1 **Title**

2 The Epstein-Barr virus major tegument protein BNRF1 is a common target of cytotoxic CD4+

3 T cells

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5 **Running title**

6 Immunogenicity of EBV BNRF1 to CD4+ T cells

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Abstract

Cellular immunotherapy is a proven approach against Epstein-Barr virus (EBV)-driven lymphoproliferation in recipients of hematopoietic stem cells. Extending the applicability and improving the response rates of such therapy demands improving the knowledge base. We studied twenty-three healthy donors for specific CD4+ T cell responses against the viral tegument protein BNRF1 and found such T cells in all seropositive donors, establishing BNRF1 as an important immune target in EBV. We identified eighteen novel immune epitopes from BNRF1, all of them generated by natural processing of the full-length protein from virus-transformed lymphoblastoid cell lines (LCL). BNRF1-specific CD4+ T cells were measured directly *ex vivo* by a cytokine-based method, thus providing a tool to study interaction between immunity and infection, in health and disease. T cells of cytotoxic Th1 type inhibited the proliferation of autologous LCL as well as virus-driven transformation. We infer that they are important in limiting reactivations to sub-clinical levels during health and reducing virus propagation during disease. The information obtained from this work will feed into datasets that are indispensable in the design of patient-tailored immunotherapeutic approaches, thereby enabling the stride towards broader application of T cell therapy and improving clinical response rates.

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Importance

Epstein-Barr virus is carried by most humans and can cause life-threatening diseases. Virus-specific T cells have been used in different clinical settings with variable success rates. One way to improve immunotherapy is to better suit T cell generation protocols to viral targets available in different diseases. BNRF1 is present in viral particles and therefore likely available as a target for T cells in diseases with virus amplification. Here, we studied healthy EBV-carriers for BNRF1 immunogenicity and report our results indicating BNRF1 to be a dominant target of the EBV-specific CD4+ T cell response. BNRF1-specific CD4+ T cells were found to be cytotoxic and capable of limiting EBV-driven B cell transformation *in vitro*. The findings of this work contribute to forwarding our understanding of host-virus interactions during health and disease and are expected to find direct application in the generation of specific T cells for immunotherapy.

63 **Introduction**

64 Epstein-Barr virus (EBV) is a WHO-classified carcinogenic human herpesvirus (1) associated
65 causally or otherwise, with several diseases including cancers like Burkitt and Hodgkin
66 lymphoma. Following primary infection, EBV elicits a strong cellular immune response that
67 is generally believed to be instrumental in keeping potential virus-related disorders in
68 healthy carriers under check (2). EBV transforms B cells *in vitro* to generate so-called
69 lymphoblastoid cell lines (LCL) that serve as efficient stimulators to EBV-reactive T cells in
70 the peripheral blood of immune individuals, thereby providing a laboratory model to study
71 immunogenicity of infected B cells, and serving as readily available stimulators for selectively
72 activating and expanding virus-specific T cells for clinical use (3).

73 *In vitro* EBV-transformed B cells express a maximum of nine so-called latency proteins (4)
74 with a small percentage of cells undergoing spontaneous lytic replication. Early studies on
75 the cellular immune response to EBV focused largely on understanding cytotoxic CD8+ T cell
76 responses to the latency proteins of the virus and yielded several antigenic epitopes in
77 different viral proteins (5). The body of knowledge on the immune responses against the
78 virus has immensely expanded over the years but CD4+ T cell responses have remained less
79 well studied (6).

80 The early understanding of the cellular immune responses was successfully translated to
81 clinical application in the form of adoptive transfer of T cells for the prevention as well as
82 treatment of some EBV-associated clinical disorders, most prominently in the context of
83 post-transplant lymphoproliferation disorders (PTLD) in recipients of hematopoietic stem
84 cell transplants (HSCT) (7). The challenge now is to extend and improve the applicability and
85 success of T cell therapy to further clinical disorders such as Hodgkin lymphoma, and

nasopharyngeal carcinoma (8). Two important factors that are relevant to this pursuit are i) the application-specific relevance of the antigen specificity of the T cell preparations and ii) the CD4+ component in clinically used T cell preparations (9).

Improving the response rates to immunotherapy is likely to depend on tailoring immunotherapy to the viral antigen expression context of the disease. The changes in protocols used to prepare T cells for therapy reflect this. Whereas early protocols generally used LCL as stimulators of EBV-specific T cells used for immunotherapy, approaches that are more recent have incorporated the use of antigenic peptides (10, 11). In face of the rise to prominence and continuing improvements in T cell receptor transfer technologies, the rather laborious and time-consuming protocols using *in vitro* stimulation of T cells can be expected to be complemented or even replaced by tailored approaches using receptor transfer (12-14). This would be of special value in transplantations involving EBV-negative donors, where the donor lacks in naturally primed EBV-specific T cells (15). A requisite for such advancement is the expansion of our knowledge of T cell epitopes and receptors that target them.

We have observed in the past that B cells can efficiently extract structural proteins from virions following receptor-mediated uptake and present derivative peptides to CD4+ T cells (16). Viral particles contain over thirty different proteins of the virus (17). The immunogenicity of most of them remains largely unexplored. In this background, we systematically studied CD4+ T cells responses against the virion structure protein encoded by the BNRF1 gene of the virus. We chose BNRF1 for several reasons. Number one, in the past we have observed that BNRF1 is an immunodominant target in more than one LCL-stimulated CD4+ T cell line as well as virus-like particle-stimulated CD4+ T cell line from healthy virus carriers (18, 19). Number two, in a patient with PTLD that had received EBV-

derived peptide specific T cells we observed that CD4+ T cells targeting a peptide derived from BNRF1 expanded upon transfer with the peak of the peptide-specific T cell numbers correlating with the drop in viral load (10). Number three, BNRF1 is highly conserved across different strains of EBV, an important aspect to consider, given the recent realization that field strains of EBV can carry variant proteins leading to variability in known T cell epitopes (20). Number four, BNRF1 has been suggested to be expressed in latently infected cells, making it an interesting candidate to target in EBV-driven malignancies (21).

The aim of this study was to assess the breadth of BNRF1-specific CD4+ T cells in healthy virus carriers, and in the process define clinically relevant target epitopes.

Our study establishes BNRF1 as a common target of CD4+ T cells. We identified eighteen novel peptide epitopes of BNRF1 that are immunogenic to CD4+ T cells. Importantly, these epitopes are generated from full-length protein and thus are potentially clinically relevant. We expect the identified peptide epitopes to be useful in future clinical settings and suggest the approach used in this work for future studies into the immunogenicity of further EBV proteins.

Results

1. CD4+ T cells with BNRF1 reactivity can readily be expanded *in vitro* from the peripheral blood of EBV seropositive individuals

For our search of BNRF1-specific CD4+ T cells in healthy carriers, we used a protein stimulation-based enrichment approach. Recombinant BNRF1 protein was expressed in HEK293T cells with a C-terminal 6x His-tag to allow purification using nickel (Ni) -NTA agarose beads and detection using anti-His antibody (Figure 1A).

T cell lines were initiated using PBMC pulsed with recombinant BNRF1 protein as stimulators from twenty-two healthy volunteers, twenty of them EBV-seropositive and two EBV-seronegative. At the end of two weeks, the cultures were magnetically sorted using CD4+ microbeads and the positive fractions were stimulated again with irradiated PBMC pulsed overnight with recombinant BNRF1. Such stimulation was repeated every two weeks and the growing cultures tested for specific response against BNRF1. As shown in Figure 1B, twenty of the twenty-two T cell lines showed specific recognition of BNRF1 (depicted in the figure as a ratio of the IFN-γ signal obtained against PBMC pulsed with BNRF1 compared to PBMC pulsed with a recombinant protein expressed similarly and used in similar amounts). The two cases where we found no BNRF1 specific responses were from EBV-negative individuals. These T cell lines were restimulated fortnightly for up to nine times but no consistent selective BNRF1-specific activity could be established.

