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The Memory T Cell Response to West Nile Virus in Symptomatic Humans following Natural Infection Is Not Influenced by Age and Is Dominated by a Restricted Set of CD8⁺ T Cell Epitopes

Robin Parsons,* Alina Lelic,* Lisa Hayes,* Alexandra Carter,* Laura Marshall,* Carole Evelegh,* Michael Drebot,† Maya Andonova,† Curtis McMurtrey,‡ William Hildebrand,‡ Mark B. Loeb,* and Jonathan L. Bramson*‡

We examined the West Nile virus (WNV)-specific T cell response in a cohort of 52 patients with symptomatic WNV infections, including neuroinvasive and non-neuroinvasive disease. Although all virus proteins were shown to contain T cell epitopes, certain proteins, such as E, were more commonly targeted by the T cell response. Most patients exhibited reactivity toward 3–4 individual WNV peptides; however, several patients exhibited reactivity toward >10 individual peptides. The relative hierarchy of T cell reactivities in all patients showed a fixed pattern that was sustained throughout the 12-mo period of the current study. Surprisingly, we did not observe any relationship between age and either the breadth or magnitude of the T cell response following infection. We also did not observe a relationship between disease severity and either the breadth or magnitude of the T cell response. The T cell epitopes were distributed in a non-random fashion across the viral polyprotein and a limited number of epitopes appeared to dominate the CD8⁺ T cell response within our cohort. These data provide important new insight into the T cell response against WNV in humans. The Journal of Immunology, 2008, 181: 1563–1572.

West Nile virus (WNV) emerged in North America as a significant human pathogen following an outbreak in New York in 1999. Since that time, annual outbreaks of WNV have occurred across the continent. In the U.S., there were 4,256 documented cases of WNV infection in 2006 and ~24,000 cases and 1,000 fatalities have been reported since 1999 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm). Approximately 20% of those infected with WNV develop symptoms, which can range from “West Nile fever,” characterized by symptoms including headaches, myalgias, rash, and fever, to neuroinvasive disease including meningitis, encephalitis, and acute flaccid paralysis. Since it has been estimated that <1% of all WNV infections produce neurological complications, many cases of WNV infection go unnoticed or are ascribed to other causes. As such, WNV likely infects a much broader population than can be ascribed by the clinical reports. As an example, although there were only 9,862 documented reports of clinical infection with WNV in the U.S. during 2003, surveillance data from blood donor screening programs estimated that there may have been as many as 735,000 infections in that same period, the majority of which went undiagnosed (1). Given the burden of WNV infection, greater understanding of the pathobiology of this infection is necessary to develop preventive and therapeutic strategies.

T cells play a major role in controlling virus infections and data from murine models support an important role for both CD8⁺ and CD4⁺ T cells in the resolution of WNV infection. CD8⁺ T cells control viremia following infection with WNV and mediate clearance of the virus from the CNS (2–7). CD4⁺ T cells are also necessary in these processes through their function as helpers for B cell and CD8⁺ T cell development (8). Although most reports from murine models support a protective role for CD8⁺ T cells during WNV infection, evidence from studies with the Lineage II Sarafend strain indicates that CD8⁺ T cells may also contribute to immunopathology in the CNS (3). Characterization of T cell immunity in naturally infected patients who experienced mild and severe illness following WNV infection will provide new insights into the potential role of T cells in disease outcome and pathology. Such information may also be of value to other flavivirus infections, such as dengue, where T cells have similarly been associated with protection and immunopathology (9, 10).

Advanced age is a key risk factor for the development of severe pathology following WNV infection (11). Although the mechanisms that underlie this age-associated outcome are unknown, it is quite possible that immune deficits are the cause. Immune senescence is a well-described phenomenon where the innate and adaptive immune systems show progressive impairment with age (12). These immunological impairments manifest themselves as reduced...
allowing us to explore the relationship between age, disease parameters, and acute flaccid paralysis) ranging in age from 29 to 82 years, in order to understand the role of T cell immunity in WNV infection. Our patient population included individuals with both mild (no neurological complications) and severe disease (encephalitis, meningitis, meningoencephalitis, and acute flaccid paralysis) ranging in age from 29 to 82 years, enabling us to explore the relationship between age, disease parameters, and acute flaccid paralysis.

