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Longitudinal Studies of Clonally Expanded CD8 T Cells Reveal a Repertoire Shrinkage Predicting Mortality and an Increased Number of Dysfunctional Cytomegalovirus-Specific T Cells in the Very Elderly[1]

Sine Reker Hadrup,[2]*† Jan Strindhall,† Tania Køllgaard,* Tina Seremet,* Boo Johansson,‡ Graham Pawelec,§ Per thor Straten,* and Anders Wikby†

The age-associated decrease in functionality of the human immune system is thought to have a negative impact on the capacity to provide protection against infection, in turn leading to increased incidence of mortality. In a previous longitudinal study of octogenarians, we identified an immune risk phenotype (IRP) in the very elderly defined by an inverted CD4/CD8 ratio, which was associated with increased mortality and persistent CMV infection. In this study, we analyzed the CD8 clonal composition of nonagenarians and middle-aged individuals. An increased number of CD8 T cell clones was observed in the nonagenarians, and was associated with CMV-seropositivity. Surprisingly, CMV-seropositive nonagenarians with the IRP had a significantly lower number of clones compared with non-IRP individuals. The decrease in clone numbers in IRP individuals was associated with shorter survival time. MHC/peptide multimer staining indicated that the frequency of CMV-specific T cells was higher in nonagenarians than in the middle-aged, but the ratio of functionally intact cells was significantly lower. The lowest ratio of functional CMV-specific T cells was found in an IRP individual. A thorough longitudinal analysis of the CMV-specific T cells in nonagenarians showed a stable pattern with respect to frequency, phenotype, and clonal composition. We hypothesize that the number of different CD8 T cell clonal expansions increases as the individual ages, possibly, as a compensatory mechanism to control latent infections, e.g., CMV, but eventually a point is reached where clonal exhaustion leads to shrinkage of the CD8 clonal repertoire, associated with decreased survival. *The Journal of Immunology,* **2006, 176: 2645–2653.**

Age-associated changes in the immune system result in decreased immune function and increased incidence and severity of infections (1, 2). This leads to increased morbidity and mortality (3, 4). An immune risk phenotype (IRP),[3] characterized by an inverted CD4:CD8 ratio (CD4:CD8 ratio <1) and a poor T cell proliferative response to Con A, was initially identified to be predictive of 2-year mortality in a longitudinal study of octogenarians using a cluster analysis approach (5–7). Additional studies have shown that the IRP can be defined using only the inverted CD4:CD8 ratio because this sole marker was found to be significantly associated with the IRP (8). This was confirmed in a U.K. study, the Healthy Ageing Study, showing that the inverted CD4:CD8 ratio predicts survival in a sample of elderly people (9). Thus, the CD4:CD8 ratio can be used as a surrogate marker to define the IRP, which has also been found to be associated with increased numbers of CD28-negative CD8 T cells, as well as seropositivity for CMV (10, 11).

CD8 T cells represent an important immunological component of protection against viral infections. Upon antigenic stimulation, CD8 T cells differentiate from naive to memory T cells that can respond to reinfection and convert to effector T cells, which secrete cytokines and possess cytolytic capabilities (12–14). Thus, in healthy individuals persistent viral infections are controlled by virus-specific T cells. Latent viral agents including CMV and EBV coexist lifelong with the host and only manifest clinically in certain immunosuppressed states (15). This can occur despite the presence of increased numbers of virus-specific T cells (12–15).

Studies of Ag-specific CD8 T cells have been greatly facilitated by the introduction of MHC class I/peptide multimeric complexes. Use of such constructs for flow cytometry analyses of peptide-specific T cells has provided immunologists with important new insight regarding the biology of Ag-specific CD8 T cells (16). Multimer complexes have been combined with analyses of the expression of cell surface markers leading to the proposal of a sequential model of T cell differentiation (17, 18). The model operates with four differentiation states of Ag-specific CD8 T cells: naive T cells, central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and terminally differentiated T cells (T_{EMRA}). Naive T cells are characterized by expression of CD45RA, CCR7, and usually also the costimulatory molecules CD28 and CD27. Upon Ag stimulation naive T cells differentiate into T_{CM} or T_{EM}, which both express CD45RO, but are distinguished by the expression of the lymphocyte homing receptor, CCR7. Further differentiation leads

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[3] Abbreviations used in this paper: IRP, immune risk phenotype; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, terminally differentiated T cell; NONA, longitudinal study of nonagenarians; DGGE, denaturing gradient gel electrophoresis; BV, β variable.

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to the T_{EMRA} phenotype, which is characterized by expression of CD45RA, but lack of CCR7. The expression of the costimulatory molecules, CD28 and CD27, tends to decrease with T cell differentiation (17, 18). Examination of the phenotype of virus-specific CD8 T cells in the acute and chronic phases of CMV and EBV infections has shown that virus-specific T cells vary in differentiation phenotype. EBV-specific T cells are predominantly T_{EM} and CMV-specific T cells predominantly T_{EMRA} (19).

Clonal expansions of nonmalignant CD8 T cells have been detected in healthy old individuals in both humans and mice (20, 21). However, the etiology and function of such clonally expanded T cells in humans remain unknown, although accumulating evidence indicates that they are associated with persistent viral infections, predominantly CMV (22). It is unknown, however, whether there is a health-related effect associated with this phenomenon. In mouse models, the age-related T cell expansions within the CD8 compartment are known to impair the efficacy of antiviral T cell reactivity, and contribute to immunodeficiency by immunological senescence (23). In humans, the accumulation of CMV-specific T cells has been observed to reduce T cell immunity toward EBV infection (24) and influenza vaccination (25). In elderly individuals, CMV-specific T cells may comprise very high percentages of the CD8 population (up to 45%), with the predominant phenotype being T_{EMRA}, combined with low CD28 expression (22, 24). The accumulation of Ag-specific T cells seems to be age-associated (24, 26); however, the formal proof for this notion is still lacking. Moreover, very little is known concerning the dynamics and functional capacity of these CMV-specific CD8 T cell expansions.

In this study, we describe CD8 T cell clonality and CMV-specific T cell precursor frequencies, phenotypes, and functionality in very old individuals included in a longitudinal study of nonagenarians (NONA). These data provide new knowledge regarding the impact of CMV in promoting CD8 T cell clonal expansions, and describe the characteristics of the CMV-specific T cells in very old individuals over a time period of 2 years. Furthermore, the data show that a decrease in the number of expanded CD8 T cell clones in IRP individuals is associated with increased mortality.

Materials and Methods

Sample and design

We included 39 nonagenarians and 9 middle-aged individuals who were all part of the NONA longitudinal study, Jönköping, Sweden, which is a population-based investigation without exclusion criteria. Individuals were selected randomly from the population register of Jönköping in 1999. Blood samples were collected biennially in fall 1999 (N1), 2001 (N2), and 2003 (N3). We used material from the N2 and N3 collections.

