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Evolution of Epitope-Specific Memory CD4⁺ T Cells After Clearance of Hepatitis C Virus

Andrew J. Godkin, Howard C. Thomas, and Peter J. Openshaw

The generation of memory lymphocytes is one of the hallmarks of the specific immune response. The CD4⁺ T cell response is of critical importance in maintaining long-term protective immunity after clearing many infections. However, the characterization of these memory CD4⁺ T cells has relied mainly on mouse studies and is poorly understood in humans. We have detected and counted epitope-specific populations of CD4⁺ memory cells in patients who have cleared hepatitis C virus. The kinetics of the recall response and the expression of the chemokine receptor CCR7 suggested the presence of distinct populations. A population of memory cells measured in an ex vivo IFN-γ ELISPOT assay steadily declined after viral clearance. However, memory CD4⁺ T cells only characterized after short-term culture with Ag and IL-2, and, recognizing the same epitopes, developed into a long-term stable population. Depletion of CCR7⁺ cells from PBMCs markedly reduced the responses in the culture-positive population while having little effect on the ex vivo responses. The demonstration of these key memory subsets in man opens the way to defining their role in protective immune responses.


The CD4⁺ T cell response is thought to be of critical importance in determining the fate of many infections, including the noncytopathic viruses such as hepatitis C virus (HCV) (reviewed in Refs. 1 and 2). After clearance of a pathogen, the characterization of the memory CD4⁺ T cell response has relied mainly on mouse studies and is poorly understood in humans (6–8). HCV is an RNA virus that often causes chronic infection in humans without treatment (9, 10). There are several lines of evidence that support a key role for CD4⁺ T cells in orchestrating clearance of HCV, including the association of viral clearance with the class II major histocompatibility Ag HLA-DR11 (A1*0101, B1*1101) (reviewed in Ref. 11), the correlation of proliferative responses during the acute infection with a favorable outcome (12), and loss of these proliferative responses with recurrence of the virus (13). A recent report on the long-term follow-up of a cohort of patients who had been infected with the same strain of HCV and subsequently cleared the virus revealed a persistent CD4⁺ proliferative T cell responses to one or more viral proteins in the majority of patients, although the serological response waned (14).

The generation of memory lymphocytes is one of the hallmark proteins of the specific immune response (reviewed in Ref. 8). It enables a quantitatively and qualitatively different response on subsequent rechallenge by the same pathogen. The peripheral repertoire of T cells consists of fixed pools of naive and memory cells. The function and maintenance of naive and memory T cells appears to be quite independent of each other (6, 7). When naive cells encounter pathogenic epitopes expressed by MHC proteins in lymphoid tissue they undergo profound changes, multiplying severalfold and differentiating into effector cells. After clearance of the pathogen, the majority of the expanded pool of T cells undergoes activation-induced cell death, but a percentage will survive long term as memory cells. The selection of epitope-specific cells, with the most favorable TCR-MHC interaction, leads to the preservation of the same specificities in the memory population (4, 15). Apart from their enhanced responses on antigenic rechallenge, these memory cells have a variety of features that distinguishes them from naive cells. Memory cells do not seem to require the continual interaction with self MHC molecules to survive, in contrast to peripheral naive T cells. Adoptive transfer experiments show that memory cells, but not naive cells, can survive in knockout mice that do not express any class I or class II molecules (16, 17).

Memory T cells also develop particular surface markers such as CD45 isoforms and express a novel range of adhesion molecules and chemokine receptors facilitating their passage through different tissues (reviewed in Ref. 18).

However, the factors that govern the generation and maintenance of memory cells remains largely a mystery. The CD4⁺ T cell response is of critical importance in maintaining long-term protective immunity after clearing many infections (1, 2). The presence of long term Ab-mediated protection is well documented, but the development and maintenance of CD4⁺ T cell memory have been very difficult to study in man. In mice, CD4⁺ T cells have been found to be of central importance in the maintenance of protective immunity (2). We have recently used an epitope prediction program (19) that allowed the accurate identification of HLA-DR11-restricted HCV epitopes (20). Using highly sensitive ELISPOT assays to identify T cells recognizing these epitopes, we have detected and counted distinct populations of CD4⁺ memory cells in HLA-DR11⁺ patients who have cleared HCV infection. The study of the evolution of these T cell populations offers a novel insight into CD4⁺ T cell memory development in humans after recovery from a natural virus infection.