To date, there have been no published reports of T cell immunity to WNV in humans. In the current manuscript, we describe changes in T cell immunity within the peripheral blood of a cohort of 52 symptomatic WNV-infected patients using a comprehensive approach that permits the examination of most, if not all, epitopes present within the viral polyprotein. Our patient population included individuals with both mild (no neurological complications) and severe disease (encephalitis, meningitis, meningoencephalitis, and acute flaccid paralysis) ranging in age from 29 to 82 years, allowing us to explore the relationship between age, disease pathology, and T cell immunity.
Materials and Methods

Cell culture materials

All plasticware used for cell culture was purchased from Falcon. RPMI 1640 powder was purchased from Invitrogen and prepared under sterile conditions at McMaster University. Cells were cultured in complete RPMI 1640 (cRPMI) consisting of RPMI 1640 with 10% FBS, 2 mM l-glutamine, 50 μg/ml 2-ME, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Staphylococcus enterotoxin B was purchased from Toxin Technologies and used for T cell stimulation at a final concentration of 0.5 μg/ml.

Patients and PBMC preparation

Fifty-two patients were enrolled into the study following detection of serum WNV IgM by public health laboratories after presenting with symptoms of WNV infection. This trial was reviewed and approved by the Research Ethics Board at McMaster University. Patients were recruited into this study over three seasons (2003–2005). Serology for WNV and dengue virus was assessed by plaque reduction neutralization test as described previously (16). In general, patients were entered into our study within 1 mo following the onset of symptoms (median 28 days; ranging from 9 to 99 days post-onset of symptoms). In brief, the population consisted of 27 females and 26 males with an average age of 51.8 years (median 49 years; ranging from 29 to 82 years). Details of the patient characteristics are available in Table I. HLA genotypes were determined using DNA sequence analysis at the Hamilton Health Sciences Histocompatibility Laboratory (Hamilton, ON) and Pure Transplant Solutions (Austin, TX). Blood samples were drawn into heparanized tubes once the patients were enrolled in our study and monthly thereafter for the 12 mo of the study. PBMC were isolated from the blood samples by centrifugation on Ficoll (Amersham Pharmacia) and cryopreserved in RPMI 1640 containing 12.5% human serum albumin (Sigma-Aldrich) and 10% DMSO according to the method described by Disis et al. (17).