The nonagenarians. At the N2 collection, the mean age of the analyzed participants was 92 years: 10 men (26%) and 29 women (74%), of whom 65% lived in their own homes and 35% in sheltered housing or an institution. Overall health status assessments of these nonagenarians ($n = 39$) revealed that only one individual (2.6%) was rated as perfectly healthy according to the European SENIEUR protocol criteria (27). Five nonagenarians (13%) met the criteria of the OCTO immune study, i.e., not residing in an institution, not being demented, and not using medication known to affect the immune system (27). The majority (85%), however, did not meet the above health criteria (27), but there was no discernible association between the health status of the nonagenarians and the parameters analyzed in this study. Eight nonagenarians had a CD4:CD8 ratio <1 at the time of N2 collection, i.e., displayed the IRP. Three IRP individuals survived from N2 to N3, four displayed the IRP at the time of N3 collection, and one of the analyzed individuals progressed from non-IRP to IRP during the study period.

Middle-aged individuals. The mean age of analyzed middle-aged individuals was 51 years, with women constituting 67%. The middle-aged group was part of the full-time staff of Ryhov Hospital, and these individuals participated throughout the entire study. Because younger people in Jönköping are more mobile, the use of a younger control group for longitudinal comparisons was not desirable.

The present project was approved by the local ethics committee.

Preparation of PBMC

PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed Diagnostika). The PBMC were washed three times in Dulbecco's PBS and resuspended in complete medium of RPMI 1640 (Flow Laboratories), supplemented with 10% heat inactivated FCS (Flow Laboratories), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Life Technologies). Subsequently, the PBMC were cryopreserved.

Flow cytometry

CD4/CD8 counts were analyzed on whole blood after RBC lysis. mAbs, including the appropriate isotype controls, were purchased from BD Biosciences. Data were acquired using a FACScan (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

All other flow cytometric analyses were performed on isolated PBMC using FACSAria (BD Biosciences) with FACSDiva software (BD Biosciences). MHC multimers with the HLA-A2 peptides: CMV_{NLV} (NLVP MVATV), EBV_{GLC} (GLCTLVAML), and HIV_{ILK} (ILKEPVHGV) were purchased as Pro5 MHC-pentamers from ProImmune and MHC-dextramers from DakoCytomation. PBMC were stained with PE-conjugated MHC-pentamers or MHC-dextramers in PBS with 2% FCS for 20 min at 5°C in the dark, followed by washing. Next, the PBMC were stained with the following panel of directly conjugated mAbs: CD8-allophycocyanin-Cy7, CD45RA-PE-Cy5, CD45RO-FITC, CD28-allophycocyanin, and CCR7-PE-Cy7. The phenotype of CMV_{NLV}-specific CD8 T cells was determined according to expression of the following markers: T_{NAIVE}: $CD45RA^+$, $CCR7^+$; T_{CM}: $CD45RO^+$, $CCR7^+$; T_{EM}: $CD45RO^+$, $CCR7^-$, T_{EMRA}: $CD45RA^+$, $CCR7^-$. The frequency of CMV_{NLV}-specific T cell clones were quantified by anti-TCR-BV-FITC mAb (Beckman Coulter) staining combined with CD8-allophycocyanin and MHC-dextramer-PE.

CMV and EBV serology

Two different immunoassays: MEIA (ABBOT Scandinavia) and NOVITEC, EBMA-1 IgG Ab Test (HiSS Diagnostic) were used to detect anti-CMV and anti-EBV Ig G Abs in plasma, according to the manufacturer's instructions.

TCR clonotype mapping by denaturing gradient gel electrophoresis (DGGE)

RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel). cDNA synthesis and quantification of cDNA in each sample were done as previously described (28).

For TCR clonotype mapping analyses, cDNA was amplified using a primer panel amplifying the 24 β variable (BV) region families of the TCR (29). The resulting DNA fragments were separated by DGGE, and clonally expanded T cells were identified as distinct bands in the gel (30).

For DGGE, 10-μl aliquots were loaded onto a denaturing gradient gel containing 6% polyacrylamide and a gradient of urea and formamide ranging from 20 to 80%. Gels were run at 160 V for 4.5 h in 1× Tris-acetate EDTA buffer kept at a constant temperature of 56°C. Twenty minutes before termination of electrophoresis additional 10-μl aliquots of the respective PCRs were loaded in the appropriate lanes. These late-loaded products migrate only a short distance in the gel, and therefore will not reach a position that allows melting of the products. After electrophoresis the gel was stained with SYBR Green I (Molecular Probes) and visualized using the FLA-3000 fluorescence detection system (FUJI film; Science Imaging Scandinavia). The numbers of expanded TCR clonotypes/distinct bands in the gel were quantified using the Image Gauge software (FUJI film). For quantification analyses, an arbitrary threshold limit was set for the definition of clonal expansions. The intensity of each distinct band was calculated as a percentage of the total amount of unmelted DNA in each BV region. Bands exceeding 10% were defined as clonal expansions. This procedure was applied to secure identical definition of clonal expansions in all gels, and to avoid the influence of any intensity variation between the gels.

Comparative DGGE

CMV_{NLV}-specific T cells from seven HLA-A2-positive individuals (six nonagenarians and one middle-aged individual) were sorted by FACS after staining with Pro5 MHC-pentamers (ProImmune) or MHC-dextramers (DakoCytomation), and subjected to TCR clonotype analyses. The CMV_{NLV}-specific T cell clones were tracked to the CD8 population by loading the TCR-BV transcripts from the two populations in adjacent lanes.

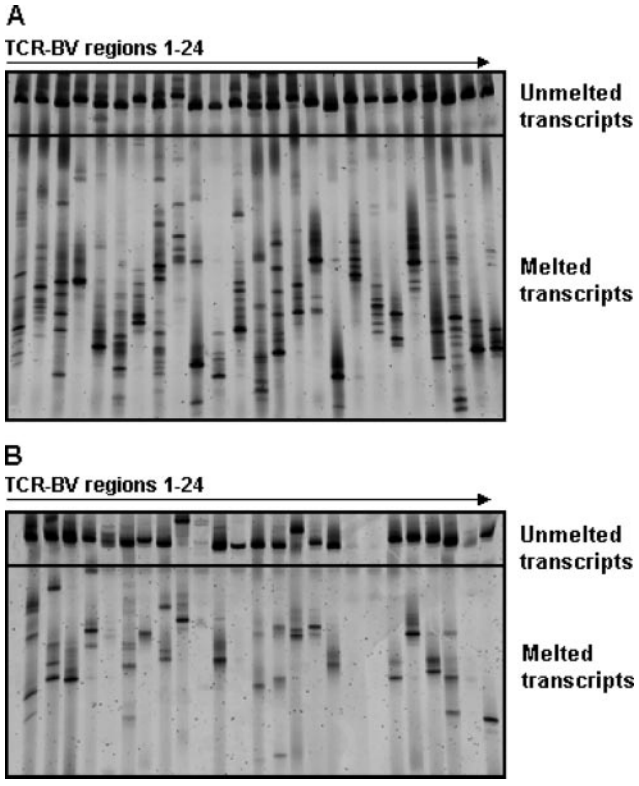

FIGURE 1. TCR clonotype mapping of CD8 T cells. CD8 T cells were analyzed by TCR clonotype mapping and the TCR transcripts were subsequently separated by DGGE. The *upper part* of the gels shows the unmelted bands of TCR transcripts for each TCR-BV region after 20-min electrophoresis, and the *lower part* of the gels shows the melted TCR transcripts after 4.5 h of electrophoresis. Each band in the *lower part* represents a clonally expanded T cell with the given TCR-BV region. The number of clonal expansions was counted as described in *Materials and Methods*. *A*, A CD8 TCR clonal map from a nonagenarian with 47 clonal expansions. *B*, A CD8 TCR clonal map from a middle-aged individual with 11 clonal expansions.

