Induction and Evolution of Cytomegalovirus-Specific CD4 + T Cell Clonotypes in Rhesus Macaques


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Induction and Evolution of Cytomegalovirus-Specific CD4+ T Cell Clonotypes in Rhesus Macaques


CMV infection induces robust CD4+ T cell responses in immunocompetent hosts that orchestrate immune control of viral replication, dissemination, and disease. In this study, we characterized the clonotypic composition of CD4+ T cell populations specific for rhesus CMV (RhCMV) in chronically infected adult rhesus macaques (RM) and in juvenile RM undergoing primary RhCMV infection and subsequent secondary challenge with RhCMV. In adult RM with established chronic infection, RhCMV-specific CD4+ T cell populations exhibited stable, pauciclonal structures with skewed hierarchies dominated by two or three clonotypes. During primary infection, in contrast, the initial RhCMV-specific CD4+ T cell populations were highly polyclonal and progressive evolution to the chronic pattern manifest in adults occurred over the ensuing 2–3 years. Clear patterns of clonal succession were observed during this maturation process, such that clonotypes present in the acute phase were largely replaced over time. However, rechallenge with RhCMV expanded virus-specific CD4+ T cell clonotypes identified solely during acute infection. These findings indicate that, during persistent viral infection, substantial selection pressures and ongoing clonotype recruitment shape the specific CD4+ T cell repertoire and that rapidly exhausted or superseded clonotypes often remain within the memory T cell pool. The Journal of Immunology, 2008, 180: 269–280.

Cytomegalovirus is a ubiquitous betaherpesvirus that has successfully coevolved with its host species (1). In immunocompetent individuals, persistent infection is almost always clinically benign; however, serious disease can occur in immunocompromised hosts such as neonates, transplant recipients, and HIV-coinfected individuals (2, 3). The adaptive immune system devotes substantial resources to the control of CMV in vivo. Indeed, many studies have demonstrated that CMV-specific CD4+ and CD8+ populations dominate the memory T cell compartment in seropositive individuals (4–9). Specific evidence that CD4+ T cell responses contribute to the immune control of CMV comes from several sources. In humans, the loss of CMV-specific CD4+ T cell responses in the setting of HIV-1 infection has been correlated with the presence of CMV-associated end organ disease (10). Furthermore, persistent viral shedding in children was associated with impaired CMV-specific CD4+ T cell responses in one study (11), while the presence of such responses has been shown to protect against disease during primary CMV infection (12). In mice, studies have identified roles for CMV-specific CD4+ T cells in the clearance of virus from salivary glands and the prevention of reactivation (13, 14). However, the precise nature of the protective effects mediated by responding CD4+ T cell populations remains elusive, although both traditional helper roles (15) and direct antiviral effector functions have been described (16–18).

The typically asymptomatic nature of primary CMV infection has proved a major barrier to the longitudinal study of CMV-specific cellular immune responses in humans. Although some studies have examined CMV-specific CD4+ T cells during primary infection (11, 12, 19), there have been relatively few opportunities to dissect the clonotypic attributes of these emergent populations and their subsequent evolution (17). The homology between human CMV and rhesus CMV (RhCMV)4 genomes (20), together with the patterns of infection and pathology (21–24), humoral (24), and cellular (25) immune responses indicate that the rhesus macaque (RM) model is ideally suited for the investigation of primary CMV-specific CD4+ T cell induction and the generation of long-term memory T cell populations. In this study, we undertook a molecular analysis of expressed TCRB genes in functional RhCMV-specific CD4+ T cells to define the clonotypic architecture of these populations directly ex vivo in RM with acute and chronic RhCMV infection to provide insight into the processes that shape the Ag-specific memory repertoire during persistent infection.