Where the T cell lines became BNRF1-specific, the number of restimulations that were required for specific response varied between individuals, perhaps indicating the difference in the precursor frequencies in the peripheral blood. Thus, whereas T cell lines from some donors demonstrated specificity within two rounds of stimulations, others required up to

148 seven rounds. As shown in figure 1C, the response of the T cells to BNRF1 as compared to
149 the response against PBMC pulsed with an irrelevant protein improved over restimulations.
150 This was not the case with T cell lines from EBV-negative donors D21 and D22.

151

152 **2. Breadth of the CD4+ T cell response to BNRF1**

153 The selective BNRF1-reactivity of the T cell lines from EBV-positive individuals indicated that
154 BNRF1 is immunogenic to CD4+ T cells. The successful establishment of specific T cell lines
155 from all seropositive donors suggested that the immunogenicity of BNRF1 to CD4+ T cells
156 spreads across a broad range of MHCII molecules. The donors were MHC genotyped. In
157 order to further characterize the T cell lines we established LCL and miniLCL from available
158 donors by infecting peripheral blood B cells either with wild type virus or with BZLF1-
159 deficient virus, respectively. The latter efficiently infects B cells which then transform to
160 miniLCL that are deficient in lytic replication. Such miniLCL were used as antigen presenting
161 cells in some experiments for subsequent T cell characterization, since our previous work has
162 shown that miniLCL are recognized by BNRF1-specific T cells only after pulsing with antigen.

163 We tested the T cell lines for responses against BNRF1 peptides. 327 15mer peptides were
164 synthesized to cover the length of the BNRF1 protein sequence from B95.8 strain of EBV. The
165 peptides were subdivide into seven pools with similar numbers of peptides. The T cell lines
166 were then tested for responses against the peptide pools. As shown in figure 2A, specific T
167 cell lines showed above background responses against at least one of the pools, suggesting
168 that most of the donors had developed T cell responses against more than one target
169 peptide.

To identify the target peptide of the T cell lines, subpools were created. Individual peptides that were contained in positive subpools were tested as single peptides. In some cases two consecutive peptides elicited similar responses from the T cells. Figures 2B and 2C show representative results of experiment using the described approach.

3. BNRF1-specific CD4+ T cells can be detected directly *ex vivo*

Given the above results, we attempted to quantify BNRF1-specific T cells directly *ex vivo*. EBV-specific CD4+ T cells have generally been described to be present in numbers around ten times lower than CD8+ T cells (6). Therefore, CD8-depleted PBMC are generally used in ELISPOT assays (22). We exposed CD8-depleted PBMC to recombinant BNRF1 and the control protein transaldolase, after washing them following overnight rest.

After 16 hours of incubation with the antigen, the ELISPOT plates were processed, and IFN-γ spot forming units were counted. The results are shown in Figure 3. The results indicated that BNRF1-reactive T cells can be detected directly *ex vivo* by ELISPOT assay but the frequency of BNRF1-specific CD4+ T cells in peripheral blood differed widely across donors and did not correlate with the number of restimulations required to detect BNRF1-specific reactivity in the T cell lines. EBV-seronegative donors were clearly negative in the ELISPOT assays validating this approach.

4. Identification of MHCII molecules presenting the epitopes

For subsequent studies, we performed limiting dilution cloning on the T cell line and tested outgrowing cultures for BNRF1 recognition. Shown in Figure 4A are results of cytokine

192 secretion assays using outgrowing clones from T cell lines from two donors. Outgrowing T
193 cells included both BNRF1- specific and not BNRF1-specific clones, indicating that T cell lines
194 were not constituted of BNRF1-specific T cells only. We expanded the BNRF1-specific T cells
195 for further characterization, starting with identification of the MHCII restriction element.

196 Different approaches were used to identify the restricting MHCII molecules. Where
197 available, we tested BNRF1-pulsed miniLCL from different allogenic donors that shared part
198 of the MHCII profile with the T cell donor for recognition by the T cells. This allowed
199 identification of the candidate MHCII molecules that were responsible for presenting the
200 antigen to T cells in some of the cases. Two examples are shown in Figure 4B for the T cell
201 clones D14#5 recognizing peptide 125 (upper panel), and D12#3 recognizing peptide 281
202 (lower panel), respectively. For the T cell clone recognizing peptide 125, we identified the
203 restricting MHCII molecule as HLADRB3*0202. By this approach, for the T cell clone
204 recognizing peptide 281, we could only narrow down the candidates to one of either
205 HLADRB1*0101 or HLADQB1*0501.

206 Another approach involved the blocking of MHCII molecules. Preliminary experiments
207 suggested this approach to work reliably for DP molecules. Therefore, we applied this
208 approach to test for HLA DP-restriction of some T cell clones. An example is shown in Figure
209 4C.

210 For some T cell clones for which candidate MHCII transient expression plasmids were
211 available, such plasmids were transfected into DG75 cells alone or along with a BNRF1-
212 encoding plasmid, and transfected DG75 cells used for identifying the presenting MHCII
213 molecule. An example is depicted in Figure 4D for the T cell clone #1 from donor D37. The
214 above approaches were combined with using the MHCII binding prediction program

NetMHCII (http://www.cbs.dtu.dk/services/NetMHCIIpan/). A combination of the mentioned approaches allowed the identification of single MHCII allotype as restriction element for the majority of the BNRF1-derived antigenic peptides recognized by T cell clones from different donors. In one case, the MHCII presenting molecule could not be identified.

Table 1 presents the BNRF1 peptides and the presenting MHCII molecules, as well as the position of those peptides in the full-length protein sequence. Where two adjacent peptides were recognized as potential targets in T cell cytokine release assays, the combined sequence was used to predict binding and the core sequence identified. For such cases, the core sequence plus three upstream and three downstream amino acids are presented as the epitope. Figure 5 shows the amino acid sequence of BNRF1 from the B95.8 viral strain with identified epitope regions marked. As depicted in Table 1, BNRF1 was presented in a variety of MHCII contexts. Thus, BNRF1 is immunogenic to CD4+ T cells across a broad set of antigen-presenting molecules, with no obvious epitope "hotspots" within the protein (Figure 5). In the same individual, we often identified more than one BNRF1 peptide recognized by T cells, indicating that BNRF1 is indeed a frequent target for EBV-specific CD4+ T cells.

5. CD4+ T cells against BNRF1 are cytotoxic Th1 type T cells

Others and we have described EBV-specific cytotoxic CD4+ T cells as cytotoxic (16, 23, 24). In terms of the cytokine phenotype, Th1 as well as Th2 type CD4+ T cells have been described (25). All BNRF1-specific T cells secreted IFN-γ and, as shown in Figure 6A, secreted perforin as well as granzyme B upon recognition of their targets, thereby establishing themselves as Th1 type cytotoxic T cells. For a more direct demonstration of cytotoxicity, we used Calcein-AM release-based cytotoxicity assay. For such assays, we used autologous miniLCL as antigen

presenting cells. Prior to labelling with the dye, the antigen-presenting cells were pulsed with either a control peptide or the cognate BNRF1-derived peptide. Peptide-pulsed, dye-labelled cells were incubated with BNRF1-specific T cells. The T cells consistently caused up to 80% specific lysis of targets loaded with the cognate antigenic peptide but not with a control peptide (Figure 6B).

6. BNRF1-specific T cells recognize, kill, and inhibit the proliferation of lymphoblastoid cell lines capable of lytic replication and counteract EBV-driven primary B cell transformation

A small percentage of wild type EBV-transformed B cells undergo lytic viral replication (6), thereby package BNRF1 into viral particles and release them. We therefore tested all available BNRF1-specific T cell clones for recognition of wild type EBV-transformed LCL. While lytic replication-deficient miniLCL were not recognized by the T-cells, lytic replication-competent LCL were readily recognized (Figure 7A). The degree of recognition of LCL varied from donor to donor, but in general corresponded to the extent of lytic replication as measured by viral load in the supernatants of the LCL. BNRF1-specific T cells from donor D6 responded to autologous LCL at only background levels and low viral load in the supernatant supported the finding of the cytokine ELISA.