Identical clonotype transcripts resolve at identical positions in the gel, according to their melting temperature, and are easily compared when loaded in adjacent lanes. The sequence identity of DNA molecules resolving at identical positions in the gel was previously verified by direct sequencing (31).

ELISPOT assay

Ninety-six well nitrocellulose plates (Multiscreen MAIP N45; Millipore) were coated with 7.5 μg/ml mouse anti-IFN-γ mAb (1-D1k; Mabtech) in 75 μl of PBS overnight at room temperature. After six washes and blocking with 200 μl of X-Vivo medium (BioWhittaker), lymphocytes were added in three concentrations: 3×10^5, 1×10^5, and 3×10^4 cells/well, together with CMV_{NLV} peptide (5 μg/ml). After an overnight incubation, the plates were washed, and biotinylated secondary anti-IFN-γ mAb was added (7-B6-Biotin; Mabtech). After a 2-h incubation period, the plates were washed and avidin-enzyme conjugate (Invitrogen Life Technologies) was added to each well. After 1 h of incubation at room temperature, the plates were washed and enzyme substrate (DakoCytomation) was added for 5–10 min. When dark purple spots emerged the reaction was terminated by addition of water. Finally, the number of spots was assessed using a digitalized ELISPOT counter (Immunospot; CTL Incorporated).

Data analyses

In this study a blinded setup was used. Thus, at the time of data collection the investigator had no knowledge of the IRP status of the included individuals. A CD4:CD8 ratio <1.00 was used to define the IRP in the present study. Student's *t* test, correlation analysis, and quadratic regression analysis were conducted using SPSS II for Windows (SPSS).

Results

The influence of age, CMV infection, and IRP status on CD8 T cell clonal expansions

CD8 T cells from nonagenarians ($n = 39$) and middle-aged individuals ($n = 9$) were analyzed by DGGE-based clonotype mapping and the number of expanded clones was counted. Representative images of clonotype maps from a nonagenarian and a middle-aged individual are shown in Fig. 1.

The mean number of expanded clones was significantly higher in nonagenarians compared with middle-aged individuals (mean = 19.4 and 10.1, respectively; $p = 0.003$; Fig. 2*A*), despite a large interindividual variation. Among the nonagenarians, CMV infection was found to be associated with a significantly higher number of different clones. The mean clone number was 22.6 for CMV-positive ($n = 33$) and 7.4 for CMV-negative ($n = 6$; $p = 0.008$) individuals (Fig. 2*B*). This association was not found among the middle-aged individuals (data not shown). The number of expanded clones among the middle-aged individuals was similar to the number found in the CMV-negative nonagenarians.

A further division of the CMV-positive nonagenarians into an IRP ($n = 8$) and a non-IRP ($n = 25$) group showed that the number

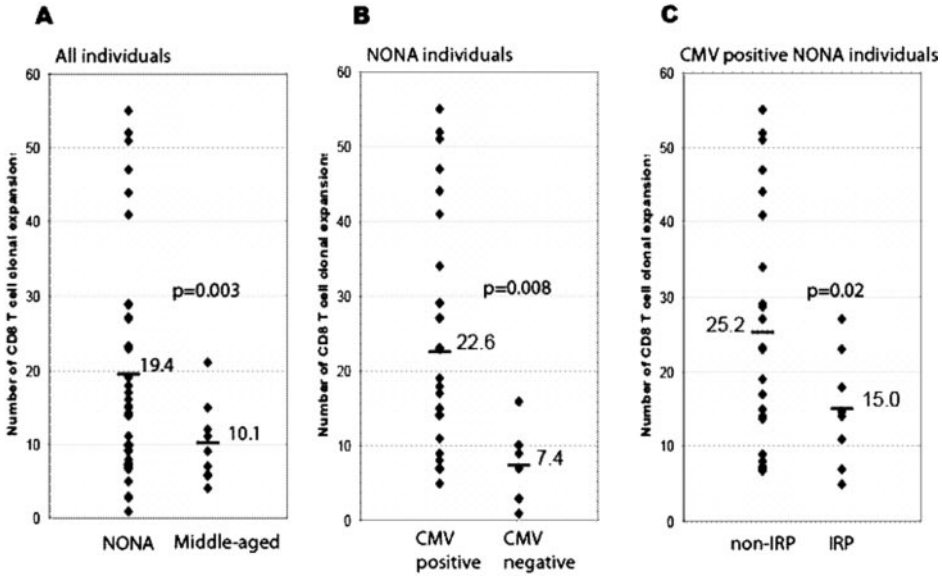

FIGURE 2. The number of clonal expansions in the CD8 repertoire in different groups of individuals. The figure compares the number of clonal expansions determined by TCR clonotype mapping of CD8 T cells from (*A*) NONA ($n = 39$) and middle-aged individuals ($n = 9$; *t* test, $p = 0.003$); (*B*) nonagenarians who were CMV-positive ($n = 33$) compared with CMV-negative ($n = 6$) (*t* test, $p = 0.008$); and (*C*) the non-IRP ($n = 25$) and IRP group ($n = 8$) of CMV-positive nonagenarians (*t* test, $p = 0.02$).

of clonal expansions was significantly lower in the IRP group compared with the non-IRP group (mean = 15.0 and 25.2, respectively; $p = 0.02$; Fig. 2*C*). A similar division of the CMV-negative nonagenarians into an IRP and a non-IRP group was not possible, because all IRP individuals were CMV-positive.

The relationship between CD8 T cell clonal expansions, CD4:CD8 ratios, and survival

The decreased number of clonal expansions in the IRP group compared with the non-IRP group led us to investigate the association between the number of clonal expansions and the CD4:CD8 ratio in more detail. A quadratic association ($p = 0.02$) between the number of expanded clones and the natural logarithm of the CD4:CD8 ratio is shown in Fig. 3*A*. The curve shows a maximum number of clones for CD4:CD8 ratios between 1 and 4 (log(CD4:CD8) between 0 and 1.4). The detected number of clones declined from this maximum level as the CD4:CD8 ratio changed; both when it increased above 4 and when it fell below 1, leading to a bell-shaped appearance of the curve. The decline in the number of clones with increasing CD4:CD8 ratios was associated with CMV status, because CMV-negative individuals exhibited both low clone numbers and high CD4:CD8 ratios. The decline in clone number with decreasing CD4:CD8 ratios was associated with the IRP status, because IRP individuals had a low number of clones. Importantly, the decline in the number of expanded clones observed in the IRP individuals was found to be associated with an increased mortality (Fig. 3*B*). There was no association between clone number and mortality in the group of non-IRP individuals (data not shown). Thus, when the CD4:CD8 ratio decreases, expanded CD8 T cell clones seem to accumulate in the elderly, but upon entry into the IRP, the clone number decreases and this is associated with increased mortality.

