

# MHC II tetramers visualize human CD4<sup>+</sup> T cell responses to Epstein–Barr virus infection and demonstrate atypical kinetics of the nuclear antigen EBNA1 response

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**Virus-specific CD4<sup>+</sup> T cells are key orchestrators of host responses to viral infection yet, compared with their CD8<sup>+</sup> T cell counterparts, remain poorly characterized at the single cell level. Here we use nine MHC II–epitope peptide tetramers to visualize human CD4<sup>+</sup> T cell responses to Epstein–Barr virus (EBV), the causative agent of infectious mononucleosis (IM), a disease associated with large virus-specific CD8<sup>+</sup> T cell responses. We find that, while not approaching virus-specific CD8<sup>+</sup> T cell expansions in magnitude, activated CD4<sup>+</sup> T cells specific for epitopes in the latent antigen EBNA2 and four lytic cycle antigens are detected at high frequencies in acute IM blood. They then fall rapidly to values typical of life-long virus carriage where most tetramer-positive cells display conventional memory markers but some, unexpectedly, revert to a naive-like phenotype. In contrast CD4<sup>+</sup> T cell responses to EBNA1 epitopes are greatly delayed in IM patients, in line with the well-known but hitherto unexplained delay in EBNA1 IgG antibody responses. We present evidence from an in vitro system that may explain these unusual kinetics. Unlike other EBNA1 and lytic cycle proteins, EBNA1 is not naturally released from EBV-infected cells as a source of antigen for CD4<sup>+</sup> T cell priming.**

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Abbreviations used: CM, central memory; conc s/n, concentrated supernatant; E, early; EBV, Epstein–Barr virus; EM, effector memory; IE, immediate early; IM, infectious mononucleosis; L, late; LCL, lymphoblastoid cell line.

The cellular immune system has evolved to control infections with intracellular parasites, particularly viruses. Efficient control of such infection typically requires the cooperative action of virus-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells recognizing viral peptides in the context of MHC I and MHC II molecules, respectively (Swain et al., 2012). Although CD8<sup>+</sup> T cells typically act as effectors of the acute cellular response, CD4<sup>+</sup> T cells play a critical role, providing help for T cell-dependent antibody responses and maintaining the functional competence of CD8<sup>+</sup> T cell memory. Current understanding of the size, kinetics, and phenotype of virus epitope-specific CD8<sup>+</sup> T cell responses has been greatly enhanced by the advent of MHC I tetramer technology. However, a paucity of MHC II tetramers has delayed parallel studies on CD4<sup>+</sup> T cell responses to viral infections (Nepom, 2012).

So far, in man, such reagents have been used in a limited way to visualize influenza vaccine-induced CD4<sup>+</sup> T cell responses (Danke and Kwok, 2003), the small, often transient, response to hepatitis C virus infection (Day et al., 2003; Lucas et al., 2007; Schulze Zur Wiesch et al., 2012), and changes in the CD4<sup>+</sup> T cell response in HIV patients following ART therapy (Scriba et al., 2005). Here, we report the first tetramer-based analysis of human CD4<sup>+</sup> T cell responses to a viral pathogen that is not only genetically stable but also naturally highly immunogenic to the T cell system. The agent of choice, Epstein–Barr virus (EBV) was selected for three reasons:

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(1) a range of CD4<sup>+</sup> T cell epitopes, many restricted through relatively common MHC II alleles, have now been defined in EBV latent and lytic cycle antigens (Leen et al., 2001; Hislop et al., 2007; Long et al., 2005, 2011a); (2) the virus's association with infectious mononucleosis (IM) provides a rare opportunity to examine primary T cell responses and to follow their evolution over time; and (3) EBV was the viral system in which MHC I tetramers first revealed the strength of epitope-specific CD8<sup>+</sup> T cell responses to acute virus infection in man (Hislop et al., 2007).

EBV is orally transmitted and replicates in permissive cells in the oropharynx, expressing a large array of immediate early, early, and late proteins of the virus lytic cycle. Thereafter, the virus spreads through the B cell system as a latent growth-transforming infection, driving the expansion of infected cells through coexpression of six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C, and -LP) and two latent membrane proteins (LMP 1 and 2), just as seen during virus-induced B cell transformation to lymphoblastoid cell lines (LCLs) in vitro (Rickinson and Kieff, 2007). This rich array of viral proteins elicits a spectrum of immune responses (Hislop et al., 2007). By the time IM patients present with symptoms (estimated to be 4–6 wk after acquiring the virus), they have already developed high IgG antibody titers to many lytic cycle proteins, as well as to latent proteins such as EBNA2, the EBNA3 family and EBNA-LP (Rickinson and Kieff, 2007). However, for reasons that are still not clear, the IgG response to EBNA1 is unexpectedly delayed until weeks or months after the resolution of symptoms but thereafter retained for life (Henle et al., 1987; Hille et al., 1993). Likewise, IM patients in acute disease show large expansions of activated CD8<sup>+</sup> T cells specific for lytic and latent cycle antigens, with individual epitope responses showing marked hierarchies in size that tend to be retained as IM symptoms resolve and virus-specific CD8<sup>+</sup> T cell numbers fall to the lower values of the virus carrier state (Hislop et al., 2007). Among lytic cycle proteins, immediate early and some early antigens are the immunodominant targets (Pudney et al., 2005). Among the latent proteins, the large EBNA3 proteins often induce the strongest responses (Khanna et al., 1992;

Murray et al., 1992), but on certain MHC I backgrounds other antigens can be dominant (Blake et al., 2000).

Compared with the dramatic shifts in CD8<sup>+</sup> T cell numbers over the course of IM, any effect on the circulating CD4<sup>+</sup> T cell population is much less marked and little is known of the size, kinetics, or epitope specificity of the primary CD4<sup>+</sup> T cell response (Tomkinson et al., 1987; Miyawaki et al., 1991; Precopio et al., 2003; Callan, 2004). However several groups, including our own, have detected CD4<sup>+</sup> memory T cells to EBV in the blood of healthy carriers and find that they are heavily skewed toward a TH1 cytokine phenotype (Khanna et al., 1995; Bickham et al., 2001; Leen et al., 2001; Paludan et al., 2005; Adhikary et al., 2006). Screening such carriers with peptide pools in cytokine assays shows that the number of circulating CD4<sup>+</sup> T cells specific for any one epitope is at least 10-fold lower than for an average CD8<sup>+</sup> T cell epitope. Frequencies fall within a much narrower range and CD4<sup>+</sup> T cell epitopes are spread more widely throughout all the EBNAs and throughout immediate early, early, and late proteins of the lytic cycle (Leen et al., 2001; Long et al., 2005, 2011a). In the course of these studies, we generated CD4<sup>+</sup> T cell clones to several latent and lytic cycle epitopes and identified their MHC II-restricting alleles. This has paved the way for the construction of a panel of MHC II-peptide tetramers capable of detecting such epitope responses by surface staining, thereby avoiding any reliance on ex vivo functional assays or in vitro expansion protocols.

RESULTS

Specificity of EBV-specific MHC II tetramers and their application to long-term EBV carriers

Table 1 describes the 9 MHC II-peptide tetramers used in this work, each representing a different MHC II allele-EBV epitope combination. Three tetramers contain epitopes from EBNA2 and two contain epitopes from EBNA1, whereas the remaining four contain epitopes from different lytic cycle proteins, BZLF1 (immediate early) plus BMRF1, BaRF1, and BFRF1 (all early). Overall, these are presented through four different MHC II-restricting alleles, DRB1\*07:01 (DR7; four

Table 1. MHC II tetramer reagents

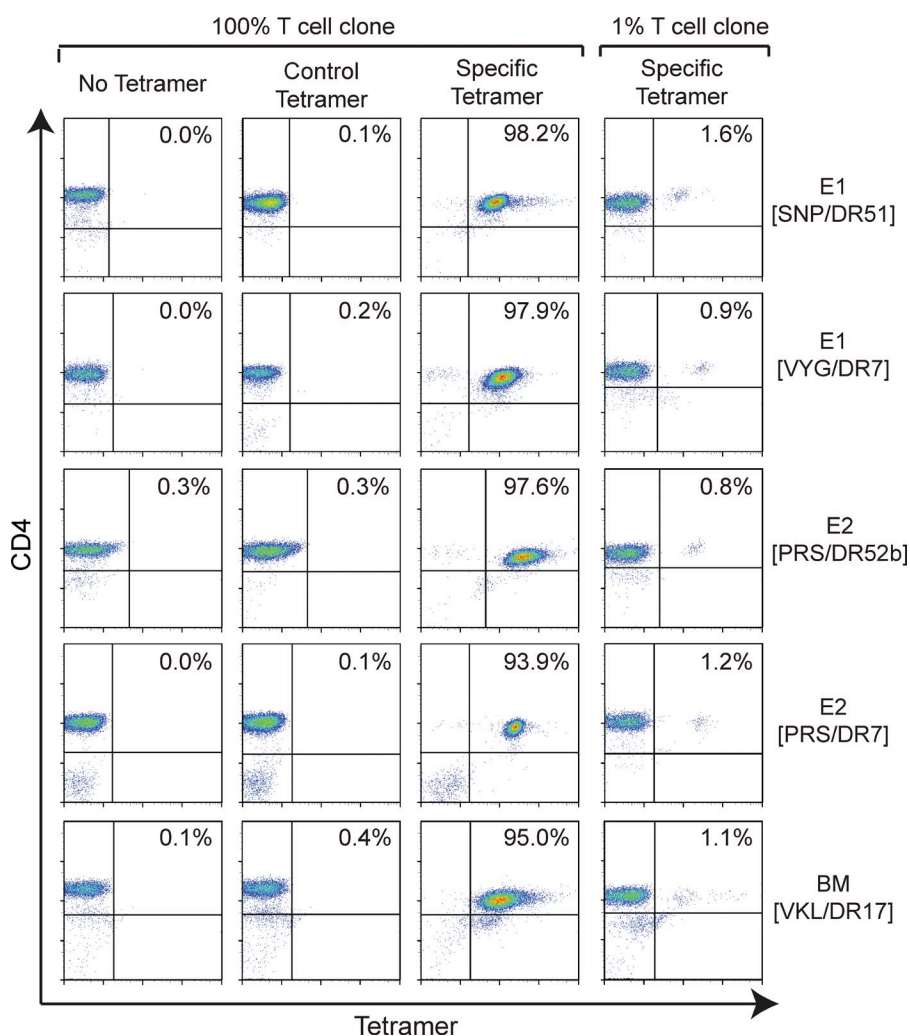
Virus phase	Protein	Coordinates	Epitope <sup>a</sup>	MHC II restriction <sup>b</sup>
Latent	EBNA1 (E1)	474–493	<u>SNPK</u> FENIAEGLRVLLARSH	DRB5*01:01 (DR51)
Latent	EBNA1 (E1)	509–528	<u>VYGG</u> SKTSLYNLRRGTALAI	DRB1*07:01 (DR7)
Latent	EBNA2 (E2)	276–295	<u>PR</u> SPTVFYNIIPMPLPSSL	DRB1*07:01 (DR7)
Latent	EBNA2 (E2)	276–295	<u>PR</u> SPTVFYNIIPMPLPSSL	DRB3*02:02 (DR52b)
Latent	EBNA2 (E2)	301–320	<u>PAQ</u> PPPGVINDQQLHHLPSG	DRB1*03:01 (DR17)
Lytic	BZLF1 (BZ)	61–75	<u>LTAY</u> HVSTAPTGSWF	DRB3*02:02 (DR52b)
Lytic	BMRF1 (BM)	136–150	<u>VKLT</u> MEYDDKVKSKSH	DRB1*03:01 (DR17)
Lytic	BaRF1 (Ba)	185–199	<u>SR</u> DELLHTRAASLLY	DRB1*07:01 (DR7)
Lytic	BFRF1 (BF)	125–139	<u>MLG</u> QDDFIKFKSPLV	DRB1*07:01 (DR7)

<sup>a</sup>All epitopes are hereby referred to by the first three amino acids of their sequence, as underlined.

