Decreased EBNA-1-specific CD8+ T cells in patients with Epstein–Barr virus-associated nasopharyngeal carcinoma

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The Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA-1) is potentially a universal target for immune recognition of EBV-infected normal or malignant cells. EBNA-1-specific CD8+ T-cell responses have been assessed against a few epitopes presented on a limited number of HLA class I alleles. We now assess CD8+ T-cell responses to a complete panel of EBNA-1 peptides in an HLA-characterized population. We detected EBNA-1-specific CD8+ T cells in 10 of 14 healthy donors by analysis of peripheral blood mononuclear cells and EBV-specific T-cell lines. The frequent detection of CD8+ T-cell responses was confirmed by mapping EBNA-1 epitopes and demonstrating HLA class I presentation to CD8+ T cells in 6 of 6 donors, including 2 new EBNA-1 epitopes presented by HLA A0206 and A6802. Importantly, EBNA-1-specific CD8+ T cells were significantly less frequent in EBV-specific T-cell lines from patients with EBV-associated nasopharyngeal carcinoma (3 out of 22, $P = 0.0003$), whereas the frequency of LMP2-specific responses (14 out of 22) was not significantly different from healthy donors (11 out of 14). EBNA-1-specific CD8+ T-cell responses were rescued in approximately half of nasopharyngeal carcinoma patients by peptide and cytokine stimulation of peripheral blood mononuclear cells, suggesting these EBNA-1-specific CD8+ T cells were functionally defective in their response to EBV-infected cells. These results indicate that humans normally mount a significant EBNA-1-specific CD8+ T-cell response to EBV infection, but the immune response to this tumor antigen has been significantly altered in nasopharyngeal carcinoma patients. Overcoming this defect in EBV-specific immunity may prevent or enhance treatment of EBV-associated nasopharyngeal carcinoma.

cancer | cytotoxic T lymphocytes | immunotherapy

Nearly all humans are persistently infected with Epstein–Barr virus (EBV), and EBV infection generates significant CD8+ T-cell responses against a variety of viral proteins expressed in latently and productively infected cells. CD8+ T-cell responses against EBV are important for immune containment of EBV-infected cells.

Latent EBV infections are associated with lymphocyte and epithelial cell malignancies. Nasopharyngeal carcinoma (NPC) is the most frequent EBV-associated malignancy (1). EBV-encoded proteins, EBV nuclear antigen-1 (EBNA-1), LMP1, and LMP2, are expressed in NPC tumor cells (2–4). Current research is focused on how NPC escapes from EBV-specific immune destruction and developing novel strategies for immune intervention. Infusion of in vitro-stimulated EBV-specific T cells has been associated with tumor regression in some NPC patients (5–7), and attention has focused on the well-characterized LMP2-specific CD8+ T cells as potentially important effector cells (8).

EBNA-1-specific CD8+ T cells have received little evaluation for a role in EBV immunity because of early studies indicating that EBNA-1 proteosomal processing and peptide presentation is inhibited by the EBNA-1 glycine-alanine repeat (9). However, multiple laboratories have now demonstrated that EBNA-1-specific CD8+ T cells can recognize peptides derived from newly synthesized EBNA-1, resulting in recognition and control of EBV-infected cells in vitro (10–12). Knowledge of EBNA-1-specific CD8+ T-cell responses is limited to 6 EBNA-1 epitopes described to vary between 100% recognition in HLA B3501-positive individuals (HPV epitope; 19 out of 19 donors) (13, 14) to 0% and 9% in HLA-B07-positive donors (RPQ and IPQ epitope; 0 out of 32 and 2 out of 32 donors, respectively) (14, 15). Because EBNA-1-specific CD8+ T cells can recognize EBNA-1-expressing cells and EBNA-1 is present as a universal tumor antigen for EBV-associated malignancies, we asked whether humans with persistent EBV infection mount a significant EBNA-1-specific CD8+ T-cell response and compared this with responses in patients with EBV-associated NPC.

Results

EBNA-1-Specific CD8+ T-Cell Responses in Peripheral Blood Mononuclear Cells from Healthy EBV-Immune Subjects. We looked for evidence of EBNA-1-specific CD8+ T-cell responses using 2 different approaches, directly from peripheral blood mononuclear cells (PBMC) and in EBV-specific T-cell lines. First, EBNA-1-specific T-cell responses in PBMC from healthy adult donors were measured ex vivo by IFN-γ ELISPOT assay after exposure to an EBNA-1 peptide pool containing 102 overlapping 15-mer peptides spanning the unique EBNA-1 sequence. PBMC from 13 healthy subjects (Table 1, donors *B* through *N*) showed a high frequency of responses to the EBNA-1 peptide pool with 12 out of 13 subjects scoring greater than or equal to the threshold of 50 spot-forming cells (SFC)/10^6 PBMC. The magnitude of the response to the EBNA-1 peptide pool by PBMC was also fairly robust, with 8 out of 12 positive donors having more than 200 SFC/10^6 PBMC and a mean of 364 SFC/10^6 PBMC for all positive donors.

