters of heparinized blood was obtained at acute illness (V1) and 12–16 days (V2) and 28–35 days (V3) after symptom onset. The University of Rochester Research Subjects Review Board and the RGH Clinical Investigation Committee approved the study.

Laboratory Methods

Peripheral Blood Mononuclear Cell Collection

Blood was collected in heparinized tubes and processed within 4 hours. Peripheral blood mononuclear cells were isolated by centrifugation on a ficoll-hypaque gradient at $1500 \times g$ for 30 minutes at 20°C and washed 3 times with phosphate-buffered saline and centrifugation at $300 \times g$ for 10 minutes at 4°C . Viable cells, assessed by trypan blue exclusion, were suspended in 90% fetal calf serum (FCS)/10% dimethyl sulfoxide (DMSO), frozen, and stored in liquid nitrogen.

In Vitro Restimulation

Peripheral blood mononuclear cells were rapidly thawed in Roswell Park Memorial Institute 1640 medium (Cellgro, Manassas, VA), supplemented with penicillin (50 IU/mL)-streptomycin (50 $\mu\text{g/mL}$), 10 $\mu\text{g/mL}$ DNase (Sigma-Aldrich, St.

Table 1. Demographic and Clinical Characteristics of Study Subjects

Characteristics	Outpatients N = 43	Hospitalized N = 40	P Value	Total N = 83
Age, years; mean $\pm$ SD (range)	58.6 $\pm$ 16 (26–91)	68.9 $\pm$ 14.3 (43–96)	.003	63.6 $\pm$ 16.0 (26–96)
Sex; male, no. (%)	26 (60)	26 (65)	NS	52 (63)
Race; white	40 (93)	32 (80)	NS	72 (87)
Underlying Conditions; No. (%)				
COPD	9 (21)	21 (53)	.003	30 (36)
Congestive heart failure	0 (0)	12 (31)	.0003	12 (14)
Coronary artery disease	3 (7)	12 (31)	.02	15 (18)
Asthma	3 (7)	10 (25)	.02	13 (16)
Home O ₂ use	4 (9)	10 (25)	.07	14 (17)
Corticosteroids Before Infection				
Systemic	2 (5)	6 (15)	.14	8 (10)
Inhaled	11 (25)	15 (38)	NS	26 (31)
Evaluation; days since symptom onset; mean $\pm$ SD	2.9 $\pm$ 1.8	5.1 $\pm$ 3.2	.0002	4.1 $\pm$ 3.1
RSV group; A:B:unknown	21:21:1	13:26:1	NS	34:47:2
Days of illness; mean $\pm$ SD	16.4 $\pm$ 5.8	24.8 $\pm$ 9.3	<.0001	20.3 $\pm$ 8.7
Subjects treated with steroids	2 (5)	30 (75)	.0001	32 (39)
Symptoms				
Cough	38 (86)	38 (95)	.06	76 (92)
Sputum	23 (52)	33 (85)	.001	56 (67)
Dyspnea	12 (27)	37 (95)	.0001	49 (59)
Wheezing	7 (16)	35 (90)	.0007	42 (51)
Physical Findings				
Rhinorrhea	32 (73)	18 (46)	.05	50 (60)
Wheezing	5 (12)	32 (82)	.0001	37 (45)
Rales	2 (5)	13 (33)	.001	15 (18)

Abbreviations: COPD, chronic obstructive pulmonary disease; NS, nonsignificant; RSV, respiratory syncytial virus; SD, standard deviation.