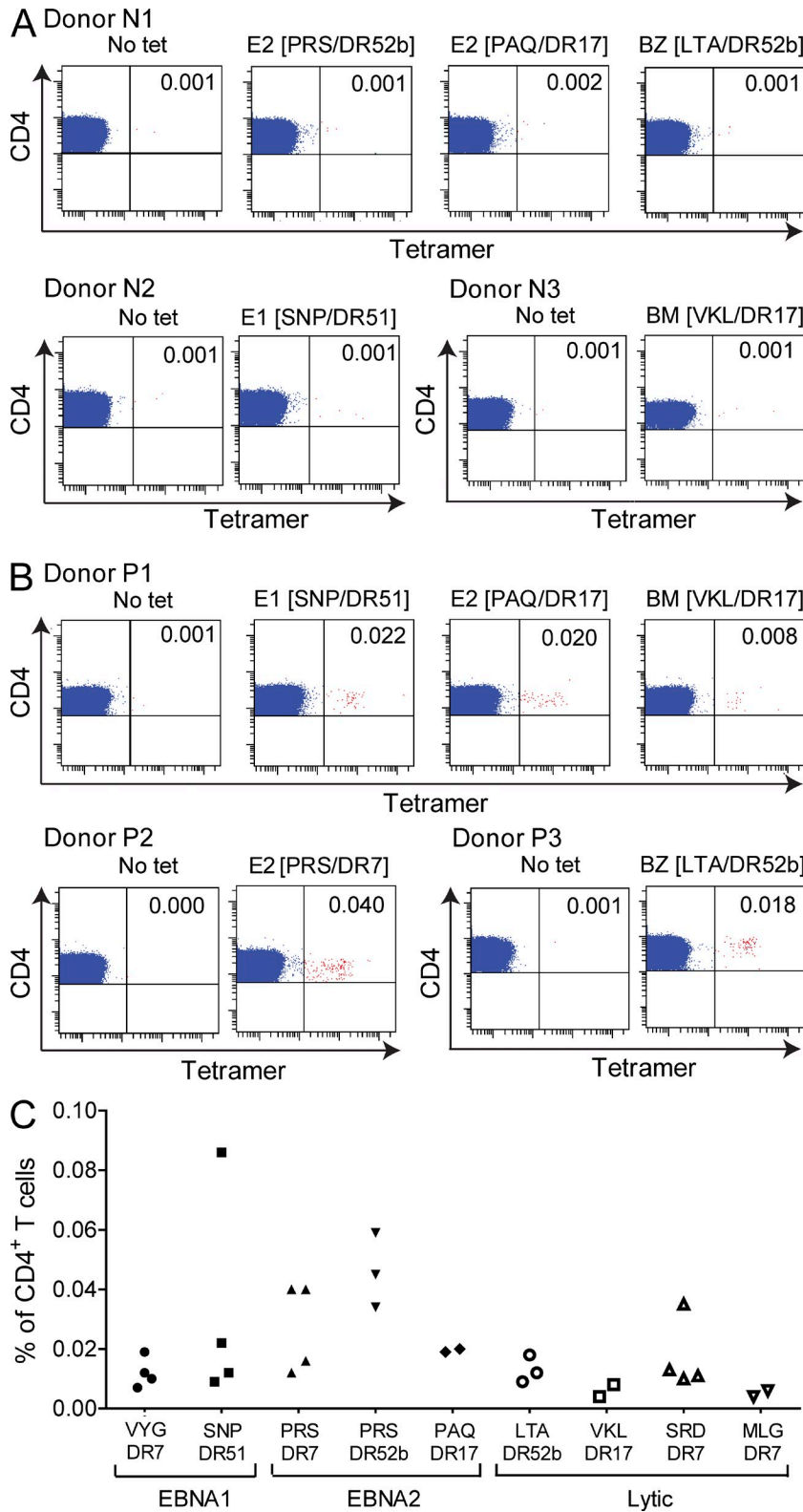
<sup>b</sup>PCR and serological MHC II types (brackets) are given for each restriction.

epitopes), DRB1\*03:01 (DR17; two epitopes), DRB3\*02:02 (DR52b; two epitopes), and DRB5\*01:01 (DR51; one epitope). In an initial series of experiments, the specificity of each of these nine tetramer reagents was confirmed by screening against CD4<sup>+</sup> T cell clones recognizing either the tetramer's cognate MHC II-peptide complex, or a different MHC II/EBV peptide complex. Thereafter, we checked each tetramer's sensitivity by testing its ability to detect cells of a cognate CD4<sup>+</sup> T cell clone present as 1% of a mixture with an irrelevant clone. Fig. 1 shows typical results from an experiment screening five such tetramer reagents, two specific for epitopes in EBNA1, two specific for epitopes in EBNA2, and one in BMRF1. In every case, >93% cells of the relevant clone were bound by the appropriate MHC II-peptide tetramer, whereas there was no detectable staining of the same clone with an irrelevant tetramer. Furthermore, all the MHC II tetramers could clearly discern small numbers of epitope-specific T cells when spiked into T cell populations of different MHC II-peptide specificity (Fig. 1, right column). Similar results were also obtained with the four other MHC II-peptide tetramers described in Table 1 (unpublished data).

Reassured by these screening assays, we went on to measure the frequency of tetramer-binding CD4<sup>+</sup> T cells in ex vivo preparations of CD8<sup>+</sup> T cell-depleted PBMCs, from healthy donors whose MHC II type and EBV serologic status were known. Fig. 2 A shows examples from such assays on 3 EBV-seronegative donors, in each case using tetramers relevant to their MHC II type. In these 3 donors, and in 2 further EBV-seronegative donors (unpublished data), we never detected any significant populations of MHC II tetramer-positive CD4<sup>+</sup> T cells. However, in parallel assays using the same tetramers on three appropriately MHC II-matched EBV-seropositive donors (Fig. 2 B), there was clear evidence of tetramer-stained CD4<sup>+</sup> T cell populations that, in every case, was above the background seen in the no-tetramer control. The numbers of stained cells ranged from 0.040% CD4<sup>+</sup> T cells (Donor P2, E2 [PRS/DR52b]) to 0.008% CD4<sup>+</sup> T cells (Donor P1, BM [VKL/DR17]). These values, although low, were robust on repeated testing and were always based on the flow cytometric analysis of a mean of 10<sup>6</sup> cells per sample. Assays were performed on a panel of 10 EBV-seropositive healthy donors; allowing each tetramer to be tested on two to four



**Figure 1. Specificity of EBV-specific MHC II tetramers.** Epitope-specific CD4<sup>+</sup> T cell clones were either unmanipulated or exposed to PE-conjugated MHC II tetramers of irrelevant or relevant MHC II-peptide combination. (right column) Epitope-specific CD4<sup>+</sup> T cell clones were added at 1% to a nonspecific CD4<sup>+</sup> T cell clone, and the mixture was stained with MHC II tetramer followed by anti-CD4. Values refer to the percentage of cells that stained with both CD4<sup>+</sup> and tetramer (top right quadrant). Results are representative of three independent experiments.



**Figure 2. Frequency of EBV MHC II tetramer-specific cells in healthy long-term EBV carriers.** CD8<sup>+</sup> T cell-depleted PBMCs from EBV-seronegative donors N1-N3 (A) and EBV-seropositive donors P1-P3 (B) tested with EBV MHC II tetramers appropriate for their individual MHC types and with no tetramer as a control. Values in the top right quadrants refer to the percentage of total CD4<sup>+</sup> T cells that stained with the tetramer in each case. Results are representative of 5 EBV-seronegative donors and 10 EBV-seropositive donors. (C) Summary of percentage frequencies of MHC II tetramer-positive CD4<sup>+</sup> T cells in 10 healthy long-term EBV carriers with no history of IM. Unpaired Student's *t* test with Welch's correction was performed in GraphPad Prism 5.

donors with a relevant MHC type. A summary of the results is shown in Fig. 2 C, each symbol giving the mean percentage of tetramer-stained cells per epitope in a single donor. Interestingly, we found that frequencies of CD4<sup>+</sup> T cells specific for

latent antigens were significantly higher ( $P = 0.015$  by unpaired Student's *t* test with Welch's correction) than for lytic antigens presented by the same MHC II allele, a trend that is the opposite of that seen for latent versus lytic CD8<sup>+</sup> T cell responses.