Because it is known that EBNA-1-specific $CD4^+$ T cells are present in PBMC from EBV-immune donors (16, 17), we tested PBMC with and without CD8+ T-cell depletion from a subset of donors to determine whether the EBNA-1 peptide pool was detecting any EBNA-1-specific responses from CD8+ T cells. As shown in Table 1, the response to the EBNA-1 peptide pool was completely inhibited by CD8+ T-cell depletion in 4 out of 7 donors (see Table 1, *E*, *J*, *M*, and *N*) and by >60% in 6 of the 7 donors studied. CD8+ T-cell responses could not be detected by depletion studies in donor *C*, where the initial response was just above the cutoff. The CD8+ T-cell depletion studies confirm the presence of EBNA-1-specific CD8+ T-cell responses in at least 6 of the healthy donors.

Comparison with LMP2-Specific CD8+ T-Cell Responses in PBMC from Healthy EBV-Immune Subjects. For comparison, LMP2-specific responses in the peripheral blood of the same healthy subjects were evaluated. A pool of 98 overlapping 15-mer peptides spanning the

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The authors declare no conflict of interest.

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Table 1. EBNA-1- and LMP2-specific IFN-γ ELISPOT responses in PBMC and in EBV-specific T cell lines from 14 healthy EBV-seropositive donors

	EBNA-1			LMP2		
	PBMC		T cell line	PBMC		T cell line
Donor	Whole pool	% Inhibition following CD8 depletion	Whole pool	Whole pool	% Inhibition following CD8 depletion	Whole pool
A	*nd*		<50	*nd*		<50
B	<50		<50	<50		2,880
C	56	0%	<50	155	*nd*	1,560
D	352	*nd*	*nd*	<50		*nd*
E	215	100%	120	73	100%	1,380
F	250	*nd*	140	145	100%	2,540
G	152	*nd*	304	<50		<50
H	113	*nd*	860	<50		3,080
I	75	*nd*	1,080	<50		7,640
J	272	100%	1,620	90	100%	3,120
K	730	85%	2,140	<50		1,280
L	1,095	60%	2,460	50	100%	360
M	400	100%	5,420	<50		10,280
N	660	100%	8,140	378	100%	5,080

nd, not done

entire LMP2 sequence was synthesized and used to stimulate LMP2-specific responses by PBMC in IFN-γ ELISPOT assays. As shown in Table 1, 6 out of 13 subjects (*C*, *E*, *F*, *J*, *L*, and *N*) responded to the LMP2 peptide pool with greater than or equal to 50 SFC/10^6 PBMC. CD8+ T-cell depletion completely abrogated the IFN-γ response in 5 out of 5 subjects with an ex vivo LMP2 response (see Table 1), confirming CD8+ T cells as the source of these LMP2-specific responses.

EBNA-1-Specific CD8+ T-Cell Expansion in Vitro. To evaluate EBNA-1-specific CD8+ T cells more stringently, EBV-specific T-cell lines were generated from 13 healthy donors by repetitive stimulation with the autologous EBV-immortalized lymphoblastoid cell line (LCL). The protocol is similar to the manufacturing process used for immunotherapy products demonstrated to have clinical activity in some NPC patients (5). T-cell lines were successfully generated from all 13 subjects, and FACS analysis showed that the T-cell lines contained predominantly CD8+ T cells (average CD3+, CD8+ cells = 79%; range: 47%–96%).

EBNA-1-specific responses were detected in 10 out of 13 T-cell lines (see Table 1, donors *E*–*N*) using the whole pool of EBNA-1 peptides in IFN-γ ELISPOT assays. All 10 T-cell lines containing EBNA-1 responses came from donors who also had detectable responses to the EBNA-1 peptide pool in PBMC. Six of the 10 T-cell lines came from donors where EBNA-1 responses in PBMC were inhibited by CD8+ T-cell depletion (see Table 1, donors *E*, *J*, *K*, *L*, *M*, and *N*). The 3 T-cell lines with no detectable EBNA-1-specific responses (see Table 1, donors *A*–*C*) were from donors with untested, undetectable, or borderline EBNA-1-specific responses in PBMC, consistent with a lack of significant EBNA-1-specific CD8+ T-cell responses in these donors. LMP2-specific responses were detected by IFN-γ ELISPOT assays in 11 out of 13 T-cell lines from healthy donors (see Table 1), but unlike EBNA-1, LMP2-specific T-cell responses were also successfully expanded in T-cell lines from 5 donors who started with undetectable LMP2-specific T-cell activity in their PBMC (see Table 1, donors *B*, *H*, *I*, *K*, and *M*).