Materials and Methods

Animals

A total of 15 purpose-bred, healthy male RM (Macaca mulatta) of Indian genetic background and free of Cercocephaline herpesvirus 1, D-type simian retrovirus, simian T lymphotrophic virus type 1, and SIV infection were used in this study. Steady-state RhCMV-specific CD4+ T cell responses were evaluated in 8 adult RhCMV-seropositive RM that naturally acquired RhCMV infection in their first year of life. For the study of primary RhCMV infection, 7 RhCMV-seronegative RM from an expanded specific

4 Abbreviations used in this paper: RhCMV, rhesus CMV; RM, rhesus macaque; BAL, bronchoalveolar lavage; qPCR, quantitative PCR.
pathogen-free colony were infected by s.c. injection of 10^7 PFU of Rh-CMV strain 68.1. Four of these RM were reinjected with 10^7 PFU of the same RhCMV strain at postinfection day 224. Bronchoalveolar lavage (BAL) was performed as described previously (26). All RM were housed at the Oregon Primate Research Center in accordance with the standards of the Center’s Animal Care and Use Committee and the “National Institutes of Health Guide for the Care and Use of Laboratory Animals.”

Cell preparation and Ag stimulation

PBMCs were isolated from citrated venous blood by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich). Cells were then washed twice in Ca²⁺/Mg²⁺-free HBSS (Fisher Scientific) and resuspended in RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated FCS (HyClone), 2 mM t-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 50 μM 2-ME (Sigma-Aldrich) (complete medium). For Ag stimulation, PBMCs were placed in 17 × 100 mm polypropylene tissue culture tubes (Falcon) at 1 × 10^6 cells/ml in complete medium with appropriately titrated “whole” RhCMV viral preparations and the costimulatory mAbs anti-CD28 and -CD95 (0.5 μg/ml each). In all cases, a negative control tube that contained complete medium and costimulatory Abs in the absence of Ag was set up in parallel. For live cell sorts, the cultures were incubated at 37°C in a humidified 5% CO2 atmosphere for 48 h. For intracellular cytokine staining, the stimulation was performed for 6 h, and cytokines were released except that for 6 h in the presence of brefeldin A (for the last 5 h). After incubation, cells were harvested by washing in cold (4°C) Dulbecco’s PBS (Invitrogen Life Technologies) containing 0.1% BSA (Roche Biochemicals) and processed for immediate staining.

Ags and Abs

RhCMV (Cercopithecine herpesvirus 8) Ag preparations were derived from 68.1 strain (American Type Culture Collection VR-677) infected mononuclear cells of either primary or tolerantized RM fibroblasts. After a 90–100% cytopathic effect was reached, infected cells were scrapped off the flasks. Cells and medium were clarified by centrifugation at 1,000 g for 10 min; pellets were resuspended in medium, frozen and thawed three times, sonicated for three cycles (30 s/cycle), and then clarified by centrifugation at 3,840 × g for a further 10 min. Supernatants from both the original culture medium and lysed cell pellets were combined and the virus was pelleted at 12,400 × g for 1 h. For use as immunostimulatory Ag in vitro, viral pellets were resuspended in medium (2% of the original culture volume) and tiered by intracellular cytokine flow cytometry. For viral stocks used in animal inoculations, the resuspended viral pellets were centrifuged over a 30% sorbitol cushion at 4°C; virus was then resuspended in medium and tiered by plaque assay on RM fibroblasts.

The mAbs L200 (anti-CD4; AmCyan, allophycocyanin, PerCP-Cy5.5), SP34-2 (anti-CD3; FITC, PerCP-Cy5.5), CD28.2 (anti-CD28; PE, PerCP-Cy5.5), BD17 (anti-CD69; PE, PerCP-Cy5.5, unconjugated), BD5 (anti-CD25; FITC, PE, allophycocyanin), MAB11 (anti-TNF-α; allophycocyanin), 2A3 (anti-CD25; FITC, PE, allophycocyanin), and isotyped matched controls were obtained from Invitrogen Life Technologies.