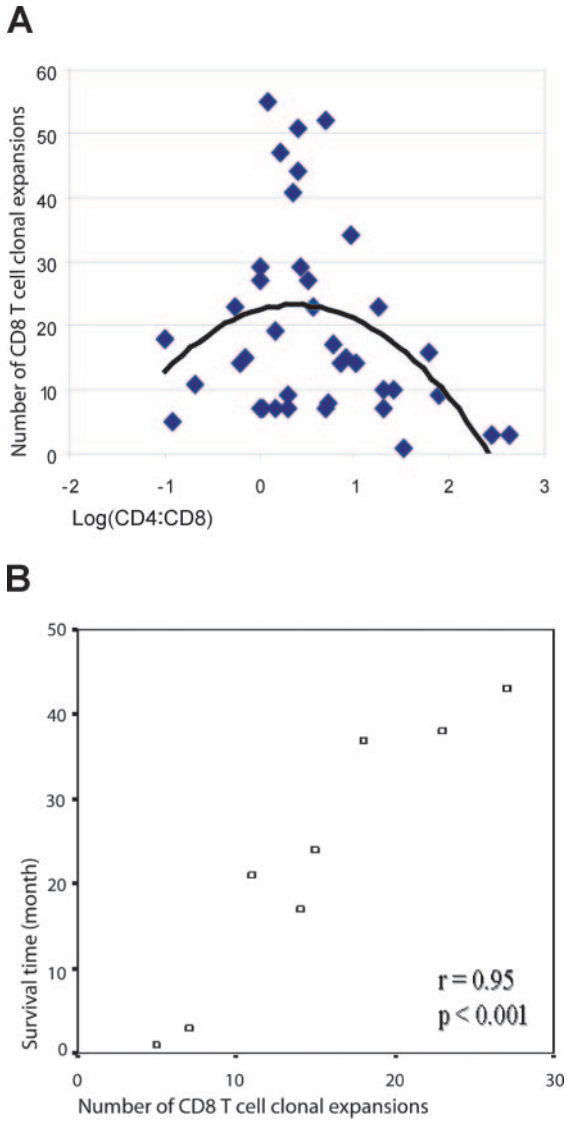

FIGURE 3. The association between CD8 T cell clonal expansions, CD4:CD8 ratio, and survival. *A*, The diagram depicts the association between log(CD4:CD8 ratio) and the number of clones observed in each individual. The dots represent the exact combination of log(CD4:CD8 ratio) and clone number for each nonagenarian ($n = 39$). A fitted quadratic line illustrates the association between log(CD4:CD8 ratio) and the clone number (black line). *B*, The diagram depicts the association between the number of expanded clones and the survival time of the nonagenarians with the IRP ($n = 8$) ($r = 0.95$; $p < 0.001$).

HLA-multimer analyses of EBV- and CMV-specific CD8 T cells

All individuals included in the study were analyzed for HLA-A2 expression by PCR (32). This class I allele was expressed by 21 of the nonagenarians (17 non-IRP and 4 IRP) and 6 of the middle-aged individuals (data not shown). All HLA-A2-positive individuals were investigated by FACS using HLA-A2 multimers to determine the frequency of CD8 T cells specific for the dominant HLA-A2 epitopes from CMV_{NLV} (NLVPMVATV) and EBV_{GLC} (GLCTLVAML). Three representative dot plots of CD8 vs CMV_{NLV} MHC-dextramer from a nonagenarian (at N2 and N3 collection) and a middle-aged individual are shown in Fig. 4*A*.

The frequencies of CMV_{NLV}- and EBV_{GLC}-specific T cells were determined longitudinally using data from the N2 and N3 collections. First, data from the N2 collection were analyzed to compare the Ag-specific T cell frequencies between the different subgroups: nonagenarians vs middle-aged and IRP vs non-IRP. Second, data from the two time points were used to perform a longitudinal analysis.

The frequency of CMV_{NLV}-specific T cells in the nonagenarians at the N2 collection was between 0.2 and 10.1% (mean = 2.9%; $n = 21$; Fig. 4*B*). This was significantly higher than the frequency of CMV_{NLV}-specific T cells in the middle-aged group, ranging from 0.1 to 2.1% (mean = 0.7%; $n = 6$; $p = 0.048$; Fig. 4*B*). The mean frequency of CMV_{NLV}-specific T cells in the IRP subgroup (3.2%; $n = 4$) was not significantly different from the non-IRP subgroup (2.8%; $n = 17$; Fig. 4*B*). However, there were very few individuals in the former group.

The EBV_{GLC} T cell frequency at the N2 collection ranged from 0.1 to 1% (mean = 0.4%; $n = 21$). There was no difference in EBV_{GLC} T cell frequency between the nonagenarians (mean = 0.4%; $n = 21$) and the middle-aged individuals (mean = 0.4%; $n = 6$) or between the IRP and the non-IRP group of the nonagenarians (data not shown).

A longitudinal analysis of the CMV_{NLV}- and EBV_{GLC}-specific T cell frequencies was performed using data from individuals who survived the 2-year period from the N2 to the N3 collection. CMV_{NLV}-specific T cells were significantly more frequent than the EBV_{GLC}-specific T cells at both the N2 and the N3 collections (mean CMV = 2.9% (N2) and 2.1% (N3); mean EBV = 0.4% (N2) and 0.3% (N3); $p < 0.001$; Fig. 4*C*). Only minor fluctuations were observed, with no significant differences in the frequency of CMV_{NLV}-specific T cells when comparing the two time points (Fig. 4*C*). The frequency of EBV_{GLC}-specific T cells declined in almost all nonagenarians during the 2-year time period (Fig. 4*C*).

