Identification of a TAP-Independent, Immunoproteasome-Dependent CD8⁺ T-Cell Epitope in Epstein-Barr Virus Latent Membrane Protein 2

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We have identified an HLA-A2-restricted CD8⁺ T-cell epitope, FLYALALLL, in the Epstein-Barr virus (EBV) latent membrane protein 2 (LMP2), an important target antigen in the context of EBV-associated malignancies. This epitope is TAP independent, like other hydrophobic LMP2-derived epitopes, but uniquely is dependent upon the immunoproteasome for its generation.

Immune intervention against virus-associated malignancies requires detailed knowledge of not only the immune response to the virus involved, but also the components of the antigen processing machinery required for efficient presentation of viral T-cell epitopes. Epstein-Barr virus (EBV), a B-lymphotropic gammaherpesvirus, is associated with a number of human malignancies, including posttransplant lymphoproliferative disease (PTLD), Hodgkin’s disease (HD), and a tumor common in Southern Chinese populations, nasopharyngeal carcinoma (NPC) (21). In healthy individuals, EBV infection is kept under control by a strong virus-specific T-cell response, principally consisting of HLA class I-restricted CD8⁺ cytotoxic T lymphocytes (CTLs) (22). The dominant targets for these EBV-specific CTLs are the Epstein-Barr nuclear antigen 3 (EBNA3) family of proteins (12, 18, 22). Importantly, these antigens are expressed in most cases of EBV-positive PTLD, and the success of adoptive T-cell therapy in this context has become the paradigm for immune intervention against a tumor (24). However, in EBV-positive HD and NPC, virus gene expression, and therefore the available targets for T-cell recognition, is restricted to EBNA1 and the latent membrane proteins 1 and 2 (LMP1 and -2, respectively) (2, 20, 21). Of these, EBNA1 is protected from processing and presentation via the conventional HLA class I pathway, due to the presence of an internal glycine/alanine domain (1, 16), and LMP1 has thus far proven to elicit CD8⁺ T-cell responses only very rarely (13).

Attention has therefore focused on LMP2 as a therapeutic target (15). Here, we report the identification of a novel HLA-A2-restricted CD8⁺ T-cell epitope in LMP2, which is presented in a TAP-independent manner, but requires the immunoproteasome for its generation.