Immunofluorescence staining, flow cytometric analysis, and cell sorting

Initial flow cytometric analyses were performed on a BD Biosciences FACSCalibur instrument using FITC, PE, PerCP-Cy5.5, and allophycocyanin as the four fluorescent parameters. Subsequently, an LSR II BD instrument with Pacific Blue, AmCyan, FITC, PE, PE-Texas Red, PE-Cy7, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, and Alexa 700 as the available fluorescent parameters was used. Five-parameter FACS was performed using a FACS Vantage SE flow cytometer (BD Biosciences); the mean purity of the sorted cell populations was 98% (SD, 1.99%; SE, 0.06%). Instrument set-up, data acquisition, and data analysis procedures were performed as described previously (27, 28). Determination of RhCMV-specific CD4⁺ T cell frequencies was accomplished with intracellular cytokine flow cytometry (25, 29). Briefly, after Ag (or control) stimulation, cells were evaluated for cell surface expression of CD3 and CD4 and intracellular expression of CD69 and TNF-α, with responding CD4⁺ T cells defined by up-regulation of both CD69 and TNF-α. In parallel, PBMC were evaluated for the fraction of memory cells within the CD4⁺ T cell compartment using CD28 and CD95 expression patterns as described previously (29). Response frequencies within the CD4⁺ T cell memory population were calculated first by subtraction of background responses from Ag-induced responses and then by dividing the net response by the memory fraction as described previously (25). For RNA-based molecular analyses, Ag-stimulated cells were stained in the absence of fixation with pretitrated mAbs (30 min at 4°C) to detect surface expression of CD4, CD25, and CD69. Viable CD4⁺CD25⁻CD69⁺ cells were sorted into 1.5-ml Sarstedt tubes containing 100 μl of RNAlater (Ambion), centrifuged at 13,000 × g for 8 min and stored overnight at −80°C. For RNA-based molecular analyses, which are not dependent on cell viability and therefore compatible with fixation/permeabilization protocols, Ag-stimulated cells were sorted according to intracellular TNF-α and CD69 expression as described above, and then stored at −80°C.

RhCMV quantification using real-time quantitative PCR (qPCR)

RhCMV in urine was concentrated by centrifugation at 16,000 × g for 1 h in 4°C. DNA from urine pellets, PBMC, and BAL cells was purified using a Puregene DNA Isolation kit (Gentra Systems) according to the manufacturer’s instructions. Purified DNA was analyzed in duplicate for each sample using real-time qPCR. Primers (forward: GGGCATCCTCAGGAGTG; reverse: CGACACAAAGAGGATG; and a fluorescently labeled probe (6FAM-ACTCGGAAGACCCAG-C3 TAMRA) specific for the RhCMV IE2 sequence were designed using Primer Express software (Applied Biosystems). The 5’ end of the probe sequence was labeled with the reporter dye FAM and the 3’ end with the quencher dye TAMRA. Each 25-μl reaction contained TagMan Universal PCR Mastermix (Applied Biosystems), 100 pmol each of the forward and reverse primers, 5 pmol probe, and 10 μl of purified DNA. The RhCMV IE2 gene was cloned and analyzed over a range of 25–1,000,000 copies to create a standard curve for each assay. As one-quarter of each sample was assayed per reaction and the results were normalized to the entire sample for representation purposes, the lowest limit of detection for the assay was 100 copies of IE2. Reaction parameters were one cycle of 2 min at 50°C, one cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C followed by 1 min at 60°C.

Analysis of TCRB gene expression

mRNA was extracted from viably sorted cells using the Oligotex Direct mRNA minikit (Qiagen) according to the manufacturer’s protocol. An unbiased strand-switch anchored RT-PCR using a 3’ C region primer was used to amplify all expressed TCRB gene products as described previously (30, 31). Gel-purified amplification products were subcloned by ligation into the pGEM-T Easy Vector (Promega) followed by transformation of JM109 High Efficiency Competent Cells (Promega). Individual recombinant colonies were amplified by PCR with HiFi Platinum Taq (Invitrogen Life Technologies); products were treated with exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences), and then sequenced from both 5’ and 3’ ends using the ThermoTaq dye TAMRA. MacVector software (Accelrys) was used to analyze the sequences. A minimum of 50 clones was analyzed per sample.

Clonotype-specific semiquantitative PCR

CD4⁺ T cell populations sorted on the parameters described above were aliquoted in microtube tubes and pelleted by centrifugation at 13,000 rpm for 3 min. A total of 50 μl of 10 mM Tris-HCl (pH 7.4), containing PCR-grade proteinase K (50 μg/ml; Roche) was added to each cell pellet and the lysate was incubated for 4 h at 56°C. Proteinase K was then inactivated at 95°C for 10 min. For PCR, 5 μl of cell lysate was combined with PCR mix containing buffer (20 mM Tris-HCl (pH 8.0) and 50 mM KCl), 0.2 mM dNTP mix (Invitrogen Life Technologies), 5 μl of (α-32P)dCTP (Amer- sham Pharmacia Biotech), 1.5 mM MgCl₂, 0.5 μM each of clonotype-specific 5’ and 3’ primers designed to anneal in the TCRBV and CDR3