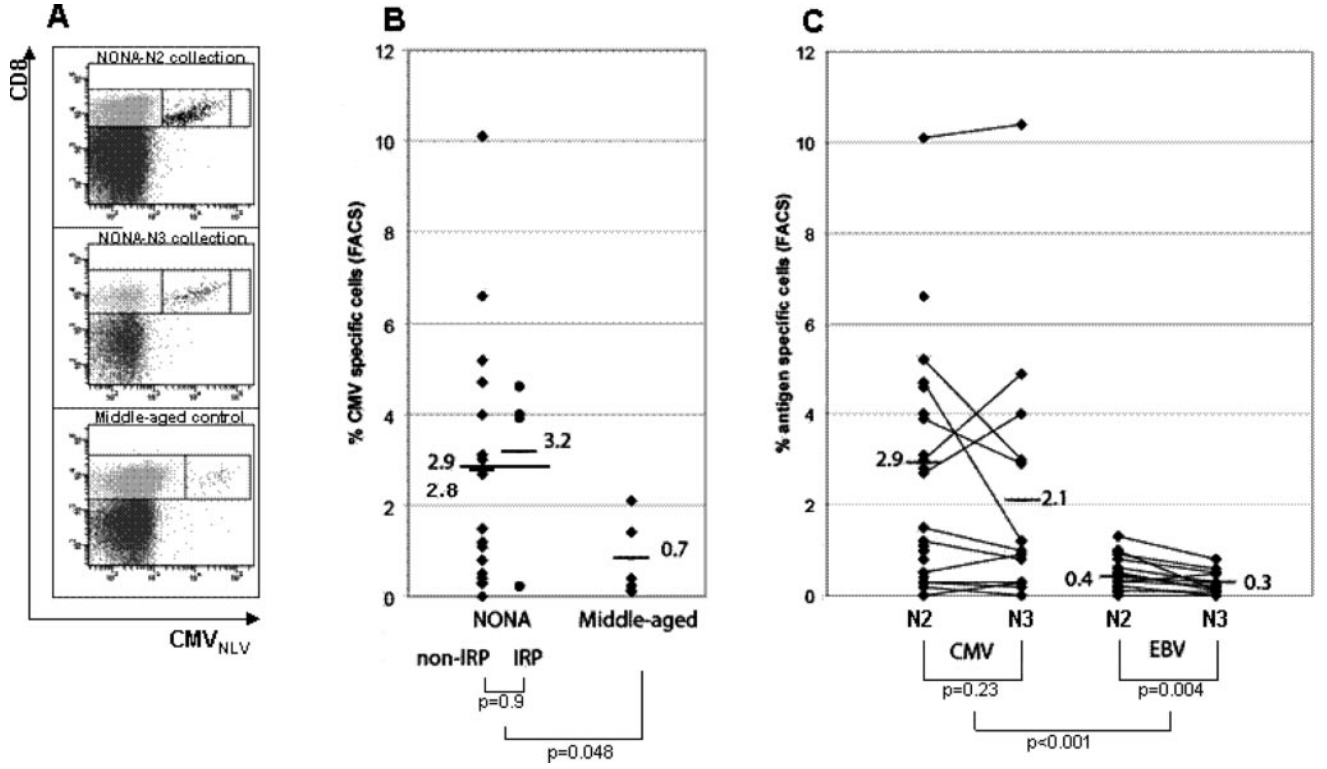

FIGURE 4. The frequency of CMV- and EBV-specific T cells monitored by flow cytometry. *A*, Representative dot plots of CD8 (allophycocyanin-Cy7) vs CMV_{NLV} MHC-dextramer (PE) from a nonagenarian at N2 and N3, and a middle-aged individual. *B*, The percentage of CMV_{NLV}-specific T cells in nonagenarians (mean = 2.9%; $n = 21$) compared with middle-aged individuals (mean = 0.7%; $n = 21$) (*t* test, $p = 0.048$). The nonagenarians were further separated into non-IRP (squares) and IRP (circles) (mean = 2.8 and 3.2%, respectively, *t* test, $p = 0.9$). *C*, The frequency of CMV_{NLV}- and EBV_{GLC}-specific T cells in nonagenarians at the time of N2 and N3 collection. CMV_{NLV}-specific T cell frequencies (mean = 2.9% (N2) and 2.1% (N3)), were higher than EBV_{GLC}-specific T cell frequencies (mean = 0.4% (N2) and 0.3% (N3)), at both time points (*t* test, $p < 0.001$). There was no significant difference in the frequency of CMV_{NLV}-specific T cells between the N2 and N3 collection (*t* test, $p = 0.23$), but a decrease in frequency of EBV_{GLC}-specific T cells was observed (*t* test, $p = 0.004$).

Clonal composition and stability of CMV-specific CD8 T cells

The association between the number of expanded clones and CMV seropositivity suggests that CMV is a driving force for large clonal expansions in the CD8 T cell compartment in nonagenarians. Furthermore, CMV_{NLV}-specific T cells are more frequent among the nonagenarians than the middle-aged individuals. Therefore, we aimed at analyzing the clonal composition of the CMV_{NLV}-specific T cells to determine the number of CMV_{NLV}-specific expanded clones within the CD8 repertoire, and to determine the persistence of these clones over the 2-year period from the N2 to the N3 collection.

CMV_{NLV}-specific T cells from six nonagenarians and one middle-aged individual were sorted by FACS for subsequent TCR clonotype mapping analyses. From the nonagenarians each CMV_{NLV}-specific T cell population was composed of two to nine (mean = 5.8; $n = 6$) distinct clonotypes. By comparative DGGE, the CMV_{NLV}-specific T cell clones were tracked to the total CD8 repertoire, to identify which clones in the CD8 repertoire were CMV_{NLV}-specific. Moreover, after identifying these in the CD8 repertoire, we were able to determine that 7–33% (mean = 19%; $n = 6$) of the total CD8 clonal expansions were CMV_{NLV}-specific. Two representative examples of comparative DGGE are shown in Fig. 5, *A* and *B*, *left* and *middle lanes*, within each TCR-BV region. One of the nonagenarians included in this analysis displayed the IRP. This individual had the lowest clonal diversity observed among any of the CMV_{NLV}-specific T cell populations analyzed. The CMV_{NLV}-specific T cells consisted of only two clones–one of them being a large clonal expansion in the CD8 population (data not shown)

A similar analysis was performed on a middle-aged individual. The CMV_{NLV}-specific T cell population was composed of four clonally expanded T cells (Fig. 5*C*). These clonal expansions were quantified by staining with anti-TCR-BV mAbs. The majority (83%) of the CMV_{NLV}-specific T cells expressed TCR-BV3. However, the other TCR-BV regions identified by TCR clonotype mapping were also represented: BV2 (0.7%), BV14 (1%), and BV22 (7.5%) (Fig. 5*D*). TCR-BV16 was used as a negative control. None of the CMV_{NLV}-specific T cells expressed this TCR-BV region. As seen by clonotype mapping, the CMV_{NLV}-specific T cells expressing TCR-BV3 represent a single clone. It is not possible to determine the frequency of the clones from the clonotype mapping of the sorted cells, because even a low frequent T cell clone will be efficiently amplified by PCR in this restricted population, but will be undetectable in the CD8 repertoire. Accordingly, only the TCR-BV3 and BV22 clones were detected in the clonal map of the CD8 repertoire, whereas the BV2 and BV14 CMV_{NLV} clonal expansions were undetectable in this population. The TCR-BV3 and BV22 CMV_{NLV}-specific T cells clones occupy 3 and 0.3% of the CD8 repertoire, respectively, but only the TCR-BV3 CMV_{NLV}-specific T cell clone exceeded the threshold limit to be classified as a large clonal expansion on the CD8 clonal map.