Louis, MO), and 8% FCS and rested overnight at 37°C in 5% CO₂. On the day of assay, samples with >80% viability by trypan blue dye exclusion were plated into 96-well V-bottom plates (BD, Franklin Lakes, NJ) at 1–2 × 10⁶ cells/well and restimulated with the following antigens: culture supernatant of A2 RSV grown in HEp-2 cells (10⁶ plaque forming units/mL), or peptide (18-mers overlapping by 3) pools representing RSV full-length F, G, M, and NP proteins (designated F5, G5, M10, NP5) diluted in DMSO. Uninfected HEp-2 cell supernatant and DMSO were negative controls, and Staphylococcal endotoxin B (1 µg/mL; Sigma-Aldrich) was the positive control. After restimulation for 2 hours, Golgi block was added for 8 hours followed by permeabilization-fixation buffer (Becton-Dickinson) and staining with a pool of fluorescence-conjugated antibodies for Ki67, CD3, CD4, CD8α, CD45RA, CD69, IL2, IL4, IL10, IL13, IL17A, tumor necrosis factor (TNF)α, and IFNγ (Supplementary Table 1). Cells were analyzed on an LSR-II flow cytometer running DiVa software (Becton-Dickinson), and data were analyzed using FlowJo (TreeStar) and manual gating of cell populations (Supplementary Figure 1). Viable lymphocytes were identified by forward- and side-scatter, single-cell, and live/dead measurements. T cells were identified as CD3⁺, CD4⁺, CD8⁺, CD45RA⁺. Ki67 and CD69 staining, respectively, distinguished recently proliferated and activated T cells.

Statistical Analysis

For all analyses, cell counts were adjusted to cells per 1 million CD4 or CD8 T cells. Results reported are total counts minus background counts in negative control wells. Comparisons in demographics between groups were analyzed using *t* test for normally distributed data or χ^2 tests for categorical data. Wilcoxon rank-sum test was used for group difference in cytokines. Repeated analysis of covariance (ANCOVA) was applied to study effects of underlying respiratory disease, age, gender, and hospitalization status on responses. Cytokines were positively skewed and thus underwent log transformation before model fitting. We also performed stepwise multivariate analyses with entry criteria for each variable at 15% significance level. A *P* value of 0.05 was considered significant.

RESULTS

Study Population

During 3 winters, 111 subjects infected with RSV were enrolled. Sufficient live PBMCs were recovered from the first time point (V1) in 83 subjects. Of these, 36 had samples from 3 time points (V1, V2, V3), 16 from the first and third time points, 19 from the first 2 visits, and 12 from the first time point only.

Subject demographic and clinical characteristics are shown in the Table 1. There were 40 hospitalized patients and 43 outpatients, with a mean age of 63.6 ± 16.0 years (range, 26–96 years). Hospitalized subjects were significantly older with more underlying diseases, notably chronic obstructive

pulmonary disease (COPD) (53% vs 21%) and congestive heart failure (31% vs 0%), and hospitalized subjects more frequently used inhaled or low-dose systemic glucocorticosteroids and home oxygen before RSV infection. The hospitalized group more commonly had lower respiratory tract symptoms, especially sputum production, dyspnea, and wheezing. Illness duration was significantly longer for the hospitalized group (24.8 vs 16.4 days) who more commonly received systemic glucocorticosteroids during illness (75% vs 5%). In the absence of defined criteria for disease severity, subjects were considered severely ill if hospitalized and mild-moderately ill if managed as outpatients.

Age-Related CD4 and CD8 T-Cell Response

To optimally assess age-related T-cell responses during infection, we first analyzed subjects with mild disease (ie, outpatients) by comparing those <65 years of age to those ≥65 years of age. This was done because hospitalized subjects had more underlying medical conditions and frequently received systemic steroids during admission, both of which could alter immune parameters. After live virus stimulation of PBMCs from the acute illness visit (V1), there were no age-related differences in CD69⁺CD4⁺ T cells secreting IFNγ, IL2, IL4, IL10, IL13, IL17, and TNFα (Figure 1 and Supplementary Figure 2) and at subsequent visits (Supplementary Figure 3). Likewise, there were no age-related differences in secretion of the same cytokines by CD69⁺CD4⁺ T cells among hospitalized subjects (Figure 2 and Supplementary Figure 4), or when inpatients and outpatients are combined (data not shown). Because T-cell responses are dynamic after antigenic stimulation, we also performed multivariate analysis to adjust for sample timing after onset of symptoms. In these analyses, subject age remained nonsignificant for each cytokine. The frequencies of IL13 and IL17 producing T cells were very low (mean 5–10 per million, median 0 per million). There were no differences by age when PBMCs were stimulated with any of the peptide pools (data not shown). We previously noted that older adults develop greater antibody responses to infection than young adults, and therefore we evaluated the relationship between magnitude of CD4⁺ T-cell responses and height of the antibody response [37]. However, we saw no relationship (data not shown).