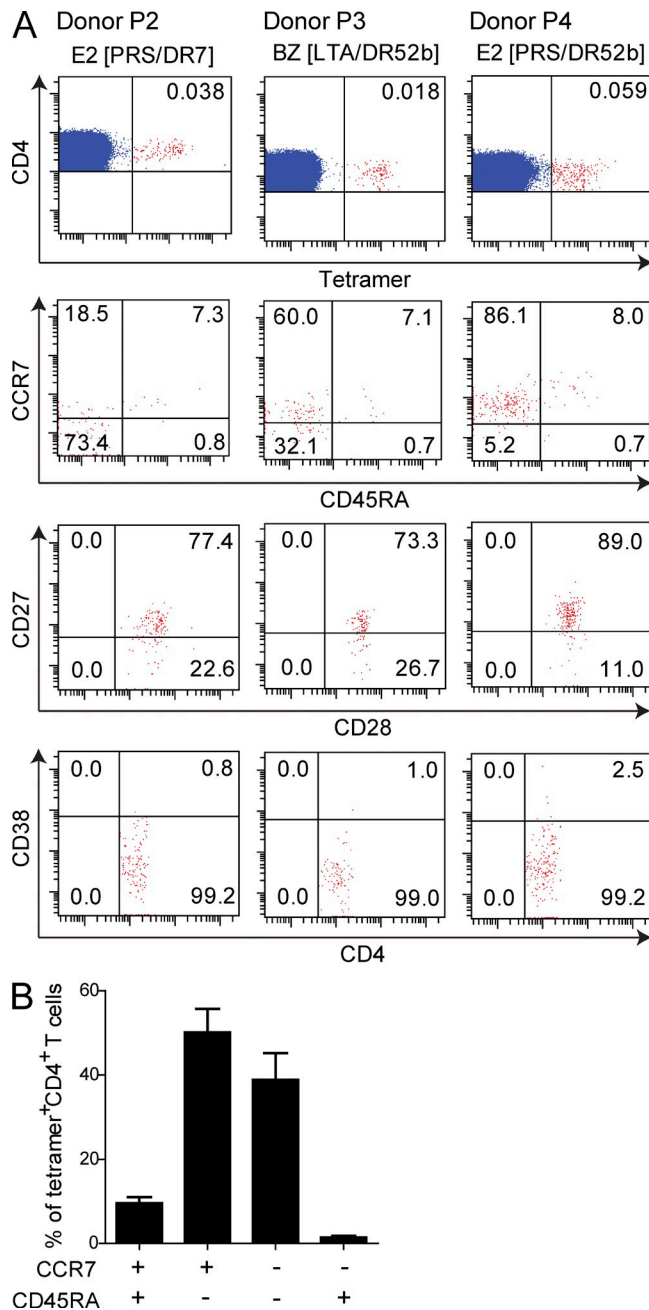


### Phenotype of EBV epitope-specific CD4<sup>+</sup> memory T cell populations

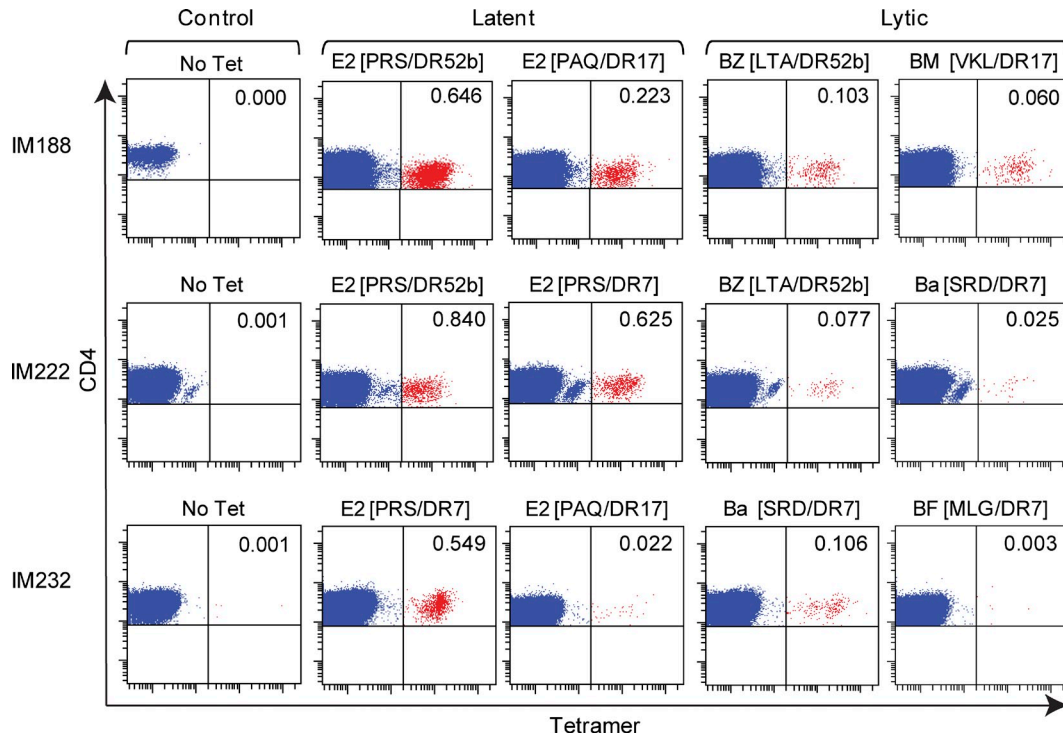
Previous studies, using cytokine responses to virus lysate, virus proteins, or epitope peptides as the readout, suggest that EBV-specific CD4<sup>+</sup> T cells in the blood of healthy carriers lay in the CD45RA<sup>-</sup> CD45RO<sup>+</sup> CCR7<sup>-</sup> effector memory (EM) and CD45RA<sup>-</sup> CD45RO<sup>+</sup> CCR7<sup>+</sup> central memory (CM) compartments. Here, we analyzed the phenotype of MHC II tetramer-positive cells using a panel of antibodies against cell surface differentiation, co-stimulatory, and activation markers in multicolor flow cytometry. Fig. 3 A illustrates the phenotype of E2 [PRS/DR7], BZ [LTA/DR52b], and E2 [PRS/DR52b] tetramer-staining cells present within the CD8<sup>+</sup> T cell-depleted PBMCs of EBV-seropositive donors P2, P3, and P4, respectively. As expected of a memory T cell population (Amyes et al., 2003), the tetramer-staining cells were uniformly positive for one co-stimulatory marker CD28, mostly positive for another, CD27, and lacked the activation marker CD38<sup>hi</sup>. Likewise the great majority of tetramer-positive cells had a CD45RA-negative, CD45RO-positive phenotype typical of memory cells (Fig. 3 A, second row, and not depicted). These cells were reliably split between CCR7<sup>+</sup>CD45RA<sup>-</sup> CM and CCR7<sup>-</sup>CD45RA<sup>-</sup> EM populations, although the proportions of cells falling into these two memory subsets did vary between individuals; in that regard Fig. 3 A shows a typical distribution with CM cells slightly in the majority (Donor P3), but also examples of outliers where memory is dominated by EM cells (Donor P2) or by CM cells (Donor P4). More importantly, however, in all three donors there was a small subset, 7–8%, of tetramer-positive cells that were CCR7<sup>+</sup>CD45RA<sup>+</sup>, a population not previously detected in cytokine response assays (Amyes et al., 2005). Overall, in the 28 tetramer-positive populations analyzed in 10 healthy EBV carriers, the epitope-specific CD4<sup>+</sup> T cells were split relatively evenly into the two major memory subsets, CM and EM (Fig. 3 B), but in every case we also detected a subset of tetramer-positive cells (mean 9.6%) with a CCR7<sup>+</sup>CD45RA<sup>+</sup> phenotype. These cells, which are also CD62L<sup>+</sup> and PD-1<sup>-</sup> (unpublished data), display surface markers conventionally associated with the naive CD4<sup>+</sup> T cell subset. However, we infer that they may be genuine antigen-experienced memory cells because we never detected any tetramer<sup>+</sup> cells of this phenotype in equivalent EBV-naive donors.

### Expansion of activated EBV MHC II tetramer-specific CD4<sup>+</sup> T cells during acute IM

We next explored the primary CD4<sup>+</sup> T cell response to EBV infection in IM patients. Overall, using the tetramer panel, we analyzed CD8<sup>+</sup> T cell-depleted PBMC preparations from 21 acute IM donors with one or more relevant MHC II alleles. Fig. 4 provides examples of results from three patients IM188, IM222, and IM232; each had MHC II types, allowing analysis with two different EBNA2 tetramers and with two different lytic antigen tetramers. The frequencies of tetramer-positive CD4<sup>+</sup> T cells were, in most cases, strikingly increased compared to those seen in the



**Figure 3. Phenotype of EBV MHC II tetramer-specific CD4<sup>+</sup> memory populations.** (A) Analysis of EBV epitope-specific CD4<sup>+</sup> T cells from three EBV seropositive donors stained with relevant MHC II tetramers, followed by anti-CD4 and indicated cell surface phenotyping antibodies. The top profiles show the percentages of CD4<sup>+</sup> T cells that stained with the relevant tetramer in each case. The bottom three profiles show the percentages of epitope-specific CD4<sup>+</sup> T cells staining for the markers in question, gated on the CD4<sup>+</sup> Tetramer<sup>+</sup> T cells in the upper profile. Results are representative of tetramer-staining populations from 10 individual donors. (B) Summary of percentage CCR7 and CD45RA expression on 28 MHC II tetramer-staining populations of all relevant specificities from 10 healthy EBV seropositive donors. The graph shows the mean values with SEM.

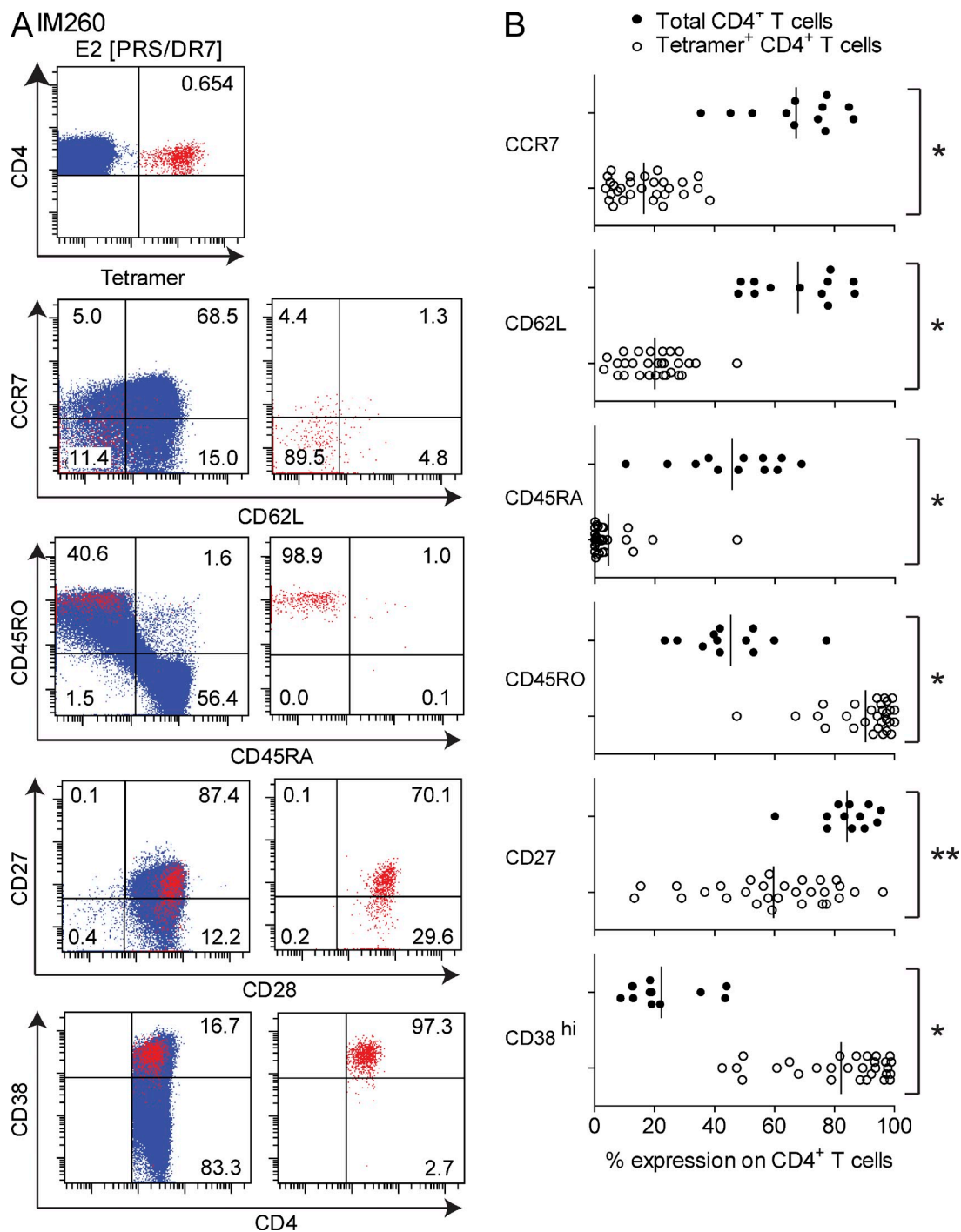


**Figure 4. Expansion of EBV MHC II tetramer-specific CD4<sup>+</sup> T cells during acute IM.** Analysis of CD8<sup>+</sup> T cell-depleted PBMCs from donors IM188, IM222, and IM232 that were cryopreserved during the acute phase of IM. Cells were stained with individual tetramers relevant for each patient's MHC II type, or with no tetramer, followed by anti-CD4. Values in the top right quadrants refer to the percentage of total CD4<sup>+</sup> T cells that stained with the tetramer in each case. The results shown are representative of experiments performed on 21 acute IM donors. Note that corresponding ELISpot assays of IFN- $\gamma$  release (Long et al., 2005) gave the following frequencies of spot forming cells per 10<sup>6</sup> CD8<sup>+</sup> T cell-depleted PBMCs in response to epitope peptide: IM188, 539 against PRS, 295 against PAQ, 40 against LTA, and 59 against VKL; IM232, 848 against PRS and 76 against PAQ (SRD and MLG not tested).

blood of healthy donors, with examples of >10-fold elevations in all three of these patients (Fig. 4 versus Fig. 2, B and C). In each case, responses were strongest against the EBNA2 epitopes, with the combined EBNA2 response accounting for 0.5–1.5% circulating CD4<sup>+</sup> T cells and with individual EBNA reactivities exceeding lytic antigen responses restricted through the same MHC II allele; compare, for example, the E2 [PRS/DR52b] versus BZ [LTA/DR52b] responses in IM188 and IM222, and the E2 [PRS/DR7] versus Ba [SRD/DR7] response in IM232. The expansion of EBV epitope-specific CD4<sup>+</sup> T cells and the predominance of EBNA2 over lytic responses was also seen in parallel ELISpot assays of IFN- $\gamma$  release. However, just as with EBV epitope-specific CD8<sup>+</sup> T cells in acute IM blood (Hislop et al., 2005), the ELISpot assay underestimated the actual number of epitope-specific CD4<sup>+</sup> T cells by 5–10-fold (see Fig. 4 legend).