Peptides

The sequences for a library of 847 15-mer overlapping peptides spanning the full WNV polyprotein were generated by the PeptGen application (www.hiv.lanl.gov/content/sequence/PEPTGEN/PepGen.html). Separate peptide sets were produced for each individual protein (C, E, M, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The peptides were generated with 11 residues overlapping for all proteins except NS3, where the overlap was 10 residues, and NS5, where the overlap was 9 residues. We also generated a peptide set covering the C-terminal/N-terminal junctions between the individual viral proteins in the event that epitopes might be generated from the polyprotein before processing (termed the junction pool). A library comprising 91 MHC class I epitopes from EBV with broad
FIGURE 4. Both CD8⁺ and CD4⁺ T cells were identified in our screens. A, PBMC from patient 11201 were restimulated with minipool M-6, DMSO (negative control), or a pool of defined CD8⁺ T cell epitopes from EBV (positive control). The samples were subsequently stained with Abs to CD3, CD8, CD4, IFN-γ, TNF-α, and IL-2 as described in Materials and Methods. Top row, The data was gated on live cells and CD3. The number in the left-hand corner reflects the % IFN-γ⁺/CD8⁺ cells of total CD8⁺ cells (shown by the elliptical gate). Middle row, The data was gated on live cells, CD3 and CD8. The number in the upper, left-hand quadrant is the % IFN-γ⁺/CD8⁺ cells of total CD8⁺ cells; the bracketed number in the lower, left-hand quadrant is the % IFN-γ⁺/IL-2⁺/CD8⁺ cells of total CD8⁺ cells. B, PBMC from patient 77313 were restimulated with minipool E-13, DMSO (negative control), or Staphylococcus enterotoxin B (positive control). The samples were subsequently stained with Abs to CD3, CD8, CD4, IFN-γ, TNF-α, and IL-2 as described in Materials and Methods. Top row, The data was gated on live cells and CD3. The number in the left-hand corner reflects the % IFN-γ⁺/CD4⁺ cells of total CD4⁺ cells (shown by the elliptical gate). Middle row, The data was gated on live cells, CD3 and CD4. The number in the upper, left-hand quadrant is the % IFN-γ⁺/TNF-α⁺ cells of total CD4⁺ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ⁺/TNF-α⁺ cells of total CD4⁺ T cells; the number in the lower, left-hand quadrant is the % IFN-γ⁺/IL-2⁺/CD4⁺ cells of total CD4⁺ T cells. Bottom row, The data was gated on live cells, CD3 and CD4. The number in the upper, left-hand quadrant is the % IFN-γ⁺/TNF-α⁺ cells of total CD4⁺ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ⁺/TNF-α⁺ cells of total CD4⁺ T cells; the number in the lower, left-hand quadrant is the % IFN-γ⁺/IL-2⁺/CD4⁺ cells of total CD4⁺ T cells; the number in the lower, left-hand quadrant is the % IFN-γ⁺/IL-2⁺/CD4⁺ cells of total CD4⁺ T cells.

Intracellular cytokine staining
PBMC were thawed and placed immediately into cRPMI prewarmed to 37°C and cultured overnight in cRPMI at 37°C. The cells were subsequently harvested, counted, and viability was assessed by trypan blue exclusion. Cells were aliquoted (1–2 × 10⁵ cells/tube) into Falcon 2057 tubes, peptides were added to a final concentration of 2 μg/ml, and the cells were incubated for 2 h. Brefeldin A was then added to a final concentration of 5 μM and the cells were incubated 4 h further. At the end of this period, cells were pelleted and washed in 10 μM EDTA. The cells were subsequently surface stained with either anti-CD8-PE-Cy7 or anti-CD4-PE-Cy7 and anti-CD28-PE-Cy5, permeabilized with Cytoperm, and intracellular cytokines were identified using anti-CD8-PerCP-Cy5.5, anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE-Cy7, and anti-TNF-α-APC-Cy7. Flow cytometry reagents were obtained from BD Pharmingen. In some cases, samples were stained with both anti-CD8-PE-Cy7 and anti-CD4-PE-Cy7. Fluorescence data were acquired using a FACS Canto and 200,000 events based on the live lymphocyte gate were collected per sample.
FIGURE 5. The breadth and magnitude of the T cell response does not correlate with age or disease pathology. A, The scattergram reflects the number of minipools recognized by individual patients as a function of their age. Each point represents a single patient. The dotted line represents a linear regression curve modeled on the data. B, The scattergram reflects the number of minipools recognized by individual patients separated into groups defined by disease severity. Each point represents a single patient. C, The scattergram reflects the magnitude of the T cell response at 3–4 mo post-onset of symptoms as defined by the total number of SFC as a function of age. Each point represents a single patient. The dotted line represents a linear regression curve modeled on the data. D, The scattergram reflects the magnitude of the T cell response at 3–4 mo post-onset of symptoms as defined by the total number of SFC separated into groups defined by disease severity. Each point represents a single patient.

Statistical analysis
The data are presented as mean ± SEM. All statistics (SEM, Students t test and regression analysis) were calculated using Microsoft Excel 2004 for Mac.

