A Role for Intercellular Antigen Transfer in the Recognition of EBV-Transformed B Cell Lines by EBV Nuclear Antigen-Specific CD4$^+$ T Cells[1]

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The CD4$^+$ T cell response to EBV may have an important role in controlling virus-driven B lymphoproliferation because CD4$^+$ T cell clones to a subset of EBV nuclear Ag (EBNA) epitopes can directly recognize virus-transformed lymphoblastoid cell lines (LCLs) in vitro and inhibit their growth. In this study, we used a panel of EBNA1, 2, 3A, and 3C-specific CD4$^+$ T cell clones to study the route whereby endogenously expressed EBNAs access the HLA class II-presentation pathway. Two sets of results spoke against a direct route of intracellular access. First, none of the clones recognized cognate Ag overexpressed in cells from vaccinia vectors but did recognize Ag fused to an endo/lysosomal targeting sequence. Second, focusing on clones with the strongest LCL recognition that were specific for EBNA2- and EBNA3C-derived epitopes LCL recognition was unaffected by inhibiting autophagy, a postulated route for intracellular Ag delivery into the HLA class II pathway in LCL cells. Subsequently, using these same epitope-specific clones, we found that Ag-negative cells with the appropriate HLA-restricting allele could be efficiently sensitized to CD4$^+$ T cell recognition by cocultivation with Ag-positive donor lines or by exposure to donor line-conditioned culture medium. Sensitization was mediated by a high m.w. antigenic species and required active Ag processing by recipient cells. We infer that intercellular Ag transfer plays a major role in the presentation of EBNA-derived CD4 epitopes by latently infected target cells. *The Journal of Immunology,* **2006, 177: 3746–3756.**

Epstein-Barr virus is a B lymphotrophic herpesvirus which has potent B cell growth-transforming activity and is linked to several B cell malignancies, yet is carried by the great majority of individuals as an asymptomatic infection. A role for the host T cell response in the long-term control of persistent EBV infection is clear from clinical observation. Thus, T cell-immunocompromised patients, in particular transplant recipients given high doses of T cell-suppressive drugs, are at greatly increased risk of EBV-positive posttransplant lymphoproliferative disease (PTLD)[4] (1–3). Most PTLD lesions express the full spectrum of EBV latent proteins, that is the EBV nuclear Ags (EBNAs) 1, 2, 3A, 3B, 3C, and LP and the latent membrane proteins 1 and 2, and in this respect resemble the lymphoblastoid cell lines (LCLs) generated when EBV transforms normal B cells in vitro. Such PTLD tumors are susceptible to immunological attack by adoptively transferred T cell populations produced in vitro by LCL stimulation of EBV latent-Ag-specific memory T cells either from the patient or from a HLA-matched donor (1–3). Virus-specific CD8$^+$ T cells are thought to be the main effectors in this regard and indeed LCL-stimulated populations tend to be dominated by cytotoxic CD8$^+$ T cell clones reactive to one or more immunodominant epitopes, most often derived from the EBNA 3A, 3B, 3C subset of proteins (4, 5). However, these same populations also contain CD4$^+$ T cells and this has stimulated increasing interest in latent-Ag-specific CD4$^+$ T cell responses. Such responses may have a dual role. First, they are likely to be important in the maintenance of virus-specific CD8$^+$ T cell surveillance in the host (6); second, because virally transformed B cells express HLA class II molecules and have HLA class II-processing function (7), EBV-specific CD4$^+$ T cells may be capable of recognizing latently infected cells directly and thereby acting as effectors in their own right.

The first CD4$^+$ T cell clones to EBV latent proteins, specific for EBNA1-and EBNA2-derived epitopes respectively, were identified as rare components of LCL-reactivated memory T cell preparations (8, 9). Of these, only the EBNA2- specific clone appeared to be capable of recognizing LCL cells directly in cytotoxicity assays (9). Since then, CD4$^+$ recall responses to more epitopes have been generated by a variety of protocols. Most work in this area has focused on EBNA1 as a CD4$^+$ T cell target and has produced conflicting reports as to the ability of Ag-specific CD4$^+$ clones to recognize LCL cells endogenously expressing the EBNA1 protein from the resident EBV genome (10, 11). In a recent study, we widened the analysis to include responses against a range of epitopes in the EBNA1, EBNA2, EBNA3A, and EBNA3C proteins, and found that the capacity for LCL recognition was highly epitope specific (12). Indeed, for any one of the above Ags, responses to individual epitopes differed markedly in their level of LCL recognition. Thus, while EBV infection naturally elicits CD4$^+$ T cell responses to a range of different EBNA-derived epitopes, only a subset of these responses are likely to have

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[4] Abbreviations used in this paper: PTLD, posttransplant lymphoproliferative disease; LCL, lymphoblastoid cell line; EBNA, EBV nuclear Ag; 3-MA, 3-methyladenine; Ii, invariant chain; GAr, glycine-alanine repeat; moi, multiplicity of infection; MVA, modified vaccinia Ankara; CytC, cytochrome *c*; BL, Burkitt's lymphoma.