Table I. RhCMV-specific CD4⁺ T cell responses during chronic infection

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regions, respectively, and 2.5 U of Platinum TaqDNA polymerase (Invitrogen Life Technologies). Amplification parameters were 94°C for 2 min (1 cycle), followed by 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (35 cycles) with a final extension at 72°C for 2 min. The PCR products were loaded on a 6% polyacrylamide gel (Invitrogen Life Technologies) and visualized by exposure to a phosphor screen (Amersham Pharmacia Biotech). Data analysis was performed using ImageQuant software (Amersham Pharmacia Biotech).

Results

Clonotypic architecture of RhCMV-specific CD4+ T cell responses during chronic infection

Unless special precautions are taken, essentially 100% of captive RM become chronically infected with RhCMV in the first year of life. From our colony, four young adult (median age, 8 years; range, 6–9 years) and four old (median age, 24.75 years; range, 23.5–33 years) RM were selected for further analysis based on the presence of RhCMV-specific CD4+ T cell responses determined by the dual expression of intracellular CD69 and TNF-α on exposure to RhCMV Ag preparations. As shown in Table I, substantial RhCMV-specific CD4+ T cell responses were detected in these RM regardless of age; similarly robust CMV-specific CD4+ T cell responses have been observed in chronically infected humans (5, 7, 9).

To examine the clonal nature of RhCMV-specific CD4+ T cells during chronic infection, the responding populations were isolated viably by flow cytometry based on up-regulation of the activation markers CD25 and CD69 following stimulation for 48 h with whole RhCMV lysate. A modified strand-switch anchored RT-PCR was then used to amplify all expressed TCRB gene products without bias (30, 31), and a minimum of 50 subclones was sequenced for each RhCMV-specific CD4+ T cell population. The RhCMV whole virus-specific CD4+ T cell populations contained remarkably few clonotypes in both the young adult (median, 5.5 clonotypes; range, 4–11 clonotypes) and old (median, 7 clonotypes; range, 4–11 clonotypes) RM, with no significant correlation between response magnitude and clonotypic diversity (Fig. 1). Furthermore, the clonal hierarchies were highly skewed and dominated by two or three individual clonotypes (Fig. 1). In confirmatory experiments, a DNA-based PCR incorporating clonotype-specific primers was used to amplify individual rearranged TCRB genes from RhCMV-specific CD4+ T cell populations sorted according to the detection of intracellular CD69 and TNF-α; these data demonstrated the validity of the anchored RT-PCR approach in conjunction with function-based cell-sorting parameters (Fig. 2). To examine the stability of these RhCMV-specific CD4+ T cell repertoires, we conducted a repeat evaluation of the four young adult RM at a second time point, in each case from between 3 and 7 mo after the first analysis. The data demonstrate that the clonotypic composition and hierarchical organization of RhCMV-specific CD4+ T cell populations during chronic infection is extremely stable (Fig. 1A). These findings are consistent with previous studies in humans with established CMV infection (32).

Evolution of RhCMV-specific CD4+ T cell clonotypes during primary infection

To investigate the processes that precede establishment of the clonotypically stable RhCMV-specific CD4+ T cell populations that prevail during chronic infection, three juvenile RhCMV-seronegative RM from an expanded specific pathogen-free colony were infected with RhCMV as described in Materials and Methods and serially monitored for 938 days. A real-time qPCR assay was used to detect and quantify RhCMV DNA in PBMC and urine at frequent intervals following viral inoculation. Although cell-associated viremia was not detected in these RM at any time point, primary RhCMV infection was manifest by the appearance of virus in urine between days 56 and 91 postinfection, and by the development of RhCMV-specific CD4+ T cell responses (Fig. 3). This specific CD4+ T cell response was measurable in all three RM by 14 days after inoculation, and was maintained thereafter; in two of three RM, an abrupt increase in RhCMV-specific CD4+ T cell frequency occurred after day 100 postinfection and subsequently stabilized.