Results
Characterization of the T cell response to WNV
The design of the ELISPOT pools is shown in Fig. 1. Using this strategy, we were able to screen the entire WNV polyprotein on a single 96-well ELISPOT plate. We observed that the results of this screen were highly reproducible and stable over time. A total of 15 patients were screened with the full library using 2 different samples obtained at least 30 days apart (data not shown). For 12 patients, the pattern of reactivity was the same at both time points. For the remaining three patients, the reactivity measured with the later samples was too weak to compare with the data from the earlier time point. In no case did we observe a change in the pattern of reactivity. In general, we
observed the strongest ELISPOT results with the samples drawn closest to the onset of symptoms.

Forty-one patients were screened with the entire peptide library. The results were subsequently deconvoluted and individual minipools containing putative epitopes were selected for further screening. Each minipool comprised an average of six consecutive overlapping peptides (described in Materials and Methods and Fig. 1), which span a region of 40–50 residues of the polyprotein. All of the patients showed reactivity to at least two independent minipools. The median number of minipools for which a patient showed reactivity was 4 with a range of 2 to 12 (Fig. 2A). It should be noted that when a patient showed reactivity to neighboring pools, we counted this as only one reactive pool since, in all cases, we later discovered that the target epitope was shared by both pools (see below).

We found that not all proteins were equally targeted by the T cell response (Fig. 2B). Peptides from the E protein produced reactivity in the highest frequency of patients as almost 80% of the population exhibited some responsiveness against E. By contrast, NS2B was the protein least frequently targeted by the T cell response and only 2 of the 41 patients displayed some reactivity to NS2B. Although it appears that protein size may be related to immunogenicity, other factors must also be responsible (Fig. 2B). For example, NS4B, which is 255 residues long, elicited responses in a greater number of individuals than NS1 (355 aa), NS3 (619 aa), and NS5 (905 aa) (Fig. 2B). Likewise, the M protein, which is only 167 residues in length, elicited immunity in a comparable number of individuals as NS3 and NS5, which are 3.5 and 4.5 times larger (Fig. 2B).

When we examined reactivities to individual minipools, we observed two things: 1) there were many regions of the polyprotein that did not produce any reactivity in the ELISPOT assay and 2) several minipools were stimulatory in an unexpectedly high proportion of individuals (Fig. 3) [NOTE: throughout these studies, we observed that patients reactive to minipool E1 were also reactive to minipool E-2. Likewise patients reactive to E-21 were reactive to E-22 and patients responsive to M-5 were also responsive to M-6. Therefore, for the remainder of the text, we refer to these pools as E-1/-2, E-21/-22, and M-5/-6]. E was clearly the most immunogenic protein as three separate minipools (E-1/-2, E-11, E-21/-22) were stimulatory in >20% of the patients. In all other cases of common reactivity, only a single minipool from any of the other proteins was stimulatory in >20% of the individuals (C-5, M-5/-6, NS3-19, NS4B-1, and NS5-17). Strikingly, we identified minipools in E, M, and NS4B (E-21/-22, M-5/-6, and NS4B-1, respectively) that were stimulatory in ~40% of the individuals that we tested (Fig. 3).

The T cells that were responsive to E-21/-22, M-5/-6, and NS4B-1 were determined to be CD8+ using flow cytometry (an example of the flow cytometry data is shown in Fig. 4A). In fact, most of the minipools identified by the ELISPOT assay contained CD8+ T cell epitopes (data not shown), although we did identify minipools containing CD4+ T cell epitopes (an example of the flow cytometry is shown in Fig. 4). The CD4+ T cells differed from the CD8+ T cells in terms of their cytokine production. At the time points shown (1–3 mo following onset of symptoms), only a fraction of the IFN-γ-secreting CD8+ T cells also produced TNF-α (<50%) and fewer still produced IL-2 (<10%) (Fig. 4A, middle and lower rows). By contrast, most of the IFN-γ-secreting CD4+ T cells produced TNF-α (>80%) and a larger fraction produced IL-2 (20%) (Fig. 4B, middle and lower rows). Thus, we can distinguish between CD8+ and CD4+ T cells based on both phenotype and cytokine production at early time points. At later time points (6 mo following infection and beyond), the West Nile-spe
cific CD8+ T cells secreted high levels of both IFN-γ and TNF-α, although they remained poor producers of IL-2 (data not shown).

