Integration of apoptosis and telomere erosion in virus-specific CD8⁺ T cells from blood and tonsils during primary infection

Maria Vieira D. Soares, Fiona J. Plunkett, Caroline S. Verbeke, Joanne E. Cook, Jeff M. Faint, Lavina L. Belaramani, Jean M. Fletcher, Nicholas Hammerschmitt, Malcolm Rustin, Wolfgang Bergler, Peter C. L. Beverley, Mike Salmon, and Arne N. Akbar

Human-virus–specific CD8⁺ T cells that are found during primary infection have been studied almost exclusively in the peripheral blood, and it is unclear whether these cells are regulated in the same way as those in secondary lymphoid tissue. We investigated, therefore, the control of apoptosis and telomere erosion of Epstein-Barr virus (EBV)–specific CD8⁺ T cells found in the blood and tonsils of the same patients during acute infectious mononucleosis (AIM). Although the clonal composition of CD8⁺ T cells as determined by heteroduplex analysis was similar in both compartments, there was greater CD28 expression in the tonsil population, indicating that they were less differentiated. EBV-specific CD8⁺ T cells in both tissue types were extremely susceptible to apoptosis related to low Bcl-2 expression and were dependent on exogenous cytokines such as interleukin-2 (IL-2), IL-15, and interferon-α/β (IFN-α/β) for survival. In both compartments, however, these cells maintained their telomere lengths through telomerase induction. Thus, apoptosis-prone EBV-specific CD8⁺ T cells found during acute infection have to be rescued from death to persist as a memory population. However, signals that induce telomerase ensure that the rescued cells retain their replicative capacity. Significantly, these processes operate identically in cells found in blood and secondary lymphoid tissue. (Blood. 2004;103:162-167) © 2004 by The American Society of Hematology

Introduction

After primary infection in animals, virus-specific T cells found in different tissues are not identical. In addition, the extensive lymphadenopathy that occurs during Epstein-Barr virus (EBV) and other primary viral infections in humans suggests that the lymphoid tissue is a major site at which the virus-specific CD8⁺ T cells may be activated. However, most studies on EBV-specific CD8⁺ T cells during primary infection in humans have been performed on populations in peripheral blood only, and the relationship of these cells to those found in secondary lymphoid tissue is unknown. Important questions that remain unanswered are whether virus-specific CD8⁺ T cells of the same specificity are found in different anatomic sites, whether they are at the same stage of differentiation, and whether mechanisms that control their survival and persistence operate identically in different lymphoid compartments.

Apoptosis induced by cytokine deprivation and telomere erosion are 2 mechanisms that control the survival and replicative capacity of virus-specific CD8⁺ T cells after the primary response. Telomeres are repeating hexameric units of DNA and protein found at the ends of chromosomes. In the absence of compensatory mechanisms, repeated cell division results in critically short telomeres that signal growth arrest or replicative senescence. Previous studies have shown that peripheral blood populations of EBV-specific CD8⁺ T cells are susceptible to apoptosis. It is unknown, however, whether these cells are destined for clearance and those found in lymphoid tissues are preferentially selected for survival. In addition, it has been shown previously that there is considerable proliferation of the EBV-specific peripheral blood pool of CD8⁺ T cells during acute infection, and it has been proposed that the extent of this proliferation is sufficient to result in loss of replicative capacity caused by telomere erosion. Although EBV-specific CD8⁺ T-cell populations in peripheral blood may avoid this by inducing telomerase activity, it is unclear whether this is a general phenomenon that also applies to cells found in secondary lymphoid tissue during acute infectious mononucleosis (AIM).

For an efficient memory pool to persist after primary infection, not only must specific CD8⁺ T cells escape apoptosis, they must retain enough replicative capacity to allow subsequent proliferation in response to virus restimulation throughout life. In this study we demonstrate a close clonal relationship between EBV-specific CD8⁺ T cells in the blood and tonsils of the same patients during AIM. We also show that mechanisms that prevent apoptosis and telomere erosion operate equally in the CD8⁺ T cells from both compartments, and we suggest that both sets of signals have to be

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engaged during primary infection to enable the selection of specific cells that persist to form the memory pool.