Similar to findings with CD4⁺ T cells, we did not see age-related differences in IFNγ-secreting CD69⁺CD8⁺ T cells after stimulation with either live RSV or peptide pools in mildly ill and severely ill subjects combined (Supplementary Figure 5). Although not significant, there was a trend toward higher peak nasal viral titer with more IFNγ producing CD4 or CD8 T cells in acute blood samples (*P* = .12) (Supplementary Figure 6). Taken together, these data do not support the contention that there are age-related declines in T-cell responses to RSV during active infection.

Figure 1. Comparison of CD69⁺CD4⁺ T-cell responses between younger (<65; n = 24) and older (≥65; n = 16) respiratory syncytial virus (RSV)-infected adults with mild illness at the acute illness time point (V1). Peripheral blood mononuclear cells were stimulated with live RSV or with uninfected cell culture media. Values shown are with background subtracted. Bars represent median and interquartile ranges. Shown are number of CD69⁺CD4⁺ cells/million expressing (A) interferon (IFN) γ , (B) interleukin (IL)2, (C) IL10, and (D) tumor necrosis factor (TNF) α . All comparisons are not significantly different.

Illness Severity and CD4 T-Cell Responses

CD4⁺ T cells making IFN γ or IL10 and Th2 cells have been associated with differences in disease severity during RSV infection in infants [28, 38, 39]. Because no age-related differences were noted, we next compared CD4⁺ T-cell cytokine responses for all subjects in relation to disease severity (ie, outpatients vs inpatients). After live virus stimulation, PBMCs from subjects with severe illness had significantly greater numbers of CD69⁺CD4⁺ T cells secreting IFN γ ($P < .0001$), IL2 ($P < .0001$), IL4 ($P = .02$), IL10 ($P < .001$), and TNF α ($P < .001$) than PBMCs from subjects with mild disease (Figure 3 and Supplementary Figure 7). In addition, hospitalized subjects had more proliferating CD69⁺CD4⁺ cells, as indicated by a significantly greater number of Ki67⁺ cells ($P = .002$) (Supplementary Figure 7C). There was no difference in secretion of IL13 or IL17 by CD69⁺CD4⁺ cells according to

disease severity. Notably, there was a significantly greater IL4 response in severely ill subjects, although overall number of IL4⁺ T cells was relatively low (Figure 3C). There were no significant differences between the 2 groups in cytokine-secreting CD4 T cells at the later visits, and responses were lower (Supplementary Figure 8). We also sought to assess the influence of other covariates using repeated ANCOVA analysis. After adjustment for effects of age, presence of asthma and/or COPD, inpatient or outpatient status, and visit number (V1, V2, V3), each of the differences according to severity remained significant. In addition, there were significant gender differences in CD69⁺CD4⁺IL2⁺ ($P = .04$), CD69⁺CD4⁺IL4⁺ ($P = .02$), and CD69⁺CD4⁺Ki67⁺ ($P = .03$) cells, with higher responses in men. These observations suggest that effector Th1-like CD4⁺ T-cell responses to RSV may be more robust in subjects with severe disease, with no indication

Figure 2. Comparison of CD69⁺CD4⁺ T-cell responses between younger (<65; n = 18) and older (≥65; n = 17) respiratory syncytial virus (RSV)-infected adults with severe illness (hospitalized group). Peripheral blood mononuclear cells were stimulated with live RSV or with uninfected cell culture media. Values shown are with background subtracted. Bars represent median and interquartile ranges. Shown are number of CD69⁺CD4⁺ cells/million expressing (A) interferon (IFN)γ, (B) interleukin (IL)2, (C) IL10, and (D) tumor necrosis factor (TNF)α. All comparisons are not significantly different.

of a defective regulatory (IL10) response. They also provide evidence of a modest Th2 response, as measured by IL4 production.