Investigating the influence of age on T cell immunity to WNV

A key hypothesis that we sought to address is whether aging is associated with a decline in the T cell response to WNV infection, which, in turn, leads to increased severity of the illness. Within our cohort, the incidence of neuroinvasive disease was 30% among the patients aged <40 years old, 42% among the patients aged 41–50 years, 50% among the patients aged 51–60 years, and 85% among the patients >61 years old. Despite a clear relationship between age and disease severity, we did not observe any relationship between age and the diversity of T cell response (Fig. 5A). Even the
FIGURE 8. Patients with reactivities to dominant minipools were responding to the same peptides. PBMC from patients with reactivity to minipools E-1/-2 (A), E-21/-22 (B), M-5/-6 (C), NS3-19 (D), NS4B-1 (E), E-11 (F), and NS5-17 (G) were stimulated with individual peptides from those minipools. The data was normalized to the maximum signal produced by any peptide within the set for an individual PBMC sample. Each bar represents a single patient. Peptides are listed on the x-axis. A–E, the sequences listed correspond to the two peptides with the greatest stimulatory activity and the bolded letters reflect the minimal epitope as described in Table II. F and G, two discrete sets of peptides appeared to be stimulatory within each minipool. Open bars and solid bars discriminate between the distinct reactivities.

two most elderly individuals, aged 82 years, were reactive to five and seven distinct minipools (median for the population was four minipools; see Fig. 2). When we modeled these data, the point estimate of the linear regression coefficient was 0.018 with 95% confidence intervals ranging from 0.042 to 0.078. At the extremes of the confidence interval, a slope of −0.042 would represent a negligible reduction in reactive minipools with age, while at the other extreme 0.078 would be mild positive relationship. Likewise, we did not observe any relationship between disease severity and the diversity of the immune response (Fig. 5B). We also compared the magnitude of the T cell response to patient age and disease severity. However, this analysis was constrained by the fact that patients were accrued at different times following symptom onset. The earliest time point where we had obtained sufficient samples to carry out this analysis on the majority of our cohort was 3 mo following onset of symptoms. The total number of SFC for a given patient were tallied using samples obtained between 3 and 4 mo following onset of symptoms, and, again, we did not observe any relationship between age and the magnitude of the ELISPOT results (Fig. 5C). When we modeled these data, the point estimate of the regression coefficient for SFC was 0.009 and the 95% confidence intervals ranged from −0.037 to 0.056. Similarly, we did not observe a significant difference between patients with West Nile fever and neuroinvasive disease, although we did observe a trend toward greater total reactivity in the patients with neuroinvasive disease (Fig. 5D).

Characterization of epitope dominance and HLA-restriction

As expected, we observed a hierarchy of reactivity where a small number of epitope-specific T cell populations dominated the ELISPOT response in individual patients and represented a higher fraction of the total response. Fig. 6 shows an example of the dominance patterns in four individual patients over time. We found that the patterns were stable over the first year following infection for the dominant reactivities. In all cases, the predominant T cell populations that accounted for the highest fraction of the SFC were identified as CD8+ by flow cytometry (data not shown). In some cases, we observed codominance where T cell populations with two distinct reactivities were present at similar frequencies (Fig. 6, lower right panel).

Surprisingly, immune dominance was restricted to a limited number of minipools (Fig. 7A). Only 18 minipools were dominant among the 41 patients we studied. Strikingly, dominant T cell responses to E peptides were observed in ~40% of the population (Fig. 7A) and T cell reactivity to an epitope contained within the E-21/-22 minipools was dominant in approximately one-quarter of
the population. Furthermore, five minipools were responsible for >70% of the dominant reactivities in this population (Fig. 7A, gray area). Cumulatively, these results demonstrate that the T cell response following WNV infection is somewhat constrained (Fig. 3) and dominated by a limited number of epitopes (Fig. 7). Samples from an additional 11 patients (making a total of 52 patients) were evaluated for reactivity to the most common minipools (C-5, E-1/-2, E-11, E-21/-22, M-5/-6, NS3-19, NS5-10, and NS5-17). The incidence of minipool reactivity in this extended cohort was consistent with the data presented in Fig. 3 (data not shown).