We next analyzed the phenotype of the expanded tetramer-positive CD4<sup>+</sup> T cells detected in acute IM, comparing this with the total CD4<sup>+</sup> T cell population. Fig. 5 A shows a representative example from a different patient, IM260, who has >0.6% CD4<sup>+</sup> T cells reactive with the E2 [PRS/DR7] tetramer. Here, as in a normal healthy individual, the circulating CD4<sup>+</sup> T cell population as a whole (left column) is split between CD45RO<sup>+</sup> and CD45RA<sup>+</sup> cells, and is heterogeneous with respect to the lymphoid-homing markers CCR7 and

CD62L. Also the vast majority of CD4<sup>+</sup> T cells express the co-stimulatory marker CD28, and of these, >80% coexpress CD27. Interestingly, however, some 16.7% of total CD4<sup>+</sup> T cells express the activation marker CD38<sup>hi</sup> above the 5.1% mean seen in our healthy individuals (unpublished data). In contrast, the E2 [PRS/DR7]-staining cells within the CD4<sup>+</sup> T cell population all display a classical activated phenotype. Thus, they were almost all CD45RO<sup>+</sup>, CD45RA<sup>-</sup> and CD38<sup>hi</sup>, had lost expression of both lymphoid homing markers CCR7 and CD62L, and, although retaining CD28, had in some cases down-regulated the co-stimulatory surface protein CD27. A summary of the phenotype data, derived from 12 acute IM patients and comparing their overall CD4<sup>+</sup> T cell phenotypes with that of 29 resident tetramer-positive populations, is depicted in Fig. 5 B. Overall, the MHC II tetramer-positive CD4<sup>+</sup> T cells in acute IM displayed an activated phenotype that was clearly significantly different from that of the total CD4<sup>+</sup> T cell populations ( $P < 0.001$  or  $P < 0.0001$  by unpaired Student's *t* test with Welch's correction for all markers). Interestingly, however, in some IM donors we noticed a marked preponderance of CD45RO<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>-</sup>, and CD62L<sup>-</sup> cells in the total CD4<sup>+</sup> population, coincident with some 30–40% CD4<sup>+</sup> T cells being CD38<sup>hi</sup>; furthermore, these were also the patients with the largest tetramer-positive CD4<sup>+</sup> T cell expansions (unpublished data). This suggests that in at least some



**Figure 5. CD4<sup>+</sup> T cell expansions in acute IM have an activated phenotype.** (A) Analysis of E2 [PRS/DR7]-specific CD4<sup>+</sup> T cells in CD8<sup>+</sup> T cell-depleted PBMCs from donor IM260 that were cryopreserved during acute IM. The percentage of total CD4<sup>+</sup> T cells that bound the tetramer is shown in the top profile. The bottom four rows show the percentages of cells staining for the stated markers within the total CD4<sup>+</sup> T cell population (left hand column) and within the tetramer<sup>+</sup> CD4<sup>+</sup> T cells (right hand column). Non-tetramer<sup>+</sup> CD4<sup>+</sup> T cells are shown in blue and tetramer<sup>+</sup> CD4<sup>+</sup> T cells are shown in red. The results shown are representative of assays performed on 12 acute IM donors. (B) Summary of percentage expression of cell surface phenotypic, homing, and activation markers on total CD4<sup>+</sup> T cells in 12 acute IM donors (filled circles) and on 29 EBNA2 and lytic antigen MHC II tetramer-specific populations of appropriate MHC II restriction in those donors (open circles). Unpaired Student's *t* tests with Welch's correction comparing marker expression between total CD4<sup>+</sup> T cells and tetramer positive cells were performed in GraphPad Prism 5; \*, *P* < 0.0001, \*\*, *P* < 0.001.

cases the combined expansions of all EBV epitope-specific CD4<sup>+</sup> T cells in acute IM are large enough to skew the overall CD4<sup>+</sup> T cell phenotype.

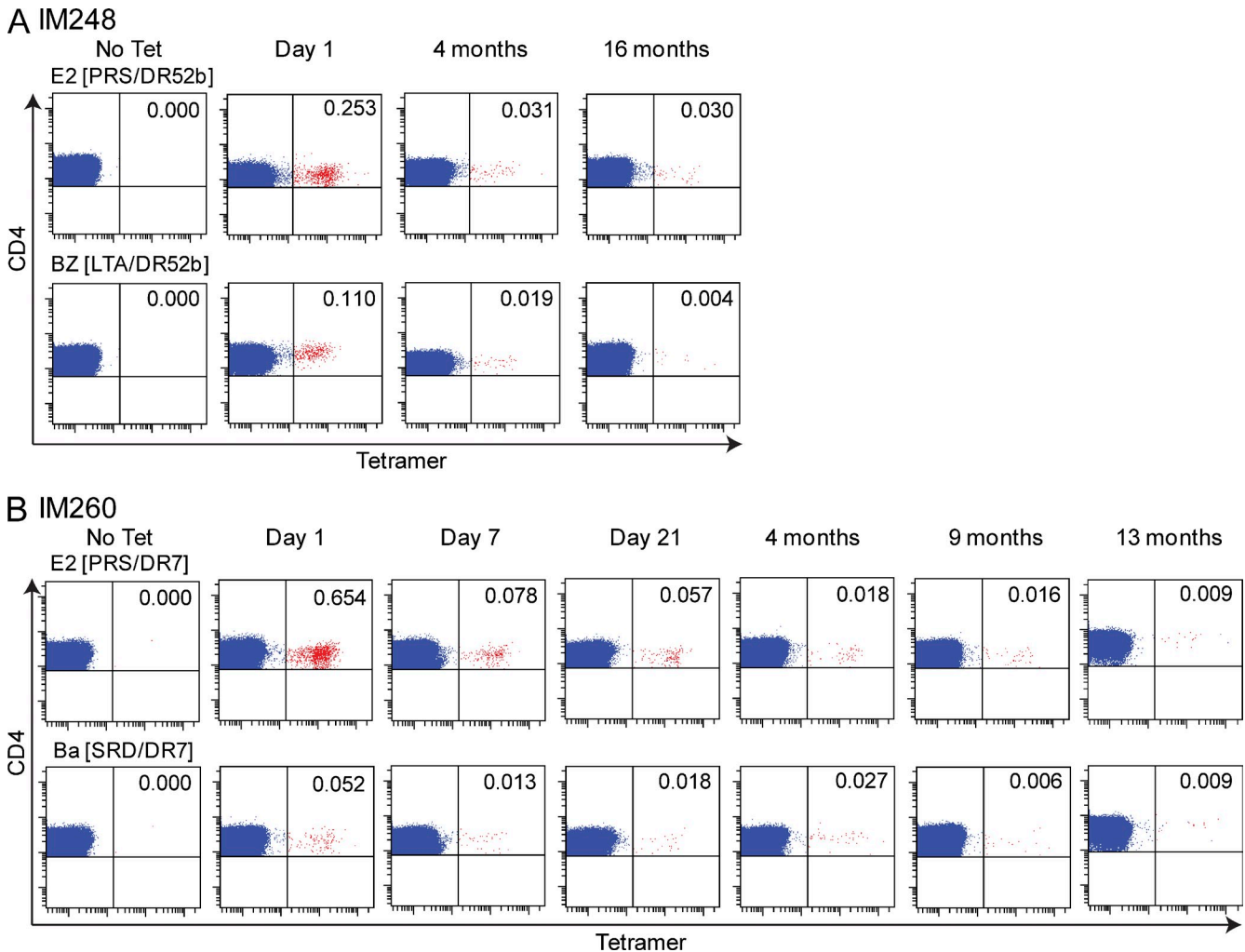
**Prospective studies of the response to EBV MHC II tetramers during IM convalescence**

Of the 21 IM patients studied in acute primary infection, 11 donated blood samples on up to 6 further occasions over a period of up to 2 yr, allowing us to follow their epitope-specific CD4<sup>+</sup> T cell responses longitudinally. Fig. 6 A shows data from two IM patients. In IM248, primary responses to E2 [PRS/DR52b] and BZ [LTA/DR52b] account for 0.253% and 0.110% of the CD4<sup>+</sup> T cell pool in the acute disease but, 4 mo later, both responses had decreased to levels <0.030%, and remained at these low levels until at least 16 mo after infection. Sampling patients more regularly showed that CD4<sup>+</sup> T cell responses decreased rapidly even during the disease course

itself and, often within weeks, had stabilized at the same low frequencies as detected in healthy EBV seropositive donors. For example, in IM260 (Fig. 6 B) the marked primary response against the E2 [PRS/DR7] epitope in the acute donation had rapidly contracted almost 10-fold within only 7 d; thereafter the rate of contraction decreased. The smaller primary response to the Ba [SRD/DR7] epitope likewise fell rapidly, in this case very soon approaching its low longer-term level. Furthermore, coincident with that culling, we found that the phenotype of the tetramer-positive CD4<sup>+</sup> T cells in post-convalescent IM patients quickly reverts to that illustrated in Fig. 3 for memory cells in healthy donors (not depicted).

**Delayed kinetics of the EBNA1-specific CD4<sup>+</sup> T cell response to EBV infection**

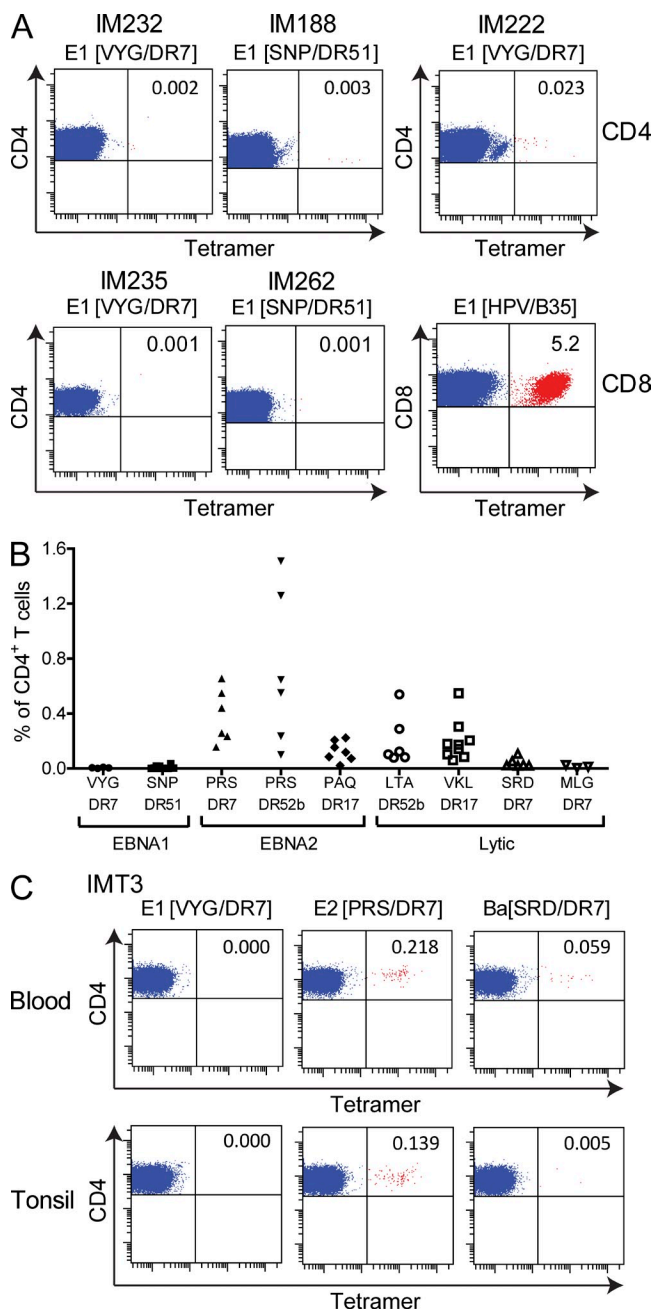
Given the well-documented delay in development of anti-EBNA1 antibodies in IM patients, we next analyzed the



**Figure 6.** Kinetics of EBV MHC II tetramer-specific CD4<sup>+</sup> T cell responses during IM PBMCs cryopreserved from. (A) Donor IM248 and (B) Donor IM260 during acute IM and at various time-points thereafter, were CD8<sup>+</sup> T cell-depleted and stained with MHC II tetramers appropriate for the donor MHC type, and with no tetramer as a control followed by anti-CD4. Flow cytometric plots present the percentage of tetramer-bound cells within the total CD4<sup>+</sup> T cell population at each time point. Results are representative of 11 IM donors who donated additional blood samples over a period of up to 2 yr.