High Frequency of EBNA-1-Specific CD8+ T-Cell Responses Confirmed by Epitope Mapping. These results were consistent with a high frequency of EBNA-1-specific, HLA class I-restricted, CD8+ T-cell responses in healthy donors. However, EBNA-1 is also known to contain HLA class II-restricted $CD4^+$ T cell epitopes. Therefore, EBNA-1-specific epitopes were mapped in a subset of donors to test whether CD8+ T cells were consistently the source of EBNA-1-specific responses in the EBV-specific, and predominantly CD8+, T-cell lines. T-cell lines from 7 donors were available to test for the presence of HLA class I-restricted presentation of EBNA-1 peptides to CD8+ T cells.

First, the individual EBNA-1-peptides recognized by each donor were identified. In brief, multiple peptide pools were constructed so that each individual peptide was represented in a unique combination of 2 pools (18). EBV-specific T-cell lines were screened for responses using the pools of EBNA-1 peptides in IFN-γ ELISPOT assays, and positive responses were confirmed by repeat testing with individual peptides. Eight individual peptide responses were mapped from the 7 T-cell lines (Table 2, donors *E*, *F*, *I*, *J*, *L*, *M*, and *N*), so the breadth of the EBNA-1-specific T-cell response was relatively narrow in each EBV-specific T-cell line (average 1.1 epitopes per donor). In 4 instances (donors *F*, *L*, *M*, and *N*), the EBNA-1 response was mapped to peptides containing previously described EBNA-1 epitopes known to be presented by HLA class I molecules, the donors were all positive for the appropriate HLA allele [supporting information (SI) Table S1], and response to the published optimal HLA class I-binding peptide was confirmed. Therefore, HLA class I-restricted presentation of EBNA-1-peptides to CD8+ T cells was confirmed in these 4 donors.

Donor *E* recognized peptide 88, which contained the sequence of a previously described HLA A0203-binding peptide, but the donor was not A0203-positive and did not respond to the optimal A0203-restricted peptide, indicating the presence of a different EBNA-1 epitope in peptide 88. The other 3 EBNA-1-specific responses mapped to peptides not containing any previously described HLA class I-binding peptides (peptides 85/86, 63/64, and 71/72 in donors *I*, *J*, and *M*).

The responses to these peptides containing newly described EBNA-1 epitopes were characterized further to confirm CD8+ T-cell recognition by intracellular cytokine staining. For these assays, EBNA-1-specific T cells were briefly expanded in vitro by stimulating PBMC from donors *E*, *I*, and *M* individually, with either peptide 88, 85/86, or 71, respectively (cells from donor *J* were unavailable for further studies). After 10 days of culture, the T cells were restimulated with either the same peptide or an irrelevant control peptide, and then stained for intracellular IFN-γ expression. As shown in Fig. 1*A*, intracellular IFN-γ was induced in a small, but

Table 2. Breadth and specificity of EBNA-1 responses in EBV-specific T-cell lines generated from 7 healthy EBV-seropositive donors

Donor	Response to whole peptide pool[a]	Number of distinct peptides recognized	Identity[b] of peptide recognized		Response to individual peptide[a]	HLA restriction
			Known	New		
E	1,270[c]	1		88	610	nd[d]
F	140	1	87/88 (VLK)		270	A*0203
I	1,080	1		85/86	2,000	A*0206
J	1,620	1			440	na[e]
L	2,460	1	46/47 (HPV)		3,200	B*35
M	5,420	2	46/47 (HPV)	71/72	2,260 200	B*53 A*6802
N	8,140	1	46/47 (HPV)		7,980	B*35

[a]SFC/10^6 cells measured by IFN-γ ELISPOT

[b]Peptides are identified by serial number assigned to individual 15-mers constituting the peptide pool. First 3 residues for previously defined EBNA-1 T-cell epitopes contained within a given 15-mer are indicated in parentheses.

[c]EBV-specific T-cell line stimulated with LCL generated using an EBV deleted for the GA repeat (dGAR) was used to map the EBNA-1 peptide (28). Peptide 88 was recognized by donor E T-cell lines stimulated with either wild-type or dGAR-derived LCL.

[d]Not determined

[e]Donor not available for further analysis

significant number of CD8+ T cells from donors *E*, *I*, and *M* after stimulation with peptides 88, 85/86, and 71, respectively, but not with the control peptides. In addition, there was little or no IFN-γ induced in the CD8- T cell population, confirming CD8+ T cells as the source of the EBNA-1-specific responses.

To determine whether the new EBNA-1 epitopes were MHC class I-restricted epitopes, the minimal peptide sequence recognized within peptides 85/86 and 71/72 was mapped using short overlapping peptides in IFN-γ ELISPOT assays (Fig. 1*B*). Peptide-stimulated lines, depleted of $CD4^+$ T cells, produced IFN-γ in response to a single 9-mer (**LQT**HIFAEV) contained within the overlapping region of peptides 85 and 86 in donor *I* and an 11-mer (**FVY**GGSKTSLY) within the overlapping region of peptides 71 and 72 in donor *M* (see Fig. 1*B*).