The recognition of autologous LCL by BNRF1-specific T cell clones from almost all donors raised the question as to what percentage of cells in a LCL culture could be targeted by the T cells and whether the recognition of LCL could lead to detectable killing. To address this we tried to enumerate the number of cells in a LCL targeted by the T cells using ELISPOT assays. T cell clones from two donors, recognized over ten percent of cells in a LCL culture. However,

even in the LCL in which around twenty percent of the cells were recognized by BNRF1-specific T cells, we were unable to detect BNRF1 by Western Blot in cell lysates (unpublished data). BNRF1 could only be detected in lysates of the Burkitt Lymphoma cell line Akata in which lytic replication had been induced by anti-IgM treatment (unpublished data). These findings substantiate our previous finding that antigen transfer by virions is the major contributor to MHCII presentation of BNRF1 by LCL (16, 19).

Recognition of LCL led to their killing (Figure 7B right) that was detectable in 4-hour cytotoxicity assays already at T cell to target cell ratios of 2:1.

We next tested the T cells for inhibition of LCL proliferation. Autologous LCL were incubated in serial dilutions with or without T cells. In the presence of T cells, three to 30-fold higher starting cell numbers were required to obtain a proliferating LCL culture, except for donor D6 whose T cells failed to demonstrate a measurable inhibition of LCL proliferation. T cells from none of the donors inhibited the proliferation of miniLCL and in one case where T cells against a non-EBV antigen were available, that T cell clone was not able to limit the proliferation of LCL (Figure 7C) indicating that the inhibition of LCL proliferation by BNRF1-specific CD4+ T cells was target antigen specific.

Whereas B95.8 is a representative laboratory strain, it has been recognized over the last few years that viral strains in healthy carriers and in diseased people can be deviant from the laboratory reference strain. Therefore, we tested spontaneously outgrowing EBV transformed B cell cultures that were available from three healthy individuals. Although the sample size is small, experiments with these “spontaneous LCL” with field strains of the virus demonstrated that the lytic replication in these spontaneous LCL was enough to sensitize the cells for recognition by the T cells (Figure 7D).

These findings illustrated that BNRF1-specific T cells can recognize B cells transformed by the laboratory EBV strain B95.8 as well as by the individual field EBV strain. Given the presence of the BNRF1 protein in viral particles in amounts sufficient to trigger recognition by the T cells, we were interested whether such T cells were also able to inhibit the transformation of B cells by EBV. For these experiments, we enriched B cells in PBMC by positively sorting CD19+ cells using magnetic beads. Sorted B cells were then exposed to wild type EBV at a multiplicity of infection of 0.1 and after two hours of incubation B cells were seeded onto 96-well plates in serial dilutions starting at 30,000 in the presence or absence of BNRF1-specific clonal T cells (at a constant number of 10,000). Half the media in these co-cultures was replaced once a week. In order to limit the T cell activity to the initial phase in which virus binds to and enters B cells, no cytokine support was afforded to the T cells. After a month of incubation, the plate was read for EBV-driven transformation. Flow cytometry was performed to confirm the outgrowing cells to be B cells. There was a donor to donor difference in the results but in all donors tested the presence of BNRF1-specific T cells led to inhibition of EBV-driven transformation, as evidenced by the requirement of higher starting numbers of EBV-exposed primary B cells (Figure 7E).

Discussion

The optimism generated by the success of EBV T cell therapy in HSC-associated PTLD has driven exploration of ways to advance adoptive therapy towards improving current response rates and extending its clinical indications (26). Tailoring T cell therapy to suit the need of the patient at hand demands knowledge of the immunogenicity of the disease in question, of the target viral antigens available for presentation, as well as of antigenic epitopes generated by infected cells and presented to T cells in the given MHC context. Findings from *in vitro* studies, mouse models of PTLD-like tumors as well as from the clinical application of EBV-specific T cells have established CD4+ T cells as important components of the immune response against EBV-driven PTLD (9, 27, 28). However, studies into the antigen repertoire recognized by EBV-specific CD4+ T cells are still limited. In this work we studied the major EBV tegument protein BNRF1, identified 18 naturally processed antigenic peptide epitopes presented to CD4+ T cells across a number of MHCII molecules and demonstrated antiproliferative effects of BNRF1-specific CD4+ T cell clones.

We were able to establish BNRF1-specific CD4+ T cell lines from 20 out of 20 EBV-seropositive individuals studied in this work. A study into the CD4+ response against EBV latency proteins in healthy carriers has found a maximum of 75% donors to respond against EBNA1, the most ubiquitously expressed viral protein (22). A similar study into CD4+ T cells against EBV lytic cycle proteins did not investigate BNRF1, but among three late lytic proteins studied the best donor response rates was found for BXLF2, CD4+ T cells against which were detected in 10 out of 14 healthy carriers (24). Against this background, our finding of all EBV-positive donors studied having CD4+ T cells against BNRF1, a structural protein of the virus, was unexpected. One possible explanation for our finding is that BNRF1 might also be expressed in viral latency, as has been recently suggested, and that BNRF1 is available for

presentation even in the healthy carrier state (21). However, we have consistently observed that miniLCL, EBV transformed cells genetically deficient in supporting lytic replication, cannot trigger a response from BNRF1-specific CD4+ T cells, unless exposed to an exogenous source of antigen (19). Therefore, if it is indeed a latency protein, BNRF1 in EBV latency either is expressed in insufficient amounts to trigger cytokine and cytotoxicity responses from CD4+ T cells or is not accessible to the MHCII antigen-presentation pathway in the absence of productive lytic replication.

Our ELISPOT assay results show that using the CD8-depletion approach, BNRF1-reactive CD4+ T cells can be measured directly *ex vivo*. However, this is not true in all donors, in spite of the fact that all seropositive donors had BNRF1-specific T cells as evidenced by the specificity of the BNRF1-reactive T cell lines. This discrepancy indicates that direct ELISPOT assays *ex vivo,* as is generally employed, may not be sensitive enough to identify all positive individuals and that donor positivity data obtained from such ELISPOT studies may under-report specific T cell frequencies and/or diversities. Indeed, whereas the aforementioned peptide-based ELISPOT approach by Long et al. identified EBNA1-specific CD4+ T cells in only 75% of the donors studied (24), the *in vitro* stimulation approach by Munz et al. detected EBNA1-specific CD4+ T cells in all of ten seropositive donors studied (29). These findings together imply that *in vitro* stimulation approaches might be more reliable in detecting CD4+ T cells against EBV.

It is generally believed that *de novo* EBV-infected cells are predominantly targeted by CD8+ T cells with CD4+ T cells being better at recognizing latently infected cells (6). The findings of this work add further evidence to our previous proposition that CD4+ T cells might actually be involved as prominent players during *de novo* infection too (20). As we have observed in the past for BLLF1 and BALF4, two glycoproteins of the viral envelope, here we

demonstrated that another viral structural protein, namely BNRF1, can mediate the inhibition of B cell transformation and of LCL proliferation by CD4+ T cells.

Individuals latently infected with herpesviruses demonstrate subclinical virus reactivation from latency from time to time (30-33). It has been suggested that chronic, virus replication, a common phenomenon in the persistent carrier state, may be essential for the maintenance of latency (31). Such a low-level replication, if not promptly controlled, could amplify to produce subclinical reactivations or even full-fledged clinical disease. It is thus conceivable that virion protein-specific CD4+ T cells deliver a major contribution in keeping the likely base-line reactivation under check and thus maintain a controlled latent infection in healthy carriers. EBV-associated diseases are often associated with high viral loads (34, 35) and viral strains associated with cancers are likely to be associated with enhanced lytic replication (36), suggesting an increased availability of virion proteins like BNRF1. Efficient targeting of EBV-driven diseases likely requires targeting latently infected cells but also cells in lytic replication as well as freshly infected cells. The importance of the immune control of B cells that have picked up virus and are in the process of transformation into continuously proliferating colonies cannot be exaggerated. Our findings support the notion that T cell immunotherapy against EBV-driven diseases could draw benefits from the conscious inclusion of virion-protein-specific CD4+ T cells in T cell preparations. Such CD4+ T cells are likely to be able to play a crucial role during amplification of lytic viral activity, which is a potential key stage in the establishment and or maintenance of viral driven disease(s) states.