Analysis of TCRB gene usage within the whole Ag-responsive CD4+ T cell populations was conducted periodically in each case (Fig. 4). Several notable features emerged from these longitudinal studies. First, at the earliest time points in each of the three RM (postinfection days 182 or 203), the RhCMV-specific CD4+ T cell populations were quite polyclonal (median, 22 clonotypes; range, 15–28 clonotypes); at the latest time points studied (postinfection day 938), the corresponding populations were pauciclonal (median, 4 clonotypes; range, 3–5 clonotypes) with highly skewed distributions reminiscent of the profiles observed in chronically infected adult RM (Fig. 4). Thus, a progressive narrowing of the Ag-specific clonotypic repertoire accompanies the natural history

![FIGURE 1](http://jimmunol.org/) Analysis of RhCMV-specific CD4+ T cell clonotypes during chronic infection. The CDR3 amino acid sequences, TCRBV and TCRBJ usage, and relative frequency of each RhCMV-specific CD4+ T cell clonotype are shown for four young adult RM at two separate time points (3–7 mo apart) (A) and for four old RM at a single time point (B). Colored boxes in the “% frequency column” indicate clonotypes that are common between time points within an individual RM. The total number of sequences represented for each RhCMV-specific CD4+ T cell population is indicated at the bottom of each panel.
of RhCMV infection from the acute phase. As above, no significant correlations between response magnitude and clonotypic diversity were evident either within individual RM or across the data set as a whole. Second, the clonotypic representation at successive time points in each individual RM exhibited some consistent features and a substantial degree of variability. The dominant clonotypes induced in the early phases of infection tended to persist at later time points, albeit at lower frequencies. However, there was also a substantial apparent loss of individual clonotypes and recruitment of new clonotypes at successive time points within each individual RM. Newly recruited clonotypes either appeared transiently at single time points or persisted for longer periods and exhibited clear patterns of clonal succession; these distinct phenomena are most apparent in RM nos. 22046 and 22063, respectively (Fig. 4). Thus, a dynamic “maturation” process occurs that leads to the generation of the clonotypic structures characteristic of chronic infection in RM and humans (Fig. 1; Refs. 17 and 32).

Clonal dynamics, CD4+ T cell responses, and viral replication during the natural progression of RhCMV infection

To further investigate the impact of viral kinetics on clonotype usage during the development of cognate RhCMV-specific CD4+ T cell populations over time, we studied a second cohort of four seronegative RM that were experimentally infected with RhCMV. In this cohort, qPCR was used to detect and quantify RhCMV DNA in PBMC, BAL and urine (Fig. 5, A–C). Cell-associated virus was detected transiently in PBMC at day 42 postinfection in three of four RM, and rarely at other time points. However, in BAL, RhCMV DNA was detectable after 28 days in two of four RM and somewhat later in the other two; virus load peaked in all cases at substantially higher levels when compared with cell-associated RhCMV DNA in PBMC, and subsequently declined over time (Fig. 5B). Urine was positive for RhCMV DNA by day 56 postinfection in all RM, and this virus shedding was sustained through to the end of the study (Fig. 5C). The corresponding RhCMV-specific CD4+ T cell responses in PBMC from these three RM with experimental primary RhCMV infection. Results are presented as the percent of CD4+ memory T cells responding to RhCMV lysate with CD69 and TNF-α dual up-regulation within the overall CD4+ memory T cell compartment.

Analysis of TCRB gene expression in the RhCMV-responsive CD4+ T cell populations isolated from PBMC revealed a polyclonal picture consistent with that observed in the first cohort of RM with acute infection (Fig. 6). At the first time point examined, day 91 postinfection for RM no. 20296 and day 112 postinfection for the other RM, a median of 23.5 clonotypes (range 22–28) constituted the whole virus-specific response; at a later time point (day 182 postinfection for three of four RM, and day 203 postinfection for RM no. 21046), the corresponding median number of clonotypes was 14 (range 7–51). Thus, with the notable exception of RM no. 21046, the clonotypic repertoire within the RhCMV-specific CD4+ T cell responses exhibited a biphase-like pattern with an initial transient expansion detectable at day 14 postinfection, followed by a contraction and a secondary expansion from day 56 postinfection or later. Subsequently, these responses stabilized at between 0.5 and 2% of the total memory CD4+ T cell population.
Clonal dynamics, CD4+ T cell responses, and viral replication in infected macaques after RhCMV challenge