CD4<sup>+</sup> T cell response to EBNA1 in 16 acute IM donors of relevant HLA II type using MHC II tetramers for the EBNA1-derived epitopes VYG and SNP. Strikingly, EBNA1-specific CD4<sup>+</sup> T cells were completely absent in the acute



**Figure 7. Absence of EBNA1-specific CD4<sup>+</sup> T cell responses in acute IM.** (A) Analysis of CD4<sup>+</sup> T cell responses to the EBNA1 MHC II tetramers E1 [VYG/DR7] and E1 [SNP/DR51] in CD8<sup>+</sup> T cell-depleted PBMCs from five acute IM donors. For donor IM222, an aliquot of undepleted PBMCs from the same cryopreserved PBMCs were stained with a B35 MHC I HPV tetramer (E1 [HPV/B35]) followed by anti-CD8 antibody. The numbers in the top right quadrants refer to the percentage of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells staining with the tetramer in each case. Results are

representative of 16 acute IM donors. (B) Summary of percentage frequencies of CD4<sup>+</sup> T cells staining with MHC II tetramers of relevant specificity in 21 acute IM donors. (C) Analysis of EBV epitope-specific CD4<sup>+</sup> T cells in matched blood (top) and tonsillar mononuclear cells (bottom) prepared from an IM tonsillectomy patient. Cells were stained with E1 [VYG/DR7], E2 [PRS/DR7], or Ba [SRD/DR7] tetramers followed by anti-CD4. Values in the top right quadrants refer to the percentage of total CD4<sup>+</sup> T cells that stained with the tetramer in each case. Note that, due to limited availability of cells, these analyses were performed on  $2 \times 10^5$  cells per tube. Results are representative of two donors.

bleeds in 13/16 cases and were detectable at very low numbers in the other 3 cases. Fig. 7 illustrates these findings with data from 5 IM patients. In 4/5 cases there was no significant staining with the E1 [VYG/DR7] or E1 [SNP/DR51] tetramers, despite the fact that all four of these patients did have a detectable, often large, response to EBNA2 and to lytic cycle epitopes in the same bleeds (Fig. 4 and not depicted). Notably, this cannot be caused by a deficiency in EBNA1 expression in IM because EBNA1-specific CD8<sup>+</sup> T cell responses can be detected in patients with relevant MHC I types, in particular MHC-B\*3501. Indeed, as shown in Fig. 7, IM222 (MHC B\*3501<sup>+</sup>DR7<sup>+</sup>), made a barely detectable CD4<sup>+</sup> T cell response to the VYG/DR7 epitope, yet 5.2% of this patient's highly expanded CD8<sup>+</sup> T cell population was specific for the B\*3501-restricted HPV epitope. A summary of the frequencies of EBNA1, EBNA2, and lytic antigen MHC II tetramer-positive CD4<sup>+</sup> T cells detected in the peripheral blood of our 21 acute IM patients is shown in Fig. 7 B. During the acute phase of illness, responses to individual reactivities could reach >1.5% CD4<sup>+</sup> T cells for EBNA2 epitopes and >0.5% CD4<sup>+</sup> T cells for lytic antigen-derived epitopes. Yet, in the same panel of donors, only rarely were detectable responses to the EBNA1 tetramers seen. To address the possibility that EBNA1 CD4<sup>+</sup> T cells may have been recruited to the site of primary EBV infection within oropharyngeal lymphoid tissues, we compared the frequencies of EBV-specific CD4<sup>+</sup> T cells in matched blood and tonsil mononuclear cell preparations from patient IMT3, a DR7<sup>+</sup> patient who was tonsillectomized during acute IM (Hislop et al., 2005). This patient had the anticipated high virus loads of >10<sup>4</sup> and >10<sup>6</sup> genome copies per 10<sup>6</sup> cells in the blood and tonsil, respectively. Just as in our main panel of acute IM patients (Fig. 7 B), the blood of IMT3 contained expanded populations of E2 [PRS/DR7] and Ba [SRD/DR7] MHC II tetramer-positive CD4<sup>+</sup> T cells, but no E1 [VYG/DR7]-specific cells (Fig. 7 C). Importantly, tonsillar mononuclear cells provided a similar picture. The EBNA2 and lytic antigen responses were again detectable, although, as noted with primary CD8<sup>+</sup> T cell responses (Hislop et al., 2005), they were present at lower frequencies than in the blood. Crucially, however, there was no detectable EBNA1 response. In a second donor, we were similarly unable to detect the presence of any CD4<sup>+</sup> T cells specific for the E1 [SNP/DR51] MHC II tetramer within tonsillar mononuclear cells (unpublished data).

### Prospective studies of the CD4<sup>+</sup> T cell and IgG antibody responses to EBNA1

Prospective studies on IM patients in whom EBNA1-specific CD4<sup>+</sup> T cell responses were either undetectable or extremely low in acute phase, showed that the response eventually appeared but only after a significant delay. Examples shown in Fig. 8 A include donor IM253, in whom a CD4<sup>+</sup> T cell response to E1 [SNP/DR51] was barely detectable in the primary bleed and expanded slowly through the 2-mo bleed until it reached its long-term stable level by 7 mo. Similarly, in IM265, there was no E1 [SNP/DR51]-specific response detectable either in acute IM or 21 d later, but the response had reached its stable long-term level by 3 mo. The third example is IM260, an individual who made easily detectable primary responses to DR7-restricted epitopes in the latent EBNA2 and lytic BaRF1 proteins that were subsequently culled (Fig. 6 B). This patient had no significant response to the DR7-restricted EBNA1 epitope VYG either in the acute bleed or at any of the early time points; however, a small memory population had appeared by 13 mo. A summary of the prospective studies performed on all 11 donors from whom we were able to obtain follow up samples is shown in Fig. 8 B. The different kinetics of the epitope-specific responses to EBNA1 compared with EBNA2 and the lytic proteins is clearly apparent (note the different scales involved). Thus, the EBNA1-specific CD4<sup>+</sup> T cell responses increase from undetectable or extremely low in acute IM to values >6 mo later (0.003–0.061%) that are similar to those seen in long-term virus carriers. In contrast, in the same donors over the same time frame, the expanded frequencies of CD4<sup>+</sup> T cells specific for EBNA2 and the lytic antigens present in acute IM decrease substantially, again falling to levels typical of the carrier state in individuals with no history of IM.

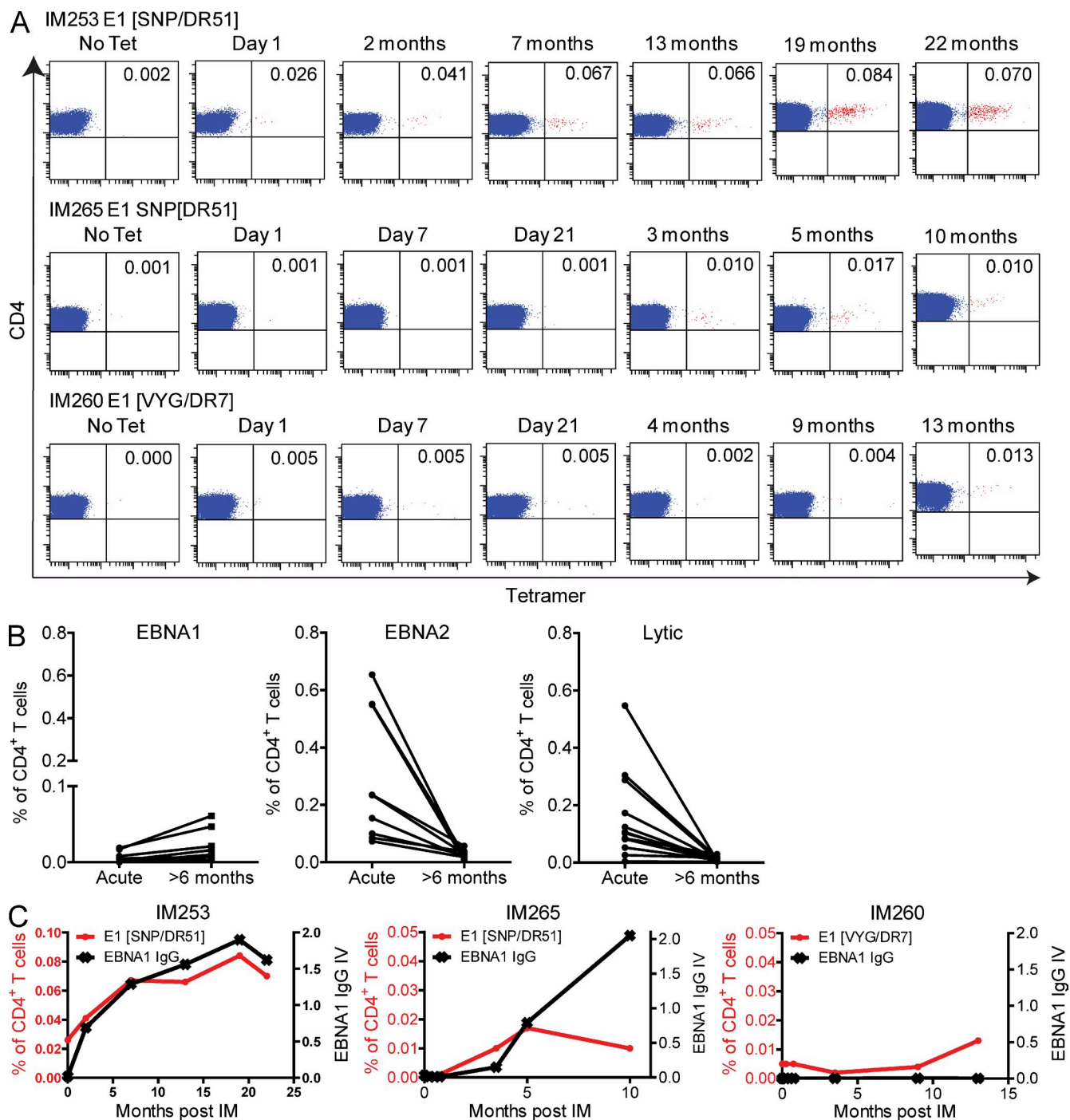
We were intrigued by this delayed EBNA1-specific CD4<sup>+</sup> T cell response, and the possibility that a lack of antigen-specific CD4<sup>+</sup> T cell help could explain the documented delay in the EBNA1-specific IgG antibody response (compared with rapid EBNA2 and lytic antibody responses) that is reported in IM patients (Henle et al., 1987; Hille et al., 1993). We therefore investigated the EBNA1 IgG response in our panel of IM donors, allowing direct comparison of the EBNA1-specific CD4<sup>+</sup> T cell and antibody responses in the same patients. The results for three patients are shown in Fig. 8 C. The first example shows IM253, in whom EBNA1-specific IgG antibodies were undetectable in the acute phase of the disease, but rose over the following months, in line with the appearance of the EBNA1-specific CD4<sup>+</sup> T cell response. Similar results were obtained for IM265, where both anti-EBNA1 IgG and EBNA1-specific CD4<sup>+</sup> T cells were both first detected after 4 mo. Interestingly patient IM260, where a significant population of E1[VYG/DR7]-specific CD4<sup>+</sup> T cells was not detected until 13 mo, remained anti-EBNA1 antibody-negative even after this prolonged follow up. Furthermore, of the prospectively studied IM patients, IM260 reported the most severe symptoms during primary infection, and an extended clinical course. In total, we measured

EBNA1-IgG responses in 8 IM donors and never detected any response during acute infection; however, 7/8 patients did develop EBNA1-specific IgG antibodies before the latest time point analyzed (10–39 mo).