Furthermore, the clonal composition of the CMV_{NLV}-specific T cells was analyzed longitudinally to examine the stability of the response over a 2-year period using material from the above analyzed individuals who survived from the N2 to the N3 collection (three nonagenarians and one middle-aged). CMV_{NLV}-specific T

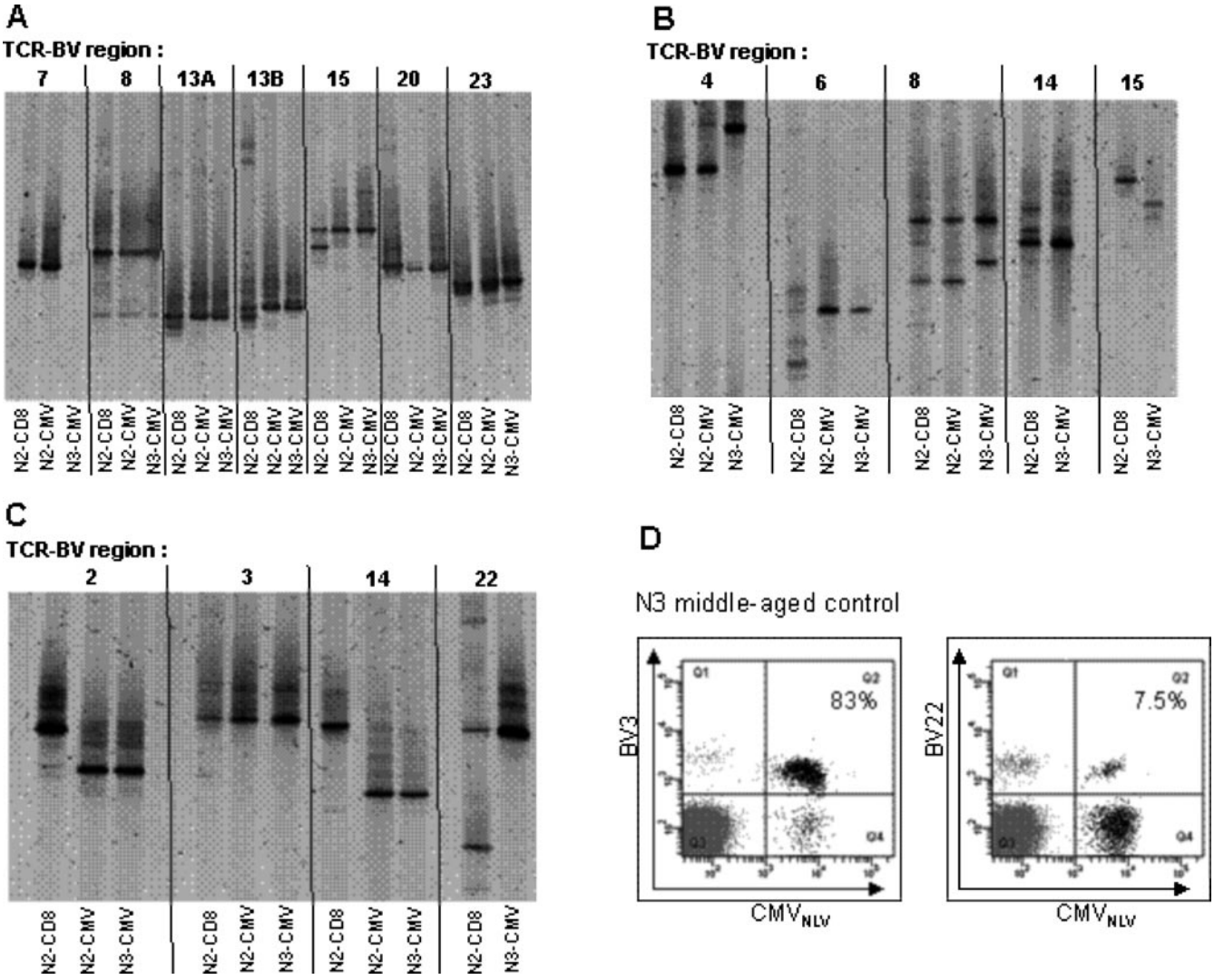

FIGURE 5. The clonal composition of CMV_{NLV}-specific T cells. CMV_{NLV}-specific T cells were sorted by FACS and subjected to TCR clonotype analyses. Three different cell populations were analyzed: the whole CD8 repertoire (N2-CD8), the CMV_{NLV}-specific T cells sorted at the N2 collection time point (N2-CMV), and the CMV_{NLV}-specific T cells sorted at the N3 collection time point (N3-CMV). The diagram shows a comparative DGGE for these three cell populations from three representative individuals: *A* and *B*, nonagenarians; *C*, a middle-aged individual. For each BV region, the position of the bands can be compared between the three cell populations. Bands that resolve at identical positions in the gel represent identical clones. By analyzing the N2-CMV and N3-CMV populations separately for the three individuals, the clonal composition at the two time points can be determined. A comparison of the N2-CD8 and the N2-CMV provides direct proof of which T cell clones in the CD8 repertoire are CMV_{NLV}-specific. The fluctuations in the clonal composition can be determined by comparing N2-CMV and N3-CMV lanes for each BV region family. *D*, A combined TCR-BV-FITC, CD8-allophycocyanin and MHC-dextramer-PE staining of PBMCs from the middle-aged control. The dot plots are gated on CD8 T cells, and show CMV_{NLV} MHC-dextramer vs BV3 or BV22.

cells from the N3 collection time were sorted and analyzed by TCR clonotype mapping. The clonal composition of the N3 CMV_{NLV}-specific T cell population was compared with the N2 CMV_{NLV}-specific T cell population by comparative DGGE (Fig. 5, *middle* and *right lanes*, for each TCR-BV region). For all analyzed CMV_{NLV}-specific T cell populations, 60–100% of the clones were found to persist during the 2-year period, indicating a relatively stably maintained response to CMV in both nonagenarians and middle-aged individuals.

Phenotype of CMV-specific T cells

We analyzed the phenotype of CMV_{NLV}-specific T cells from both nonagenarians and middle-aged individuals collected at the N2 and N3 time points to asses any differences over time or between the different groups of individuals. Phenotype analyses revealed that on average 62% of the CMV_{NLV}-specific T cells from nonagenarians were characterized by a terminal effector memory phenotype, T_{EMRA} (Fig. 6*B*), and the majority (72%) of these were negative for CD28 (data not shown). The other prominent phenotype among the CMV_{NLV}-specific T cells from nonagenarians was the effector memory phenotype, T_{EM} ($CD45RO^+$, $CCR7^-$), which was expressed by 28% of the CMV_{NLV}-specific T cells (Fig. 6*B*), with 62% of these negative for CD28 (data not shown). The phenotype of the CMV_{NLV}-specific T cells among middle-aged individuals was subject to large intraindividual variation. For two middle-aged individuals, the CMV_{NLV}-specific T cell phenotype was predominantly T_{EMRA}, whereas in two other individuals the phenotype was predominantly T_{EM}. There was no significant difference in the distribution of the various phenotypes between the nonagenarians and the middle-aged individuals (Fig. 6*C*).