In several cases, we found a clear association between HLA expression and responsiveness to the specific minipools, suggesting strongly that the patients were reactive to a common epitope within these pools. This association revealed an unexpected dominance of minipools E-21/-22 and NS3-19. Reactivity to the E-22 minipool was the predominant response in 70% of the HLA-A*02-positive individuals. Reactivity to NS3-19 was found to be dominant in only a small fraction (3/17) of the HLA-A*01-positive patients. Interestingly, five minipools were responsible for reactivity to the most common minipools (C-5, E-11, E-21/-22, M-5/-6, NS3-19, NS4B-1, NS4B-11, NS5-10, and NS5-17). Reactivity to the E-22 minipool in this extended cohort was consistent with the data presented in Fig. 3 (data not shown).

Table II. Definition of minimal peptide epitopes for the most commonly recognized peptides

<table>
<thead>
<tr>
<th>Stimulatory Peptides</th>
<th>Putative Epitope Peptides</th>
<th>Sequence</th>
<th>Reactivity</th>
<th>Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#4/E#5</td>
<td>E41–23</td>
<td>EGVSGATW</td>
<td>+ + +</td>
<td>Undetermined</td>
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<td></td>
<td>E41–26</td>
<td>SGATWVDLV</td>
<td>+ + +</td>
<td></td>
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<tr>
<td></td>
<td>E41–26</td>
<td>VSGATWVDLV</td>
<td>+ +</td>
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<td></td>
<td>E41–26</td>
<td>FLEGVSAT</td>
<td>-</td>
<td></td>
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<tr>
<td>E#124/E#125</td>
<td>E110–148</td>
<td>WMDSTKATRY</td>
<td>+ + +</td>
<td>HLA-A*02</td>
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<tr>
<td></td>
<td>M11–120</td>
<td>MDSKATRY</td>
<td>-</td>
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<td>M11–119</td>
<td>WMDSTKATR</td>
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<td></td>
<td>M11–118</td>
<td>AMWSTKAT</td>
<td>-</td>
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<td>NS4B1–24</td>
<td>SLFGRGIEV</td>
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<td></td>
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<tr>
<td></td>
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<td>HLA-A*02</td>
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<td>MPNGLAIQFY</td>
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<td></td>
<td>NS3504–512</td>
<td>GLAQTPQGP</td>
<td>-</td>
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| *Stimulatory peptides were identified as described in Fig. 8. Putative epitopes were identified using predictive algorithms. The numbers in subscript represent the numerical position of amino acid residues within the protein sequence. Reactivity was defined as follows: - - - , no reactivity; + + + , 70%–99% of maximal reactivity; + + , 50%–70% of maximal reactivity; + , <50% maximal reactivity; -, no reactivity. HLA restriction was defined based on the predicted binding of the epitope that produced the maximal response and the HLA genotype of the patients who were reactive to the defined epitope. This E450–458 epitope was identified by mass spectrometry.
typing for all these individuals, so it is possible that this epitope is either carried by HLA-C or exhibits promiscuous binding activity. Based on a recent report demonstrating broad promiscuity among well-defined HIV and EBV epitopes (19), we suspect that E<sub>17-26</sub> is likely a dominant epitope with promiscuous behavior.