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direct therapeutic potential as effectors against EBV-driven lymphoproliferations in vivo.

Given these findings, we set out to study the processing mechanisms that lead to LCL sensitization. There are multiple examples where indicator Ags have been expressed endogenously within LCL cells and appear to have gained direct intracellular entry into the HLA class II-processing pathway, apparently bypassing the normal exogenous pathway in which Ag is taken up from the extracellular milieu before being processed in endo/lysosomal compartments. Many of these examples involve membrane or secreted proteins (13–16) which are thought to engage immature MHC class II molecules during transit through the endoplasmic reticulum. However, others involve long-lived cytoplasmic proteins, stably expressed in cells by gene transfection, which appear to enter the endosomal compartment either by a specialized chaperone-mediated route (17) or by a pathway that was blocked by 3-methyladenine (3-MA) (18), a known inhibitor of autophagy (19). Most recently, two reports have suggested that nuclear Ags may also access the MHC class II-processing pathway by an autophagic route. In one case, boosting autophagy in LCL cells by nutrient starvation increased the representation of nuclear protein-derived peptides complexed with HLA class II molecules on the cell surface (20). In another, evidence was presented that in LCL cells, the endogenously expressed EBNA1 protein was processed and presented to EBNA1-specific $CD4^+$ T cell clones via a 3-MA-sensitive autophagic route (21). In this study, we use $CD4^+$ T cell clones to a panel of EBNA-derived epitopes and show that, for those epitopes mediating the strongest LCL recognition and therefore representing the best targets for T cell-directed therapy, most if not all of this recognition depended upon intercellular Ag transfer occurring within the LCL culture.

Materials and Methods

Target cell lines and T cell clones

EBV-transformed LCLs were generated from normal B cells using the prototype 1 strain B95.8 or the prototype 2 strain Ag876, or a B95.8 recombinant virus lacking the immediate early gene *BZLF1* and therefore incapable of lytic virus replication (22); all LCLs expressed the full panel of EBV latent proteins. The EBV (type 1)-positive Burkitt's lymphoma (BL) lines Kem-BL and Oku-BL express, respectively, EBNA1 only and EBNAs 1, 3A, 3B, and 3C as described (23); the BL41 line is EBV genome negative. All lines were routinely cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 2 mM glutamine, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 10% FCS. All cell lines and T cell clones were regularly checked using immunofluorescence (Ridascreen) and the Mycoalert Mycoplasma Detection kit (Cambrex) to confirm the absence of mycoplasma contamination. $CD4^+$ and $CD8^+$ T cell clones specific for defined epitopes within EBNA 1, 2, 3A, or 3C were generated as described (12, 24). Overall, the experiments involved $CD4^+$ T cell clones to nine different EBNA-derived epitopes identified along with their HLA class II-restricting alleles in Table I. Also shown for each epitope is the efficiency with which epitope-specific $CD4^+$ T cells recognize the autologous LCL target; this is determined by IFN-γ release and expressed as a percentage of the IFN-γ release induced by the same target LCL loaded with an optimal concentration of epitope peptide (12). T cell recognition experiments also included (as internal controls) $CD8^+$ T cell clones to the following epitopes: the HLA-B35-restricted EBNA1 407–417 epitope HPV (25), the HLA-B38-restricted EBNA 2 14–23 epitope YHL (26), the HLA-B35-restricted EBNA3A 458–466 epitope YPL (27), the HLA-B27-restricted EBNA3C 258–266 epitope RRI (28), and the HLA-A11-restricted EBNA3B 399–408 and 416–424 epitopes AVF and IVT (29).

Table I. *Summary of epitope-specific $CD4^+$ T cell clones*

Ag	Epitope	Epitope Coordinates	HLA Restriction	% Recognition of LCL[a]
EBNA1	PQC	529–543	DR14	0
	NPK	475–494	DP	0
EBNA2	PAQ	301–320	DR17	3
	PRS	276–295	DR52b	35
EBNA3A	GPW	780–799	DR1	1
	EDL	364–383	DR15	4
EBNA3C	ILC	141–155	DR13	0
	PHD	100–119	DR16	3
	SDD	386–400	DQ5	7

[a] Recognition of the unmanipulated autologous LCL, as measured by IFN-γ release, is expressed as a percentage of the IFN-γ release value seen in the same assay against the same LCL optimally loaded with epitope peptide. The value for each epitope is the mean from assays on several epitope-specific clones (except for PHD where only one clone was available).