Materials and Methods

Study population

Patients were diagnosed with past HCV infection by the clinical history, the presence of anti-HCV Abs, at least three negative HCV PCRs (separated by 6–12 mo), and normal liver function tests. The HLA typing was performed using allele-specific primer PCR to obtain the genotype. These
tests were performed by the hospital reference laboratories. Ethical committee approval (St. Mary’s National Health Service Trust) was granted for obtaining blood samples from patients.

Peptide synthesis

The peptides were designed according to the sequence of viral genotype 1a based on Simmond’s classification. The candidate peptides 15 or 16 aa in length were synthesized commercially (ECHAZ Micro Collections, Tubingen, Germany). The sequence and purity of selected peptides was confirmed by HPLC and tandem mass spectrometry analysis.

ELISPOT assays

The biological effect of peptides on T cells was studied in either a direct ex vivo assay or on short-term cultured T cells using single-cell cytokine release as a measure of Ag-specific effector function in a highly sensitive ELISPOT assay (anticytokine Abs and streptavidin-alkaline phosphatase obtained from Mabtech, Nacka, Sweden). The concentrations of Abs used and washing steps were according to the manufacturer’s instructions. The method has been previously described (21). For direct ex vivo analysis, $4 \times 10^5$ PBMCs (extracted from heparinized whole blood by centrifugation over Lymphoprep (Nycomed, Oslo, Norway)) were added per well of a standard polymer-backed 96-well filtration plate (Millipore, Mosheim, France). These cells were incubated at 37°C with 5% CO$_2$ for 18 h. Previous titration experiments revealed ex vivo culture from 6 to 18 h yielded the same results (data not shown). For measuring responses in short-term lines 2.5 $\times 10^5$ cultured T cells (grown as described below) in R-5 (RPMI with 5% heat-treated FCS) were added to each well. The peptides were tested at a final concentration of 10 $\mu$g/ml and compared with control wells with no peptide. The plates were either assessed by an independent observer blinded to the well contents and/or read using an automated ELISPOT reader (Autoimmun Diagnostika, Strasburg, Germany). Epitope-specific spot-forming cells per well were calculated by subtracting background values from wells with no peptide (majority of background values, 0–10 spots per well).

Short-term propagation of epitope-specific CD4$^+$ T cells

A total of 2 $\times 10^5$ PBMCs at a concentration of 2 $\times 10^5$ cells/ml were grown in 96-well round-bottom plates with 10 $\mu$g/ml peptide using R-10 (RPMI plus 10% human AB serum plus antibiotics/L-glutamine supplement) and incubated in a humidified atmosphere containing 5% CO$_2$ at 37°C. On days 3, 6, and 9 the medium was supplemented with IL-2 (10% Lymphocult T; Biostest Diagnostics, Denville, NJ) and fresh R-10 on days 6 and 9. On day 12 the lines were washed three times in RPMI and assayed as described above. To confirm presentation by HLA-DR11, matched and mismatched EBV-transformed B cell lines were used along with anti-HLA-DR mAb L243 (22) and anti-DR1 a1a3 (23) as described before (24).

Depletion of CCR7$^+$ and CD8$^+$ T cells

Magnetic cell sorting of CD8$^+$ T cells was performed using MACS CD8 MicroBeads and MS$^+$ Separation Columns according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). The non-neutralizing mouse IgM anti-CCR7 Ab derived from clone 2H4 (purchased from BD PharMingen, Oxford, U.K.) was used for depletion of CCR7$^+$ T cells. PBMCs were purified as described above and washed in cold PBS containing 1% human albumin. The cells were incubated on ice for 30 min with 30 $\mu$g of Ab/10$^7$ cells. After washing twice, rat anti-mouse IgM MicroBeads were added (50 $\mu$L of beads/10$^7$ cells) and incubated at 4°C for 20 min. The cells were then washed and sorted as above. FACS analysis revealed the purified population was $>$99% CCR7$^+$ cells (data not shown).

Results

Screening of HLA-DR11$^+$ nonviremic HCV patients for cognate memory CD4$^+$ T cells

A previously validated prediction program for identifying HLA-DR epitopes (19, 21, 24) has enabled us to identify HLA-DR11-restricted T cell epitopes (24). Using a series of epitopes derived from four different viral proteins (core, NS3, NS4, NS5), we screened HLA-DR11$^+$ patients who had successfully cleared HCV for cognate CD4$^+$ memory T cells using IFN-γ ELISPOT assays comparing ex vivo analyses with short-term cultures. The results are shown in Table I. All the patients showed positive responses to one or more epitopes after short-term culture; although the overall range of epitopes recognized and the strength of re-

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$^a$ Epitope-specific T cell responses were measured by IFN-γ release using ELISPOT assays. The responses to individual HCV peptides were measured ex vivo and after 12-day cultures with IL-2 and peptide. For the cultured responses, initially 5–10 short-term lines were assayed: positive line defined as an increase of more than two times background. Strong responses more than three times background, weak response one to three times background.