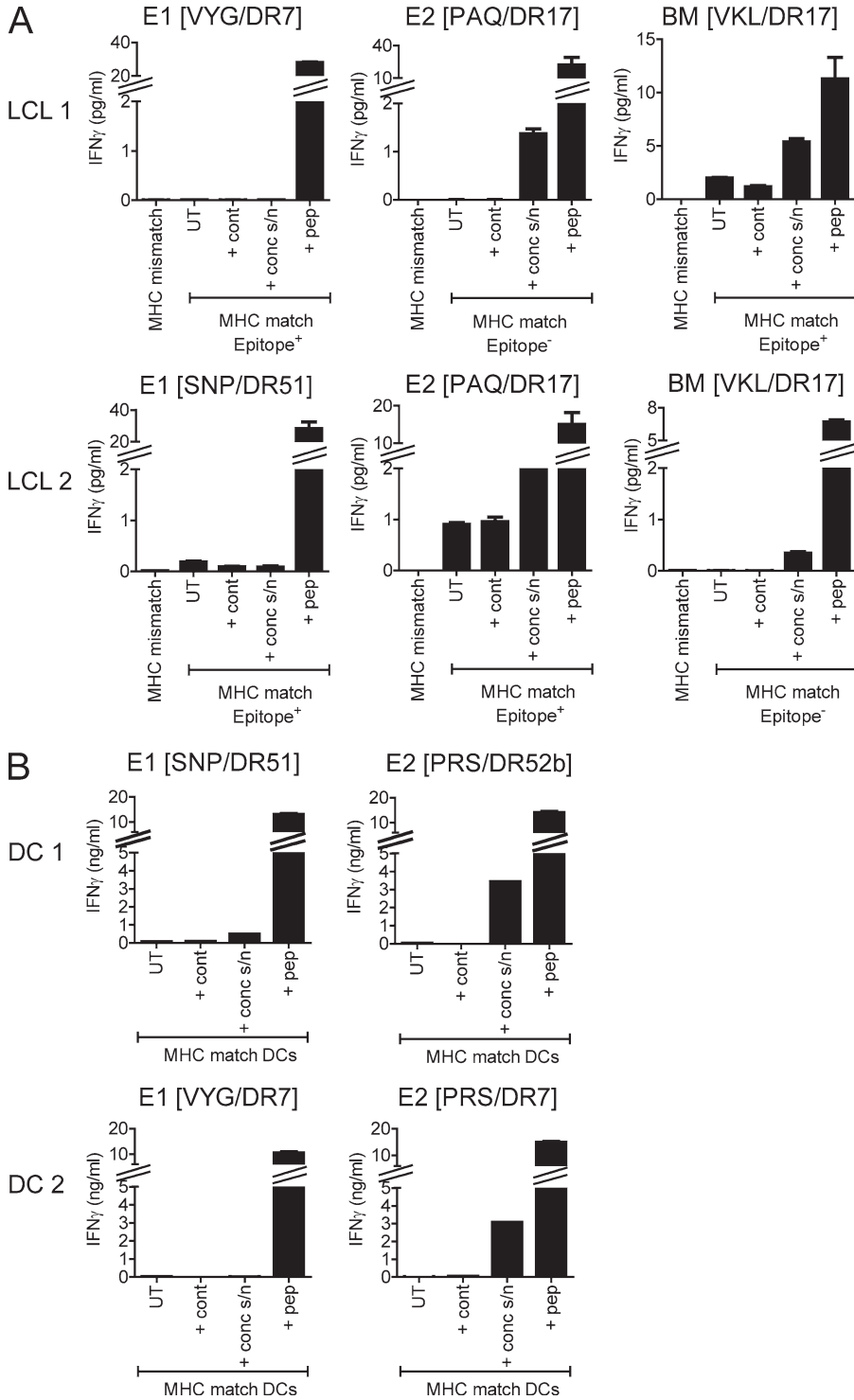
### The delayed CD4<sup>+</sup> T cell response to EBNA1 and the release of exogenous antigen from infected cells

A possible explanation as to why CD4<sup>+</sup> T cell responses to EBNA1 were delayed arose when reviewing the results of our earlier work assaying EBV epitope-specific CD4<sup>+</sup> T cell clones for their recognition of MHC II-matched, EBV-transformed LCLs. Such LCLs are typically composed of a major population of growth-transformed cells expressing only the latent antigens and a minor population (2–5%) of lytically infected cells expressing all the lytic antigens.

In two separate studies, we showed that the latent antigen EBNA2 (Taylor et al., 2006) and a range of IE, E, and L lytic antigens (Long et al., 2011a) can gain access into the MHC II presentation pathway by release into culture supernatant followed by uptake and processing as exogenous antigen by neighboring cells, thereby leading to substantial epitope presentation to CD4<sup>+</sup> T cells. However, in a subsequent study, the EBNA1 protein seemed to behave differently in this regard. Thus, although some EBNA1 epitopes may be displayed at very low levels on the infected B cell surface via endogenous presentation through autophagy (Leung et al., 2010), for every MHC II-epitope combination studied to date, epitope presentation via intercellular antigen transfer was never detected for EBNA1, even when the protein was overexpressed. We therefore set out to verify these findings by conducting experiments in which antigen transfer of EBNA1, EBNA2, and lytic cycle proteins from infected cells to co-resident antigen-presenting cells could be tested in the same assays using the same LCL combinations. In each case, concentrated supernatant (conc s/n) from a cognate antigen-expressing donor LCL was used as a source of exogenous antigen for a recipient LCL which had the appropriate MHC II-restricting alleles for T cell recognition, but whose base-line presentation of the cognate epitope was either very low or undetectable. We conducted these assays using CD4<sup>+</sup> T cells against the same latent and lytic EBV epitopes as covered by the present panel of MHC II tetramers. Fig. 9 A (top) shows one such assay with a DR7<sup>+</sup>DR17<sup>+</sup> recipient LCL (LCL 1) whose resident virus strain (Ag876) encodes a variant EBNA2 protein lacking the E2 [PAQ/DR17] epitope (Dambaugh et al., 1984). This was exposed overnight to 25-fold conc s/n from a donor LCL (B95.8 strain) expressing cognate EBNA1, EBNA2, and lytic cycle proteins. Internal controls were untreated recipient LCL or LCL treated with control supernatant from an EBV<sup>-</sup> B cell line, or loaded with exogenous cognate peptide. Although the recipient LCLs were clearly sensitized to recognition both by EBNA2 and lytic antigen-specific effectors, no sensitization to EBNA1-specific CD4<sup>+</sup> effectors was ever observed. Fig. 9 A (bottom) shows another such assay involving a DR51<sup>+</sup>DR17<sup>+</sup> recipient LCL (LCL 2) carrying a lytic cycle-defective virus strain (B95.8 BZLF1 k/o; Feederle et al., 2000)



**Figure 8. Delayed kinetics of the EBNA1-specific CD4<sup>+</sup> T cell responses to EBV infection.** (A) PBMCs cryopreserved from Donors IM253, IM265, and IM260 during acute IM and at various time points thereafter, were CD8<sup>+</sup> T cell depleted and stained with E1 MHC II tetramers appropriate for the donor MHC type and with no tetramer as a control. All cells were subsequently stained with anti-CD4. Flow cytometric plots present the percentage of tetramer-bound cells within the total CD4<sup>+</sup> T cell population at each time point. Results are representative of seven IM donors. (B) Summary of percentages of CD4<sup>+</sup> T cells staining with EBNA1, EBNA2, and lytic antigen MHC II tetramers in PBMCs from a total of 11 IM donors of appropriate MHC II type, which were cryopreserved at the time of primary EBV infection and at the latest available time point >6 mo later. (C) Graphical representation of the data presented in part A showing the percentage of E1 MHC II tetramer-bound cells within the total CD4<sup>+</sup> T cell population detected by flow cytometry at each time point (red, left axis). The same graphs show the EBNA1 IgG Index Value (arbitrary units) within the plasma collected from the same samples (black, left axis). All serial plasma samples from each patient were analyzed at the following dilutions, which were determined by prior titration assays as described in Materials and methods: 1 in 800 for IM253, 1 in 400 for IM265, and 1 in 100 for IM260.



**Figure 9. Intercellular transfer confers CD4<sup>+</sup> T cell recognition of EBNA2 and lytic antigens, but not EBNA1.** (A) T cell assays involving recipient (LCL 1) MHC DR7<sup>+</sup>DR17<sup>+</sup> LCLs transformed with Ag876 strain EBV and (LCL 2) MHC DR51<sup>+</sup>DR17<sup>+</sup> LCLs transformed with a recombinant B95.8 BZLF1 k/o virus. Recipient cells were incubated overnight with 25-fold conc s/n medium from a MHC II-mismatched B95.8-strain LCL (+ conc s/n); controls were the same line either left untreated, incubated overnight with 25-fold concentrated control medium from the EBV-negative cell line BJAB (+ cont) or exposed to 5  $\mu$ M epitope peptide for 1 h. After incubation, all cells were washed and used as targets in T cell assays, along with a MHC II mismatched target LCL as a negative control, at 5  $\times$  10<sup>4</sup> cells/well with VYG-, SNP-, PAQ-, or VKL-epitope specific CD4<sup>+</sup> T cell clones (10<sup>4</sup> cells/well). Results, which are expressed as IFN- $\gamma$  release from the T cells (ng/ml), are representative of three replicate experiments and are the mean  $\pm$  1SD of triplicate wells, as determined by ELISA. (B) T cell assays involving recipient MHC DR51<sup>+</sup>DR52b<sup>+</sup> (DC 1) and MHC DR7<sup>+</sup> (DC 2) DCs either untreated (UT), or incubated for 6 h with 25-fold conc s/n medium from a MHC II-mismatched B95.8-strain LCL (+ conc s/n) or with medium from the control EBV-negative cell line BJAB (+ cont) before 2 d maturation, or exposed to 5  $\mu$ M epitope peptide for 1 h after 2 d maturation. After incubation, all cells were washed and used as targets in T cell assays as in A with SNP, PRS, or VYG-specific CD4<sup>+</sup> T cell clones.

and exposed to the conc s/n from the donor B95.8 LCL, again expressing cognate EBNA1, EBNA2, and the lytic cycle antigens. Here again, the recipient LCL was sensitized to recognition by EBNA2 and lytic antigen-specific effectors, but not to recognition by EBNA1-specific cells.