**Patients, materials, and methods**

**Patient and control tissues**

Tonsil specimens were obtained from 26 patients in whom AIM was diagnosed. Fourteen patients with chronic tonsillitis with lymphofollicular hyperplasia were used as controls. Peripheral blood mononuclear cells (PBMCs) from 12 of the AIM patients and 6 of the control patients at the time of tonsillectomy were also studied. This study received ethical approval from the Royal Free Hampstead National Health Service (NHS) Trust (London) and the Ethics Committee of the Faculty of Clinical Medicine Mannheim, Ruprecht-Karls University of Heidelberg, where the tonsillectomies were performed.

**Sample isolation, preparation, and storage.**

Fresh tonsil specimens were cryopreserved in liquid nitrogen or were formalin-fixed and embedded in paraffin or were disaggregated with isolated mononuclear fractions cryopreserved in liquid nitrogen. PBMCs were prepared from heparinized peripheral blood at the time of tonsillectomy, and CD8+ T cells were isolated by negative selection using VARIO MACS (Miltenyi Biotech, Surrey, United Kingdom). EBV-specific CD8+T cells were isolated by positive selection using phycoerythrin (PE)-restricted peptide ligand RAKFKQLL. -restricted ligands GLCTLVALM and epitopes used were the HLA-A2

**Histologic analysis of tonsil samples**

Paraffin-embedded tonsillectomy specimens of 14 AIM patients (age range, 14-41 years; mean age, 22 years; 8 male/6 female) and 8 controls (age range, 12-34 years; mean age, 18.5 years; 4 male/4 female) with chronic tonsillitis were analyzed using immunoperoxidase staining for expression of Bcl-2, Bax, Bcl-x, and anti–Bcl-2 Fab fragments and CDP-Star substrate (Roche Diagnostics, Mannheim, Germany). Identical clones within CD8+ T-cell populations from blood and tonsils were detected as heteroduplex bands with identical migration patterns.22 The presence of heteroduplexes has previously been shown to relate to the presence of an expanded clone, as determined by sequencing of the PCR product.22

**Results**

**Relationship between EBV-specific T cells in blood and tonsils during primary infection**

We found expanded populations of EBV-lytic, epitope-specific CD8+ T cells in blood and tonsil compartments during AIM (Figure 1A). However, these cells constituted a greater percentage of CD8+ T-cell populations in blood than in tonsils in 5 of 6 patients investigated (not shown). We next compared CD8+ T cells during AIM in both tissue types using the heteroduplex technique (Figure 1B). We examined 23 different TCR Vβ families in each of 3 patients. With rare exceptions, no clones were found in CD8+ T-cell populations from healthy age-matched controls.22 A total of 237 expanded clones were detected in CD8+ T cells from the blood of 3 patients, whereas 270 clones were detected in the tonsils of the same patients. Of these, 92% ± 8% of

**Assessment of clonality of AIM T cells by heteroduplex analysis**

The clonal relationship between CD8+ T cells in blood and tonsils from AIM patients was determined by comparing the CDR3 region of different T-cell receptor (TCR) Vβ families in paired blood and tonsil CD8+ T-cell samples of patients. PCR was performed on 26 Vβ families using specific Vβ and common Cβ primers. Each sample PCR was mixed with a Vβ-matched DNA carrier, denatured, and renatured. The product was blotted and hybridized with a probe to the external Cβ region of the carrier. The probe was detected using anti–digoxigenin-AP Fab fragments and CDP-Star substrate (Roche Diagnostics, Mannheim, Germany). Identical clones within CD8+ T-cell populations from blood and tonsils were detected as heteroduplex bands with identical migration patterns.22

**Figure 1. Clonal analysis of expanded CD8+ T cells from AIM blood and tonsil samples.** (A) Representative dot plot of CD8 versus tetramer (RAK) staining in matched blood and tonsil samples. Heteroduplex analysis was used to assess the clonality of purified CD8+ T cells from matched blood and tonsil samples taken from 3 AIM patients. PCR reactions were performed for 23 different Vβ families in the blood and tonsils of each patient. (B) Data from a representative Vβ family showing heteroduplex bands shared between CD8+ T cell blood and tonsils, as indicated by solid arrows, are shown. Dashed arrow indicates the homoduplex band.
clones found in blood CD8+ T cells were also found in tonsil CD8+ populations. Conversely, 82% ± 10% of clones in tonsils were also found in blood. Collectively, these results indicate close identity between virus-specific CD8+ T cells in blood and tonsils during primary EBV infection.