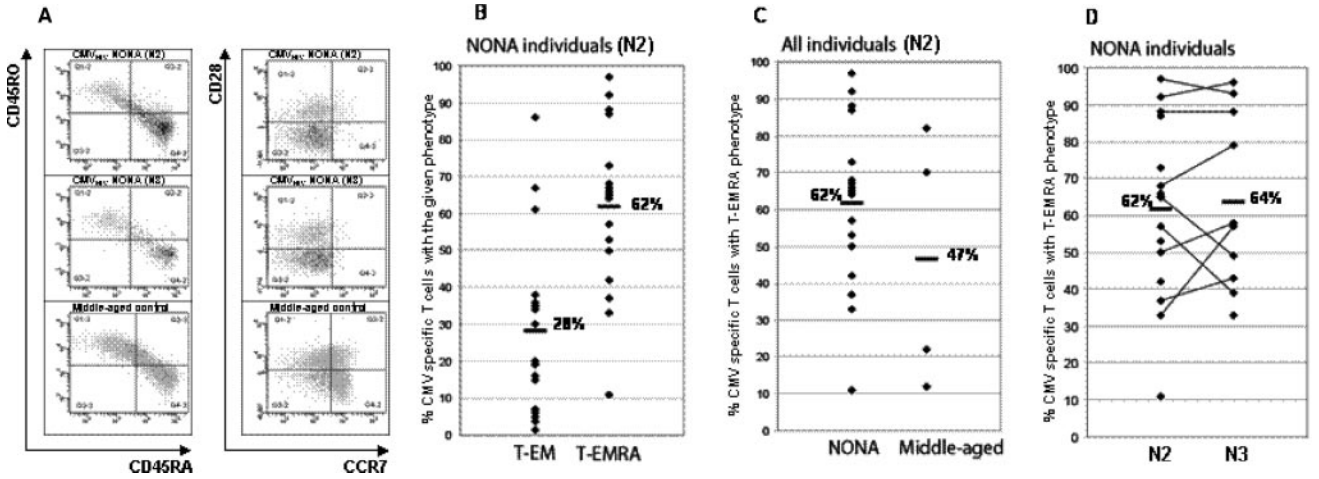

FIGURE 6. The phenotype of CMV_{NLV}-specific T cells. *A*, Representative examples of dot plots gated on CMV_{NLV}-specific T cells from a nonagenarian (at N2 and N3) and a middle-aged individual. All PBMC samples were stained with the following mAb panel: CD8-allophycocyanin-Cy7, CD45RA-PE-Cy5, CD45RO-FITC, CD28-allophycocyanin, CCR7-PE-Cy7, and CMV_{NLV} MHC-dextramer-PE. *B*, The diagram depicts the percentage of CMV_{NLV}-specific T cells from nonagenarians with the phenotypes T_{EM} (mean = 28%) and T_{EMRA} (mean = 62%). *C*, The plot shows the percentage of CMV_{NLV}-specific T cells with the T_{EMRA} phenotype in nonagenarians (mean = 62%) compared with middle-aged individuals (mean = 47%) (*t* test, $p > 0.05$). *D*, The plot shows the percentage of CMV_{NLV}-specific T cells with the T_{EMRA} phenotype at N2 compared with N3 collection (mean = 62 and 64%, respectively; *t* test, $p > 0.05$).

The CMV_{NLV}-specific T cell phenotype was also analyzed longitudinally. There was no significant difference in the CMV_{NLV}-specific T cell phenotype between the N2 and the N3 collection, neither concerning the nonagenarians (Fig. 6*D*), nor the middle-aged individuals (data not shown). Thus, the two middle-aged individuals with CMV_{NLV}-specific T cells of predominant T_{EM} phenotype showed a stable expression of this phenotype over the 2-year period, indicating that this phenotype cannot be explained solely by recent infection.

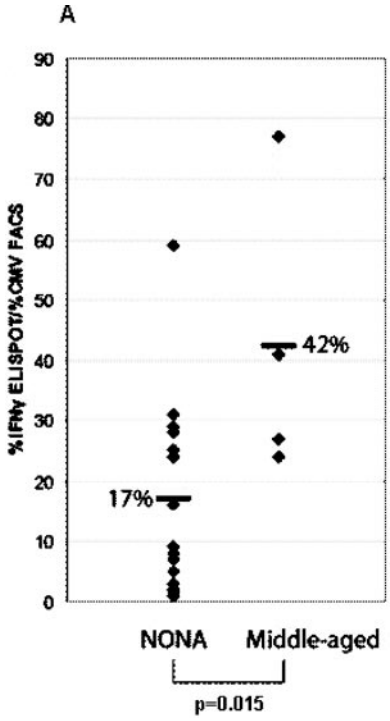

FIGURE 7. The functionality of CMV_{NLV}-specific T cells. IFN-γ secretion in response to CMV_{NLV} peptide stimulation was determined by ELISPOT. The functionality of the CMV_{NLV}-specific T cells was determined by comparing the number of IFN-γ-secreting cells in the ELISPOT assay with the CMV_{NLV} T cell frequency determined by FACS. The diagram shows the percentage of IFN-γ-secreting cells among the CMV_{NLV}-specific T cells, for a group of nonagenarians ($n = 20$) and middle-aged individuals ($n = 4$) (mean = 17 and 42%, respectively; *t* test, $p = 0.015$).

IFN-γ secretion from CMV_{NLV}-specific T cells

ELISPOT analyses were conducted to compare the functional capacity of the CMV_{NLV}-specific T cells accumulated in the nonagenarians with those found in middle-aged individuals. ELISPOT analyses were performed on PBMC from 17 nonagenarians and 4 middle-aged individuals. It was demonstrated that specific T cells from nonagenarians were able to respond to CMV_{NLV} peptide-stimulation by secreting IFN-γ. However, the fraction of CMV_{NLV}-specific T cells that were able to secrete this cytokine was lower in nonagenarians compared with middle-aged individuals. On average, 17% of the CMV_{NLV}-specific T cells from nonagenarians secreted IFN-γ in response to peptide stimulation compared with 42% from the middle-aged individuals ($p = 0.015$; Fig. 7). One IRP individual was included in the ELISPOT analyses. This individual displayed the lowest frequency of functional Ag-specific T cells with only 1.5% of the CMV_{NLV}-specific T cells secreting IFN-γ. The CMV_{NLV}-specific T cells constituted 4.6% of the CD8 population in this individual.

Discussion

Susceptibility to infection increases significantly with age, and substantial research over the past decades has aimed at characterizing the cellular and molecular mechanisms associated with decreased immune capacity. In the present study, we analyzed PBMC from nonagenarians included in the Swedish NONA longitudinal study for several immune parameters. At the time of blood collection, the nonagenarians were intensively studied for numerous immune and health parameters, including CMV status and CD4:CD8 ratios defining the IRP and being predictive of mortality (8, 11).

Data from TCR clonotype mapping demonstrated the presence of increased mean numbers of clonally expanded CD8 T cells in nonagenarians compared with the middle-aged (Fig. 2*A*). Such clonal expansions in the CD8 compartment in the elderly have been described previously by others–and occur in mice as well as

humans (21, 33). The numbers of CD8 T cell clonal expansions are distributed over a large range, in particular among nonagenarians. Overall, however, the CD8 T cell expansions significantly exceed the numbers found in middle-aged individuals (Fig. 2*A*). Assuming that the increased clonality is followed by a decrease in diversity of the total CD8 repertoire, these data point toward a scenario similar to that observed for CD4 T cells, where a dramatic decrease in diversity is observed as a cause of aging (34).