Stimulation of peripheral blood mononuclear cells (PBMCs) with cells of the autologous EBV-transformed lymphoblastoid cell line (LCL) has already identified a total of 11 CD8⁺ T-cell epitopes in LMP2, presented in the context of a range of HLA alleles, including HLA-A*0201, -A11, -A24, -B27, -B40, and -B67 (14, 22). In this study, a similar reactivation of PBMCs from an EBV-seropositive individual (donor A) carrying the HLA-A*0201, -A11, -B27, and -B40 alleles generated a number of CTL clones that did not recognize any of the previously identified CD8⁺ epitopes. As shown in the chromium release assay in Fig. 1A by using one such CTL clone, there was clear recognition of A*0201-positive LCLs overexpressing LMP2 from a vaccinia virus vector, but no recognition of either of the previously characterized A*0201-restricted LMP2 epitopes CLGGLL TMV and LLWTLVVVL (henceforth designated by the first three letters of their amino acid sequence). To identify the new epitope, such clones were screened in a T-cell–T-cell killing assay (3) against a panel of overlapping 14- and 15-mer LMP2 peptides, and their reactivity was mapped to the overlapping 14-mers, LMP2 amino acid positions 353 to 366 and 357 to 370. As shown in Fig. 1B, titration of these 14-mers and smaller peptides from within this region identified the minimal epitope as FLYALALLL (FLY; LMP2 amino acids 356 to 364); interestingly this sequence does not lie entirely within the original overlap so that the recognition of the position 357-to-370 14-mer was possible, even though it lacked the phenylalanine residue that forms position 1 of the optimal recognition sequence. Importantly, this epitope sequence is conserved in the LMP2 gene of all Caucasian and Chinese EBV isolates so far sequenced, including the viruses present in the tumor cells of EBV-positive HD and NPC (data not shown). Furthermore, analysis by enzyme-linked immunospot (ELISPOT) assay for peptide-induced gamma interferon (IFN-γ) release revealed that CD8⁺ T cells specific for the FLY epitope are present in a high proportion of EBV-seropositive, HLA-A*0201-positive donors at levels (up to 168/10⁶ PBMCs) that were generally as strong as the response to CLG and stronger than the response
to LLW in the same individuals (Table 1) (5). Clearly therefore the FLY epitope is immunogenic in vivo, and FLY-specific responses are likely to play a significant role in the physiologic control of EBV infection in A*0201-positive individuals. It is worth pointing out that the FLY T-cell clone illustrated in Fig. 1, like CD8+ T-cell clones expanded in vitro against several different EBV epitopes, including CLG and LLW, shows little if any baseline killing of autologous EBV-transformed LCL targets in conventional 5-h chromium release assays; only cells overexpressing the target antigen from a vaccinia virus vector elicit strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, again, like clones against other epitopes, good recognition of the unmanipulated LCLs elicited strong killing (5, 10). However, given the unique pattern of results obtained with FLY effectors in the assays described above, we sought to determine whether the requirements for processing of LMP2 to generate the FLY epitope were in some way different. In this context, we had earlier shown that all other LMP2-derived epitopes (both TAP dependent and TAP independent) appeared to be generated by proteasomal degradation, since specific inhibitors of the proteasome, such as lactacystin and epoxomicin, blocked
their presentation in LMP2-expressing cells (14); this is indeed the pathway by which most CD8+ epitopes are produced from endogenously expressed antigens (4, 6, 23). The catalytic core unit of the proteasome, the 20S proteasome, has a cylindrical structure composed of four stacked rings made up of 14α and 14β subunits (30). Importantly, the catalytic activity of this constitutively expressed complex has been mapped to three β subunits, β1, β2 and β5. In professional antigen-presenting cells, such as B cells and certain other cell types following IFN-γ induction, the subunit composition of the proteasome alters such that the active site β subunits are replaced by three IFN-γ-inducible subunits, low-molecular-weight protein 2, low-molecular-weight protein 7, and MECL-1, resulting in the formation of the immunoproteasome (11, 19). We noted that the major histocompatibility complex (MHC) region deletion that rendered T2 cells TAP negative has also removed the genes encoding two of the IFN-γ inducible immunoproteasome subunits, low-molecular-weight proteins 2 and 7 (here referred to as ip-lmp2 and ip-lmp7, respectively) (25). Recent studies have shown that these subunits, either individually or collectively, are required for efficient generation of particular CD8+ epitope sequences from native antigen (8, 26, 27). In an attempt to study this further, we took advantage of a series of TAP and ip-lmp transfectants that had been generated on the processing function and was in fact the line from which the T2 clone was originally derived. However, these experiments showed that coexpression of the human TAP1 and TAP2 proteins plus either ip-lmp2 or ip-lmp7 was insufficient to rescue presentation of the FLY epitope (data not shown). Because there was no line which express TAP but not ip-lmp subunits had been restored, we moved to the use of epithelial or fibroblast targets (i.e., to cells in which the immunoproteasome subunits could be induced by IFN-γ).

The first experiments of this type involved the TAP-positive, HLA-A*0201-positive melanoma cell line MEL-275, which preliminary work had identified as negative for the immunoproteasome subunits. As shown in Fig. 3A, assays with CLG-specific effectors first confirmed that after infection by vLMP2, these cells can efficiently present the CLG epitope to levels almost equivalent to those of peptide-pulsed targets. In contrast, the same target cells were not recognized by FLY-specific effectors; however, good recognition was achieved if the cells were first treated with IFN-γ (Fig. 3A), and such treatment was associated with the induction of significant levels of the ip-lmp2 and ip-lmp7 subunits (Fig. 3B). This presentation of FLY by IFN-γ-induced cells was completely blocked by pretreatment with the proteasomal inhibitor lactacystin (Fig. 3A) and by a second specific inhibitor, epoxomicin (17) (data not shown), while such IFN-γ-induced and drug-treated cells retained the ability to present exogenously added FLY peptide. This strongly suggests that the IFN-γ-induced immunoproteasome is necessary for generation of the FLY epitope.