The data described above suggest a relationship between viral kinetics and the development of RhCMV-specific cellular immune responses in the CD4+ T cell compartment. To test this possibility, each RM in the second primary infection cohort was challenged with a further inoculum of RhCMV (10^7 PFU) at day 224 postinfection; at this time point, viral loads in BAL had subsided and RhCMV-specific CD4+ T cell frequencies were stabilizing. In all four RM, there was a substantial boosting of RhCMV-specific CD4+ T cell frequencies in PBMC, followed by a period of fluctuation, and ultimately higher steady-state response frequencies (Fig. 5D). Notably, despite the 10-fold higher inoculation dose, no cell-associated virus was detected in the PBMC from any of these RM and viral loads detected in BAL were considerably smaller than those observed during primary infection (Fig. 5, A and B). Overall viral secretion in urine was unchanged following secondary challenge (Fig. 5C); however, it should be noted that reinfection studies with genetically marked RhCMV strains indicate that the second virus appears in urine with similar kinetics to primary infection and continues to be secreted indefinitely (J. Edgar and L. Picker, manuscript in preparation). Thus, although RhCMV can reinfect previously infected hosts, viral replication during reinfection was substantially attenuated compared with primary infection.

In two of four RM, studied 112 days after secondary challenge, we observed a clear increase in the overall clonality of the corresponding RhCMV-specific CD4+ T cell populations isolated from PBMC; in the other two animals, clonality either decreased (RM no. 21046) during this same time frame, or stayed the same, albeit

FIGURE 4. Evolution of RhCMV-specific CD4+ T cell clonotypes during primary infection. The CDR3 amino acid sequences, TCRBV and TCRBJ usage, and relative frequency of each RhCMV-specific CD4+ T cell clonotype are shown for RM nos. 22063 (top panels), 22052 (middle panels), and 22046 (lower panels) at the time points indicated in the figure. Colored boxes in the “% frequency” column indicate clonotypes that are common between time points within an individual RM. The total number of sequences represented for each RhCMV-specific CD4+ T cell population is indicated at the bottom of each panel. PID, Postinfection day.
with more pronounced skewing of the repertoire (RM no. 20955; Fig. 6). In all cases, many new clonotypes were recruited into the RhCMV-responsive CD4+ T cell population, and many clonotypes were lost. These clonal dynamics are perhaps not unexpected in light of the dramatic proliferation and expansion within the RhCMV-specific CD4+ T cell pool (Figs. 5D and 7). Indeed, detailed

FIGURE 5. Comparison of the virologic and immunologic response to primary RhCMV infection and subsequent challenge. A real-time qPCR assay was used to detect cell-associated RhCMV copies in PBMC (A) and BAL (B), and RhCMV copies in urine (C), following RhCMV primary infection and subsequent RhCMV challenge at postinfection day 224 in four RM. Blank time points reflect target IE2 DNA levels below the limits of detection. D, Intracellular cytokine flow cytometry was used to evaluate the frequencies of RhCMV-specific CD4+ T cells in PBMC from these experimentally infected RM. Results are presented as the percent of CD4+ memory T cells responding to RhCMV lysate with CD69 and TNF-α dual up-regulation within the overall CD4+ memory T cell compartment.
longitudinal analyses revealed that Ki-67 expression levels paralleled the biphasic expansion of RhCMV-specific CD4+ T cells postchallenge, with initial peaks occurring in all four RM between days 7 and 14 and secondary peaks occurring in three of four RM between days 56 and 70 (Fig. 7); these data indicate that viral replication and consequent Ag exposure dictate clonal dynamics, although formally we cannot exclude the possibility of redistribution from anatomically distinct compartments. Regardless, the most striking feature of the clonotypic analysis at the first time point after challenge was the re-emergence and dominance of

### FIGURE 6

Analysis of RhCMV-specific CD4+ T cell clonotypes during acute infection and subsequent virus challenge. The CDR3 amino acid sequences, TCRBV and TCRBJ usage, and relative frequency of each RhCMV-specific CD4+ T cell clonotype are shown for RM nos. 19997, 20296, 20955, and 21046 at two time points after primary infection and at two time points after virus challenge at day 224 postinfection. Colored boxes in the "% frequency column" indicate clonotypes that are common between time points within an individual RM. The total number of sequences represented for each RhCMV-specific CD4+ T cell population is indicated at the bottom of each panel. PID, Postinfection day.
RhCMV-specific CD4+ T cell clonotypes that had been induced at high frequency during primary infection and subsequently disappeared during the contraction process noted above. This phenomenon was apparent in all four RM (Fig. 6). In two of four RM (nos. 20296 and 20955), these recrudescent clonotypes were maintained within the RhCMV-specific CD4+ T cell repertoire; in the other two RM (nos. 22147 and 21046), these clonotypes were no longer detected (Fig. 6).