Further assessment of the data demonstrated that a high number of CD8 clones was strongly associated with CMV seropositivity (Fig. 2*B*) in concordance with previous observations (21, 22). However, compared with previous studies, the NONA study group is larger, and comprises older individuals. Although CMV seropositivity appears to be one of the driving forces for induction of CD8 T cell clonality this is clearly not detectable in the middle-aged group. Thus, the influence of CMV on clonality only becomes relevant–at least at a detectable level–in the elderly. However, factors other than age may also influence the development of CD8 clonality. In this respect, very little is known regarding asymptomatic CMV reactivation in healthy individuals. The frequency of reactivation might play an important role (35).

In the CMV-positive group, high clone numbers were significantly more predominant among non-IRP compared with IRP individuals (Fig. 2*C*). In addition, low clone numbers in the IRP group were found to be associated with increased mortality (Fig. 3*B*). Considering that increased CD8 clone numbers and development of IRP is presumably a gradual process; these data point toward a scenario in which sequential phases are initiated by increasing CD8 clone numbers and skewing of CD4:CD8 ratios. Progressive skewing of the CD4:CD8 ratio gradually leads to the development of an IRP. Individuals will enter the IRP category with high numbers of CD8 T cells, many of which are clonally expanded. Gradually, the clonal diversity in IRP individuals will start to shrink, and the number of clonal expansions begins to decrease without diminishing the size of the CD8 T cell pool (Fig. 3*A*). This clonal attrition leads to a decrease in the clonal heterogeneity of, e.g., the CMV repertoire. Although the direct proof for this hypothesis is still lacking, the diminished diversity of the CD8 T cell pool coincides with the increased mortality observed in IRP individuals, which is most likely due to increased susceptibility to pathogens (8). Loss of control of CMV infection itself might also affect the mortality; however, overt clinical manifestation of CMV infection was not observed in any of the IRP individuals. Analyses of future NONA samples may help clarify these issues by monitoring the CD4:CD8 ratios and the number of CD8 clonal expansions of currently non-IRP individuals upon entrance into the IRP.

Using HLA/peptide multimer complexes, we scrutinized the frequency and phenotype of CMV-specific T cells in the NONA study group. The data from these analyses revealed that relatively large fractions of the CD8 T cells are specific for the analyzed HLA-A2-restricted CMV-derived peptide (NLVPMVATV), confirming previous findings (Fig. 4, *A* and *B*) (22, 24, 26). The choice of this peptide for analyses relates to the immunodominance of this epitope for recognition on HLA-A2, as well as the frequent expression of this allele. However, immunodominance depends not only on the peptide, but is also to a large extent dependent on the HLA alleles that are coexpressed in the individual, e.g., HLA-B7 often seems to be dominant over HLA-A2 in regard to CMV reactivity (24). From combined analyses of several HLA alleles the CD8 response against CMV has been suggested to comprise up to 45% of the CD8 repertoire (24). To this end, the T cell response against CMV is broader than reflected by analyzing for reactivity against the commonly measured peptides from the pp65 protein (36). A wide range of peptides from several proteins is involved in the recognition of CMV and yet uncharacterized peptides may very well be engaged in the natural reactivity against CMV (37).

In concordance with previous data, the predominant phenotype of the CMV-specific T cells in the nonagenarians was the T_{EMRA}, having variable but mostly low CD28 expression (Fig. 6) (19). Furthermore, CMV-specific T cells were previously shown to have high levels of Bcl-2 expression, suggesting resistance to apoptosis (22). Possibly, this renders these cells resistant to activation-induced cell death which is one of the mechanisms involved in clearance of Ag-specific T cells during late phases of a successful immune response (38). Moreover, apoptosis resistance may offer an advantage in the competition for nutrients and growth factors among the CD8 T cells. Such competition may be further increased in the IRP group compared with non-IRP individuals due to the high numbers of CD8 T cells.

Three individuals (one nonagenarian and two middle-aged) were characterized by harboring CMV-specific T cells of the divergent T_{EM} phenotype together with expression of CD28. This particular phenotype was previously described in newly infected individuals, but was observed to differentiate into T_{EMRA} at time of convalescence with an almost fully changed phenotype 8 wk after infection (14). By comparing the CMV-specific T cells at the two time points in this longitudinal study, we were able to show that the frequency and phenotype of these T cells in general remained stable. Individuals, in whom the CMV-specific T cells were characterized by the divergent T_{EM} phenotype at the first time point (N2), still retained this phenotype for 2 years, suggesting that a recent infection does not fully explain this observation.

Sorting CMV_{NLV}-specific T cells enabled these cells to be subjected to TCR clonotype mapping. This revealed that the CMV_{NLV}-specific T cell populations were oligoclonal, consisting of a mean number of six expanded clones (Fig. 5, *A* and *B*), each of which may comprise variable fractions of the population, as clearly shown in our data concerning the middle-aged control (Fig. 5, *C* and *D*). Even though these analyses were limited to HLA-A2-restricted CMV reactivity, ~19% of the clonal expansions found in the total CD8 repertoire were CMV_{NLV}-specific. Moreover, considering that these cells presumably represent only a fraction of the entire CD8-mediated response against CMV, restricted by a single HLA allele, it seems quite plausible that CMV-specific T cell clonotypes occupy very large parts of the clonally expanded CD8 T cells in the elderly. These data provide direct proof of CMV infection causing appearance of clonally expanded T cells in the elderly.

In addition, comparison of the clonality of CMV-specific T cells from nonagenarians at 2-year intervals demonstrated that the clonal composition of CMV-specific T cells remained relatively stable with only few fluctuations (Fig. 5, *A* and *B*). Clearly, as discussed above, changes in the number of different expanded T cell clones do occur upon entry into the IRP phenotype, at least as can be judged from the one IRP individual among the nonagenarians, who had the lowest clonal diversity of CMV-specific T cells.

Functional analyses confirmed that the CMV-specific T cells from the nonagenarians are characterized by a decreased functional capacity compared with similar cells from the middle-aged individuals (Fig. 7). This suggests that increased numbers of CMV-specific T cells could be the result of a compensatory mechanism enabling control of CMV despite lower functional capacity. Possibly, the decreased functionality leads to viral reactivation for extended periods which in turn leads to activation and outgrowth of yet more CMV-specific T cells. Future longitudinal studies would benefit from combining data on viral reactivation and primary infection with immunological monitoring (39).

In conclusion, the data presented herein provide direct proof that many of the expanded CD8 T cell clones found in elderly are specific for CMV. Thus, our data suggest that CMV is one of the driving forces for acquiring the IRP. Strikingly, individuals in the IRP category–all of whom were CMV-positive–were characterized by a significant decrease in numbers of expanded CD8 clones which was associated with increased mortality. Importantly, the frequency, phenotype, and clonal composition of the CMV_{NLV}-specific T cells remain stable in the studied 2-year interval.

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Disclosures

The authors have no financial conflict of interest.

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