Tonsillar homing of Epstein-Barr virus–specific CD8+ T cells and the virus-host balance

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Patients with infectious mononucleosis (IM) undergoing primary EBV infection show large expansions of EBV-specific CD8+ T cells in the blood. While latent infection of the B cell pool is quickly controlled, virus shedding from lytically infected cells in the oropharynx remains high for several months. We therefore studied how responses localize to the tonsil, a major target site for EBV, during primary infection and persistence. In acute IM, EBV-specific effectors were poorly represented among CD8+ T cells in tonsil compared with blood, coincident with absence of the CCR7 lymphoid homing marker on these highly activated cells. In patients who had recently recovered from IM, latent epitope reactivities were quicker than lytic reactivities both to acquire CCR7 and to accumulate in the tonsil, with some of these cells now expressing the CD103 integrin, which mediates retention at mucosal sites. By contrast, in long-term virus carriers in whom both lytic and latent infections had been controlled, there was 2- to 5-fold enrichment of lytic epitope reactivities and 10- to 20-fold enrichment of latent epitope reactivities in tonsil compared with blood; up to 20% of tonsillar CD8+ T cells were EBV specific, and many now expressed CD103. We suggest that efficient control of EBV infection requires appropriate CD8+ T cell homing to oropharyngeal sites.

Introduction

EBV, a γ1-herpesvirus widespread in the human population, is the causative agent of infectious mononucleosis (IM), a self-limiting lymphoproliferative disease, and is etiologically linked to a number of lymphoid and epithelial malignancies (reviewed in ref. 1). Primary infection occurs by the oral route and, at least as witnessed in IM patients, is characterized by extensive replication of the virus in permissive cells, probably mucosal epithelial cells closely associated with pharyngeal lymphoid tissues (2); this leads to the release of high titers of infectious virus into the throat. At the same time, the virus initiates latent growth-transforming infections within the B cell pool, best visualized in tonsils from IM patients, where immunohistochemical staining has revealed large numbers of latent antigen-positive B lymphoblasts in extrafollicular areas of the tissue (3, 4). This virus-driven expansion generalizes the infection and establishes a lifelong reservoir of latently infected memory B cells that preferentially populate the mucosal lymphoid tissues of the Waldeyer ring (5, 6). The majority of these infected cells have by then returned to the resting state and have downregulated expression of most if not all EBV latent cycle proteins. However their occasional reactivation into the productive (lytic) cycle is thought to reseed foci of virus replication at oropharyngeal mucosal sites, which explains why many asymptomatic virus carriers continue to shed low levels of infectious virus detectable in throat washings; furthermore, virus produced at these sites is thought also to initiate new growth-transforming infections in locally infiltrating B cells (7).

These events appear to be controlled in part by immune T cell surveillance. Thus, primary infection as seen in IM patients is associated with a large expansion of CD8+ T cell numbers in the blood, and recent work has shown that many of these activated CD8+ T cells are EBV specific. The most abundant responses have been mapped by HLA class I tetramer staining to particular viral epitope peptides derived from proteins of the EBV lytic cycle, with smaller responses directed against latent protein epitopes (8, 9). As the infection subsides, these epitope-specific CD8+ T cell populations in the blood fall to lower numbers and switch from the original phenotype of activated effector cells (expressing the activation marker CD38 and lacking central memory markers such as CCR7) to a resting CD38-negative phenotype. We and others (9–12) have found that lytic and latent epitope responses not only show different kinetics of expansion and contraction over the course of primary infection but also, with resolution of symptoms, rapidly acquire somewhat different memory cell phenotypes. These differences are also apparent in memory populations in the blood of long-term virus carriers. Thus, the lytic epitope–specific population tends to retain more cells in the CCR7-effector memory compartment (with some of these cells switching from CD45RO to CD45RA expression) and have fewer cells entering the CD45RO+CCR7+ central memory pool.

Correlating these detailed parameters of the immunological response with coincident virological events is difficult because, as in most studies of virus infection in humans, all available

Nonstandard abbreviations used: CLA, cutaneous lymphocyte-associated antigen; EBNA, EBV nuclear antigen; ELISpot, enzyme-linked immunospot; IM, infectious mononucleosis; SFC, spot-forming cell.

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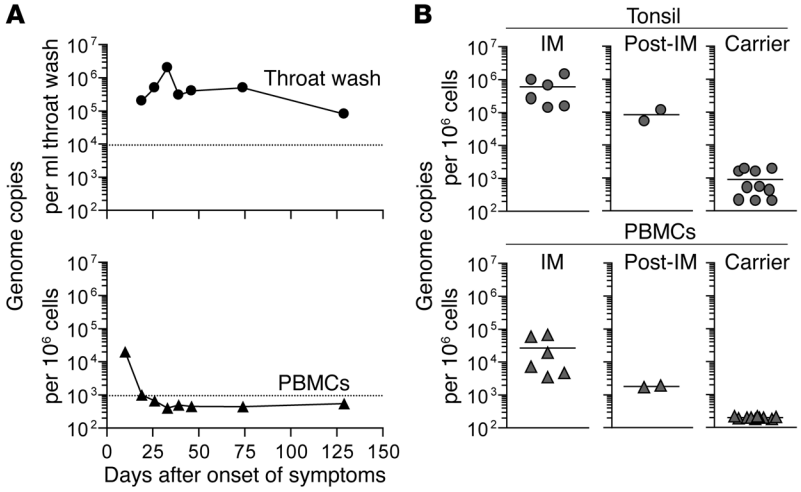

Figure 1
EBV genome load quantitation in IM patients, post-IM patients, and long-term carriers. (**A**) EBV genome copies in 1 ml of concentrated throat washing (upper panel) and 10^6 PBMCs (lower panel) from an IM patient (IM-171) from whom samples were taken during acute disease (days 0–14) and at intervals up to 4 months later. Upper limits of genome levels in throat washing and blood samples from healthy carriers are indicated by the dashed lines. (**B**) EBV genome copies were estimated in 10^6 tonsillar cells (upper panels) or 10^6 PBMCs (lower panels) from tonsillectomy patients who were either undergoing acute IM or recently recovered from IM (post-IM) or were long-term carriers.

immunological readings come exclusively from peripheral blood and not from the principal anatomical site of virus infection. The limitations of assays solely on blood are best illustrated by recent studies of herpes simplex virus (HSV), an agent that typically replicates in skin epidermis. These show that HSV-specific $CD8^+$ T cells are low in peripheral blood but highly enriched within the $CD8^+$ T cell subset expressing the cutaneous lymphocyte-associated antigen (CLA) and selectively homing to the skin (13, 14). The issue of T cell homing is particularly germane to EBV infection, wherein virus replication and also latent infection preferentially involve cells in the Waldeyer ring. Little is known about immune controls at this location. However, we suspected that such controls may be suboptimal in acute IM because IM patients have large numbers of lytic epitope–specific $CD8^+$ T cells in the blood, yet viral shedding in the throat reportedly remains high for several months, long after the resolution of disease symptoms (15). Here we compare $CD8^+$ T cell responses to EBV in the blood with those seen at 1 of the major oropharyngeal sites of infection, the tonsil, in paired samples from 3 different types of donor: acute IM patients, recently recovered post-IM patients, and long-term carriers. In all 3 cases, the situation in tonsil is different from that in the blood, and in each case, the tonsillar picture provides a much more informed view of the virus-host balance.