**FIGURE 6.** (continued)

**FIGURE 7.** Virus-specific CD4+ T cell proliferation after RhCMV challenge. A, PBMCs from RM no. 19997 were stimulated in vitro with RhCMV lysate and costimulatory mAbs in the presence of brefeldin A as described in Materials and Methods. Cells were stained for cell surface CD3 and CD4, and for Ki-67 and TNF-α after permeabilization. Expression of Ki67 identifies cells that have entered S phase of the cell cycle within the preceding 7 days. Panels are gated on CD3+CD4+ small lymphocytes, with the responding cells highlighted in black and the nonresponding cells colored gray. The percentage of Ki-67+ cells in the responding populations is indicated in the upper right corner of each panel. PID: postinfection day.

**B**, The percentages of RhCMV-specific CD4+ T cells expressing Ki67 after RhCMV challenge are shown for all four RM in the second primary infection cohort.
two RM (nos. 19997 and 21046), they were present only transiently after challenge (Fig. 6). Importantly, within each RM, the identity of clonotypes present at more than one time point was established at the nucleotide level in all cases. At a subsequent time point (day 420 or 441 postinfection; day 196 or 217 postinfection), the overall clonotypic repertoire of the RhCMV-specific CD4⁺ T cell populations in all four RM had decreased. This clonal expansion and contraction after reinfection is similar to that observed during acute infection (Figs. 4 and 6), and occurred after challenge despite the dampened viral replication kinetics that presumably reflect a primed and more efficient immune response (Fig. 5, A and B). Thus, in all studied RM, new or increased Ag exposure was associated with expansion of the RhCMV-specific CD4⁺ T cell memory repertoire in the periphery, whereas Ag decline and/or stabilization were associated with clonotypic contraction and skewing.

Discussion

In this report, we conducted a direct ex vivo analysis of the clonotypic structure of RhCMV-specific CD4⁺ T cells in both primary and chronic infection. The principal findings were as follows: 1) whole virus-specific CD4⁺ T cell populations induced during acute RhCMV infection are polyclonal and undergo substantial evolution with progression through to chronic infection; 2) RhCMV-specific CD4⁺ T cell populations in chronic infection are relatively oligoclonal and exhibit stable, highly skewed clonotypic structures; 3) patterns of clonotypic usage in RhCMV-specific CD4⁺ T cell populations are intimately linked to viral replication dynamics; and 4) initially dominant clonotypes appear to persist within the RhCMV-specific memory CD4⁺ T cell pool despite ongoing clonotypic recruitment and clonal succession.

The marked clonotypic evolution observed with progression from acute to chronic RhCMV infection could reflect several potentially coincident and interrelated processes. First, the recruitment of new clonotypes could be explained by temporal changes in epitope targeting. Such shifts in immunodominance and Ag recognition profile have been documented, albeit primarily for CD8⁺ T cell responses, in several systems (33–41). Mechanistically, these changes can reflect differential Ag presentation (38), clonal exhaustion (39), and mutational escape from initially targeted epitopes (41) among other phenomena. In the present study, we were unable to conduct detailed epitope mapping experiments due to limited sample availability and the magnitude of the RhCMV genome; it is therefore not possible to ascertain whether such altered epitope targeting applies to the current data set, although the re-emergence of initially recruited clonotypes after inoculation of persistently infected RM with RhCMV (Figs. 5 and 6) tends to support this possibility. Second, interclonal competition for Ag, which can occur both between clonotypes specific for the same epitope and between clonotypes specific for different epitopes derived from the same pathogen, has been shown to shape clonotypic hierarchies in vivo (42–44). In the case of CD4⁺ T cells, there appears to be an avidity threshold beyond which competitive effects are minimized (43); under these circumstances, additional factors that impact the “fitness” of individual clonotypes within the host environment are likely to assume greater importance (45). Such ongoing selection pressures could preferentially lead to the progressive selection of clonotypes with optimal “fitness” and the demise of initially recruited clonotypes that might have elicited an early advantage in the absence of limiting Ag concentrations due to stochastic effects or higher precursor frequencies within the naive pool. Third, clonal succession resulting from senescence and the preferential deletion of high-avidity clonotypes could exert a significant negative influence on the cognate CD4⁺ T cell populations that are recruited during acute infection under conditions of relative Ag excess (46–49). All of these processes are likely affected by the dynamics of viral replication, but dissection of the relative contributions of these and other formative influences at different stages of infection is beyond the scope of this study.