BNRF1-specific T cells were found to recognize wild type virus-transformed B cell lines to variable degrees that correlated with the rate of lytic replication in the LCL. The non-recognition of miniLCL but recognition of LCL even though BNRF1 could not be detected by immunoblot in miniLCL or in LCL could be explained by our previous observation that viral

particles released from cells undergoing lysis can serve as source of antigen for neighboring cells (19). In agreement with this, ELISPOT assays revealed the number of LCL recognized by T cells to be more than the generally low, limited lytic replication in LCL(6, 37). The previous indication that miniLCL can present BNRF1 to CD8+ T cells (21) raises the question whether there is a preferential presentation of intracellular BNRF1 to CD8+ T cells and, if yes whether the differential presentation can be attributed to differences in levels of expression. In order to address this further we are in the process of establishing a system to express BNRF1 intracellularly in an inducible manner. Such a system is expected to also allow the assessment of the number of cells expressing BNRF1 intracellularly that can actually be targeted by CD4+ and CD8+ T cells as well.

The lytic replication-dependent recognition of LCL suggests that one approach to optimize EBV-specific T cell therapy might be the induction of lytic replication to aid the recognition of tumor cells by EBV-specific CD4+ T cells, like those against BNRF1. Induction of lytic replication has been an avenue that has been under investigation as a way to make infected cells susceptible to antivirals like ganciclovir (38). Our findings with virion-specific CD4+ T cells raises the intriguing question of whether controlled induction of lytic replication can aid immunotherapy by increasing the availability of target antigens.

This work has identified 18 previously unknown target peptide epitopes recognized by BNRF1-specific CD4+ T cells and has established an approach to quantifying BNRF1-specific CD4+ T cells directly *ex vivo* by ELISPOT. This ELISPOT approach needs to be optimized to improve sensitivity so as to determine true precursor frequencies of such T cells in the peripheral blood. Using peptides instead of full-length recombinant protein might be one way of optimization. Peptides are purer than recombinant protein in terms of BNRF1 and can be expected to reduce the background signals, but full-length protein-elicited spots are

probably more meaningful since they indicate appropriate processing of full length BNRF1 and presentation to T cells. Therefore, these two aspects need to be weighed against each other. In PBMC from two seropositive donors, ELISPOT assays performed with individual peptides or a combination of peptides recognized by their T cell lines have shown promising results (data not shown). Such an approach could be used for assessing virus-specific CD4+ T cells in health and disease.

Healthy virus carriers may be different in terms of the basal activation rate, as has been suggested by previous studies (32). This may contribute to different degrees of activation of BNRF1-specific CD4+ T cells in different individuals, thereby producing highly variable precursor frequencies when measured in cross-section studies like done in this work. If BNRF1-specific T cells were stimulated to a different extent in different individuals due to different rates of basal viral replication, the T cells might differ in their differentiation phenotype. With the knowledge of a considerable number of target peptides presented on common MHCII molecules, the phenotype question might be systematically addressed with MHCII multimer technology as has been employed in the past by Long and colleagues for several MHCII target epitopes in other EBV proteins (39). An understanding of the effector-memory phenotype of the T cells will likely contribute to understanding virus reactivation during health.

Our findings suggest that cytotoxic CD4+ BNRF1-specific T cells can efficiently recognize and eliminate lytically infected B cells. It has indeed been noted nearly forty years ago that the suppression of EBV infection *in vitro* takes place after infection but before transformation and that viral determinants left on the surface are potential triggers of the T cell response (40). Our results support these early observations and add BNRF1 to other viral targets with

high clinical impact in the immune control of viral spreading and EBV-associated cell proliferation.

421 **Materials and Methods**

422 **Donors and primary blood cells**

423 This work used peripheral blood mononuclear cells (PBMC) and derivatives thereof. PBMC
424 were purified using standard procedures from peripheral blood obtained from healthy
425 volunteer donors. The use of material of human origin in this work was approved by the
426 ethics committee of the Technical University Munich (approvals 934/03 and 1872/07) and
427 was in accordance with the Declaration of Helsinki of the World Medical Association (last
428 amended in 2013).

429 The donors were assigned donor numbers starting with D1. All donors from whom T cell
430 lines and clones were raised as well as those from whom LCLs and miniLCLs were established
431 were HLA-typed at the Laboratory of Immunogenetics at the University Hospital of the
432 Ludwig Maximilians University, Munich. PBMC or derivatives thereof were not available from
433 all donors for all experiments. Therefore, based on availability, material derived from
434 different sets of donors were used for different experiments.

435

436 **Protein preparation**

437 Protein preparations that were used as antigens were expressed in human embryonic kidney
438 cells (HEK 293T) (ATCC® CRL3216™) from a CMV promoter (pCMV)-driven plasmid construct
439 as described before (41). The cloning allowed proteins to be expressed with a C-terminal 6 X
440 Histidine-tag and purified out of the lysates of transfected cells using Ni-NTA Agarose beads
441 (Qiagen).

442 We used wild type EBV DNA of B95.8 viral strain origin (GenBank: AJ507799.2, webpage:
443 https://www.ncbi.nlm.nih.gov/nuccore/AJ507799.2) as template for polymerase chain

444 reactions to clone the EBV protein coding genes *BNRF1* and *BZLF1*. The human protein
445 transaldolase (Uniprot Protein ID P37837, webpage:
446 https://www.uniprot.org/uniprot/P37837) used as a control in ELISPOT assays was similarly
447 expressed as EBV proteins following cloning from cDNA derived from HEK 293T cells.

448 In order to ensure consistent antigen delivery we pooled multiple preparations of
449 recombinant proteins, measured the protein concentration, and froze down appropriate
450 aliquots.

451

452 **Protein concentration, Polyacrylamide Gel Electrophoresis and Western Blot**

453 Protein concentration was measured by spectrophotometry at wavelength 595 nm using the
454 Bradford reagent and standard protocols. Western blot analysis of 6x histidine (His)-tagged
455 protein preparations was performed using the mouse monoclonal anti-His_6 antibody 3D5
456 (kindly provided by the Monoclonal Antibody Facility, Helmholtz Centre Munich, webpage:
457 https://www.helmholtz-muenchen.de/mab).

458

459 **T cell lines and clones**

460 T cells were cultured in AIM-V media (Invitrogen) supplemented with antibiotics, L-
461 glutamine and 10% heat-inactivated pooled human serum obtained from healthy donors
462 following informed consent.

463 Polyclonal T cell lines were established by exposing PBMC to recombinant BNRF1 overnight
464 followed by irradiation of the cells and culture with non-irradiated PBMC. 1-1.5 micrograms
465 of recombinant BNRF1 were used for 2 million PBMC in 2 ml media in a well of a 24 well

plate. Cultures started for T cell lines were treated with recombinant human IL-2 (50 U/ml) 24 to 48 hours after initiation of culture. Depending on proliferation, the cultures were expanded using media supplemented with IL-2. Two weeks after the initiation of T cell lines, the cells were harvested and the CD4+ fraction obtained by magnetic sorting stimulated again by co-culture with irradiated PBMC pulsed with recombinant BNRF1 protein. This mode of stimulation was repeated fortnightly. Clonal T cell lines were obtained by dilution culture of T cell lines and cultures as described before (16). T cell clones were assigned numbers that follow the donor number.