Furthermore, as shown in Fig. 1*C*, the recognition of peptide LQT by donor I was shown to be restricted by HLA A0206 using HLA null 721.221 cells transfected with individual HLA alleles to present peptide in IFN-γ ELISPOT assays. The LQT EBNA-1 epitope sequence is consistent with the HLA A0206 binding motif (glutamine in position 2 and valine in position 9). Similarly, peptide FVY was recognized by donor *M* when presented by HLA A6802 expressing 721.221 cells (see Fig. 1*C*). These data demonstrate that these EBNA-1 epitopes were presented by HLA class I molecules to CD8+ T cells.

Thus, EBNA-1 responses in the EBV-specific T-cell lines were tightly linked to CD8+ T cells, because HLA class I-restricted EBNA-1 peptide presentation to CD8+ T cells could be documented in all T-cell lines available (6 out of 6) for more detailed analyses. The presence of EBNA-1-specific CD8+ T cells in the majority of healthy, EBV-immune donors would also imply that EBNA-1 should be presented on a wide variety of HLA class I backgrounds. The HLA class I-restricted EBNA-1 epitopes identified in this group of donors provides support for that conclusion.

EBNA-1-Specific T-Cell Responses in EBV-Specific T-Cell Lines from Patients with EBV-Associated NPC. We studied NPC patients to see if EBNA-1-specific CD8+ T-cell responses were a prominent feature of EBV immunity in NPC patients. EBV-specific T-cell lines were generated from 17 patients with EBV-positive NPC evaluated at the Dana-Farber Cancer Institute in Boston, MA. The phenotype of these EBV-specific T-cell lines was comparable to those from healthy donors (CD3+, CD8+ = 73%, range 35%–96%).

The EBNA-1- and LMP2-specific responses in the EBV-specific T-cell lines were evaluated by IFN-γ ELISPOT assay after exposure to the whole peptide pools. As shown in Table 3, only 3 of 22 NPC patients had EBNA-1 responses in their EBV-stimulated T-cell lines. This was significantly lower compared to healthy donors, where EBNA-1-specific responses were present in 10 of 13 T-cell lines ($P = 0.0003$ by Fisher's exact test) (Fig. 2). In contrast, LMP2-specific responses were detected in 14 of 22 NPC patient T-cell lines, and this was not significantly lower than that observed

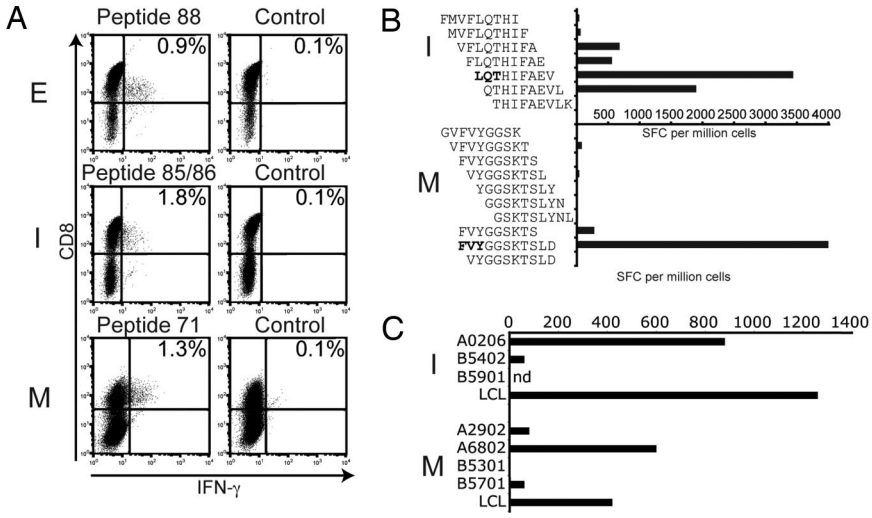

Fig. 1. CD8+ T cell recognition of EBNA-1 minimal peptides and their HLA restriction. (*A*) Intracellular cytokine staining of EBNA-1 peptide-specific cells. EBNA-1 peptide-specific cels from donors E, I, and M were stimulated with peptides 88, 85/86, or 71 respectively (*Left*) or an irrelevant peptide (*Right*) and surface stained for CD3 and CD8 followed by staining for intracellular IFN-γ. Gates were set on CD3+ live cells. (*B*) Recognition of minimal peptide by EBNA-1 peptide-specific cells. EBNA-1 peptide-specific cells, depleted of CD4+ T cells, from donors I and M were stimulated with overlapping 9- to 11-mer peptides in IFN-γ ELISPOT assays. (*C*) HLA restriction of EBNA-1 peptide-specific cells. 721.221 cells expresing individual HLA class I alleles and autologous LCL were pulsed with minimal peptides LQT and FVY and incubated with EBNA-1 peptide-specific cells, depleted of CD4+ T cells, from donors I and M respectively, in IFN-γ ELISPOT assays.