Regardless of the relative importance of the various selection pressures that operate in vivo, the viral and clonotypic dynamics of acute infection resolve to an equilibrium in which persistent RhCMV infection is associated with a remarkably stable, oligoclonal whole virus-specific CD4⁺ T cell repertoire (Fig. 1). The stability of these clonotypic hierarchies is consistent with previous studies of both CD4⁺ and CD8⁺ T cell populations in chronic human CMV (32, 44) and other persistent viral infections (50, 51). However, the apparent oligoclonoality of the chronic-phase RhCMV whole virus-specific CD4⁺ T cell populations would appear to contradict previous observations that: 1) mature CMV-specific CD4⁺ T cell responses in humans are broad, targeting multiple viral proteins and often multiple epitopes within a given protein; 2) the whole viral lyssate-specific response typically reflects this complexity; and 3) each epitope-specific CD4⁺ T cell population comprises multiple clonotypes (9, 18, 32, 52). This apparent discrepancy can be resolved by the assumption that the mature RhCMV-specific CD4⁺ T cell responses characterized in this study are not oligoclonal in an absolute sense. Thus, each functional RhCMV-specific CD4⁺ T cell population could include multiple clonotypes that are present at vastly different frequencies thereby creating highly skewed hierarchies; our characterization of TCRB gene expression, while unbiased at the molecular level, may be relatively insensitive to the detection of very infrequent clonotypes as a consequence of sampling limitations. Indeed, we demonstrated that the initial recruitment of clonotypes into the RhCMV-specific CD4⁺ T cell population in primary infection is unequivocally polyclonal. Although most of these “early” clonotypes are subsequently superseded by a few dominant clonotypes and seem to disappear from the active repertoire, at least some can be resurrected when conditions change, such a during reinfection, indicating that they persist at low frequencies.

The observation that prevalent RhCMV-specific CD4⁺ T cell clonotypes detected during acute infection re-emerged on secondary challenge was one of the most intriguing features of this study (Fig. 6). This was associated with: 1) a biphasic expansion of the whole virus-specific CD4⁺ T cell response, similar in pattern to that observed in primary infection and in other systems (53, 54), which resulted in a sustained boosting of RhCMV-specific CD4⁺ T cell frequencies (Fig. 5); 2) a general diversification of the RhCMV-specific CD4⁺ T cell repertoire, indicating an intimate relationship between viral replication dynamics and clonotype selection in the periphery; and 3) the recruitment of new clonotypes, consistent with previous reports that demonstrated ongoing priming during persistent infection in the presence of a pre-existing memory T cell population (17, 55, 56). The phenomenon of clonotypic re-emergence during periods of increased viral burden indicates that, in addition to the repertoire plasticity that provides a renewable source of virus-specific T cell clonotypes, initially primed clonotypes likely remain within the memory T cell pool despite their apparent subsequent disappearance from the Ag-specific repertoire. Taken together, these observations delineate a remarkably “layered” flexible recognition system for persistent viruses with a first line of defense comprising a small number of dominant clonotypes that are highly selected, a second line composed of a larger number of persistent minor clonotypes that expand on subsequent...
viral exposure (either reinfecion with a new strain or recrudescence of the original strain), and a third line that encompasses the recruitment of new clonotypes from the naïve pool.

In summary, we have conducted a detailed longitudinal analysis of clonotypic usage within the CD4+ T cell response to a persistent DNA virus. Our findings highlight both the stability and malleability of the clonotypic repertoire that can be mobilized in response to chronic viral replication and suggest that ongoing recruitment and retention of Ag-specific CD4+ T cell clonotypes contribute to the immune control of persistent viruses.

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References