B cell lines

LCL were established by infection of peripheral blood B cells using either the wild type B95.8 strain of EBV or laboratory strains derived from B95.8 and maintained in culture as previously described (41). For simplicity`s sake, LCL established by infection with B95.8 are called LCL, whereas those established by infection with laboratory viral strains deficient in lytic replication are called miniLCL. The laboratory strain used to generate miniLCL is genetically deficient in the Epstein-Barr virus lytic cycle switch gene BZLF1. It was kindly provided by Prof. Henri-Jacques Delecluse, at the German Cancer Research Center, Heidelberg. The same culture conditions were used for LCL and miniLCL as well as for the EBV-negative Burkitt lymphoma cell line DG-75 (ATCC® CRL-2625™). The transfection of DG-75 cells for their use in antigen presentation experiments has been described (42).

Spontaneous LCL were established by setting up PBMC culture as for the establishment of LCL, with cyclosporine A as described before (41) but without any exogenous virus. Outgrowing cultures were treated similarly as LCL.

Magnetic sorting

Magnetic sorting was performed employing the MACS sorting technology (Miltenyi) following published protocols of the manufacturer. Magnetic microbeads against human CD4, CD8, and CD19 were used.

Cytokine ELISA and ELISPOT

For characterization of T cell cytokine secretion, appropriate cell culture supernatants were used for measurement of cytokine content using commercial kits and accompanying protocols. The following kits (all from Mabtech) were used: Human Interferon gamma (IFN-γ), Granzyme B, and Perforin.

HLA-DP inhibition experiments were performed using clone B7/21 (Abcam) against HLA-DP. IFN-γ cytokine ELISPOT assays were performed using kits from Mabtech according to the accompanying protocol, and cell numbers were adjusted as described in the main text. We performed quantification of ELISPOT using in a scanner/analyzer from C.T.L. Cellular Technology Ltd. using the Immunospot® 5.0 Software.

Cytotoxicity assays

The T cell cytotoxicity assays performed in this work were Calcein-AM-based cytotoxicity assays (43) . Briefly, cells to be used as targets were labelled with Calcein-AM, cell-permeant dye (ThermoFischer) followed by co-culture with T cells. The number of target cells used was five thousand per well and the number of T cells depended upon the desired T cell to APC

ratio. After four hour of co-culture to allow for cytolysis by the T cells, we measured absorbance at 485 nm as a proxy of Calcein-AM released into the supernatant with an Infinite® F 200 PRO microplate reader (Tecan).

We recorded the reading for target cells exposed to a detergent as the maximum and the reading from supernatants where the target cells were left untreated as the minimum. The following formula was used to calculate cytotoxicity: [(reading in the presence of T cells – minimum)/ (maximum-minimum)] X 100. The result is presented as percent specific lysis.

Peptides

327 15-amino acid peptides, each 15-amino acids long were synthesized (JPT Peptide Technologies) covering the whole length of BNRF1. Two adjacent peptides overlapped by eleven amino acids, with one exception- the last two peptides at the C-terminal end of BNRF1 overlapped by twelve amino acids.

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655

Figure Legends:

Figure 1. BNRF1-specific CD4+ T cells are present in the peripheral blood of EBV-seropositive individuals.

A. Expression of recombinant proteins. BZLF1, transaldolase and BNRF1 were expressed as C-terminal 6x His-tagged proteins in HEK 293T cells and purified using Ni-NTA agarose beads. A sample of the eluate was loaded on a polyacrylamide gel and Western blotting was performed using the anti-His antibody 3D5. Molecular masses (according to www.uniprot.org) were 142,844 daltons for BNRF1, 26,860 daltons for BZLF1, and 37,540 daltons for transaldolase.

B. BNRF1-stimulated T cell lines from EBV-seropositive and EBV-seropositive donors. CD4+ T cell lines established by restimulations with protein-pulsed PBMC were tested against PBMC, either untreated or pulsed with BNRF1 or with the control protein BZLF1. The IFN-□ indices were calculated as cytokine concentration in supernatants of T cells responding to PBMC pulsed with either BNRF1 (black bars) or control protein BZLF1 (grey bars) divided each by that of T cells incubated with untreated PBMC. Shown are the IFN-□ indices for T cells from different donors after up to seven passages (indicated as D1p1 to D22p7 along the X-axis). Representative assays are shown. D21 and D22 represent EBV-seronegative donors, the remaining donors were seropositive.

C. BNRF1-specificity of T cell lines improves upon restimulation. The T cell lines were restimulated biweekly for at least seven rounds. The IFN-□ indices for BNRF1 are shown for the T cell lines from different donors (D1-22). Each data point depicts an IFN-□□index (Y-axis) for a given donor at a given passage number. The passage numbers are denoted on the X-axis (p1-7). Representative assays are shown. D21 and D22 represent EBV-seronegative donors while the remaining donors were seropositive. The T cell line from

seropositive donor D1 showed specific responses as passage 1 and was thus not restimulated further. At some passages, some T cell lines could not be tested, due either to a lack of sufficient PBMC or of T cells.

Figure 2. Identification of peptide epitopes targeted by BNRF1-specific CD4+ T cells.

A. BNRF1-specific T cell lines recognize pools of peptides derived from BNRF1. T cell lines shown here from five donors (D2, D6, D7, D13 and 19) were tested against seven peptide pools (I to VII), pools I to VI each with 47 unique peptides and pool VII with 45 unique peptides drawn out of a library of 327 15mer synthetic peptides covering the entire amino acid length of BNRF1, with neighboring peptides overlapping by at least eleven amino acids. Autologous PBMC were used as antigen-presenting cells in these studies. Representative responses from five donors are shown.

B. Identification of individual target peptides recognized by BNRF1- specific T cells. 20 peptide subpools (8 row subpools and 12 column subpools) were derived from each 96-well plate of peptides. Each peptide was present in one row subpool and one column subpool. Subpools relevant to the positive pool were tested in further T cell cytokine release assays using PBMC as antigen-presenting cells and responsive T cells as effectors. In the example depicted here the T cell line from donor D11, that was found to be responsive to pools I and II, was tested against row (r) subpools and column (c) subpools from 96-well plate number 1 (left). Once positive subpools were identified, candidate single peptides were determined and subsequently tested (right) allowing the identification of single target peptides (#19-95), which were further confirmed.

C. Identification of T cells recognizing adjacent peptides. Some T cell clones (shown for donor D1 clone#4) recognized two pools (pools II and III), which was found to be due to the recognition of two consecutive peptides (number 94 and 95) that were the last peptide in pool II and the first peptide in pool III, respectively. Some T cell clones responded against the same pool (lower panel left, clone #7 and 12 from donor D1 both

recognized pool I) but recognized different peptides in the pool. D1 clone #7 recognized peptide #21, D1 clone #12 recognized peptides #19 and #20.

Figure 3. BNRF1-specific T cells can be detected *ex vivo*.

CD8-depleted PBMC from 21 different donors (EBV-seropositive except D21 and D29) were rested overnight followed by exposure to BNRF1 or to the control protein transaldolase for 16 hours on a precoated IFN-□ ELISPOT plate. ELISPOT assays were set up with three to four replicates of each condition. Background-subtracted mean counts of spot forming units (SFU) per million cells along with the standard deviation (error bars) are shown. The mean of the number of spots in the absence of any antigen was considered as background.

715 **Figure 4. Characterization of polyclonal T cell lines and T cell clones.**

716 **A.** BNRF1-pulsed PBMC stimulated T cell lines contain BNRF1-specific as well as non-specific
717 T cells. Limiting dilution cloning yielded several outgrowing clones. Representative
718 results for nine clones (#1-9) each obtained from the line from donor D1 (top) and D12
719 (bottom) were tested in cytokine secretion assays against antigen-presenting cells
720 (miniLCL), either untreated or pulsed with BNRF1 or the control protein BZLF1.