Table 3. EBNA-1- and LMP2-specific IFN-γ ELISPOT responses (SFC/10^6 cells) in EBV-specific T-cell lines generated from NPC patients

Patient	EBNA-1	LMP2
NPC1	<50	5,160
NPC2	1,800	3,800
NPC4	589	9,579
NPC5	<50	<50
NPC6	<50	3,070
NPC7	<50	<50
NPC8	<50	<50
NPC9	<50	5,680
NPC11	<50	1,900
NPC12	<50	<50
NPC13	<50	<50
NPC14	<50	6,700
NPC15	<50	9,500
NPC17	<50	5,400
NPC18	<50	2,910
NPC19	<50	<50
NPC20	<50	<50
NPC21	<50	335
NPC26	498	2,793
NPC29	<50	725
NPC30	<50	<50
NPC34	<50	433

in healthy donors (11 out of 14, $P = 0.7$ by Fisher's exact test) (see Fig. 2).

EBNA-1-Specific CD8+ T-Cell Responses Following Peptide Stimulation of PBMC from Patients with EBV-Associated NPC. The lack of EBNA-1-specific responses in NPC patient T-cell lines may be because NPC patients do not have EBNA-1-specific CD8+ T cells or because their EBNA-1-specific CD8+ T cells did not respond to their EBV-infected cells. To distinguish these possibilities, we tested

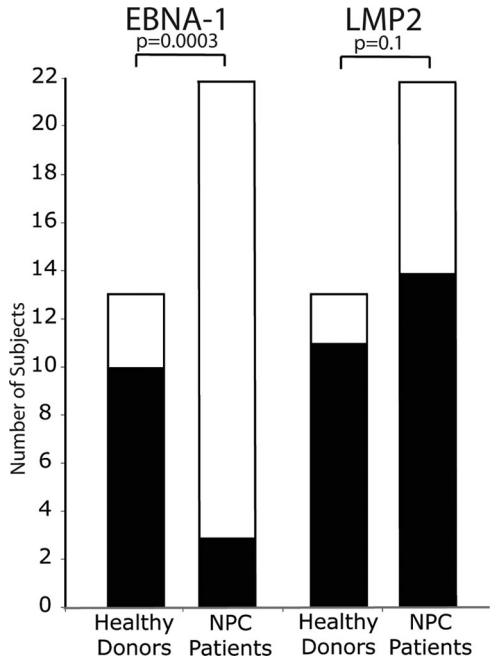

Fig. 2. Frequency of EBNA-1- and LMP2-specific responses in EBV-specific T cell lines generated from healthy donors and patients with EBV-associated NPC. Total number of subjects (white bars) and the number of subjects responding to either EBNA-1 or LMP2 (overlayed black bars) are shown. Fisher's exact *t* test was used to determine the significance.

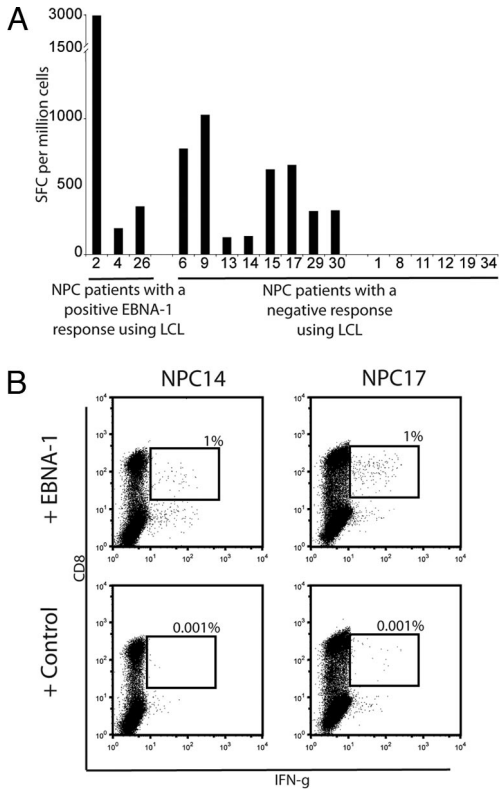

Fig. 3. Rescue of EBNA-1-specific CD8+ T cell responses from NPC patients by peptide and cytokine stimulation. (*A*) EBNA-1-specific responses in peptide-stimulated cell lines. Peptide-stimulated cell lines from 17 NPC patients were depleted of CD4+ T cells and restimulated with the EBNA-1 peptide pool for IFN-γ ELISPOT assays. (*B*) Intracellular cytokine staining of EBNA-1 peptide stimulated cells. EBNA-1 peptide stimulated cells from NPC patients 14 and 17 were stimulated with EBNA-1 peptides (*Top*) or control peptide (*Bottom*) and surface stained for CD3 and CD8 followed by ICS for IFN-γ. Gates were set on CD3+ live cells. Number in upper right of each plot is percent of CD8+ cells producing IFN-γ.