We found that the EBV-lytic, epitope-specific CD8+ T cells and total CD8+ T cells from blood and tonsils of AIM patients expressed low levels of CD45RA and the CCR7 chemokine receptor, indicating that they underwent considerable expansion (Figure 2A). In contrast, 40 ± 7 (mean ± SEM of 3 experiments) control CD8+ T cells in blood and 46 ± 12 of these cells in tonsils expressed both CD45RA and CCR7, indicating that they are less differentiated. Most EBV-specific and total CD8+ T-cell populations in the blood and tonsils of AIM patients that lose CD45RA expression retain CD27 expression (Figure 2B). Large numbers of CD8+ T cells in control blood and tonsil samples expressed both CD27 and CD45RA, again indicating that these populations are less differentiated than equivalent populations found during AIM.

The loss of the costimulatory molecule CD28 has been shown to precede that of CD27 during progressive differentiation of CD8+ T cells.4,7,23-25 We found that although the total CD8+ and EBV-specific T-cell populations from AIM tonsils expressed 70% ± 10% and 57% ± 16% of CD27 and CD28 T cells, respectively, only 28% ± 2% and 24% ± 1% of these cells in blood expressed both molecules (Figure 2C). This indicates that virus-specific CD8+ T cells from tonsils are less differentiated than those from blood. These differences appear to be intrinsic to the EBV-specific CD8+ T-cell populations because control blood and tonsils show similar proportions of cells expressing both molecules (Figure 2C).

The observation that 18% of hetroduplex-defined clones are found in tonsils but not in blood (Figure 1), together with the observation that EBV-specific CD8+ T cells from tonsils are less differentiated than their blood counterparts (Figure 2), suggests that the expansion of specific CD8+ cells occurs initially in the secondary lymphoid tissue during AIM and that more differentiated cells enter the bloodstream preferentially. We are investigating the relationship between different lytic and latent epitope-specific CD8+ populations in paired blood and tonsil samples. Our preliminary observations indicate that EBV latent epitope-specific CD8+ T cells were also less differentiated in the tonsils than in the blood of the same patients during AIM (not shown).

Extensive CD8+ T-cell proliferation in AIM is related to extent of B-cell infection

EBER-positive cells were observed mainly in the stroma underlying the crypt epithelium and in the superficial interfollicular zone (Figure 3A, left). Combined in situ hybridization for EBERs and immunostaining confirmed the EBER+ cells to be CD3+CD20+ B cells in all patients in whom this was investigated. Immunostaining for Ki-67 revealed high proliferative activity in germinal centers (GCs) but not in the interfollicular zone (IFZ) or the mantle zone (MZ) of control tonsils (Figure 3A, middle panel). In contrast, there were significantly higher numbers of Ki-67+ cells in the IFZ from tonsils of AIM patients (Figure 3A, right; 3B). More than 99% of these cells were CD3+CD20+ cells. The mean CD4/CD8 ratio was 5-fold lower than in control tonsils, indicating a disproportionate expansion of CD8+ T cells (Figure 3C). The extent of interfollicular T-cell proliferation was significantly correlated with the number of EBV-infected B cells (Figure 3D). These results indicate that preferential CD8+ T-cell expansion during primary EBV infection is directly linked to the extent of B-cell infection by the virus.

CD8+ T cells from tonsils and blood are susceptible to apoptosis during AIM

The relationship between apoptosis and extent of EBV infection during AIM is unknown. In control tonsils, apoptotic activity was
AIM patients recovered from AIM (Figure 5A). We found that apoptosis was not with the same cells taken from persons between 3 and 10 years after apoptosis when cultured without exogenous cytokines compared specifically.

Figure 5. Total CD8 and antigen-specific T cells from AIM patients are susceptible to apoptosis in vitro but can be rescued by cytokines. (A) Freshly isolated PBMCs from AIM patients or from those with previous history of AIM were cultured in complete culture medium for 5 days. Survival of total CD8 and tetramer-positive CD8+ T cells was calculated as a percentage of the initial number of CD8+ or tetramer-positive T cells present before culture. Mean ± SEM of triplicate determinations from 3 separate experiments with similar results are shown. (B) Survival of CD8+ and EBV-lytic, epitope-specific CD8+ T cells cultured in the presence or absence of IL-2, IL-15, or type I IFN for 24 hours was investigated. Mean ± SEM percentage survival of 3 separate experiments is shown. (C) Expression of Bcl-2, Bcl-x, and Bax mRNA levels were determined by RT-PCR, performed over a range of cycles. Representative blot shows the level of expression in freshly isolated CD4+ and CD8+ T cells from AIM patients and the same CD8+ T cells cultured in the presence of IL-2 for 24 hours. :