Results

Evolution of viral loads in the oropharynx and PBMCs after acute IM. In an initial series of experiments, we monitored levels of EBV infection in the oropharynx and in the circulating B cell reservoir during and after acute primary infection. Throat washing and peripheral blood samples were taken from the time of acute IM through

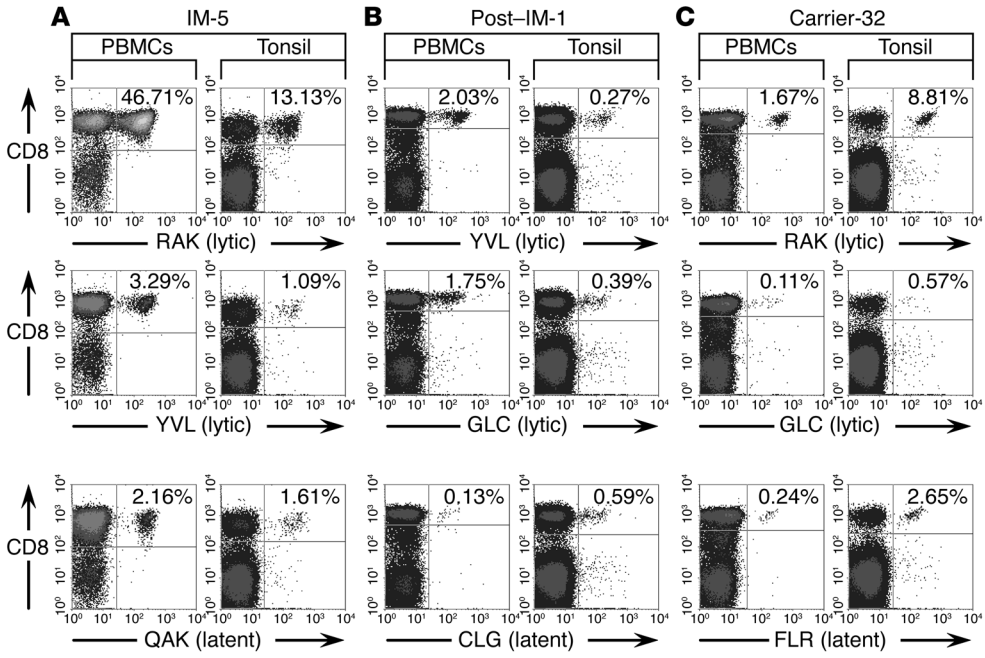

Figure 2
Frequency of EBV-specific $CD8^+$ T cells in PBMCs and matched tonsil preparations from IM, post-IM, and long-term carrier tonsillectomy patients. (**A**) PBMCs (left panels) and tonsillar cells (right panels) from acute IM patient IM-5 were stained with either HLA-B*0801 tetramers containing the lytic cycle epitope RAK or latent cycle epitope QAK peptides or the HLA-A*0201 tetramer containing the lytic cycle epitope YVL peptide and subsequently stained with anti-CD8 mAbs. Values shown refer to the percentage of $CD8^+$ T cells that stained with the tetramer. (**B**) PBMCs and tonsillar cells from post-IM-1 were stained as described above with HLA-A*0201 tetramers containing either the lytic cycle epitope YVL or GLC or the latent cycle epitope CLG peptides. (**C**) PBMCs and tonsillar cells from carrier-32 were stained as described above with the HLA-B*0801 tetramers containing the lytic cycle epitope RAK or latent cycle epitope FLR peptides or the HLA-A*0201 tetramer containing the lytic cycle epitope GLC peptide.

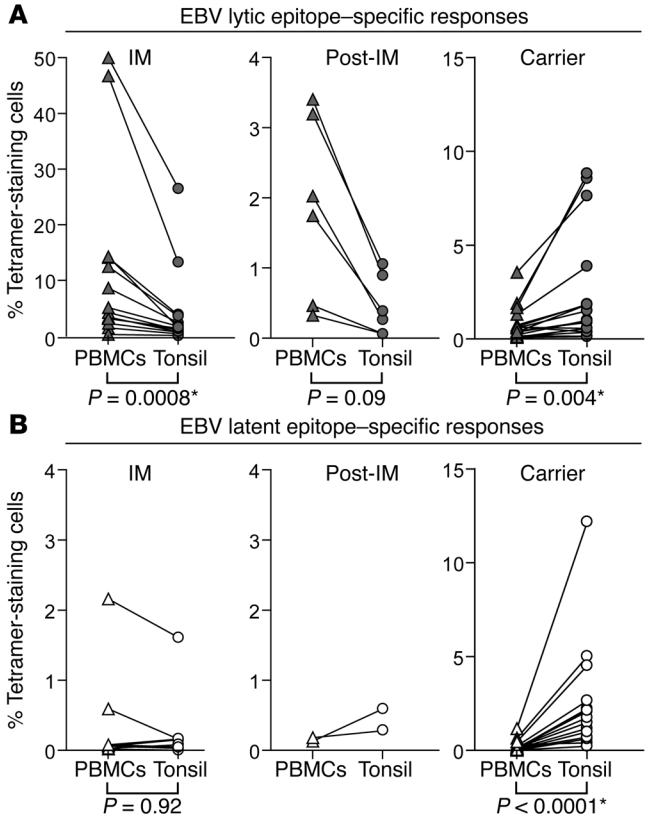

Figure 3
Summary of the frequency of EBV-specific responses in the PBMCs and tonsils of 6 IM, 2 post-IM, and 11 long-term carrier tonsillectomy patients; symbols connected by a line refer to a particular epitope-specific response in an individual donor. (**A**) Percentage of $CD8^+$ T cells staining with EBV lytic epitope–specific tetramers in IM (left panel) and post-IM (middle panel) patients and long-term carriers (right panel). (**B**) Percentage of $CD8^+$ T cells staining with EBV latent epitope–specific tetramers in IM (left panel) and post-IM (middle panel) patients and long-term carriers (right panel). *P* values shown were obtained using linear mixed model analysis as described in Methods. Significant differences are indicated by an asterisk. In addition, in acute IM, the reduction in epitope-specific T cell representation in tonsil relative to blood was significantly greater for lytic than for latent reactivities ($P = 0.004$); also, in carriers, the increase in epitope-specific T cell representation in tonsil relative to blood was significantly greater for latent than for lytic epitopes ($P = 0.003$).

convalescence and at intervals over a further 4–6 months. Figure 1A illustrates the data from a representative patient, IM-171; EBV DNA loads were determined by quantitative PCR assay in a standard volume (1 ml) of the throat washing sample and in a standard number (10^6) of PBMCs. The high viral loads detected in PBMCs during acute disease fell substantially during the next 2 weeks to a value that lay toward the top end of the normal range shown by healthy virus carriers and were subsequently maintained at this level. By contrast, the high viral loads present in throat washings during acute disease showed no significant reduction over the entire course of study, always remaining well above the normal range of virus shedding shown by healthy carriers. These results are in accord with a recent detailed study showing that high oropharyngeal levels of virus shedding are maintained for many months following IM, long after resolution of symptoms and the concomitant fall in latently infected B cell load in the blood (15).