As an additional check, we conducted similar experiments using monocyte-derived DCs as antigen recipients, given

their efficient antigen processing capacity and the likely physiological role of DCs in priming EBV-specific CD4<sup>+</sup> T cell responses in vivo. Two representative experiments involving DR51<sup>+</sup>DR52b<sup>+</sup> and DR7<sup>+</sup> DC preparations (DC 1 and DC 2, respectively) are shown in Fig. 9 B. Although exposure to the conc s/n from the donor B95.8 LCL could sensitize both DC lines to recognition by EBNA2-specific CD4<sup>+</sup> T cells, in



the same assays there was very limited sensitization of the same target DCs for recognition by EBNA1 SNP epitope-specific CD4<sup>+</sup> T cell effectors and no sensitization to EBNA1 VYG epitope-specific CD4<sup>+</sup> T cells. These assays were repeated a total of four times using DCs derived from four independent donors, with similar results obtained. Therefore, even using DCs as the sensor, EBV-infected cells once again proved to be a very poor source of released EBNA1 antigen.

## DISCUSSION

CD4<sup>+</sup> T cells are key orchestrators of virus-induced immune responses, being important for the development and/or maintenance of both humoral and CD8<sup>+</sup> T cell-mediated immunity (Swain et al., 2012). Yet we still know relatively little about the characteristics of virus-specific CD4<sup>+</sup> T cell responses at the single-cell level. This gap in our knowledge reflects the generally smaller size of CD4<sup>+</sup> T cell responses relative to their CD8<sup>+</sup> counterparts, the smaller number of CD4<sup>+</sup> T cell epitopes defined in many viral systems and, most importantly, the continued dependence on functional assays to identify individual epitope-specific CD4<sup>+</sup> T cells *ex vivo*. This latter limitation is now being overcome with the emergence of MHC II tetramer technology (Nepom, 2012), but its application to viral infections, whether in murine models (Ertelt et al., 2009; Liu et al., 2009) or in man (Danke and Kwok, 2003; Day et al., 2003; Scriba et al., 2005; Lucas et al., 2007; Schulze Zur Wiesch et al., 2012), has been relatively limited. The present work, focusing on EBV infection, represents the first tetramer-based analysis of human CD4<sup>+</sup> T cell responses to a genetically stable agent, moreover an agent already known from its association with IM to be strongly immunogenic to the CD8<sup>+</sup> T cell system. The results show that the CD4<sup>+</sup> T cell response to this virus mirrors features of the coincident CD8<sup>+</sup> T cell response in some respects, but in other respects is quite distinct.

Having confirmed the specificity of all 9 MHC II tetramers using epitope-specific CD4<sup>+</sup> T cell clones, we applied these to the circulating CD4<sup>+</sup> T cell repertoire of 10 healthy EBV carriers and 5 EBV-naïve controls, all with relevant MHC II alleles. No reactivity was seen in the naïve donors, whereas cells staining with one or more of the appropriate MHC II-matched tetramers were detectable in all 10 virus carriers. Although the frequencies of MHC II tetramer-positive CD4<sup>+</sup> T cells were low (0.005–0.085% of total CD4<sup>+</sup> T cells), all values were based on the analysis of on average 10<sup>6</sup> cells per CD4<sup>+</sup> T cell-enriched sample and in every case were reproducible upon repeat testing. In terms of size, these epitope-specific CD4<sup>+</sup> T cell populations are significantly smaller than their CD8<sup>+</sup> counterparts, where individual responses to an immunodominant epitope can account for 1–2% of the circulating CD8<sup>+</sup> T cell pool (Benninger-Döring et al., 1999; Saulquin et al., 2000; Hislop et al., 2001). They also show a different bias of distribution, with latent tending to outnumber lytic epitope reactivities in CD4<sup>+</sup> T cell memory, while the opposite is true in CD8<sup>+</sup> T cell memory (Hislop et al., 2001; Bihl et al., 2006).

Previous work, using either peptide panels, whole proteins, or lysates of EBV-infected cells as an antigen source,

have enumerated circulating CD4<sup>+</sup> memory T cells in *ex vivo* FACS or ELISpot analyses of cytokine production (Münz et al., 2000; Amyes et al., 2003; Long et al., 2005; Long et al., 2011b). Interestingly, epitope-specific CD4<sup>+</sup> T cell numbers detected here by MHC II tetramer staining exceed those seen in such functional assays by 3–10-fold (Leen et al., 2001; Long et al., 2005; Long et al., 2011a; and Fig. 4). This is not unexpected given that the capacity to mount immediate cytokine responses to antigen challenge *ex vivo* are thought to be restricted to cells (though not necessarily all cells) within the CCR7<sup>-</sup>, CD45RA<sup>-</sup> EM subset (Amyes et al., 2005; Sallusto et al., 1999). Our MHC II tetramer stains indeed show that on average 50% EBV-specific CD4<sup>+</sup> T cell memory cells in virus carriers were in the CM pool, with the remainder largely in EM. Interestingly latent- and lytic epitope-specific CD4<sup>+</sup> T cell populations showed no consistent bias in their split between these two subsets, whereas CD8<sup>+</sup> T cell memory to lytic, compared with latent, antigens is always more concentrated in the EM pool (Hislop et al., 2001). The EBV-specific CD4<sup>+</sup> memory T cell response also contained a small population of cells that had reacquired CD45RA and lost CD45RO; a similar population has also been noted within CD8<sup>+</sup> T cell memory responses but there is a crucial difference. While CD45RA<sup>+</sup> CD8<sup>+</sup> memory T cells typically have a CCR7<sup>-</sup>, CD62L<sup>-</sup>, CD28<sup>-</sup> phenotype (Hislop et al., 2001) sometimes characterized as “terminally differentiated” and hyporesponsive to reactivation *in vitro* (Champagne et al., 2001), the CD45RA<sup>+</sup> CD4<sup>+</sup> memory T cell population were CCR7<sup>+</sup>, CD62L<sup>+</sup>, CD28<sup>+</sup>, PD1<sup>-</sup>, a phenotype conventionally defined as naïve T cells (Sallusto et al., 1999). We infer that these cells may nevertheless be a genuine memory population, as we never detected such cells in the blood of EBV-naïve donors. Thus, at least for this B lymphotropic virus, a fraction of antigen-experienced CD4<sup>+</sup> T cells seem to revert to a naïve-like phenotype.

Next, we examined the CD4<sup>+</sup> T cell response in acute IM, where dramatic expansions of EBV epitope-specific CD8<sup>+</sup> T cells can lead to a 5–10-fold increase in the total size of the CD8<sup>+</sup> T cell pool (Callan et al., 1998; Catalina et al., 2001; Hislop et al., 2002). We wondered to what extent EBV-specific CD4<sup>+</sup> T cell responses might impact on the total CD4<sup>+</sup> T cell pool in IM blood, which reportedly can increase up to twofold in size in acute disease (Tomkinson et al., 1987; Miyawaki et al., 1991). MHC II tetramer analyses of 21 acute IM donors demonstrated that EBNA2 and lytic antigen-specific CD4<sup>+</sup> T cells are indeed expanded in primary EBV infection, with epitope-specific frequencies early in disease reaching >10-fold over those detectable in the blood of healthy carriers. Numerically, EBNA2 epitope-specific responses were the largest with individual frequencies reaching up to 1.5% of all CD4<sup>+</sup> T cells. Such values are small compared with some of the CD8<sup>+</sup> T cell expansions seen in acute IM, where responses to certain immunodominant lytic epitopes can account for 10–40% of the total CD8<sup>+</sup> T cell pool (Callan et al., 1998; Catalina et al., 2001; Hislop et al., 2002). However these smaller epitope-specific CD4<sup>+</sup> T cell expansions become significant when one bears in mind that,

compared with its CD8<sup>+</sup> counterpart, the CD4<sup>+</sup> T cell response is less dominated by individual reactivities and is more diverse (Long et al., 2005; Adhikary et al., 2007; Long et al., 2011a). Hence the nine epitope-specific CD4<sup>+</sup> T cell populations being visualized by tetramer staining in these studies is likely to be only a small fraction of the total EBV-induced response. An antigenically diverse response with many co-resident reactivities would indeed be consistent with the polyclonal pattern of wide TCR- $\text{V}\beta$  usage seen among CD4<sup>+</sup> T cells in IM, compared with the marked oligoclonality of the CD8<sup>+</sup> T cell response (Maini et al., 2000). Phenotypic analysis points to a similar conclusion. Thus, the great majority of MHC II tetramer-staining cells in IM blood had a phenotype (CD45RO<sup>+</sup>, CCR7<sup>-</sup>, CD62L<sup>-</sup>, CD38<sup>hi</sup>) typical of cells activated by recent antigen exposure, yet in each case the sum of these individual tetramer-stained populations was well below the total number of activated CD4<sup>+</sup> T cells in the same blood sample. On average, 22.3% CD4<sup>+</sup> T cells in the blood of the IM patients had an activated CD38<sup>hi</sup> phenotype (compared with 5.1% in healthy controls) and this value reached >40% in some patients. We suggest that this partly activated phenotype does indeed reflect the presence of multiple EBV epitope-specific responses within the total circulating CD4<sup>+</sup> T cell pool. Furthermore, where we could follow patients during the course of disease (typically comparing samples taken within 10–21 d of disease onset, and then again 1 wk later), we noted that EBNA2 and lytic antigen-specific responses were already falling very quickly. Thus, it is possible that CD4<sup>+</sup> T cell expansions are even more dramatic earlier in the disease course. In this regard, there has been one case report of primary EBV infection in a renal allograft recipient where CD4<sup>+</sup> T cells producing IFN- $\gamma$  in response to EBV lysate peaked at the time of seroconversion, 1–2 wk before the peak CD8<sup>+</sup> T cell response (Piriou et al., 2006).

In contrast to the aforementioned situation, EBNA1-specific CD4<sup>+</sup> T cell responses were extremely difficult to detect in acute IM. Among 16 acute IM patients of relevant MHC II type, 13 gave no detectable staining with either of the EBNA1 epitope tetramers, and 3 showed very small numbers of stained cells. A similar absence of EBNA1-specific CD4<sup>+</sup> T cell reactivity was detected in tonsillar mononuclear cells from two acute IM patients, despite the presence of EBNA2 and lytic antigen-specific cells in the same donor. This did not reflect a failure to generate EBNA1 responses because tetramer-stained cells appeared in the blood of 6/7 prospectively studied patients between 2 and 13 mo after IM, eventually rising to levels typical of EBNA1 epitope-specific memory in the life-long carrier state. Previous reports have looked for EBV latent and lytic antigen-specific CD4<sup>+</sup> T cell responses in IM, measuring cytokine production either *ex vivo* after stimulation with GST-fusion proteins (46) or following 12 d of *in vitro* expansion with peptide pools (Scherrenburg et al., 2008); both noted dominance of responses to lytic antigens compared with EBNA1, but the epitope specificity of these responses and their detailed kinetics was not further investigated.