predominantly observed in germinal centers (Figure 4A), and only 0.2% ± 0.07% of T cells in the IFZ were apoptotic. In contrast, a significantly higher proportion of T cells in the interfollicular T-cell zone of tonsils from AIM patients were apoptotic (1.7% ± 0.45%; P < .0001), and these cells were observed throughout this compartment (Figure 4B). This level of apoptotic activity was comparable to that observed in florid germinal centers of control tonsil tissue (1.8% ± 0.34%). EBER in situ hybridization coupled to TUNEL demonstrated that the apoptotic cells in the interfollicular T cell zone were not EBER-positive B cells (Figure 4B). In addition, many apoptotic bodies appeared to be localized together within vacuoles (Figure 4B) that were identified by CD68 staining to be the cytoplasm of macrophages (Figure 4C).

In control tonsils, the antiapoptotic molecule Bcl-2 was absent from germinal centers, whereas it was expressed at high levels by cells in the MZ and IFZ (54% ± 12%; Figure 4D). However, this protein was significantly decreased in T-cell areas but not in the MZ of AIM tonsils (33% ± 10%; P < .0012; Figure 4E). Levels of T-cell apoptosis (r = 0.65; P = .01) and Bcl-2 expression (r = 0.72; P = .003) in the in the IFZ were significantly correlated with the number of infected B cells present in 14 AIM tonsils investigated.

Cytokine-mediated rescue of CD8+ T cells from apoptosis in AIM patients

Peripheral blood populations of CD8+ and EBV-lytic, epitope-specific T-cell populations of AIM patients were susceptible to apoptosis when cultured without exogenous cytokines compared with the same cells taken from persons between 3 and 10 years after recovery from AIM (Figure 5A). We found that apoptosis was not inevitable and that cytokines such as IL-2, IL-15, and interferon-β (IFN-β) could promote the survival of total CD8+ or EBV-lytic, epitope-specific CD8+ T cells of AIM patients (Figure 5B) and of patients with chronic EBV infection (not shown). The susceptibility to apoptosis of CD8+ T cells from the peripheral blood of AIM patients was also related to low Bcl-2 expression, and rescue from apoptosis by IL-2 resulted in an increased level of Bcl-2 and a second antiapoptotic molecule, Bcl-XL, but not the proapoptotic molecule Bax (Figure 5C). This suggests that virus-specific CD8+ T cells found in blood and secondary lymphoid tissue during AIM are susceptible to apoptosis. However, some cells can be rescued from death by cytokines, and this may allow a memory pool to this virus to persist.

Extensive T-cell proliferation does not lead to telomere loss during AIM

The calculated net expansion of EBV-specific CD8+ T cells during AIM is sufficient to lead to replicative senescence resulting from telomere erosion. We next investigated whether this was a constraint on the virus-specific CD8+ T cells in the blood and tonsils of patients during AIM. In healthy persons, CD4+ T cells have longer telomeres than does the CD8+ subset. However, despite preferential CD8+ T-cell expansion (Figure 3C), CD8+ T cells from blood and tonsils had consistently longer telomeres than the CD4+ populations in the same persons (Figure 6A). This increase in telomere length relative to the CD4+ population was also observed when isolated populations of EBV-lytic, epitope-specific CD8+ T cells from blood and tonsils were compared with CD4+ T cells (Figure 6B). CD8+ T cells from AIM patients that were not
specific for the lytic epitopes investigated also had longer telomeres than the CD4+ T-cell population. This is probably because other EBV-specific CD8+ T cells with different specificities are also found within this population.19 A no-probe control (Figure 6B) that showed minimal fluorescence was also used in blood and tonsil samples to demonstrate the specificity of binding.

We found that the increase in telomere length was caused by an up-regulation of the enzyme telomerase in CD8+ T cells from blood and tonsils and in EBV-lytic, epitope-specific CD8+ T cells isolated from both tissue types (Figure 6C-D). The similar level of telomerase induction in CD8+ T cells specific for only 1 EBV protein and found in the CD8+ T-cell population as a whole in the same AIM patients suggests that most enzymatic activity observed in AIM is found within EBV-specific CD8+ T cells. Collectively these results demonstrate that preventing apoptosis and inducing telomerase are 2 mechanisms that have to be integrated when selecting the EBV-specific CD8+ T-cell pool after AIM.