The main body of experiments in the present work analyzed matching tonsil and blood mononuclear cell samples from 6 patients tonsillectomized during acute IM, 2 patients tonsillectomized at approximately 3 months after IM, and 11 tonsillectomy patients who had no history of EBV-related disease but were long-term virus carriers. Figure 1B shows the EBV DNA loads in 10^6 tonsillar lymphocytes (upper panels) and 10^6 PBMCs (lower panels) from the 3 groups of patients. The acute IM patients showed very high EBV loads at both sites, as expected. The post-IM patients showed tonsillar EBV loads around 5-fold lower than the acute IM values but still 100-fold higher than long-term carrier values, while PBMC EBV loads in post-IM patients were around 20-fold lower than the acute IM values, at the very top of the normal range. By comparison, the long-term virus carriers all had low loads both in tonsillar lymphocytes and in PBMCs, their mean values being, respectively, about 500-fold and 200-fold lower than the mean values in acute IM patients. These virological assays confirm that the cell preparations collected for immunological analysis showed levels of EBV infection consistent with those expected for the 3 different patient groups.

EBV-specific $CD8^+$ T cell numbers in blood and tonsil with evolution of the infection. We next monitored the EBV-specific $CD8^+$ T cell numbers in the same blood and tonsillar preparations by staining with HLA class I tetrameric complexes specific for EBV lytic or latent epitopes; this was followed by anti-CD8 mAb staining and flow cytometric analysis.

Matching PBMCs and tonsil preparations from 6 acute IM patients were studied. In each case, as already described in an earlier work (9), HLA class I tetramer staining of the PBMCs showed high levels of EBV-specific T cells in this compartment. However, these cells were always represented to a much lesser extent in tonsillar $CD8^+$ populations. Results for 1 representative patient, IM-5 (positive for HLA-B*0801 and HLA-A*0201) are shown in Figure 2A. In the blood, responses to the immunodominant HLA-B*0801–restricted RAK and HLA-A*0201–restricted YVL lytic cycle epitopes constituted, respectively, 46.71% and 3.29% of the $CD8^+$ T cells in PBMCs, with cells specific for the HLA-B*0801–restricted latent cycle QAK epitope constituting 2.16% of the $CD8^+$ population. When tonsillar cells from the same patient were analyzed in parallel, responses to all 3 epitopes were significantly lower than those in blood (Figure 2A, right column). Thus, RAK and YVL responses in the $CD8^+$ T cell pool were reduced 3- to 4-fold to 13.13% and 1.09%, respectively, while the latent epitope response was reduced less markedly, to 1.6%. The relative paucity of EBV-specific cells in the tonsil compared with blood of IM-5 was also apparent when the cells were tested in overnight enzyme-linked immunospot (ELISpot) assays of epitope peptide–induced IFN-γ release and results expressed as number of IFN-γ spot-forming cells (SFCs) per 10^5 $CD8^+$ T cells (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI24810DS1). Note that this functional assay is useful for comparing relative numbers of responsive cells in the 2 populations, but in each case likely underestimates the true frequency of such cells because activated primary effectors are highly prone to apoptosis in vitro. Tetramer analysis of samples from an additional 5 acute IM patients confirmed low levels of lytic and latent epitope responses in tonsil compared with blood (see Figure 3).

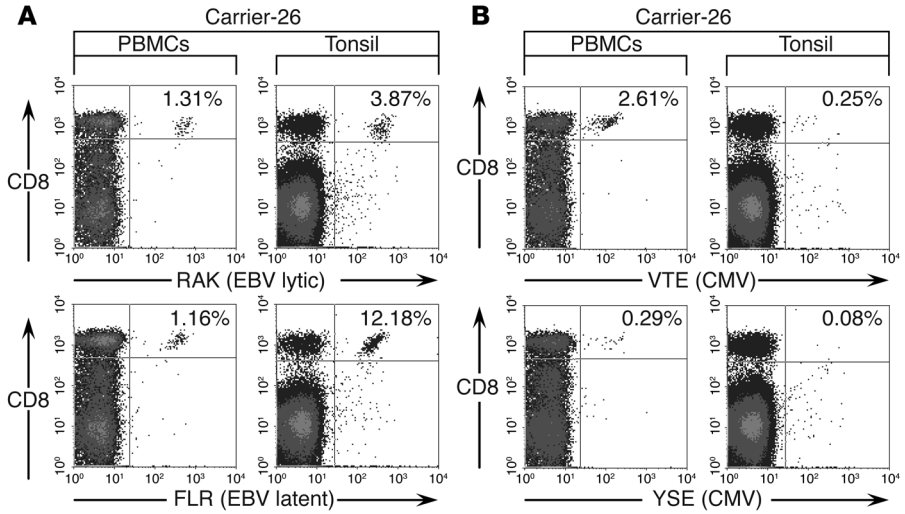

Figure 4
Representation of EBV- and CMV-specific CD8+ T cells in PBMCs and tonsil of a long-term EBV/CMV carrier. PBMCs (left panels) and tonsillar cells (right panels) were stained as before. (**A**) Cells were stained with HLA-B*0801 tetramers containing the EBV lytic epitope RAK and latent epitope FLR peptides. (**B**) Cells were stained with HLA-A*0101 tetramers containing the CMV peptides VTE and YSE. Results are presented as in Figure 2.

We next examined EBV-specific responses in 2 patients who underwent tonsillectomy 3 months following IM. They showed a consistent pattern of results, which was distinct from that seen in patients with acute disease. Data from 1 of these patients, the HLA-A*0201–positive individual post-IM-1 are shown in Figure 2B. In this case, PBMCs and tonsillar preparations were stained using the HLA-A*0201 tetramers refolded with the YVL and GLC lytic epitope peptides and with the CLG latent epitope peptide. In the blood, the lytic epitope–specific T cells constituted 2.03% and 1.75% of the CD8 population, respectively; such values are typical of those of an earlier study in which the blood of HLA-A*0201–positive patients was analyzed in the months following IM (9). Once again, as in acute IM, representation of these same responses in tonsillar CD8+ T cells was reduced, in this case by 4- to 7-fold. In contrast, the response to the latent CLG epitope, usually regarded as a subdominant response on the basis of its frequency in PBMCs, was detected at a typically low level (0.13%) among CD8+ T cells in the blood but was enriched approximately 4-fold in the tonsil. These different patterns were also apparent when post-IM PBMC and tonsillar preparations were screened in IFN-γ ELISpot assays for functional responses to epitope peptide stimulation. Representative results from post-IM-1 are shown in Supplemental Table 1. The response to the YVL lytic epitope was represented at a low level in tonsil compared with blood, whereas responses both to CLG and to a stronger latent epitope for which tetramers were not available, the HLA-B*4402–restricted EEN epitope from the EBV nuclear antigen 3C (EBNA3C), were clearly amplified in the tonsil.