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response to each epitope were unique to each subject, peptides core31–45, core141–155, NS31245–1259, and NS52268–2282 were recognized in the majority of individuals. However, the ex vivo responses were not present in all individuals and appeared to decline with time since viral clearance. Furthermore, there was not a direct correlation between an ex vivo response to a particular epitope and the cultured response to the same epitope; e.g., subject 1 had strong ex vivo responses to core31–45, core141–155, and NS52268–2282 yet showed positive cultured responses to core51–45, NS3207–221, and NS52798–2802. These results suggest that these ex vivo and cultured responses are measuring different populations of memory cells.

**Evolution of the ex vivo and cultured memory response**

The overall pattern in Table I suggests the cultured responses develop and persist after clearance of the virus but that the ex vivo responses eventually decline. To explore this notion further, two individuals who demonstrated robust ex vivo responses (subjects 1 and 4) were studied prospectively. The immediate ex vivo (Fig. 1, A and C) and cultured (Fig. 1, B and D) memory CD4+ T cell responses in these individuals were measured to three epitopes over a 24-mo period. Both subjects revealed the same pattern. Initially there was a powerful ex vivo response, with frequencies of cognate epitope-specific T cells reaching $150 \times 10^6$ PBMCs, but over the study period powerful robust responses evolved. The decline in an ex vivo epitope-specific population of memory T cells was particularly striking: initially the cultured responses were almost totally absent to all three epitopes, but over the study period powerful robust responses evolved. The declines in both subjects were gradual, as illustrated in the graphs.

**FIGURE 1.** The evolution of ex vivo and cultured epitope-specific CD4+ memory T cell changes over time. A–D, The changes in frequencies of cognate T cell responses measured by IFN-γ ELISPOTS to three HCV epitopes were recorded over 24 mo in two subjects. For both individuals there was a decline in the ex vivo responses (A and C) but a gradual increase during the same period in cultured responses to the same epitopes (B and D). Experiments 1–4 were conducted at 6- to 8-mo intervals. E, Direct comparison of the ex vivo response against the frequencies of the cultured response to the same epitope. The collective data outlined were analyzed. The two populations reveal an inverse relationship (Pearson’s correlation $-0.334$).
entering a reasonably stable population. Repeat experiments over 2 years on these subjects confirmed the persistence of these central CD4+ T cells. Furthermore, the relative contribution of each epitope to the memory pool appeared to be stable over time. A representative example with subject 7 is given in Fig. 2A. The epitope NS31245-1259 producing persistently higher frequency responses (proportion of total responses originally: 74% NS31245-1259 and 26% core141-155; proportion of total responses when repeated after 24 mo: 72 and 28%, respectively). It seems both the range of epitope-specific responses and the hierarchy indicated by frequency are reasonably stable over time. Subject 7 had been nonviremic for the longest period of time and had no ex vivo responses. The CCR7+ T cells were magnetically depleted and the epitope-specific responses to NS31245-1259 were measured after culture (Fig. 2B). The depletion reduced the response to 46% of the unsorted PBMCs, but addition of the retained CCR7+ T cells from the column restored in part the response to 76% of the original.

CCR7+ CD4+ T cells contribute substantially to the HCV cultured memory cells but not to ex vivo responses

It has been suggested that expression of the lymphotropic chemokine receptor CCR7 on memory T cells acts as a marker for central memory cells (25). These cells require Ag restimulation/IL-2 to develop effector functions, whereas the CCR7+ cells have the characteristics of immediate effector memory cells. To examine the characteristics of HCV epitope-specific memory cells further, CCR7+ T cells were depleted from PBMCs of another two subjects who had both ex vivo and cultured responses (Fig. 3). CCR7 depletion reduced the culture-positive epitope-specific responses by a similar degree (34 and 33% of unsorted PBMCs), suggesting a significant proportion of these cells share the characteristics of the previously described CCR7+ T cells. In contrast, the ex vivo responses were only marginally affected by CCR7+ cell depletion (responses 78 and 94% of original), indicating these cells are mainly CCR7−. Depletion of CD8+ cells, as a control, did not diminish the responses and confirmed we were monitoring IFN-γ production from CD4+ T cells.