NPC PBMC to see if EBNA-1 responses could be rescued after short-term peptide and cytokine stimulation. PBMC from 17 NPC patients were stimulated in vitro with the EBNA-1 peptide pool for 10 to 14 days and supplemented with IL-7 and IL-2 to expand EBNA-1-specific cells in the absence of EBV-infected cells. $CD4^+$ T cells were then depleted (average <1% $CD4^+$ T cells remaining), and the enriched CD8+ T-cell fraction was restimulated with the EBNA-1 peptide pool to measure EBNA-1-specific responses by IFN-γ ELISPOT assays.

In 3 NPC patients who were known to have EBNA-1-specific responses in their EBV-stimulated T-cell lines (NPC 2, 4, and 26) (Fig. 3*A*, *Left*), EBNA-1-specific responses were also observed in their PBMC following EBNA-1- peptide stimulation. For the remaining 14 patients who had no detectable EBNA-1 response in their EBV-stimulated T-cell lines, 8 of 14 had a significant EBNA-1 response in their PBMC following EBNA-1-peptide stimulation (see Fig. 3*A*, *Middle* and *Right*). Two-color FACS analysis and intracellular cytokine staining directly linked the EBNA-1-induced IFN-γ response to CD8+ T cells (NPC14 and NPC17) (Fig. 3*B*). Overall, the average magnitude of response to EBNA-1 following peptide stimulation of PBMC from NPC patients was 687 SFC/10^6 cells (range 127–3,000; $n = 17$). This was not significantly different from the average magnitude of response to EBNA-1 peptide stimulation in healthy donors (1,253 SFC/10^6 cells; range 180–2,635; $n = 8$; $P = 0.18$; data not shown), suggesting that the precursor frequency of EBNA-1-specific CD8+ T cells was not significantly

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different in these NPC patients compared to healthy donors. Thus, in a significant percentage of NPC patients, EBNA-1-specific CD8+ T cells were present in PBMC, but were not detected in EBV-stimulated T-cell lines, indicating a defective response of the EBNA-1-specific CD8+ T cells to EBV-infected cells in these NPC patients.

Discussion

EBNA-1-specific CD8+ T-cell responses were often present in EBV-immune healthy donors (10 out of 14 donors) and could be detected in a similar percentage of healthy donors as LMP2-specific responses. These findings differ from those of Blake *et al.* (3 EBNA-1 responders out of 16) (13) and Piriou *et al.*, (8 EBNA-1 responders out of 14) (19), who both used EBNA-1 peptide libraries to avoid bias associated with defined epitopes. Blake *et al.* found a lower frequency of EBNA-1 responders in a population of donors excluding B35-positive individuals, potentially lowering the rate of EBNA-1 responders. Piriou *et al.* found a high frequency of EBNA-1 responders, but did not provide any HLA typing or epitope mapping to determine whether this frequency was skewed by a few dominant epitopes, such as the B35 HPV epitope.

EBNA-1 is also a strong stimulus for $CD4^+$ T-cell responses (16, 17); therefore, it was important for us to demonstrate that CD8+ T cells were the source of EBNA-1-specific responses. It is important to note that our studies do not argue against the presence of strong EBNA-1-specific $CD4^+$ T-cell responses, nor do they make a relative comparison of EBNA-1-specific $CD4^+$ versus CD8+ T-cell responses. Our experiments were aimed at determining how frequently EBNA-1-specific CD8+ T-cell responses could be detected in an HLA-diverse population of healthy donors. We scored healthy donors as EBNA-1-specific CD8+ T-cell responders only if we could: (*i*) demonstrate recognition of a known HLA class I-restricted EBNA-1 epitope (donors *F*, *L*, *M*, *N*); (*ii*) demonstrate recognition of a new EBNA-1-epitope presented by an HLA class I allele (donors *I*, *M*, *N*); (*iii*) demonstrate EBNA-1-specific CD8+ T cell responses using intracellular cytokine staining (donors *E*, *I*, *M*); (*iv*) CD8+ T-cell depletion inhibited EBNA-1-specific responses by 60% or greater in PBMC responses to peptide (donors *E*, *J*, *K*, *L*, *M*, *N*); or (*v*) if, in the absence of any other criteria, the T-cell lines expanded by LCL stimulation had strong EBNA-1-specific responses (donor *G*), because 6 out of 6 other T-cell lines derived in parallel were documented to have predominantly HLA class I-restricted, EBNA-1-specific responses. These results show EBV-infected humans do mount a significant EBNA-1-specific CD8+ T-cell response.

Defects in EBV immunity may predispose to the development or progression of EBV-associated malignancies. EBNA-1-specific CD8+ T cells were significantly less frequent in T-cell lines from patients with EBV-associated NPC compared to healthy donors. In contrast, LMP2-specific CD8+ T-cell responses were not significantly reduced in the same NPC patients compared to healthy donors. These immune differences were not associated with advanced disease or recent systemic therapy. NPC patients were receiving no immunosuppressive drugs and had no immunologic diseases, second cancers, or serious comorbid illnesses at the time of blood draw. Almost all (18 out of 22) had local or regional disease, and 4 had metastatic disease (see Table S1). Thirteen had been treated with chemotherapy, but were 26 months on average from the last chemotherapy (see Table S1). Nine were studied before any therapy, and only 1 of these patients had an EBNA-1-specific response detectable in their EBV-specific T-cell line, consistent with the premise that EBV-specific immune defects contribute to NPC pathogenesis.