These findings showed that EBV-specific T cells were differentially distributed between blood and tonsil and implied that their relative distribution between these 2 sites may change over time. To further explore this idea, we next examined blood and tonsil samples from 11 long-term EBV carriers in whom both lytic and latent infections had been brought under control. In these donors, levels of EBV epitope–specific T cells in the blood were typical of those seen in earlier studies of healthy carriers (9, 16). However, all such reactivities were found to be significantly enriched in the matching tonsil specimens. Figure 2C shows FACS plots of a representative HLA-A*0201, B*0801–positive donor, carrier-32. Tetramer staining identified 1.67% and 0.11% of CD8+ T cells in the blood as specific for the HLA-B*0801/RAK and HLA-A*0201/GLC lytic epitopes, respectively, and 0.24% as specific for the latent cycle HLA-B*0801/FLR epitope. In the tonsil, these percentages were increased dramatically, by 5-fold for the 2 lytic cycle epitopes and by 11-fold for the latent cycle epitope. Collectively, therefore, these 3 responses accounted for almost 12% of tonsillar CD8+ cells in this long-term carrier. Again, we were able to confirm the enrichment of EBV-specific T cells in tonsillar tissue using the ELISpot assay of IFN-γ release as a functional readout. Representative results from the HLA-A*0201–positive carrier-25 and the HLA-B*0801–positive carrier-27 are shown in Supplemental Table 1. Carrier-25 responses to the lytic YVL and latent CLG epitopes were enriched in tonsillar CD8 populations by 6-fold and more than 40-fold, respectively; carrier-27 responses to the lytic RAK and latent FLR epitopes were enriched by 11-fold and 28-fold, respectively.

The results of tetramer staining analysis from all 3 donor groups are summarized in Figure 3. These reflect the cumulative data for responses to 5 lytic epitopes (Figure 3A) and 5 latent epitopes (Figure 3B), in each case restricted through 1 of 3 different HLA class I alleles. Each donor group showed its own specific pattern of results. In results consistent with and extending those of a previous study (17), acute IM patients showed very strong representation of lytic and to a lesser extent latent epitope responses in the blood, while these responses were decreased in the tonsil by 3- to 4-fold for the lytic reactivities and to a lesser extent for the latent reactivities. The post-IM patients showed lower levels of EBV lytic reactivities in the blood, but, again, all 6 responses analyzed were reduced by 3- to 8-fold in the tonsil; yet in the same post-IM patients, the 2 latent epitope–specific responses we were able to study both showed enrichment in tonsillar CD8 populations. In the long-term carrier group, the situation was different again. Here both sets of responses were enriched in tonsil compared with blood, by a factor of 2- to 4-fold for lytic epitope reactivities and 10- to 20-fold for latent reactivities.

Comparison of EBV-specific and CMV-specific CD8+ T cell distributions. We reasoned that the accumulation of EBV-specific CD8+ T cells in the tonsils of long-term carriers might reflect a trend common to all responses induced by persistent viruses. To check this, we studied particular donors who were persistently infected with both EBV and with CMV, a β-herpesvirus that is carried as a more generalized infection of myeloid and other cell types. Figure 4 shows the results from 1 such dually infected donor, the HLA-A*0101, B*0801–positive

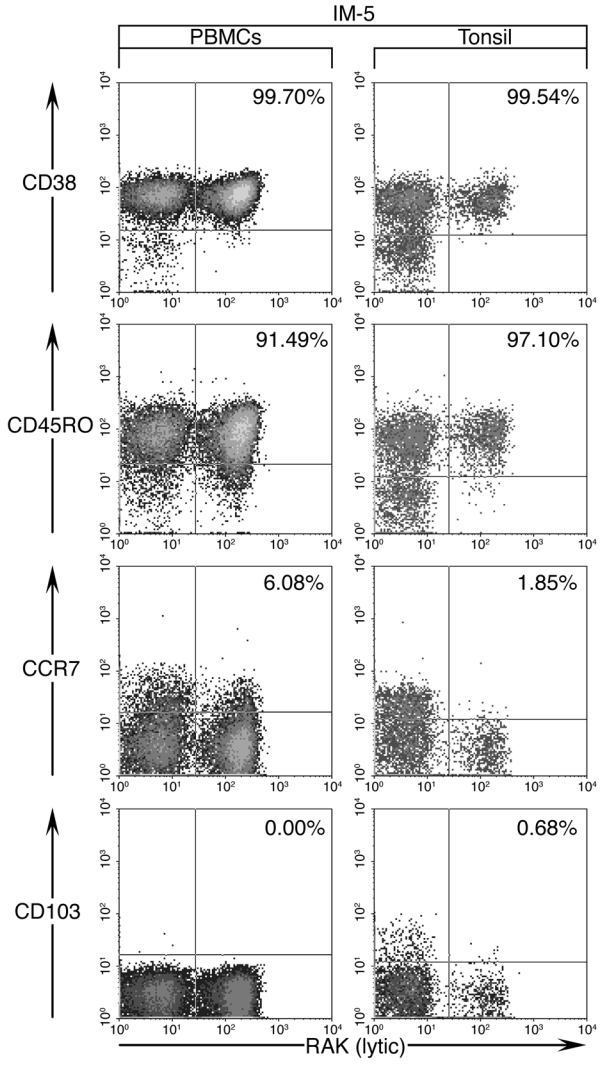

Figure 5
Analysis of EBV-specific CD8+ T cells from an acute IM patient for CD38, CD45RO, CCR7, and CD103 status. PBMCs (left panels) or tonsillar cells (right panels) from IM-5 were stained with the HLA-B*0801 tetramer containing the lytic epitope RAK peptide, followed by mAbs specific for CD8 and the relevant marker. Flow cytometric analysis was performed after gating on CD8+ cells, and profiles show tetramer staining versus the third marker (CD38, CD45RO, CCR7, and CD103). Values shown refer to the percentage of tetramer-positive cells that express the relevant marker.

carrier-26. Memory T cell responses to the HLA-B*0801–restricted EBV lytic (RAK) and latent (FLR) epitopes were enriched in tonsil compared with blood by factors of 3- and 10-fold, respectively. Indeed, a second HLA-B*0801 latent epitope (QAK) was likewise amplified almost 10-fold in tonsil (data not shown), such that collectively these 3 reactivities accounted for 20% of all CD8+ T cells in the tonsil. The contrast between this and the distribution of the CMV-specific CD8+ T cells was marked. Tetramer staining showed that memory CD8+ T cells specific for 2 HLA-A*0101–restricted CMV epitopes, the pp50-derived VTE and pp65-derived YSE, were well represented among CD8+ T cells in the blood, but were 3- to 10-fold less frequent among tonsillar CD8+ T cells.