CD4⁺ T-Cell Specificity

We sought to address RSV protein specificity of CD4⁺ T-cell responses by stimulating PBMCs with RSV F, G, M, and NP peptide pools (15–20 amino acid range) chosen to favor stimulation of CD4⁺ T cells (Supplementary Figure 9). The RSV M and F peptide pools elicited the highest frequencies of CD69⁺CD4⁺IFNγ⁺ T cells and cytokine-positive CD69⁺Ki67⁺CD4⁺ T cells (Figure 4 and Supplementary Figure 9). More severely ill hospitalized subjects had greater IFNγ ($P = .05$ for F peptides; $P = .001$ for M peptides) and Ki67 ($P = .02$ for both F and M peptides) responses than mildly ill outpatients after stimulation with F and M peptide pools (Figure 4A and B). Responses were less than frequencies seen with whole RSV stimulation. In contrast to whole RSV stimulation,

we saw no significant differences in F-specific or M-specific IL2, IL10, or TNFα-secreting CD4⁺ T cells. No other RSV peptide pool showed differences related to severity (data not shown).

Illness Severity and CD8 T-Cell Responses

CD8⁺ T cells making IFNγ have been associated with increased immune pathology in animal models [20, 40]. We therefore assessed the relationship of RSV-specific CD8⁺ T cells to disease severity. Severely ill subjects had significantly increased numbers of IFNγ-secreting CD69⁺CD8⁺ cells at the acute visit when stimulated by live RSV ($P = .0002$) and RSV NP peptides ($P = .018$) (Figure 5A and D and Supplementary Figure 10). Responses to other peptide pools did not differ based on severity.

It is probable that many diverse factors play a role in disease severity during acute respiratory infections in heterogeneous groups of older adults. In an attempt to consider several

Figure 3. Comparison of CD69⁺CD4⁺ T-cell responses between respiratory syncytial virus (RSV)-infected subjects with mild illness (O, outpatients; n = 40) and those with severe illness (I, hospitalized inpatients; n = 35). Peripheral blood mononuclear cells from the acute visit (V1) were stimulated with live RSV or uninfected cell culture media. Values shown are with background subtracted. Bars represent median and interquartile ranges. (A) interferon (IFN) γ , $P < .0001$; (B) interleukin (IL)2, $P < .0001$; (C) IL4, $P = .022$; and (D) IL10, $P = .0014$.

of these variables, we performed stepwise multivariate logistic regression analysis for each of the cytokines at the time of acute presentation (V1) considering age, sex, presence of underlying COPD/asthma, days of illness, hospitalization status, peak neutralizing antibody titer, peak viral load, and duration of viral shedding for all 83 subjects. In these multivariate analyses, hospitalization status was associated with significantly greater number of CD4 cells producing IL2, IL4, and TNF α , with a trend toward a greater number of CD8⁺IFN γ responses (Supplementary Table 2). It is interesting to note that a higher peak nasal viral load was associated with increased magnitude of response for each cytokine after stimulation with RSV with the exception of the CD69⁺CD4⁺IL4⁺ response.

Effect of Glucocorticoid Treatment on T-Cell Responses

The majority of hospitalized subjects (75%) were administered systemic glucocorticosteroids during treatment for COPD or asthma exacerbations. In almost all, steroids were administered 1–2 days before blood collection and averaged 8.8 ± 9.0 days of treatment (range, 1–27 days). Notably, hospitalized subjects treated with steroids had greater CD4 and CD8 responses at the acute time point than nontreated subjects (Figure 6 and Supplementary Figure 11), although this was not statistically significant, nor were the results at the second and third time points. Because the number of subjects was small and steroid administration was not randomized and likely driven by presence of wheezing, firm conclusions regarding steroid treatment effect cannot be made.