721 **B.** Testing T cells against target cells with partly overlapping MHCII genotype of the donor
722 allows identification or narrowing down of potential antigen-presenting molecules. T cell
723 clones D14#5 and D12#3 were tested in cytokine secretion assays against BNRF1-pulsed
724 miniLCL from different donors (marked as D14-D39 and D12-D44 on the X-axis) with
725 known, partially overlapping MHCII profiles, allowing for the identification of the
726 antigen-presenting molecule (top: DRB3*02:02) or for narrowing down the potential
727 antigen presenting molecules (bottom: DRB1*01:01 and DQB1*05:01).

728 **C.** Test for DP molecules as potential antigen-presenting molecules using an anti-DP
729 blocking antibody. T cell clones (named along the X-axis) were tested against matched
730 BNRF1-pulsed target cells, either untreated or pretreated with an anti-human DP
731 inhibitory antibody or with an IgG isotype-matched control antibody. Cytokine secretion
732 in response to antibody-treated (anti-DP or control) target cells is shown as percentage
733 of cytokine secretion in response to untreated targets.

734 **D.** Test for antigen-presenting molecule using transfection of MHCII molecules in the
735 transfection-permissive cell line DG75. For some T cell clones, the restriction element
736 was identified by expressing single MHCII molecules using expression plasmids (p). In the
737 example shown here, the EBV negative Burkitt lymphoma cell line DG75 was transfected
738 with expression plasmids coding for BNRF1 alone or along with another plasmid coding

739 for the MHCII molecules DRB1*1301, DRB1*1501, DRB5*0101 or DQB1*0603.
740 Transfected cells were tested for recognition by the T cell clone D37#1. MiniLCL, either
741 untreated, or pulsed with recombinant BNRF1 served as negative and positive controls.
742 DRB5*0101 was identified as the molecule presenting BNRF1 to D37#1 T cells.

743 **Figure 5: Relative positions of CD4+ T cell epitopes in the reference BNRF1 protein**
744 **sequence from B95.8 virus.** The BNRF1 protein contained epitope sequences recognized by
745 T cell lines from only one (red), two (bold green), or three (bold italic print face purple)
746 donors. Where epitopes overlapped, amino acids common to both epitopes are shown in
747 brown bold typeface.

748 **Figure 6. BNRF1-specific CD4+ T cells are cytolytic.**

749 A. Secretion of cytolytic molecules by BNRF1-specific T cells. T cell clones were tested for
750 secretion of perforin (top) and granzyme B (bottom) in response to miniLCL either
751 unpulsed or pulsed with cognate or control peptide. The perforin (or granzyme B) index
752 represents the perforin (or granzyme B) concentration in supernatants of T cells in
753 response to cognate (black) or control (grey) peptide in relation to that of untreated T
754 cells. The X-axis depicts the Donor number along with the T cell clone number.

755 B. Specific lysis of antigen-presenting target cells by BNRF1-specific T cells. T cell clones
756 D25#8 (top) and D23#1 (bottom) were tested for their cytolytic potential in Calcein-AM
757 cytotoxicity assays using miniLCL pulsed with the cognate peptide or a control peptide as
758 target cells. Peptide-pulsed miniLCL were labelled with Calcein-AM dye and then brought
759 out with T cells at different effector to target ratios as marked on the X-axis.

Figure 7. BNRF1-specific CD4+ T cells inhibit EBV-driven proliferation and transformation.

A. BNRF1-specific CD4+ T cells recognize EBV-transformed B cells that are permissive to lytic replication. T cell clones were tested in IFN-γ secretion assays against autologous miniLCL, miniLCL pulsed with recombinant BNRF1, or LCL.

B. BNRF1-specific T cells recognize unmanipulated EBV-transformed B cells and can efficiently kill them. Left- T cell clones (10000 cells per well) were tested in IFN-γ ELISPOT assays against autologous miniLCL or LCL brought out in serial dilutions starting at 2500 target cells per well. Right- T cells at different T cell to target ratios were used to assess cytolytic activity in 4-hour cytotoxicity assays using untreated or peptide-pulsed autologous miniLCL and untreated LCL as targets.

C. BNRF1-specific T cells restrict the proliferation of EBV-transformed LCL but not miniLCL. Autologous LCL or miniLCL (target cells) were plated in serial dilutions from 10,000 to 30 per well in round bottom 96-well plates, in four replicas, with or without T cells (10,000 per well). In all cases CD4+ BNRF1-specific T cells (BNRF1 T cells) were used, except for donor D23, where in addition to BNRF1-specific T cells, an influenza M1-specific CD4+ T cell clone (M1 T cells) was available from the same donor and was therefore also included as a control T cell clone. After a month, the plate was inspected for proliferation of miniLCL or LCL and wells with target outgrowths in the absence and presence of T cells noted. The results are expressed as ratios of the number of input target cells that led to outgrowth in the presence of T cells to the number of input target cells that led to outgrowth in the absence of T cells.

D. Donor-derived spontaneous LCL efficiently present antigen to BNRF1-specific CD4+ T cells. BNRF1-specific CD4+ T cell clones from donors D25, D1 and D23 were tested for

responses against autologous spontaneous LCL. The corresponding miniLCL served as controls.

E. BNRF1-specific CD4+ T cells inhibit EBV-driven transformation of primary B cells. Magnetically sorted CD19+ PBMC from three donors (D6, D23, D24) were exposed to B95.8 virus for two hours and then brought out in serial dilutions from 10,000 to 10 cells per well in 96-well plates, in two different conditions, either with or without BNRF1-specific T cells (10,000 per well), each condition in four replicates. After four weeks, the plate was monitored for B cell transformation by microscopy. Shown in the figure are the fold input cell numbers that led to transformation. The number of B cells that led to a transformed proliferating culture when plated without T cells was taken as fold 1.

Amino acid coordinates in B95.8 protein	Sequence of peptide recognized	Number of responsive T cell lines	Antigen presenting candidate/s	Method(s)
37-51	RLYELLSDPRSALGL	1	DRB1*14:01	Common MHCII between recognized target cells combined with binding prediction
53-67	PGPLIAENLLLVALR	1	DPB1*04:02	MHCII blocking experiments combined with binding predictions
77-91	RQERARELALVGILL	3	DPB1*104:01	MHCII blocking experiments combined with common MHCII between recognized target cells
81-95	ARELALVGILLGNGE	1	DRB1*01:02	Common MHCII between recognized target cells
133-147	QQFLRLLGATYVLRV	2	DRB1*01:02	Common MHCII between recognized target cells combined with binding prediction
171-185	NHLVLFDNALRKYDS	1	DPB1*05:01	MHCII blocking experiments combined with binding predictions
277-295	AAGTIQANCPQLFMR	1	DRB3*0101	Binding prediction

	RQHP		/ DRB5*0202	
375-389	LGAIKHQALDTVRYD	3	DRB4*01:03	Common MHCII between recognized target cells
427-441	LELFSALYPAPCISG	1	DRB1*01:01	Common MHCII between recognized target cells combined with binding predictions
449-463	SAVIEHLGSLVPKGG	1	DRB4*0103	Common MHCII between recognized target cells
492-506	MQQFVSSYFLNPACS	1	DPB1*04:01	MHCII blocking experiments combined with common MHCII between recognized target cells
497-511	SSYFLNPACSNVFIT	2	DRB3*02:02	Common MHCII between recognized target cells
548-562	LGGLNFVNDLASPVS	2	DRB3*01:01	Common MHCII between recognized target cells
915-929	LPEMFAEHPGLVFEV	1	DRB5*01:02	Expression of MHCII in trans
983-997	TWSSFASEQYECLRP	3	DPB1*04:01	Common MHCII between recognized target cells
1006-1020	VSDYGYNEALAVSPL	2	DRB3*02:02	Expression of MHCII in trans
1121-1135	LNRPDTFSVALGELG	1	DRB1*01:01 /	Binding predictions, IEDB

			DQB1*05:0 1	
1238-1252	TDAWRFAMNYPRNPT	1	DRB5*01:01	Expression of MHCII in trans

Table 1. CD4+ T cell epitopes in BNRF1 and their presenting molecules.