Although our healthy donor population was randomly selected, there was no major bias in the ethnic distribution of our 2 populations (healthy donors: 36% Asian, 57% Caucasian, 7% African-American; NPC patients: 35% Asian, 58% Caucasian, 5% African-American). However, the average age of the 2 groups differed (healthy donors, 34.5 years versus NPC patients, 49.7 years), leaving open the possibility that an age-related decline in EBNA-1-specific responses may be a factor in the observed differences.

Why are EBNA-1-specific CD8+ T-cell responses less frequent in NPC patients and why do they respond differently compared to healthy donors? Artificial antigen stimulation of PBMC using peptide and cytokines could rescue EBNA-1-specific CD8+ T-cell responses in approximately half of the NPC patients who had no detectable EBNA-1 responses in their EBV-specific T-cell lines. Thus, the most likely scenario is that EBNA-1-specific CD8+ T cells circulating in the peripheral blood of these NPC patients are less responsive to EBV-infected cells and deficient in their ability to expand in vitro after stimulation with EBV-infected cells. Mapping epitopes recognized by these NPC patients will allow the expansion of EBNA-1-specific CD8+ T cells and the formal demonstration that they are capable of recognizing EBV-infected cells. Additionally, reversing the inhibitory mechanisms would provide an alternative strategy for expanding EBNA-1-specific CD8+ T cells using EBV-infected cells and will have important implications for immunotherapy. These EBNA-1-specific CD8+ T cells may have become unresponsive because of exhaustion, suppression by Tregulatory cells (Tregs), or from the lack of $CD4^+$ T-cell help.

Exhaustion may occur in NPC patients because of chronic, high-level exposure of EBNA-1-specific T cells to antigen presented by EBV-positive tumor cells, versus the usual low-level stimulation by the rare EBV-infected B cell present in healthy, EBV-immune donors. Additionally, NPC tumors express increased mRNA for PD-1 ligand (20), so that EBNA-1-specific T cells attracted to the tumor microenvironment may become exhausted and lose activity through PD1/PD-1L interactions with the tumor cells.

Alternatively, Tregs may suppress EBNA-1-specific CD8+ T cells in NPC patients. Tregs specific for EBNA-1 have been described in healthy donors (21). Lau *et al.* (22) reported that the frequency of Tregs in the peripheral blood of NPC patients versus healthy subjects was not significantly different, but the relative expression levels of FoxP3 and GITR were increased in Tregs from NPC patients. In addition, analysis of the tumor-infiltrating lymphocytes in NPC biopsies demonstrated 11% of the cells were of the Treg phenotype (CD4+CD25hiFoxP3+) (22). Thus, Tregs may modify the ability of EBNA-1-specific CD8+ T cells to respond to antigen as they traffic through the tumor microenvironment. In tissue culture systems, Tregs might be stimulated by EBV-infected cells, resulting in an ongoing suppression of EBNA-1-specific CD8+ T cells in vitro.

$CD4^+$ T cells have an important role in the development and maintenance of CD8+ T-cell responses. Loss of $CD4^+$ T cells has been associated with decreased EBV-specific CD8+ T-cell function and progression of AIDS-associated non-Hodgkin Lymphoma (23). Thus, a lack of $CD4^+$ T cell help may be lead to decreased EBNA-1-specific CD8+ T-cell responses in NPC patients.

More extreme manifestations of these mechanisms may result in the inability to rescue EBNA-1-specific responses even after peptide/cytokine stimulation. This may explain the inability to detect EBNA-1-specific responses by any means in a significant subset of NPC patients. Alternatively, these patients may have a complete absence of EBNA-1-specific CD8+ T cells because of a lack of EBNA-1-specific CD8+ T-cell priming or a genetic background that does not enable development of an EBNA-1-specific CD8+ T-cell response. Multiple mechanisms may lead to a common phenotype where EBNA-1-specific CD8+ T-cell responses are compromised, and this loss of EBNA-1-specific CD8+ T-cell immune surveillance may be important for the development or progression of EBV-positive NPC.

Other studies in patients with NPC have suggested the presence of subtle changes in EBV-specific CD8+ T-cell immunity, but EBNA-1-specific CD8+ T-cell responses have been scarcely examined. Early studies using in vitro regression assays showed NPC patients had mild defects in their ability to control EBV-infected

cells in vitro (24), but T-cell cloning studies found that NPC patients targeted the highly immunogenic EBNA-3 proteins and had slightly, but not significantly, reduced numbers of LMP2-specific T cell clones (25). More recent studies using defined LMP2-specific epitopes have found decreased responses in NPC patients to some epitopes, but not others (25, 26). Only a single EBNA-1-specific CD8+ T-cell epitope (VLK) has been evaluated in NPC patients, and Lin *et al.* (27) found a marked decreased in the PBMC response to this peptide compared to healthy donors that did not reach statistical significance when only HLA A0203-positive individuals were considered.