Figure 4. Comparison of CD69⁺CD4⁺ T-cell responses in peripheral blood mononuclear cells stimulated with peptide pools representing respiratory syncytial virus F5, M10, NP5, and G5 proteins. Data are shown for all subjects with mild illness (O, outpatients) and severe illness (I, hospitalized inpatients) for stimulation with (A) F5 pool, (B) M10 pool, (C) NP5 pool, and (D) G5 pool. Bars represent median and interquartile ranges. *P* values for significant comparisons are as follows: F5 stimulation interferon (IFN) γ (*P* = .054) and Ki67 (*P* = .02); M10 stimulation IFN γ (*P* = .001) and Ki67 (*P* = .02). All other comparisons are not significant. Number of samples in each plot varies depending upon number of cells available for each stimulation. Abbreviation: ns, nonsignificant.

DISCUSSION

The pathogenesis of adult RSV disease is likely multifactorial, including factors such as underlying medical conditions, overall frailty, differences in viral virulence, and immune responses. Autopsy studies in infants and older adults demonstrate variable viral cytopathology, and virus is often relatively sparse and limited to the superficial layer of ciliated epithelial cells in lower airways [41, 42]. Pathologic changes are primarily related to inflammatory cell infiltration, suggesting a significant immune component to disease pathogenesis [12, 41, 42]. Thus, it has been posited that immune responses significantly influence clinical manifestations and severity of infection. Furthermore, it has been suggested that immunosenescence with a shift from Th1 to a more Th2 phenotype could account for greater susceptibility to infection, poor control of virus replication, and be causally associated with more severe RSV illness in older adults [33–35]. Although our study did not demonstrate a Th1 to Th2 shift in more severely ill subjects, the data suggest that more severe disease is associated with greater T-cell responses.

We were surprised to find that there were no age-related changes in CD4 or CD8 T-cell responses during RSV infection.

To minimize confounding effects of disease severity and steroid use, we first assessed age-related differences in the mildly ill group. However, results were similar when both mildly and severely ill subjects were combined, including when adjusting for differences in timing of sample collection. Thus, we could not demonstrate differences in T-cell responses to RSV, in terms of virus-specific T-cell numbers or effector phenotypes, as a function of age during active infection. Moreover, there was no evidence of Th2, Th13, or Th17 bias in older subjects. Our data does not support the supposition of age-related declines or skewing of function in RSV-specific T cells in RSV-infected adults. Nevertheless, it is possible that unrecognized host factors could have limited our ability to detect age-related changes in T-cell responses.

In contrast, we did find significant and consistent differences in CD4 and CD8 T-cell responses according to illness severity in univariate analysis. Severely ill subjects had greater numbers of IFN γ , IL2, IL4, IL10, and TNF α secreting CD69⁺CD4⁺ T cells during the acute phase of illness, with negligible differences by 12–16 days after symptom onset. In addition, we found evidence of a modestly greater Th2 response in hospitalized subjects, as

Figure 5. Comparison of interferon (IFN) γ secretion by CD69 $^{+}$ CD8 $^{+}$ T-cells from respiratory syncytial virus (RSV)-infected subjects with mild illness (O, outpatients) and those with severe illness (I, hospitalized inpatients). Bars represent median and interquartile ranges. Peripheral blood mononuclear cells from acute visit (V1) were stimulated with (A) live RSV $P = .0002$, (B) F5 peptide pool $P = .22$, (C) M10 peptide pool $P = .19$, and (D) NP5 peptide pool $P = .018$. Uninfected cell culture media or dimethyl sulfoxide were used as negative controls, and values shown are background subtracted. In each panel, the mild illness group is on left and severe illness group on right. Number of samples in each plot varies depending upon number of cells available for each stimulation. Abbreviation: ns, nonsignificant.