Phenotype of EBV-specific CD8+ T cells in blood and tonsil with evolution of the infection. Having mapped the distribution of EBV-specific T cells between blood and tonsil in individuals at 3 different stages of EBV infection, we now sought to determine the phenotype of these T cells in terms of markers of effector function, differentiation, and migratory capacity. These included CD38, a marker of activated effector cells (8); CD45RO, a CD45 isoform expressed by both activated cells and antigen-experienced memory cells (18); and CD45RA, an isoform historically associated with naive T cells but now also shown to be reexpressed on some antigen-experienced cells (19). Expression of the 2 isoforms was inversely related throughout, so data are only shown for CD45RO. We also stained for the costimulatory molecules CD27 and CD28 (20, 21), whose downregulation is thought to be associated with terminal differentiation (22), and for a range of migratory markers. These included CCR7 and CD62-l, whose expression identifies central memory cells with the ability to migrate to lymphoid tissues (23); CCR9 and $\alpha 4\beta 7$, both characteristic of cells migrating to gut-associated lymphoid tissue (24, 25); CCR4 and CLA, which are characteristic of cells migrating to the skin (26, 27); CCR10, a chemokine receptor whose ligands are expressed by a diverse range of epithelial cells (28, 29); and CD103, an integrin characteristic of cells retained at mucosal epithelial sites (30).

Preparations from PBMCs and tonsil were stained with EBV-specific tetramers, followed by mAbs specific for CD8 and the relevant surface marker. Figure 5 illustrates typical phenotype data obtained from acute IM patients, in this case from the HLA-B*0801–positive patient IM-5. Whether in blood or tonsil, cells specific for the immunodominant RAK lytic epitope showed a highly activated phenotype, expressing CD38 and CD45RO in the absence of CD45RA. As previously described (17), CD27 and CD28 were downregulated in a significant fraction of these EBV-specific cells in the blood, although such downregulation was less apparent in the tonsil (data not shown). These activated cells were largely devoid of homing markers, in particular the lymphoid homing markers CCR7 and CD62-l, and also lacked the CD103 marker of epithelial retention. These findings were typical not only of the large lytic epitope–specific populations but also of the generally smaller populations of latent epitope–specific cells seen in IM (data not shown).

A different picture was observed when we studied EBV-specific reactivities in the blood and tonsil of 2 post-IM patients, both 3 months after disease resolution. Figure 6 shows representative data from 1 of these patients, the HLA-A*0201–positive post-IM-1. By this time, most lytic (YVL; Figure 6A) and latent (CLG; Figure 6B) epitope-specific cells in the blood had lost CD38 expression, but interestingly, in the tonsil, 45–50% of these same epitope-specific populations remained CD38 positive, which suggests their continued antigen-driven activation. In terms of CD45 isoform status, EBV lytic epitope–specific cells in the blood were heterogeneous for expression of these isoforms, with more than half now losing CD45RO and gaining CD45RA, whereas all latent epitope–specific cells remained CD45RO positive; these differences are in accord with our earlier findings on blood samples from post-IM patients (9). In the tonsil, however, we found that both lytic and latent reactivities remained CD45RO positive. Differences were also observed in the lymphoid migration markers on lytic versus latent epitope reactivities in post-IM blood; CCR7 (and CD62-l; data not shown) were essentially absent from the

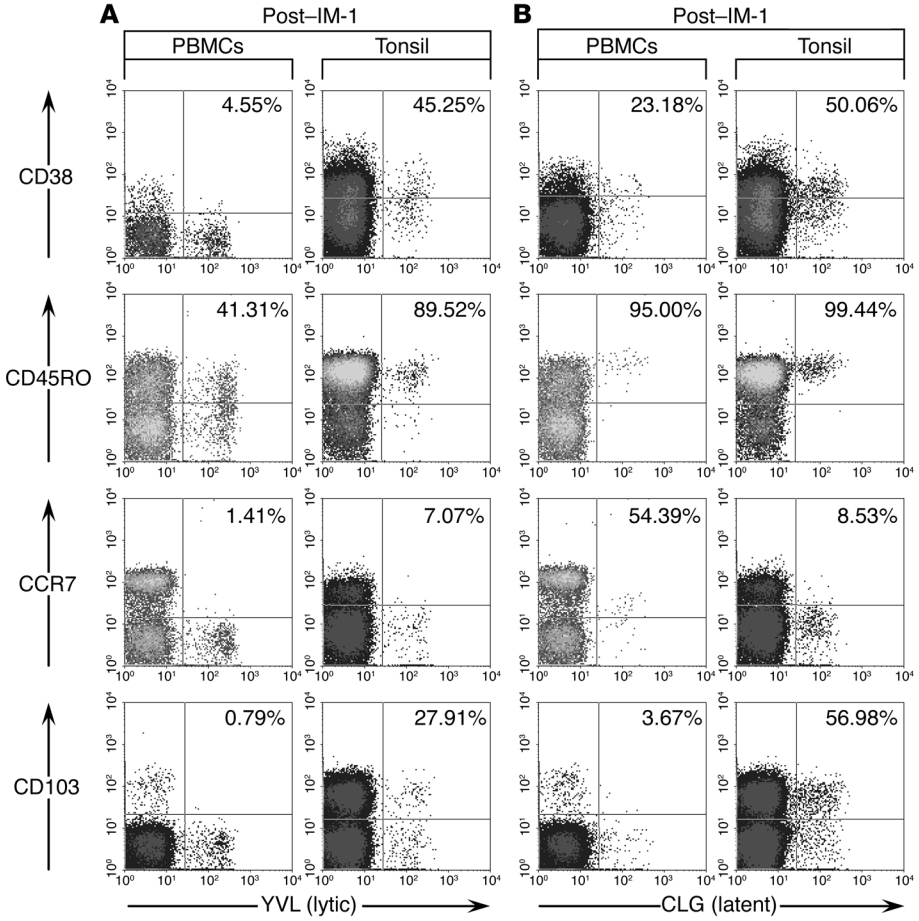

Figure 6
Analysis of EBV-specific CD8+ T cells from a patient recently recovered from acute IM for CD38, CD45RO, CCR7, and CD103 status. PBMCs (left panels) or tonsillar cells (right panels) from post-IM-1 were stained with HLA-A*0201 tetramers containing either the lytic cycle epitope YVL peptide (**A**) or the latent cycle epitope CLG peptide (**B**), as well as with antibodies specific for CD38, CD45RO, CCR7, and CD103. Flow cytometric analysis was performed after gating on CD8+ cells, and profiles are presented as in Figure 5.

lytic epitope population, whereas more than 50% of latent epitope–specific cells had reacquired both markers by this time. These differences were coincident with the increased representation of latent but not lytic epitope responses in the tonsil. Most important were the findings from CD103 staining. While very few if any EBV-specific T cells in the blood expressed this marker, in the tonsil, around 28% of the lytic epitope population and 57% of latent epitope population were clearly CD103 positive, which implied active retention of these populations at this site.

Analysis of paired samples from long-term virus carriers also gave a consistent pattern of results, here illustrated with respect to lytic (RAK; Figure 7A) and latent (FLR; Figure 7B) epitope responses in the HLA-B*0801–positive carrier-32. At this stage of infection, few lytic or latent epitope–specific cells express the CD38 activation marker, whether in the blood or the tonsil. This is consistent with the very low viral loads present at both of these sites (see Figure 1B). Differences in CD45 isoform status between lytic and latent epitope–specific populations in the blood were now less marked than those in post-IM patients, with both specificities expressing predominantly CD45RO, and again all tonsillar populations were CD45RO$^+$CD45RA$^-$. Both sets of epitope-specific memory cells in the blood were consistently negative for markers of migration to gut-associated lymphoid tissue (CCR9, $\alpha4\beta7$) or skin (CCR4, CLA) (data not shown) but did contain some cells with lymphoid homing markers. Acquisition of CCR7 (and CD62-l; data not shown) by EBV-specific cells in the blood was relatively low in this particular patient but still illustrated the consistent trend for greater movement of the latent reactivities to a CCR7-positive central memory phenotype. Finally, both lytic and latent reactivities were again CD103 negative in the blood, but a significant proportion of these cells expressed CD103 in the tonsil.