Discussion

Most previous studies of CD8<sup>+</sup> T cell immunity following flavivirus infection in humans have focused on dengue virus infection, although there have also been some reports from yellow fever virus 17D vaccinees and children infected with Japanese encephalitis virus. From the dengue literature, it is clear that CD8<sup>+</sup> T cell responses develop against most viral proteins, and, indeed, CD8<sup>+</sup> T cell epitopes have been identified in E, NS1, NS2A, NS4A, NS4B, and NS5 (9, 10, 20–24). Likewise, a study of four volunteers immunized with the yellow fever virus vaccine demonstrated CTL reactivity against E, NS1, NS2A, NS2B, and NS3 (25). Thus, it is clear that the CD8<sup>+</sup> T cell response following flavivirus infection targets against both structural and non-structural proteins. Although our data are consistent with the previous reports, our study has also revealed an unexpected bias in the specificities of the dominant CD8<sup>+</sup> T cell responses. The results presented herein suggest that E is the most immunogenic WNV protein (Fig. 2) and reactivity to two peptides from E (E<sub>17-26</sub>, and E<sub>430-438</sub>) dominated the CD8<sup>+</sup> T cell response in 40% of our cohort (Fig. 7). These data are in marked contrast to the results in dengue infection where T cell responses to NS3 are more common (9, 20, 21). This difference may be explained by either the nature of the T cell response in the infected cohorts (i.e., primary responses to WNV but secondary responses to dengue) or differences in the immunobiology of the viruses. Interestingly, reactivity to NS3 was also found to dominate the memory response following Japanese encephalitis virus infection (26, 27), suggesting that dominance of NS3 reactivity is not likely due to secondary responses. It is equally possible that ethno-geographic differences may influence the outcome, although a recent report in HIV-infected individuals found that neither geography nor ethnicity influenced the dominance of CD8<sup>+</sup> T cell responses against specific viral proteins (28).

The distribution of CD8<sup>+</sup> T cell epitopes across the polyprotein was relatively constrained and CD8<sup>+</sup> T cells specific for five epitopes dominated the response in 70% of our patients (Fig. 5A) was unexpected. Additionally, we found that reactivity to E<sub>430-438</sub>, M<sub>111-120</sub>, NS3<sub>501-509</sub>, and NS4B<sub>155-163</sub> occurred in almost every patient expressing HLA-A<sup>∗</sup>02, -A<sup>∗</sup>01, -B<sup>∗</sup>35, and -A<sup>∗</sup>02, respectively. In an examination of vaccinia virus immunity, it was observed that a broad spectrum of epitopes was uncovered without any specific epitope showing dominance (29); a phenomenon described by Yewdell as “immunodemocratic” (30). Similarly, an immunodemocratic CD8<sup>+</sup> T cell response was observed among four HLA-identical siblings infected with EBV (31). A key difference between the previous reports and the current one is that the other studies involved large DNA viruses, whereas the current study was focused on a small RNA virus with only 3433 amino acids. As an example of common reactivities produced by less complex viruses, almost all HLA-A<sup>∗</sup>02-positive individuals exhibit CD8<sup>+</sup> T cell reactivity to the M<sub>148-166</sub> epitope from influenza A (32). Similarly, a comprehensive analysis of CD8<sup>+</sup> T cell determinants in HIV-infected subjects found three peptides that produced reactivity in >50% of the study population (57 patients) including a peptide in Nef that was recognized by 47% of the population, similar to the M<sub>111-120</sub> peptide described herein (33), although the relative dominance of CD8<sup>+</sup> T cells specific for the common HIV peptides was not assessed. Thus, the somewhat restricted distribution of T cell epitopes in the WNV polyprotein may reflect its small size.