measured by IL4 secretion from CD4 T cells, although IL13- and IL17-secreting cells were similar between mild and severe illness groups. In almost all cases, the number of IL4, IL13, and IL17 secreting CD4 T cells were of low frequency, regardless of illness severity. Similar to CD4 responses, we also found greater numbers of CD69 $^{+}$ CD8 $^{+}$ IFN γ^{+} T cells in more ill subjects during the acute stage of illness. This difference was no longer evident by day 12–16 after illness onset. Our data suggests that severe disease is associated with an increased CD4 and CD8 effector T-cell response, principally with a Th1 cytokine and inflammatory pattern with increases in IFN γ , IL2-, and TNF α -secreting cells but also with some Th2 component as well. It is important to recognize that our observations do not allow us to make a causal connection between the T-cell response and disease severity, only that they are associated. However, the association of T cells and more severe disease is consistent with murine models of RSV, in which both CD4 and CD8 T-cell responses are implicated in disease severity, and where depletion of CD4 and CD8 T cells, either singly or together, was found to reduce illness [20, 43, 44]. Using the aging cotton rat model, outcomes were best when infected animals were treated

with antivirals plus anti-inflammatory agents compared with either agent alone [45]. Given the more vigorous T-cell response in the more severely ill adults, it is possible that a similar approach might be beneficial in severe adult RSV disease.

Although not significant in univariate analysis, in multivariate modeling, peak nasal viral load was associated with greater CD69 $^{+}$ CD4 $^{+}$ IFN γ^{+} T-cell response. This is analogous to findings noted in the experimental human RSV challenge model in which airway T-cell response was associated with viral load [46]. Although circulating T cells may not represent the number or function of T cells sequestered at the site of infection, similar to the human challenge model, our data suggest that RSV-specific T-cell responses in blood may be an indicator of risk for more severe disease.

Our results appear inconsistent with published data indicating age-related changes in RSV-specific memory CD4 and CD8 T cells, although an important distinction is that our results were derived from acutely infected subjects as opposed to asymptomatic subjects [33–35]. To our knowledge, this is the first study of acute naturally infected adults. Perhaps age is associated with diminished baseline memory function, placing

Figure 6. Effect of glucocorticoid treatment on CD69⁺CD8⁺IFN γ ⁺ (A) and on CD69⁺CD4⁺ cytokine-positive (B–D) T-cell responses among hospitalized subjects only. For each panel, peripheral blood mononuclear cells were stimulated with live respiratory syncytial virus and background subtracted (uninfected control). Shown in each panel are the 2 groups, no steroid treatment (N) and steroid treated (Y) for all 3 visits. Bars represent median and interquartile ranges. All comparisons are nonsignificant. Number of samples in each plot varies depending upon number of cells available for each stimulation. Abbreviations: IFN, interferon; IL, interleukin.

older persons at increased risk of infection; however, it does not result in measurable differences in response once infected. There may also be age-related differences in the kinetics of T-cell responses, sequestration in peripheral tissues, or expansion of RSV-specific T cells that account for the observed differences in illness severity in older persons.

Because administration of glucocorticosteroids is common in adults hospitalized with RSV infection, we were also interested in the potential effect of this treatment. We previously showed in these same individuals that steroids did not alter the absolute number of B cells or T-cell subsets, although RSV-specific antibody responses were modestly reduced [47]. In this study, there was no obvious effect of steroids on cytokine production by CD4 or CD8 T cells, suggesting that a short course of steroids does not produce substantial deleterious effects on RSV humoral or cellular immunity.

There are a number of important technical and analytical limitations in our study when attempting to study T-cell responses in naturally infected adults. The timing of virus

exposure and symptom onset can be difficult to precisely determine, especially in patients with chronic underlying cardiopulmonary conditions who often have daily respiratory symptoms. In addition, there is great heterogeneity of the population enrolled, as well as possible genotypic differences in the yearly circulating strains of RSV. Perhaps most important, the circulating T cells may not accurately reflect the local pulmonary response that is more likely to influence illness characteristics.

CONCLUSIONS

In conclusion, the more severe illness seen during RSV infection in older adults does not appear to be a result of age-specific changes in CD4 or CD8 T-cell function during infection, whereas increased T-cell responses appear to be associated with more severe illness and higher viral load.