793

794 Figure 1 color.tif

795 Figure 2 color.tiff

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798 Figure 5 bnw.pptx

799 Figure 6 bnw.tiff

800 Figure 7 bnw.tif

Amino acid coordinates in B95.8 protein	Sequence of peptide recognized	Number of responsive T cell lines	Antigen presenting candidate/s	Method(s)
37-51	RLYELLSDPRSALGL	1	DRB1*14:01	Common MHCII between recognized target cells combined with binding prediction
53-67	PGPLIAENLLLVALR	1	DPB1*04:02	MHCII blocking experiments combined with binding predictions
77-91	RQERARELALVGILL	3	DPB1*104:01	MHCII blocking experiments combined with common MHCII between recognized target cells
81-95	ARELALVGILLGNGE	1	DRB1*01:02	Common MHCII between recognized target cells
133-147	QQFLRLLGATYVLRV	2	DRB1*01:02	Common MHCII between recognized target cells combined with binding prediction
171-185	NHLVLFDNALRKYDS	1	DPB1*05:01	MHCII blocking experiments combined with binding

				predictions
277-295	AAGTIQANCPQLFMR RQHP	1	DRB3*0101 / DRB5*0202	Binding prediction
375-389	LGAIKHQALDTVRYD	3	DRB4*01:03	Common MHCII between recognized target cells
427-441	LELFSALYPAPCISG	1	DRB1*01:01	Common MHCII between recognized target cells combined with binding predictions
449-463	SAVIEHLGSLVPKGG	1	DRB4*0103	Common MHCII between recognized target cells
492-506	MQQFVSSYFLNPACS	1	DPB1*04:01	MHCII blocking experiments combined with common MHCII between recognized target cells
497-511	SSYFLNPACSNVFIT	2	DRB3*02:02	Common MHCII between recognized target cells
548-562	LGGLNFVNDLASPVS	2	DRB3*01:01	Common MHCII between recognized target cells
915-929	LPEMFAEHPGLVFEV	1	DRB5*01:02	Expression of MHCII in trans
983-997	TWSSFASEQYECLRP	3	DPB1*04:01	Common MHCII between

				recognized target cells
1006-1020	VSDYGYNEALAVSPL	2	DRB3*02:02	Expression of MHCII in trans
1121-1135	LNRPDTFSVALGELG	1	DRB1*01:01 / DQB1*05:01	Binding predictions, IEDB
1238-1252	TDAWRFAMNYPRNPT	1	DRB5*01:01	Expression of MHCII in trans

Table 1. CD4+ T cell epitopes in BNRF1 and their presenting molecules.

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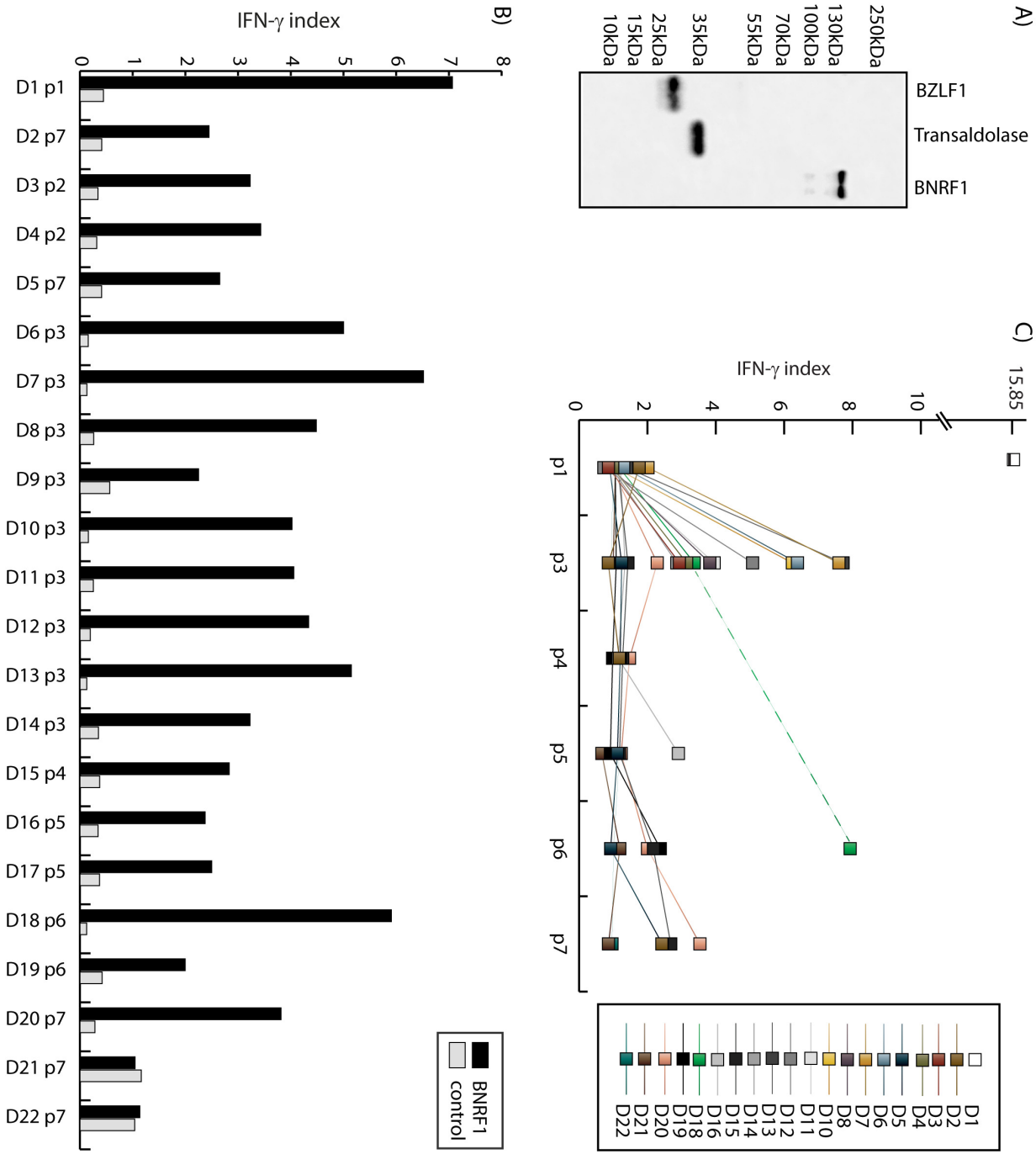