Our studies provide insights into a universal tumor-associated target of the EBV-specific immune response. We show that EBNA-1-specific CD8+ T-cell responses are present in the majority of healthy, EBV-immune donors, and EBNA-1 can be recognized by CD8+ T cells on a variety of HLA class I alleles. Furthermore, NPC patients do not have the same high level EBNA-1-specific CD8+ T-cell responses to EBV-infected cells as healthy EBV-immune donors, consistent with the hypothesis that EBV-specific immunity is important for control of their EBV-associated malignancy and could be used for effective treatment. In many patients, the immune deficiency is most likely the result of a functional defect leading to a poorly responsive EBNA-1-specific CD8+ T cell, as opposed to a total lack of EBNA-1-specific CD8+ T cells. Thus, identifying the mechanism, reversing the defect, and restoring normal EBNA-1-specific CD8+ T-cell responses could lead to more effective immunotherapy for NPC patients and provide a better understanding for how tumor-specific immune responses are regulated in cancer.

Materials and Methods

Human Subjects. Peripheral blood was obtained from 14 EBV-seropositive healthy subjects and 22 patients with EBV-positive NPC diagnosed by in situ hybridization for EBERs under an Institutional Review Board-approved protocol at the Dana-Farber Cancer Institute.

PBMC Purification and T-Cell Depletion. PBMC were isolated from heparinized blood by density gradient centrifugation. CD8+ T cells were selectively depleted from PBMC and $CD4^+$ T cells were depleted from peptide-stimulated cell lines by immunomagnetic selection (Dynal, Invitrogen). Greater than 90% CD8+ T-cell depletion and >98% $CD4^+$ T-cell depletion was confirmed by flow cytometric analysis.

EBV-Specific and Peptide-Specific T-Cell Lines. EBV-specific and peptide-stimulated cell lines were generated as previously described (5, 13). Briefly, to generate EBV-specific T-cell lines, PBMC were stimulated with irradiated autologous LCL at a ratio of 40:1 for 10 days, followed by 3 subsequent stimulations at a ratio of 4:1 on a weekly basis. IL-2 (Roche) was added at 20 IU/ml after the second stimulation and cultures fed every 3 or 4 days with fresh medium containing 20 IU/ml IL-2. Peptide-stimulated lines were generated by peptide-pulsing PBMC for 1 h, washed, and then PBMC were cultured in medium containing 25 ng/ml IL-7 (R&D systems). After 3 days, 10 IU/ml IL-2 was added, and subsequently cultures were fed every 3 days with medium containing IL-2 (10 IU/ml) and IL-7 (25 ng/ml). Cells were harvested for intracellular cytokine assays and IFN-γ ELISPOT assays after 10 to 14 days in culture.

EBNA-1 and LMP2 Peptides. EBNA-1 (102 $\times$ 15-mer peptides, overlapping by 11 residues, spanning the N-terminal 96 and C-terminal 334 residues, and excluding the Glycine-Alanine repeat) and LMP2 (97 $\times$ 15-mers and one 12-mer, overlapping by 10 residues) peptides for the B95/8 EBV were synthesized by F-moc chemistry. Peptide sequences recognized by EBNA-1-specific T cells: 63/64 GGSNPKFENIAEGLRALLA, 71/72 VAGVFVYGGSKTSLYNLRR, 85/86 CYFMVFLQTHIFAEVLKDA, 88 AEVLKDAIKDLVMTK.

Intracellular Cytokine Staining. Cells were stimulated with peptide in the presence of 1-μg/ml costimulatory antibodies, anti-CD28 and anti-CD49d (BD Biosciences). After 1 h, Brefeldin A (Sigma) was added (final concentration, 10 μg/ml). After 6 h, cells were stained with anti-CD3 and anti-CD8 (BD Biosciences), fixed, and then permeabilized. Permeabilized cells were incubated with anti-IFN-γ (BD Biosciences), washed, and analyzed on a FACScalibur (BD Biosciences).

Enzyme-Linked Immunospot. Gamma IFN (IFN-γ) ELISPOT assays were carried out using a commercially available kit (Mabtech). Various cell concentrations in complete medium were incubated overnight with peptide (1 μg/ml) or with peptide-pulsed 721.221 cells expressing single HLA class I alleles, in 96-well plates coated with anti-human IFN-γ monoclonal antibody and subsequently developed following the manufacturer's directions. Spots were counted by automated reader (Carl Zeiss Inc.) using KS ELISPOT software 4.5 (Zellnet), and background values of cells incubated with DMSO/no peptide were subtracted from all test samples.

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