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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References

- Hall CB, Weinberg GA, Blumkin AK, et al. Respiratory syncytial virus-associated hospitalizations among children less than 24 months of age. *Pediatrics* **2013**; 132:e341–8.
- Henderson FW, Collier AM, Clyde WA Jr, Denny FW. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. *N Engl J Med* **1979**; 300:530–4.
- Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med* **2005**; 352:1749–59.
- Lee N, Lui GC, Wong KT, et al. High morbidity and mortality in adults hospitalized for respiratory syncytial virus infections. *Clin Infect Dis* **2013**; 57:1069–77.
- Widmer K, Griffin MR, Zhu Y, Williams JV, Talbot HK. Respiratory syncytial virus- and human metapneumovirus-associated emergency department and hospital burden in adults. *Influenza Other Respir Viruses* **2014**; 8:347–52.
- Sundaram ME, Meece JK, Sifakis F, Gasser RA Jr, Belongia EA. Medically attended respiratory syncytial virus infections in adults aged ≥ 50 years: clinical characteristics and outcomes. *Clin Infect Dis* **2014**; 58:342–9.
- Walsh EE, Peterson DR, Falsey AR. Risk factors for severe respiratory syncytial virus infection in elderly persons. *J Infect Dis* **2004**; 189:233–8.
- Mehta J, Walsh EE, Mahadevia PJ, Falsey AR. Risk factors for respiratory syncytial virus illness among patients with chronic obstructive pulmonary disease. *COPD* **2013**; 10:293–9.
- Zhou H, Thompson WW, Viboud CG, et al. Hospitalizations associated with influenza and respiratory syncytial virus in the United States, 1993–2008. *Clin Infect Dis* **2012**; 54:1427–36.
- Fleming DM, Taylor RJ, Lustig RL, et al. Modelling estimates of the burden of respiratory syncytial virus infection in adults and the elderly in the United Kingdom. *BMC Infect Dis* **2015**; 15:443.
- van Asten L, van den Wijngaard C, van Pelt W, et al. Mortality attributable to 9 common infections: significant effect of influenza A, respiratory syncytial virus, influenza B, norovirus, and parainfluenza in elderly persons. *J Infect Dis* **2012**; 206:628–39.
- Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. *J Virol* **2008**; 82:2040–55.
- Collins PL, Fearn R, Graham BS. Respiratory syncytial virus: virology, reverse genetics, and pathogenesis of disease. *Curr Top Microbiol Immunol* **2013**; 372:3–38.
- Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. *Pediatr Infect Dis J* **2000**; 19:113–7.
- Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. *Clin Infect Dis* **2001**; 33:792–6.
- Walsh EE, Peterson DR, Kalkanoglu AE, Lee FE, Falsey AR. Viral shedding and immune responses to respiratory syncytial virus infection in older adults. *J Infect Dis* **2013**; 207:1424–32.
- Mills J 5th, Van Kirk JE, Wright PF, Chanock RM. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. *J Immunol* **1971**; 107:123–30.
- Bagga B, Cehelsky JE, Vaishnaw A, et al. Effect of preexisting serum and mucosal antibody on experimental respiratory syncytial virus (RSV) challenge and infection of adults. *J Infect Dis* **2015**; 212:1719–25.
- Openshaw PJ, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. *Clin Microbiol Rev* **2005**; 18:541–55.
- Openshaw PJ. The mouse model of respiratory syncytial virus disease. *Curr Top Microbiol Immunol* **2013**; 372:359–69.
- Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J Exp Med* **1988**; 168:1163–8.
- Connors M, Collins PL, Firestone CY, Murphy BR. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. *J Virol* **1991**; 65:1634–7.
- Ostler T, Hussell T, Surh CD, Openshaw P, Ehl S. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. *Eur J Immunol* **2001**; 31:2574–82.
- Varga SM, Wang X, Welsh RM, Braciale TJ. Immunopathology in RSV infection is mediated by a