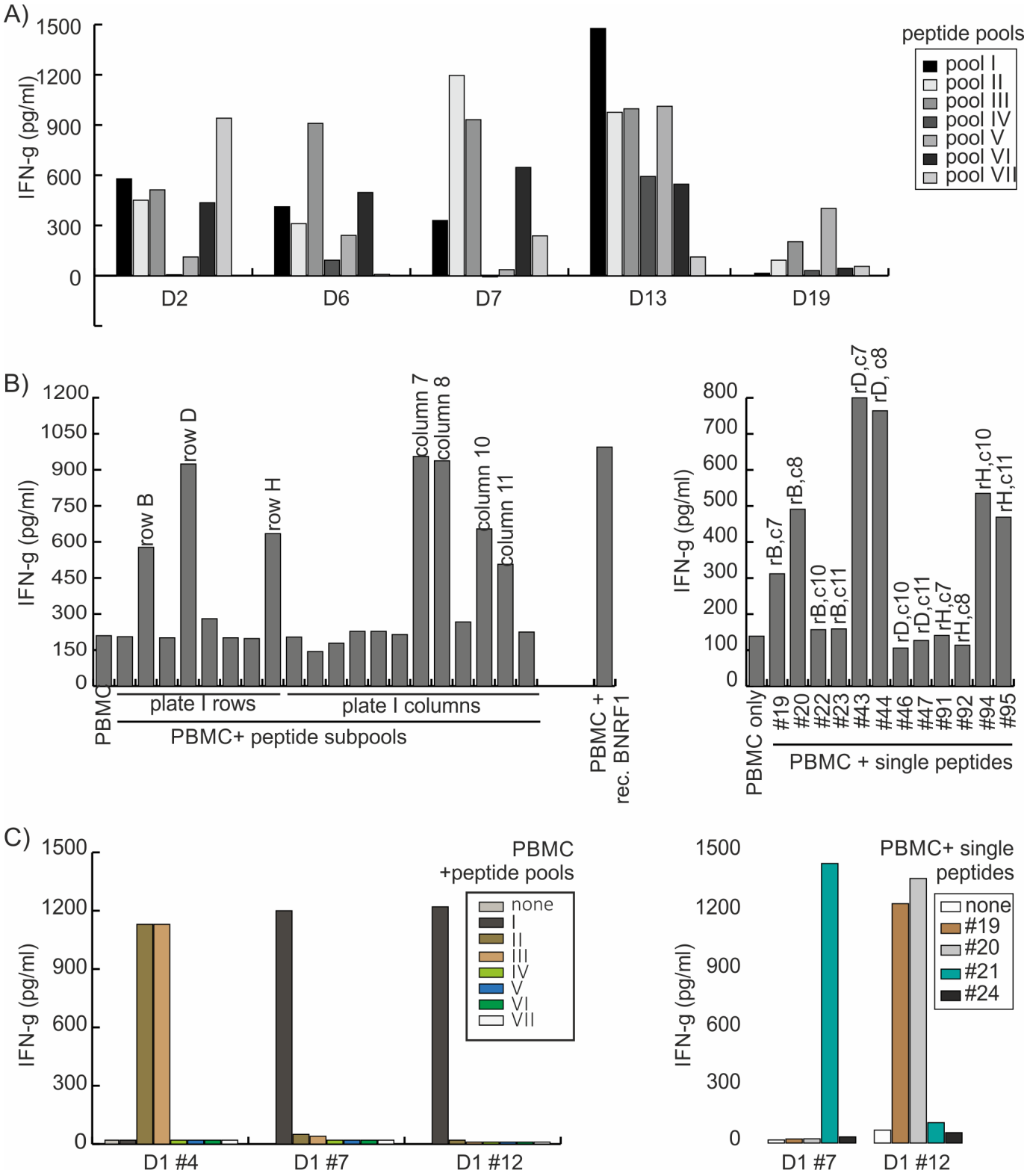

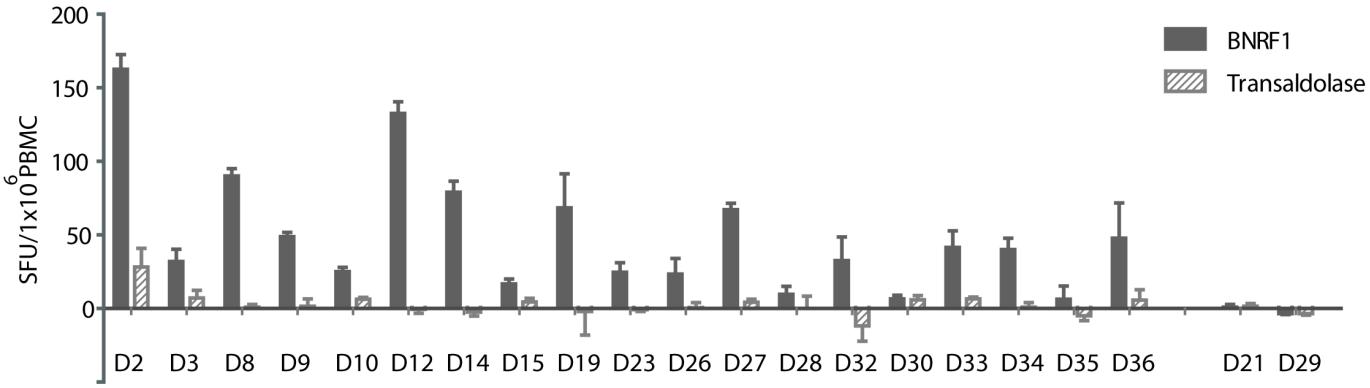

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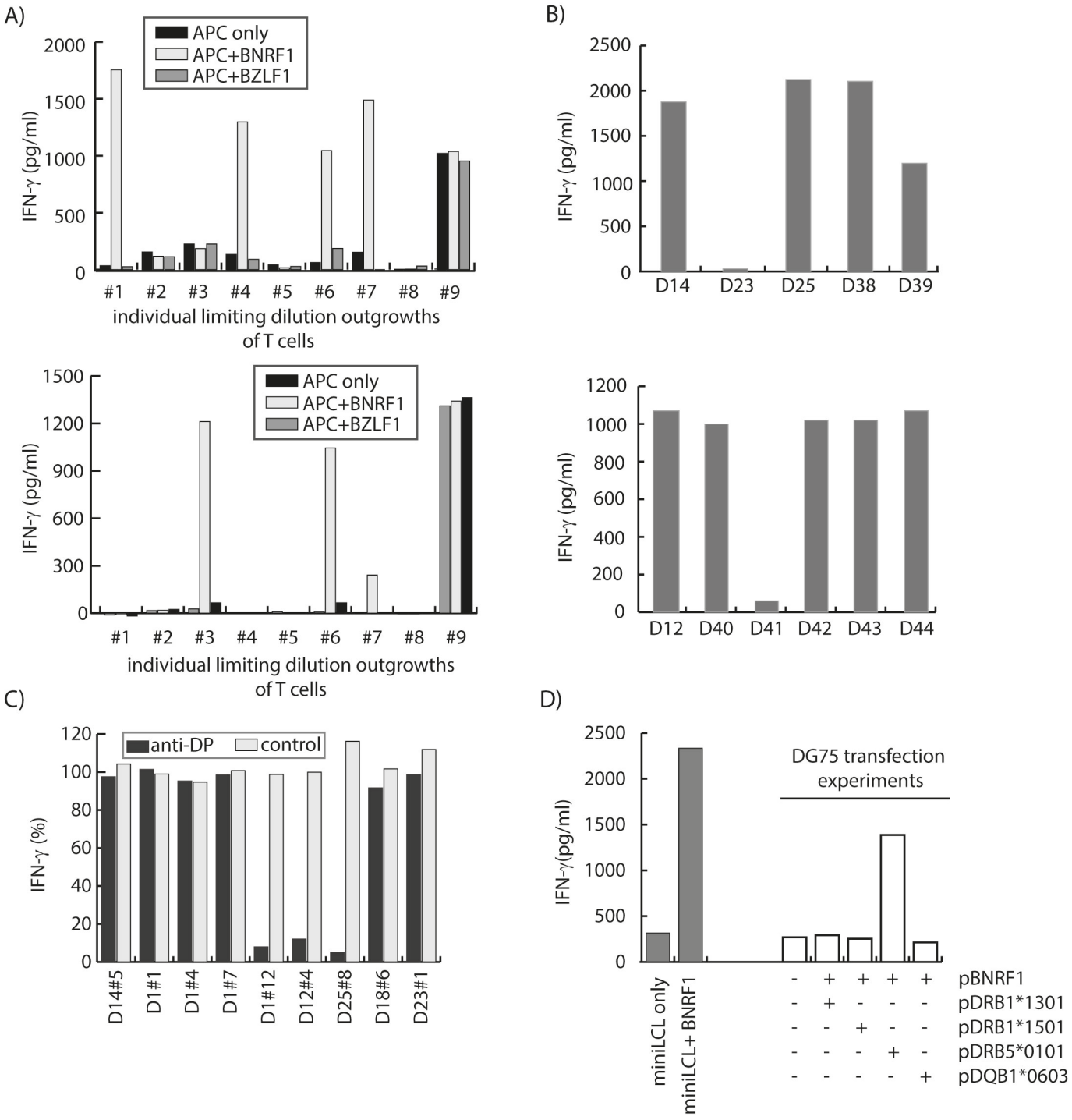

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