The essential trends in phenotype change are illustrated in Figure 8, which compiles the data from all subjects: 6 acute IM patients, 2 post-IM patients, and 11 long-term carriers. Results are expressed as the percentage of tetramer-positive cells that were also positive for the marker in question, CD38, CD45RO, CCR7, or CD103. In each case, individual symbols represent 1 epitope-specific response, with lytic epitopes indicated by filled and latent epitopes by open symbols. The CD38 activation marker was highly expressed on all cells in acute IM, whether in blood or tonsil. Within 3 months after IM, CD38 had been lost from most EBV-specific cells in the blood but was retained on about 50% of such cells in the tonsil. By contrast, in long-term carriers, most cells at both sites no longer expressed this activation marker. The CD45RO isoform was highly expressed on acute IM cells in both compartments but, immediately after IM, many lytic epitope specificities in the blood had reverted to a CD45RA$^+$CD45RO$^-$ phenotype, whereas in the tonsil, they remained CD45RO$^+$ throughout. Figure 8 illustrates important differences in the CCR7 status of lytic versus latent specificities in the blood; a second lymphoid homing marker, CD62-l, showed a very similar pattern (data not shown). While the activated cells in acute IM were all CCR7 negative, the subsequent acquisition of lymphoid homing potential was more marked among latent than among lytic reactivities, both in post-IM patients and in long-term carriers. These trends were generally not as apparent in the tonsil, where, following entry, the CCR7 and CD62-1 markers appear to have been downregulated in most cells. Finally, as expected, the CD103 marker of retention at mucosal sites was absent from the blood at all time points; in the tonsil however, while acute IM effectors lack CD103, the marker was progressively acquired as these cells accumulated at the site of infection.

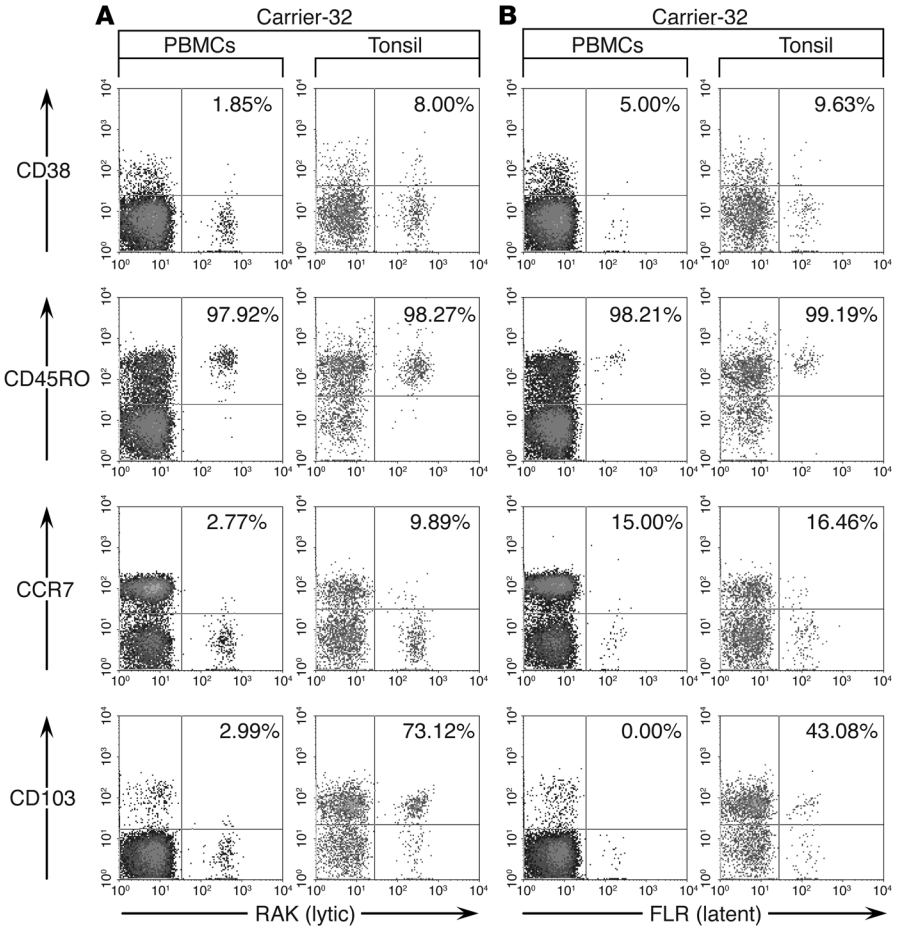

Figure 7
Analysis of EBV-specific CD8+ T cells from a long-term carrier for CD38, CD45RO, CCR7, and CD103 status. PBMCs (left panels) or tonsillar cells (right panels) from carrier-32 were stained with HLA-B*0801 tetramers containing either the lytic cycle epitope RAK peptide (**A**) or the latent cycle epitope FLR (**B**) as well as with antibodies specific for CD38, CD45RO, CCR7, and CD103. Flow cytometric analysis was performed after gating on CD8+ cells, and profiles are presented as in Figure 5.

Discussion

This study was prompted by the observation (Figure 1A and ref. 15) that EBV replication in the oropharynx remains high for some months following IM despite the large numbers of EBV lytic epitope–specific CD8+ T cells in the blood during the acute disease. Accordingly, we found that these lytic epitope reactivities were much lower (by a factor of 3- to 4-fold) in tonsillar CD8 populations than in blood. This lack of obvious recruitment of primary effectors to the main sites of infection in oropharyngeal lymphoid tissues may be a consequence of the cells' altered homing phenotype. Thus, these activated CD38+ cells have lost expression of the CCR7 and CD62-l markers that normally allow entry from peripheral blood into lymphoid tissues through high endothelial venules. Lymphocyte activation is known to be associated with CCR7 loss and the rapid shedding of CD62-l from the cell surface (23, 31). The fact that activated T cells have been observed infiltrating many body tissues in IM patients (32) highlights the nondirected nature of much of this highly expanded primary T cell response. Since EBV replication and shedding into the oropharynx is thought to involve either mucosal epithelium or mucosa-associated B cells as the main permissive cell type (1, 2), we were further interested in examining EBV-specific T cell populations for CD103 (αEβ7) expression. This integrin is induced by the epithelium-derived cytokine TGF-β and facilitates T cell–epithelial interaction through binding of the epithelial cell–specific surface adhesion molecule E-cadherin (30, 33); CD103 is therefore considered a marker of retention at mucosal epithelial sites rather than a homing molecule per se (34). The fact that primary effectors within IM tonsils fail to express CD103 is further circumstantial evidence that even those effectors that do happen to infiltrate oropharyngeal lymphoid tissues are not engaging the appropriate target sites of virus replication.