Discussion

Recently, it has been suggested that memory T cells may reveal distinct characteristics indicated by particular function, location, and phenotype (18, 25–28). These populations of cells can be differentiated in part by the presence or absence of the lymphotropic

with the accompanying development of culture-positive memory cells to the same epitope specificities suggests that in vivo these latter cells may be developing sequentially from the immediate effectors and, hence, there is a negative correlation between these two populations of cells (Fig. 1E).

The kinetics of these changes are different for particular individuals. However, the presence of robust central memory responses many years after viral clearance suggests these cells are

FIGURE 2. Stability of the central memory CD4+ T cells over time and contribution of CCR7+ T cells to this population. A, Representative experiment from subject 7, who had no ex vivo responses to these HCV epitopes, having cleared the virus >10 years previously. The cultured responses were repeated after 2 years. The same pattern emerged with epitope NS31245-1259 dominating over core141-155. B, The responses to NS31245-1259 were repeated with depletion of CCR7+ T cells. This markedly reduced the magnitude of the cultured response. Furthermore, addition of the retained CCR7+ cells from the depletion column to the cultures restored in part the responses.

FIGURE 3. The contribution of CCR7+ T cells to ex vivo and cultured responses. PBMCs were depleted of CCR7+ T cells and then assayed both ex vivo and after short-term culture. Depletion of CD8+ cells was used as a control. The results are standardized to a percentage of the control experiment (i.e., whole PBMCs). Depletion of the CCR7+ population mainly affected the cultured responses.
chemokine receptor CCR7. Memory CD4+ T cells residing in lymph nodes tend to require a period of restimulation (e.g., Ag and IL-2) before acquiring an effector function and have been termed by some as central memory T cells (25). In contrast, CCR7+ memory cells respond rapidly to Ag (demonstrating ex vivo cytotoxicity or IFN-γ release), may localize in peripheral nonlymphoid tissues, and have been termed effector memory T cells (25). It seems these nonviremic HCV patients may also develop separate populations of memory CD4+ T cells that share some of these characteristics with the measured ex vivo responses corresponding to effector memory T cells and the measured cultured responses to central memory T cells.

HCV offers a useful model to study these memory cells, because the infection can be eradicated by treatment and re-exposure to the pathogen is unlikely in the study population. HLA-DR11 has been associated with clearance of HCV in several studies (11). Having recently identified HCV-derived HLA-DR11-restricted epitopes (24), we were able to track memory cells in HLA-DR11+ patients using highly sensitive ELISPOT assays. The presence of a strong ex vivo response to particular epitope(s) did not necessarily correlate to a positive cultured response and, vice versa, ex vivo responses to these epitopes were absent in cases that were positive after culture, offering support to the concept that there are functionally distinct epitope-specific CD4+ memory T cell populations. The CCR7+ cells appear to contribute mainly to these cultured “central” memory HCV-specific T cells. Furthermore, there appears to be an inverse relationship between these two populations, with the evolution of a long-term, culture-positive population of memory T cells at the expense of the ex vivo population. The relationship and interdependence between these two categories of memory T cells is unclear. Whether long-term central memory cells develop from primary effector cells in a sequential fashion or in parallel is not known (29). Recently it has been proposed that in humans immediate effectors may develop from central memory cells (25). While this may be the case in vitro, the evolution of the responses measured prospectively suggests that the converse may apply in vivo after clearance of HCV.

There are contrasting results regarding the stability of CD4+ memory T cells after clearance of a pathogen (4, 30). However, the presence of robust central memory responses to HCV epitopes many years after viral clearance suggests these cells are entering a stable population. The range of HCV epitope-specific responses in these central memory cells, as well as the hierarchy indicated by frequency, also appear to be reasonably stable over time in this study.

A key question is whether one or both of these populations can confer protective immunity, and what factors are involved in their maintenance. It has been demonstrated for LCMV infection that activated memory cells are necessary for protection in peripheral sites, and that these cells require persistent exposure to Ag (31). It is possible that a reservoir of HCV Ag persists after viral clearance, and that this Ag maintains the effector memory population. The steady decline in the effector memory T cells and the development of the central T cells may mirror a diminishing supply of Ag. There is also indirect evidence in humans that persistent Ag aids the maintenance of long-term protective immunity against intracellular pathogens (32). Now that we have identified these populations of memory cells to a human pathogen, the challenge in the future is to identify whether these central memory cells confer long-term protection to a parenterally acquired infection like HCV.

References