The delayed development of EBNA1 CD4<sup>+</sup> T cell responses during acute IM is intriguing given the well documented lag

in the T cell-dependent IgG antibody response to this protein. Thus, by the time they develop symptoms, IM patients are strongly positive for IgG antibodies to EBNA2 and lytic antigens, yet do not become anti-EBNA1-positive until weeks or months after disease resolution (Henle et al., 1987; Hille et al., 1993). This finding has remained unexplained for almost four decades. Here we show, for the first time, a correlation between the late development of the EBNA1 IgG response and the late appearance of EBNA1-specific CD4<sup>+</sup> T cells in the blood after IM. These delays cannot be caused by a deficiency in EBNA1 expression, *per se*, because in patients with MHC I alleles such as MHC-B\*3501 that present EBNA1 epitopes, one can detect potent EBNA1-specific CD8<sup>+</sup> T cell responses (Blake et al., 1997). Rather, the discrepancy may reflect differences in the way these responses are generated and/or expanded. Thus, the range of antigens targeted by CD8<sup>+</sup> T cells in IM strongly suggests that these cells are responding to endogenously expressed antigens that are being presented by EBV-infected cells themselves; for example, antigens expressed late in the lytic cycle, when viral evasion proteins are blocking the MHC I presentation pathway (Ressing et al., 2008), are poor targets of the CD8<sup>+</sup> T cell response despite their abundant expression (Pudney et al., 2005). In contrast the much broader range of antigens targeted by EBV-specific CD4<sup>+</sup> T cells, including the late lytic cycle proteins (Long et al., 2011a), is what one would expect of a conventionally cross-primed response. We therefore reasoned that a delay in CD4<sup>+</sup> T cell responses to EBNA1, and hence a delay in the T cell-dependent EBNA1 antibody response, might reflect a delay in the availability of exogenous EBNA1 protein for uptake by antigen-presenting cells.

This possibility was intriguing given that we and others noted quite strong recognition of EBV-transformed LCLs by CD4<sup>+</sup> T cell clones whether specific for latent proteins such as EBNA2 and EBNA3A, 3B, 3C (Khanna et al., 1997; Long et al., 2005; Omiya et al., 2002; Rajnavölgyi et al., 2000) or for lytic proteins, the latter despite the small fraction of lytically infected cells in the LCL culture (Adhikary et al., 2007; Long et al., 2011a). In each case, recognition was occurring through antigen release, followed by uptake, processing, and presentation by neighboring cells (Long et al., 2011a; Taylor et al., 2006). In contrast, LCL recognition by EBNA1-specific CD4<sup>+</sup> T cell clones was either undetectable or very weak (Leung et al., 2010; Long et al., 2005) and no release of processable EBNA1 antigen could ever be detected (Leung et al., 2010). As these results came from three separate studies, we studied the cross-presentation of EBNA1 versus EBNA2 versus the lytic antigens in head-to-head experiments using not just LCL cells but also DCs as antigen recipients. The results confirm that, unlike all other antigens tested, EBNA1 is released very poorly if at all from EBV-infected cells. These findings, despite taking place in an *in vitro* system, lead us to suggest that the delayed CD4<sup>+</sup> T cell and antibody responses to EBNA1 seen in IM patients reflect the protein's limited availability to the cells that prime CD4<sup>+</sup> T cell responses.

The question as to how the EBNA1-specific CD4<sup>+</sup> T cell response is finally induced, weeks or months after IM, remains to be resolved. It may be that, despite the limitations of antigen release, eventually enough protein accumulates to trigger a CD4<sup>+</sup> T cell response. Another intriguing possibility, however, is that the true source of antigen is an infected cell type that is not represented in LCL cultures *in vitro*. In this regard, it is interesting that those rare individuals (often children) in whom primary EBV infection leads to chronic IM-like symptoms either remain anti-EBNA1 antibody-negative or have very low titers, causing an inversion of the normal EBNA1/EBNA2 ratio (Henle et al., 1987). Similarly, patients presenting with Hodgkin Lymphoma, another EBV-related disease characterized by immune dysregulation, tend to have altered EBNA1 antibody titers and decreased CD4<sup>+</sup> T cell-mediated IFN- $\gamma$  responses to an EBNA1 peptide pool (Heller et al., 2008). Such findings suggest that the development of CD4<sup>+</sup> T cell and antibody responses to EBNA1 reflects, perhaps requires, establishment and maintenance of the normal carrier state.

In summary, we report the most comprehensive analysis to date of the human CD4<sup>+</sup> T cell response to a viral infection. Using MHC II tetramers to nine EBV-coded epitopes, we show that CD4<sup>+</sup> T cell responses against five different latent and lytic cycle antigens are significantly amplified during primary infection, although individual epitope-specific responses do not approach epitope-specific CD8<sup>+</sup> T cell expansions in size. Thereafter, CD4<sup>+</sup> T cell responses contract rapidly to values typical of life-long virus carriage, where most tetramer-staining cells display conventional memory markers but some, unexpectedly, revert to a naive-like phenotype. In stark contrast, CD4<sup>+</sup> T cell responses to epitopes in EBNA1 are greatly delayed in IM patients, paralleling the well-known but hitherto unexplained delay in EBNA1 antibody responses. We present evidence from an *in vitro* system that may explain these unusual kinetics: unlike other EBNA1s and lytic cycle proteins, EBNA1 is not naturally released from EBV-infected cells as a source of antigen for CD4<sup>+</sup> T cell priming. These findings should stimulate work in other viral systems, and lead to research that can determine whether differential kinetics of antibody responses are similarly mirrored at the level of CD4<sup>+</sup> T cell responses to the same antigens, and whether such differences might be explained by antigen supply.

## MATERIALS AND METHODS

**Ethics statement and donors.** All experiments were approved by the South Birmingham Local Research Ethics Committee (reference number 07/Q2702/24), and all donors provided written informed consent for the collection of blood samples and their subsequent analysis. The donor cohort included 5 EBV seronegative donors, 10 EBV seropositive donors with no history of IM, 21 donors with acute IM infection who donated blood between 10 and 21 d after disease onset and of which 11 gave further donations through to convalescence of their disease, and two donors who were tonsillectomized during acute IM.

**T cell clones.** CD4<sup>+</sup> T cell clones were generated from healthy donors as previously described (Long et al., 2011a), and were maintained in RPMI

supplemented with 10% FCS, 1% human serum, 30% supernatant from the MLA144 cell line, and 50 U/ml recombinant IL-2.

**MHC II tetramer staining assays.** Binding of each tetramer to antigen-specific CD4<sup>+</sup> T cell clones of the relevant MHC II restriction was optimized with respect to tetramer concentration and time. Thereafter, conditions for each tetramer remained constant for the remainder of the study. PBMCs and tonsillar mononuclear cells to be analyzed by flow cytometry were all cryopreserved before use. Routine depletions of CD8<sup>+</sup> cells from PBMCs, and CD8<sup>+</sup> and CD19<sup>+</sup> cells from tonsillar mononuclear cells, were performed using anti-CD8 and anti-CD19 Dynabeads (Invitrogen) in accordance with the manufacturer's instructions. 10<sup>6</sup> CD8<sup>+</sup> T cell-depleted PBMCs or T cell clones, or 2  $\times$  10<sup>5</sup> tonsillar mononuclear cells were washed twice in ice-cold buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2), before resuspending in 50  $\mu$ l human serum. After 5 min, a pretitrated volume of MHC II tetramer (0.5–1.0  $\mu$ l) was then added to relevant cells, or no tetramer to control tubes, which were incubated at 37°C for 2 h during which the cells were resuspended periodically by agitating the tubes. After an additional two washes, the cells were stained for surface markers by incubation for 30 min on ice with predetermined saturating amounts of various combinations of the following antibodies: AmCyan-conjugated CD3 mAb (BD), FITC-conjugated CD4 mAb (BD), PerCp-Cy5.5-conjugated CD4 mAb (BD), ECD-conjugated CD4 mAb (Beckman Coulter), AmCyan-conjugated CD8 mAb (BD), AF700-conjugated CD45RA mAb (BioLegend), eFluor780-conjugated CD45RA mAb (eBioscience), Pacific blue-conjugated CD45RO mAb (BioLegend), FITC-conjugated CCR7 mAb (BD), APC-conjugated CD62L mAb (BD), eFluor780-conjugated CD27 mAb (eBioscience), ECD-conjugated CD28 mAb (Beckman Coulter), PE-Cy7-conjugated CD38 mAb (BD), and APC-conjugated PD-1 mAb (eBioscience). After two subsequent washes, stained cells were either analyzed immediately or fixed in 2% paraformaldehyde for later analysis, on either an Epics flow cytometer (Beckman Coulter) or an LSRII flow cytometer (Beckman Coulter). CD3<sup>+</sup>CD4<sup>+</sup> cells falling within the lymphocyte gate were analyzed for tetramer and surface antigen staining. All data were processed using FlowJo software (Tree Star) or BD FACSDiva software (BD).

**ELISAs of IFN- $\gamma$  release.** CD4<sup>+</sup> T cell clones (10,000/triplicate test well) were incubated overnight in V-bottom microtest plate wells with standard numbers (5  $\times$  10<sup>4</sup>/well) of MHC II-matched or control MHC II-mismatched LCL cells. MHC II matched LCLs were generated from donor B cells by *in vitro* infection with either Ag876 strain virus in which the PAQ epitope sequence is mutated (Ag876 LCLs; Dambaugh et al., 1984), or with a B95.8 recombinant virus lacking the immediate early gene *BZLF1* (BZ k/o LCLs) that is unable to enter lytic replication (Feederle et al., 2000), thereby reducing background LCL recognition by PAQ- and VKL-specific T cells, respectively, to zero. MHC-matched LCLs were unmanipulated, preexposed for 1 h to 5  $\mu$ M epitope peptide, or preexposed overnight to a 25-fold concentrate of AIM-V medium that had been conditioned by 3 d of culture using either a MHC II-mismatched B95.8 LCL (conc s/n) or a control EBV-negative B cell line, BJAB (cont). Both media were filtered through a 0.2- $\mu$ m membrane and concentrated with a Centricon centrifugal device (Millipore) with a 10-kD molecular mass cut-off. Cells exposed to the conditioned supernatant or peptide were washed before their use as targets. The supernatant medium from the T cell and LCL co-cultures was harvested after 18 h, and assayed for IFN- $\gamma$  by ELISA (Endogen) in accordance with the manufacturer's recommended protocol.

**EBNA1 IgG ELISA.** Plasma EBNA1 IgG titers were measured by ELISA using the diagnostic Diamedix EBNA-1 IgG Enzyme Immunoassay Test kit (Diamedix Corporation), which contains a purified recombinant 27-kD protein from the C-terminal third of the EBNA1 protein. Note that we analyzed for IgG responses against this region of the EBNA1 protein only, as nonspecific cross-reactive antibodies against the glycine-alanine repeat of EBNA1 are commonly seen in acute IM (McClain et al., 2003). A modified protocol was used in which doubling dilutions of patient plasma from 1:100 to 1:12,800 were prepared from the most recently collected donation, which



were analyzed by ELISA in accordance with the manufacturer's instructions. The Index Value (IV) of each sample was determined according to the following equation:  $IV = (A_{450} \text{ Sample} - A_{450} \text{ blank}) / (A_{450} \text{ Cut-Off Calibrator} - A_{450} \text{ blank})$ , where the Cut-Off Calibrator included in the kit is defibrinated human plasma that is weakly reactive for EBNA1 IgG antibodies. For each patient, the plasma dilution giving an Index Value in the center of the linear range of the assay was determined, and plasma samples from all time points were subsequently analyzed at this dilution.

**DC targets.** PBMCs were separated from healthy donors as previously described (Long et al., 2005). DCs were prepared by 6-d culture of adherent PBMCs in RPMI-1640 medium + L-glutamine (Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 5% human serum (HD Supplies), 20 µg/ml IL-4 (PeproTech) and 25 µg/ml GM-CSF (Immunex), followed by 2-d maturation in the aforementioned medium supplemented with 10 µg/ml IL-1β (PeproTech), 10 µg/ml IL-6 (PeproTech), and 10 µg/ml TNF (PeproTech). DCs were preexposed for 6 h to 25-fold B95.8 LCL concentrate (conc s/n) or control BJAB concentrate (cont) before the addition of maturation cytokines, or exposed to 5 µM epitope peptide for 1 h after 2-d incubation with maturation cytokines, or unmanipulated. DC maturation was confirmed by flow cytometric analysis by the up-regulation of surface MHC II and CD86, and down-regulation of surface CD209, and the DCs were used as targets in ELISAs of IFN-γ release.

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