As our earlier studies have shown, CD8+ T cells specific for EBV latent cycle epitopes are less abundant in acute IM blood (8, 9), and, with the available panel of paired samples from acute IM patients, comparisons between blood and tonsillar responses to latent epitopes were more limited. However, there appeared to be a more equal distribution of latent epitope–specific CD8+ T cells between the 2 sites. The contrast between lytic and latent epitope responses became clearer, however, when we looked at samples from 2 patients who had tonsillectomies in the immediate aftermath of IM. Like the samples from patients with acute disease, these showed underrepresentation of lytic epitope responses in tonsillar CD8 populations compared with blood, whereas both tetramer analysis and ELISpot assays showed that latent epitope responses had begun to accumulate in the tonsil by this stage. Since tonsils lack afferent lymphatics (35), this accumulation cannot be explained by migration back from peripheral (extranodal) sites, but must have resulted from active migration from the blood via high endothelial venules. This accords with the fact that, following primary infection, latent epitope–specific memory cells acquire the CCR7 and CD62-l lymphoid homing markers more rapidly and in a greater proportion of cells than do lytic epitope–specific memory cells (Figure 6 and ref. 9); latent epitope responses would therefore be expected to have greater access to the tonsil. Among the cells that do access this site, a significant fraction of the latent epitope response in particular have now begun to express CD103, which implies active retention in the tonsil, and many still expressed CD38, which indicates ongoing antigen stimulation. The more efficient homing of latent epitope responses to oropharyngeal tissues could explain why latent infections of the B cell pool are more rapidly controlled than are virus replicative lesions. In this context, CD8 responses to latent cycle antigens focus heavily on

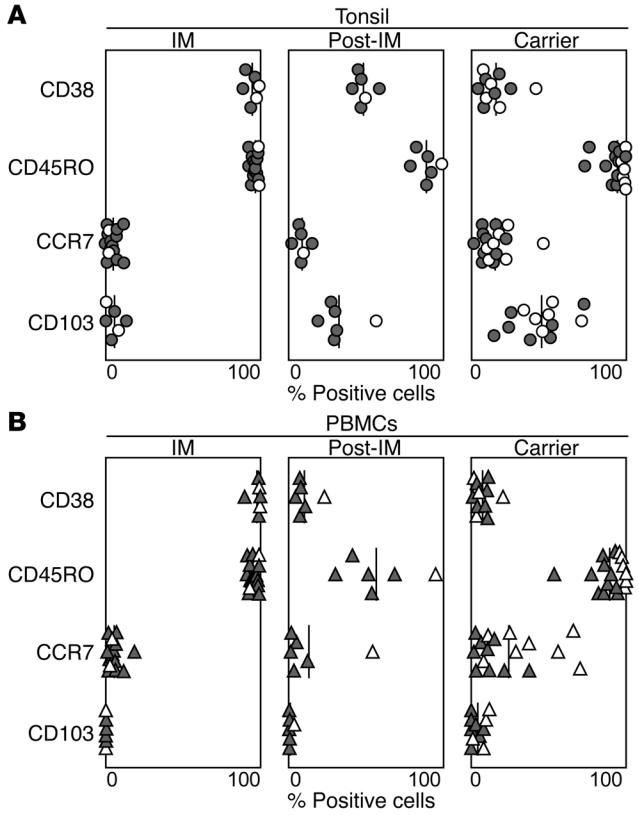

Figure 8
Compilation of results of phenotypic analysis of EBV-specific CD8+ T cells in tonsil and PBMCs from acute IM, post-IM, and long-term carrier tonsillectomy patients. (**A**) Percentage of tonsillar EBV-specific CD8+ cells expressing the relevant marker. (**B**) Percentage of PBMC EBV-specific CD8+ cells expressing the relevant marker. Left panels show results from acute IM patients; middle panels show results from post-IM patients; and right panels show results from long-term carriers. Filled symbols represent results from EBV lytic cycle epitopes, while open symbols represent results from EBV latent cycle epitopes. Statistical analysis of the data was carried out using a model similar to that described in the legend to Figure 3. CD38 expression was significantly related to infection state both in tonsil ($P = 0.0003$) and in blood ($P < 0.0001$); furthermore, CD38 was significantly higher in tonsil than in blood of post-IM patients ($P = 0.04$). CD103 expression was significantly related to infection state in tonsil only ($P = 0.05$) and was elevated in tonsil compared with blood in both post-IM patients and long-term carriers ($P = 0.008$). Significant differences in CCR7 expression were only seen in long-term carriers in whom CCR7 was significantly higher on latent compared with lytic epitope–specific cells in blood ($P = 0.003$).

the EBNA3A, -3B, -3C family of target antigens (36), proteins that are only expressed in the context of growth-transforming B cell infections (1). Such growth-transformed cells are thought to arise from de novo infections initiated by viruses produced at sites of virus replication in the mucosa (37). It is therefore to be expected that appropriate targeting of such B cell infections would localize near mucosal sites and acquire the retention markers induced by that cytokine environment.

The corresponding analysis of EBV-specific memory populations in the blood and tonsils of long-term virus carriers revealed a situation that was distinct from those seen in acute IM and in post-IM patients, though one to which post-IM patients most likely move over time. Now we found a marked accumulation of all EBV specificities in the tonsillar CD8 populations, with percentage representation being increased compared with levels in blood by a factor of 2- to 5-fold for lytic epitopes and 10- to 20-fold for latent epitopes. Remarkably, according to tetramer staining, up to 20% of all CD8+ T cells in the tonsil of these long-term carriers were directed against defined EBV epitope peptides. Functional assays of peptide-induced IFN-γ production gave the same pattern of response distribution, though absolute numbers of SFCs in ELISpot assays were always lower than the numbers of tetramer-positive cells. In this context, we have shown that immediate IFN-γ production is restricted to a subset of cells in the CCR7-effector memory population (10), and indeed, when the size of that subset was calculated for representative responses from carrier-25 and carrier-27 (Supplemental Table 1), it ranged from 13% to 47% of effector memory cells. Interestingly, however, within any one epitope response, the effector memory populations in blood and tonsil gave roughly similar values.

We believe that the tonsillar accumulation of EBV-specific responses seen in our long-term carriers is genuinely representative of tonsillar tissue in the asymptomatic carrier state and is not an artifact of the recurrent tonsillitis that led to surgery. Thus, the patients were clinically healthy, and their tonsils were not inflamed at the time of removal. Furthermore, EBV loads in the carrier tonsils were uniformly very low, far below those seen in acute IM and post-IM samples, and there was no evidence from CD38 staining of substantial T cell activation. Arguably the presence of such large numbers of these cells in oropharyngeal lymphoid tissues can prevent the outgrowth of EBV-transformed foci and can also maintain tight control over chronic lytic replication. The efficiency of control over latent growth-transforming infection is reflected by the fact that transformed cells are never detectable in the recirculating B cell pool of immunocompetent virus carriers and indeed are only seen in tonsillar preparations as short-lived transformation events occurring in B cells recently infected at sites of replication (7, 37–39). Virus replication is also well controlled, although it is interesting that many healthy virus carriers continue to shed very low levels of infectious virus into the throat, despite the presence of this local immune response. This implies that there may be an immunologically privileged site in the oropharynx where low-level replication can still occur. Alternatively, as seen with other herpesviruses (40), complete elimination of replication may be hampered by immune evasion proteins that recent results suggest may be encoded by EBV lytic cycle genes (41, 42).