**Discussion**

We have found that during primary infection, EBV-lytic, epitope-specific CD8+ T cells in blood and tonsils are closely related clonally and are regulated similarly by apoptosis and telomere erosion. Hence, EBV-specific CD8+ T cells in the circulation are representative of those in secondary lymphoid tissue during primary infection, which validates previous studies performed on blood populations only.4,4 An important observation was that both the proliferation and the susceptibility to apoptosis of the EBV-specific CD8+ T cells in tonsils was related to the extent of B-cell infection, indicating an antigen-driven component in the immune expansion and in the death of CD8+ T cells during AIM. We are performing a detailed analysis of the relationship between the extent of lytic and latent virus expression and the nature of the specific CD8+ T-cell response in these samples.

Although the expanded populations of virus-specific CD8+ T cells during AIM are programmed to die, they can be rescued by cytokines, and maintaining the telomere lengths of these cells ensures that those rescued from death will keep their replicative capacity. Nevertheless, the most highly expanded clones of virus-specific cells may be lost after primary infection, and this may be attributed to telomere erosion.15,17 This raises the possibility that telomerase induction may not compensate indefinitely for telomere loss in virus-specific CD8+ T cells,27 and is supported by the development of shorter telomeres in EBV-specific CD8+ T cells studied in the same patients during and 14 years after AIM.17

Important questions that remain to be addressed are how telomere-related replicative senescence and apoptosis are regulated in EBV-specific CD8+ T cells during chronic infection. Previous studies have shown that Bcl-2 is up-regulated on virus-specific memory CD8+ T cells, indicating that they are less susceptible to apoptosis than those found during acute infection.5,26 However, it is unclear whether the increase in Bcl-2 is caused by the influence of cytokines such as IL-2 and IL-15, which induce the survival and up-regulation of this molecule,20,29 or whether it is caused by a reprogramming of apoptosis in the virus-specific CD8+ T-cell population.8 Nevertheless, the apoptosis-resistant phenotype has also been observed in T cells that have been aged in vitro,30 suggesting that although apoptosis plays an important role during acute infection, this process may not be the major constraint on the persistence of virus-specific CD8+ T cells during the chronic phase of the disease.

The extent of apoptosis observed in the tonsils of AIM patients is dependent on the availability of antiapoptotic cytokines, and we are investigating the expression of cytokines that regulate death in tonsils from patients with AIM. However, the rate of clearance of apoptotic CD8+ T cells by macrophages determines the level of apoptosis observed at any given time.31 We have found that numerous apoptotic cells in AIM tonsils are found within macrophages, suggesting that there is a high incidence of death coupled to intensive clearance of these cells in situ.

Telomere levels in virus-specific CD8+ T cells decrease during chronic infection,17 but it is unknown whether this is sufficient to impose a limit on the persistence or replication of virus-specific memory CD8+ T cells in elderly persons. The loss of telomeres is related to the decreased ability of T cells that are repeatedly activated to up-regulate telomerase.11,32 Studies in most murine systems may not provide an accurate portrayal of the impact of telomere erosion on immune memory in humans because of the 10-fold longer telomeres found in mice33 and because of their much shorter lifespans. Nevertheless, the observation that sixth-generation telomerase-deficient mice show signs of premature aging34 and of dramatic reductions in the numbers of germinal centers formed after immunization35 does point to a role for telomere erosion in regulating immune responses.
A role for telomerase in regulating the replicative capacity of lymphocytes has been demonstrated in studies in which transfection of the catalytic unit of the enzyme prolongs replicative potential\(^{36-38}\) and restores cytotoxic function\(^{19}\) in human T lymphocytes.

The development of new murine models that have short telomeres may provide representative data on whether telomere erosion constrains memory T-cell persistence in humans.\(^{18}\) Given that the human life expectancy worldwide is increasing,\(^{40}\) the memory T-cell pool to infectious agents will have to be maintained for increasing periods in the future. An important question is whether this will ultimately lead to an increased incidence of infection caused by the development of replicative senescence in antigen-specific T-cell pools that have to be maintained for more than 8 decades in vivo.\(^{41}\)

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**References**


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