As discussed in the introduction, it is generally believed that older individuals have a diminished capacity to mount CD8<sup>+</sup> T cell responses toward novel pathogens due to the diminished availability of naive T cells and the somewhat dysfunctional nature of the available CD8<sup>+</sup> T cells (12). Yet, in our study, age did not appear to influence any of the measured immunological parameters. It is notable that much of the existing literature that characterizes the influence of age on T cell immunity has focused on either chronic/latent herpesvirus infections (i.e., CMV, EBV, and Varicella zoster virus) or responses to recurrent infections, notably influenza. As such, the data may be skewed since they focused on memory responses that are likely influenced by multiple rounds of antigenic stimulation over the lifetime of the individual. By contrast, very few individuals in Canada, the source of our cohort, have been exposed to flaviviruses. Indeed, the majority of the patients in our cohort were found to be seronegative for dengue exposure, as defined by PRNT, and those who were found to have dengue-specific Abs actually had poorer responses to WNV than those who were seronegative. Therefore, the immune response produced by WNV appears to reflect a primary response to a novel agent thereby providing a unique opportunity to examine immune function in the elderly against novel agents. Our data demonstrates that it is possible to elicit robust CD8<sup>+</sup> T cell immunity against a novel agent in the elderly with a breadth and magnitude equivalent to younger individuals. It should be noted that the earliest samples we could analyze were obtained on average 30 days after the onset of symptoms and our comparisons of magnitude were based on samples obtained 3–4 mo after infection. We compared these parameters based on the assumption that the breadth and magnitude of the CD8<sup>+</sup> T cell population during the early memory phase is a direct reflection of the breadth and magnitude of the population at the peak of the acute response. Thus, it remains possible that age-related differences in the WNV-specific CD8<sup>+</sup> T cell population occurred at the peak of the response but escaped our analysis due to limitations in the availability of patient material. Additionally, since only 20–30% of WNV infections produce symptoms of illness, it is possible that the younger individuals within our cohort have an immunological defect akin to the defect in aged individuals. To address this possibility, we would need to examine age-matched asymptomatic individuals. Unfortunately, we have no method to identify asymptomatic individuals at early points following infection, so we cannot definitively address this issue. Additionally, we cannot comment on possible age-related defects in CD4<sup>+</sup> T cell immunity to WNV at this time since we only identified a few CD4<sup>+</sup> T cell epitopes. It has been suggested that failure to produce Abs in aged mice and humans may be a result of a defect in type 2 differentiation due to excessive production of type 1 cytokines (34, 35). Since type 1 polarization supports the development of antiviral CD8<sup>+</sup> T cell responses, it is possible that age is not a factor since the immune system in older individuals is driven toward type 1 already. Oligoclonal expansion of CD8<sup>+</sup> T cell populations may be reactive to chronic infections (CMV or EBV) or represent an autoreactive population. Whether such expansions in humans impact upon the primary response to novel agents is not known. In a murine model of HSV-1 infection where the CD8<sup>+</sup> T cell response is dominated by TCRs bearing V<sub>β</sub>8 or V<sub>β</sub>10 rearrangements, diminished anti-HSV-1 immunity was observed when age-related clonal expansions occurred within T cell pools bearing V<sub>β</sub>8 or V<sub>β</sub>10 although clonal expansions of CD8<sup>+</sup> T cells bearing other V<sub>β</sub> segments did not impact the CD8<sup>+</sup> T cell response as severely
In that regard, oligoclonal expansions may only affect T cell responses that depend upon CD8+ T cells bearing specific TCR rearrangements. Since the T cell response to WNV involves an average of four distinct epitopes, it is quite possible that comparable expansions may have occurred within our cohort but the T cell populations maintain adequate diversity to respond to new Ags. As stated previously, whether WNV-specific CD8+ T cell immunity reflects novel activation of naïve precursors or reactivation of cross-reactive memory cells is unknown. Current investigations in our laboratory are addressing the relationship between general diversity of T cell clones within the elderly patients in our cohort compared with the younger patients. We are also examining the number of individual clonotypes within a given pool with a common reactivity as it is possible that the Ag-specific populations in the aged have expanded to a similar magnitude but represent a more clonally restricted population.

We have presented the first description of the T cell response to WNV in naturally infected humans. Although these investigations failed to demonstrate a relationship between the CD8+ T cell response and disease pathology, we did observe a number of unexpected findings which have more general implications for anti-viral CD8+ T cell immunity.

Disclosures
The authors have no financial conflict of interest.

References