We subsequently showed that, like MEL-275, normal fibroblasts from an HLA-A*0201-positive donor (donor B) were able to present the CLG epitope but not the FLY epitope from vaccinia-expressed LMP2, but acquired the ability to present following IFN-γ induction (Fig. 4A). This proved important in view of the availability of fibroblasts from an HLA-A*0201-positive patient (patient C) with a homozygous TAP2 gene mutation that abrogated TAP function (S.G. and V.C., unpublished data). When the experiment was repeated in this TAP-defective fibroblast background, uninduced cells again presented the TAP-independent CLG epitope from LMP2 but not the FLY epitope; importantly, however, IFN-γ induction did allow FLY to be presented (Fig. 4B). Figure 4C confirms that the immunoproteasome subunits ip-lmp2 and ip-lmp7 are indeed induced by IFN-γ in patient C fibroblasts, as is TAP1, but these cells are incapable of making TAP2. There is one report on a study using mouse RMA/S cells, also expressing only...
TAP1, that raises the possibility that a TAP1 homodimer is capable of transporting peptides (7). However, from work with T2 cells, human TAP1 has an absolute requirement for TAP2 to transport peptides (9), and so we consider it very unlikely that in patient C fibroblasts a homodimer of TAP1 could be responsible for the ability of the FLY epitope to access the HLA class I presentation pathway. The data are therefore consistent with FLY being a TAP-independent epitope. This is in accord with our proposed hypothesis that cytosolically generated peptides, if sufficiently hydrophobic, can access the endoplasmic reticulum by a novel TAP-independent pathway (14).

As to the route whereby the FLY epitope is generated from endogenously expressed LMP2, our data from both epithelial and fibroblast systems clearly show that the processing of FLY (i) is dependent upon IFN-γ induction of the target cells, (ii) is blocked by specific inhibitors of the proteasome, and (iii) is coincident with appearance of the IFN-γ-inducible immunoproteasome subunits ip-lmp2 and ip-lmp7 (Fig. 3 and 4) (data not shown). Besides the ip-lmp subunits, it is of course possible that some other IFN-γ-induced proteasomal activity is responsible for the change in processing capacity. In this context, the most likely candidate would be the proteasome modulator complex, PA28, which has been shown to influence the generation of antigenic peptides in a manner independent of
the presence of immunoproteasome subunits (28). However, we found that PA28 is present at significant levels in donor B and patient C fibroblasts even before IFN-γ induction, in addition to being present in T74 and T2 cells (data not shown). This would suggest that PA28 is not the limiting factor in the generation of the FLY epitope. We therefore infer that FLY is indeed dependent upon the immunoproteasome components ip-Lmp2 and ip-Lmp7 for its generation.

In summary, we have identified a new EBV-encoded CD8+ T-cell epitope which could prove a therapeutically useful target in that it is restricted through a relatively common HLA allele, HLA-A*0201, and is derived from LMP2, one of the few viral proteins expressed in EBV-positive malignancies such as HD and NPC. Interestingly, though the epitope displays the expected TAP-independent phenotype, it also represents the first EBV epitope whose generation has been shown to be immunoproteasome dependent. These data emphasize a more general lesson: that as more “tumor-associated” epitopes are described, one needs to study the requirements for their generation within cells and to determine whether these requirements are met in the tumor cells themselves. In the particular context of the FLY epitope, it will be important to determine whether EBV-positive HD and NPC cells, for which there are very few if any truly representative cell lines in culture, have the capacity to present this epitope from endogenously expressed LMP2 in vivo.

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