It will be important to understand further the basis of this marked tonsillar accumulation of EBV-reactive T cells. Clearly it cannot be ascribed to some nonspecific inflammatory effect generally attracting or retaining CD8+ memory cells against a range of persistent viruses. Such a nonspecific effect is seen in inflamed rheumatoid synovium, for example, where there is an accumulation of many irrelevant CD8 specificities, including those against viruses such as EBV and CMV (43, 44). By contrast, CMV-reactive cells are virtually excluded from tonsillar populations, even though they are usually more abundant in the blood than the corresponding EBV specificities. We believe that one factor favoring the access of EBV-specific cells to the tonsil, particularly EBV latent specificities, is the significant levels of CCR7

and CD62-l expression displayed by these memory populations in the blood; in contrast, circulating CMV-specific memory cells are almost entirely devoid of these markers (45, 46). However, other factors may be equally important in this context. For example, recent work in mouse models indicates that T cells receive specific migratory instructions depending upon the location in which the response is primed (47–49). Thus the fact that EBV is an agent preferentially infecting the Waldeyer ring (6) may also influence the long-term migration of EBV-specific memory. Most important, local antigen stimulation could act both to expand and then to retain EBV-specific reactivities within the tonsil once the cells have trafficked to that site. Despite these influences over the behavior of memory cells, the acute primary response to EBV infection, at least as seen in IM patients, is not efficiently targeted to the main site of virus replication, and this correlates with prolonged viral shedding into the oropharynx. By contrast, both lytic and latent memory responses do accumulate in oropharyngeal sites in long-term virus carriers, and this is associated with efficient control over the resident infection. Monitoring $CD8^+$ T cell responses at a relevant tissue site, rather than in blood, therefore gives a much more informative view of the EBV-host balance.

Methods

Tonsillar and PBMC preparations. Tonsil specimens and matching heparinized blood samples were obtained from (a) 6 patients who had undergone tonsillectomy during acute IM to relieve airway obstruction due to tonsillar inflammation; (b) 2 patients undergoing tonsillectomy 3 months after IM; and (c) 11 patients with no history of EBV-related disease who had undergone routine tonsillectomy to treat chronic tonsillitis. All patients studied were adolescent or young adults and, with the exception of those of the acute IM patients, the tonsils were not inflamed at the time of surgery. The clinical diagnosis of acute IM was confirmed by heterophile antibody positivity. Routine tonsillectomy patients were confirmed as long-term EBV carriers showing IgG anti-virus capsid antibody titers in the normal range. A standard serological assay for antiviral IgG antibody (CMVscan kit; BD Biosciences) was used per the manufacturer's instructions to identify donors who were also carriers of CMV infection. The experiments were approved by the Ethics Committee of the Faculty of Clinical Medicine Mannheim, Ruprecht-Karls University of Heidelberg, and the South Birmingham Health Authority Local Research Ethics Committee. Tonsillectomy specimens were disaggregated to single-cell suspensions by teasing apart the tissue and fine mincing. Mononuclear cells were isolated by purification over a Lymphoprep gradient (Nycomed) per the manufacturer's instructions. PBMCs were isolated in a similar manner from peripheral blood specimens taken at the time of tonsillectomy. In both cases, mononuclear cells were aliquoted, cryopreserved, and stored in liquid nitrogen. DNA was isolated from an aliquot of the tonsillar cells for HLA typing by sequence-specific oligonucleotide PCR analysis.

Flow cytometric analysis of samples. The cryopreserved mononuclear cell preparations were subjected to analysis using HLA class I tetramers, where the relevant class I molecule was refolded with β2 microglobulin and the appropriate epitope peptide and complexed with streptavidin-phycoerythrin as previously described (9). Viral epitopes are identified in the text by the first 3 letters of the peptide sequence. The EBV epitopes studied were the HLA-A*0201–restricted peptides YVLDHLIVV, GLCTLVAML, and TLDYKPLSV, derived from the lytic antigens BRLF1, BMLF1, and BMRF1, respectively (9, 43, 50, 51), and CLGGLLTMV, derived from the latent membrane protein LMP2 (52); the HLA-B*0801–restricted peptides RAKFKQLL, derived from the lytic antigen BZLF1 (53), and FLRGRAYGL and QAKWRLQTL, both derived from the latent cycle nuclear antigen EBNA3A (54, 55); and the HLA-B*3501–restricted peptides EPLPQGQLTAY, derived from the lytic antigen BZLF1 (50), and HPVGEADYFEY and YPLHEQHGM, derived from the latent nuclear antigens EBNA1 and EBNA3A, respectively (55, 56). HLA class I tetramers containing CMV epitope peptides were also constructed; these included the HLA-A*0101–restricted peptides VTEHDTLLY, derived from pp50 (57), and YSEHPTFTSQY, derived from pp65 (58). In all cases the tetramer-stained cells were subsequently stained with Tricolor-labeled anti-CD8 antibodies (CALTAG Laboratories).

Phenotypic analysis involved staining with the above reagents plus a third marker. These included FITC-labeled antibodies specific to CD45RA (Beckman Coulter), CD45RO (DakoCytomation), CD27 (BD Biosciences), CD28 (BD Biosciences), CD38 (BD Biosciences), CD62-l (CALTAG Laboratories), and CD103 (BD Biosciences). Unconjugated antibodies specific to CCR4 (BD Biosciences), CCR7 (R&D Systems), CCR9 (R&D Systems), CCR10 (Abcam Ltd.), CLA (BD Biosciences), and α4β7 (a kind gift from E. Rainger, University of Birmingham, Birmingham, United Kingdom) were also used, and binding was revealed using FITC-labeled goat anti-mouse or FITC-labeled goat anti-rat antibodies (SouthernBiotech). In all cases, cells were stained with appropriate isotype control antibodies as negative controls.

ELISpot analysis of samples. ELISpot assays were carried out to determine epitope peptide–induced IFN-γ responses as described previously (10). These assays included the HLA-B*4402–restricted peptide EENLLDFVRF, derived from the latent cycle nuclear antigen EBNA3C (59).

Virus genome load analysis. Quantitative PCR analysis was performed to estimate viral genome levels as described previously (60). DNA for genome quantitation was extracted from ultraconcentrated throat washings or 1×10^6 mononuclear cells derived from single-cell preparations of either tonsil or PBMCs using a DNeasy tissue kit (QIAGEN).

Statistical analysis. Statistical analysis was conducted using linear mixed models to model log-transformed data using SAS/STAT software (version 8.2; SAS Institute Inc.). In the mixed models, random donor effects were included together with fixed effects for epitope, tissue compartment, and health state where applicable. The *P* values were not adjusted for multiple testing.

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