- discrete oligoclonal subset of antigen-specific CD4(+) T cells. *Immunity* **2001**; 15:637–46.
25. Hall CB, Powell KR, MacDonald NE, et al. Respiratory syncytial viral infection in children with compromised immune function. *N Engl J Med* **1986**; 315:77–81.
 26. Hussell T, Baldwin CJ, O'Garra A, Openshaw PJ. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. *Eur J Immunol* **1997**; 27:3341–9.
 27. Sun J, Cardani A, Sharma AK, et al. Autocrine regulation of pulmonary inflammation by effector T-cell derived IL-10 during infection with respiratory syncytial virus. *PLoS Pathog* **2011**; 7:e1002173.
 28. Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *Am J Respir Crit Care Med* **2003**; 168:633–9.
 29. Ponnappan S, Ponnappan U. Aging and immune function: molecular mechanisms to interventions. *Antioxid Redox Signal* **2011**; 14:1551–85.
 30. Makinodan T, Kay MM. Age influence on the immune system. *Adv Immunol* **1980**; 29:287–330.
 31. Franceschi C, Capri M, Monti D, et al. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech Ageing Dev* **2007**; 128:92–105.
 32. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci* **2014**; 69 (Suppl 1):S4–9.
 33. Looney RJ, Falsey AR, Walsh E, Campbell D. Effect of aging on cytokine production in response to respiratory syncytial virus infection. *J Infect Dis* **2002**; 185:682–5.
 34. Cherukuri A, Patton K, Gasser RA Jr, et al. Adults 65 years old and older have reduced numbers of functional memory T cells to respiratory syncytial virus fusion protein. *Clin Vaccine Immunol* **2013**; 20:239–47.
 35. Cusi MG, Martorelli B, Di Genova G, Terrosi C, et al. Age related changes in T cell mediated immune response and effector memory to Respiratory Syncytial Virus (RSV) in healthy subjects. *Immun Ageing* **2010**; 7:14.
 36. Lee FE, Walsh EE, Falsey AR, et al. The balance between influenza- and RSV-specific CD4 T cells secreting IL-10 or IFN γ in young and healthy-elderly subjects. *Mech Ageing Dev* **2005**; 126:1223–9.
 37. Walsh EE, Falsey AR. Age related differences in humoral immune response to respiratory syncytial virus infection in adults. *J Med Virol* **2004**; 73:295–9.
 38. Kim CK, Kim SW, Park CS, Kim BI, et al. Bronchoalveolar lavage cytokine profiles in acute asthma and acute bronchiolitis. *J Allergy Clin Immunol* **2003**; 112:64–71.
 39. Román M, Calhoun WJ, Hinton KL, et al. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. *Am J Respir Crit Care Med* **1997**; 156:190–5.
 40. Braciale TJ. Respiratory syncytial virus and T cells: interplay between the virus and the host adaptive immune system. *Proc Am Thorac Soc* **2005**; 2:141–6.
 41. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod Pathol* **2007**; 20:108–19.
 42. Welliver TP, Reed JL, Welliver RC Sr. Respiratory syncytial virus and influenza virus infections: observations from tissues of fatal infant cases. *Pediatr Infect Dis J* **2008**; 27:S92–6.
 43. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J Clin Invest* **1991**; 88:1026–33.
 44. Tregoning JS, Yamaguchi Y, Harker J, Wang B, Openshaw PJ. The role of T cells in the enhancement of respiratory syncytial virus infection severity during adult reinfection of neonatally sensitized mice. *J Virol* **2008**; 82:4115–24.
 45. Blanco JC, Boukhvalova MS, Hemming P, Ottolini MG, Prince GA. Prospects of antiviral and anti-inflammatory therapy for respiratory syncytial virus infection. *Expert Rev Anti Infect Ther* **2005**; 3:945–55.
 46. Jozwik A, Habibi MS, Paras A, et al. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat Commun* **2015**; 6:10224.
 47. Lee FE, Walsh EE, Falsey AR. The effect of steroid use in hospitalized adults with respiratory syncytial virus-related illness. *Chest* **2011**; 140:1155–61.