Synthetic peptides and protein preparations

Epitope peptides were synthesized using 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience; University of Birmingham, Birmingham, U.K.), dissolved in DMSO, and their concentrations determined by biuret assay. The 9-mer TAMRA fluorescently labeled peptide was a gift from J. Fox (Alta Bioscience, University of Birmingham, Birmingham, U.K.). Protein preparations, provided by Dr. F. Grässer (Institut für Mikrobiologie und Hygiene, Homburg/Saar, Germany), consisted of lysates from insect cells infected with control or EBNA2 (B95.8 strain) expressing baculoviruses.

MVA recombinants

EBNA1, 2, 3A, and 3C coding sequences (from the B95.8 EBV strain) were recombined into the modified vaccinia Ankara (MVA) genome using the pSC11 vaccinia virus shuttle vector as before (5); MVA recombined with the empty pSC11 vector served as a control. The HLA class II-targeted Ag constructs were made for EBNA1 by fusion with an N-terminal signal sequence and a C-terminal LAMP1 sequence (30, 31) and for EBNAs 1, 2, 3A, and 3C by fusion with aa 1–82 of the p33 isoform of the invariant chain (Ii) (32) at the N terminus. Note that all constructs containing EBNA1 were deleted for the glycine-alanine repeat (GAr) domain because the GAr is refractory to expression from vaccinia vectors. Experiments used recombinant virus preparations purified by sucrose gradient centrifugation (33) to minimize contamination of virus stocks with nonvirion proteins (in particular, the EBNA protein) present in infected cells during production of stocks. Expression of the relevant EBNA proteins in the target cells for T cell recognition assays was confirmed by immunoblotting of protein extracts from rMVA-infected LCLs (multiplicity of infection (moi) 10; 18 h postinfection) probed with mAbs 1H4 to EBNA1, PE2 to EBNA2, E3cA10 to EBNA3C (23) and a polyclonal sheep serum to EBNA3A (Exalpha Biologicals).

T cell assays involving MVA recombinants

Target LCLs with the relevant HLA-restricting allele were exposed for 60 min to MVA virus preparations at a moi of 10, and then washed well. In some experiments, these infected LCL cells were then immediately incubated in V-bottom microtest plate wells (50,000 targets/well) with cloned $CD4^+$ or $CD8^+$ T cells specific for epitopes within the same EBNA Ag (500 T cells/well), and the assay supernatants were harvested after a total of 18 h coculture. These supernatants were then assayed for IFN-γ content by ELISA as described in earlier work (12). In other experiments, Ag876-transformed LCL cells with the relevant HLA-restricting allele were infected as above and then cultured for 24 h before addition to a microtest plate (50,000 targets/well) for a further 18 h incubation with T cells (2,500 T cells/well); therefore, these target cells had been infected for a total of 42 h overall. These longer assays also included B95.8-transformed HLA-mismatched LCL cells, infected as above and then 24 h later cocultured for a further 18 h with T cells, with or without the addition of uninfected Ag876-transformed LCL cells with the relevant HLA-restricting allele (50,000/well) before the 18 h assay. All such MVA assays included, as control targets, uninfected cells both from HLA-matched and HLA-mismatched LCLs either prepulsed for 1 h with 5 μM epitope peptide or with an equivalent concentration of DMSO solvent as a control, then washed well and used immediately in the assay.

Autophagy inhibitor experiments

The reported ability of 3-MA to inhibit autophagy in LCL cells (18, 21) was first checked in experiments in which the LCL cells were nucleofected (Amaxa Biosystems) with a plasmid pINCO-NeoR-GFP (provided by Dr. J. Mautner, GSF-National Research Centre for Environment and Health, Munich, Germany). After 2 days to allow expression of the neomycin

phosphotransferase II-GFP (NeoR-GFP) fusion protein, the cells were incubated for a further 1–4 days either in culture medium alone or in the presence of a range of concentrations (2.5–10 mM) of 3-MA. Levels of NeoR-GFP, a known target for autophagy (18), were analyzed by flow cytometry (Coulter Epics Excel Flow Cytometer; Coulter) gating on live cells. In parallel experiments, LCLs were maintained in control medium or in the presence of 3-MA as above for 3–4 days, then washed three times, fixed for 60 s in 0.05% glutaraldehyde (stopped by quenching in excess glycine), washed a further three times and used as targets in $CD4^+$ T cell recognition assays. In additional control experiments conducted to determine the half-life of pre-existing HLA class II/epitope complexes on the cell surface of such targets, LCL cells carrying EBV strains that naturally lack the relevant epitope sequences or BL cells that lack the relevant Ag were exposed for 1 h with a limiting concentration (10^{-7} M) of epitope peptide, washed well, and then cultured in normal medium for periods of up to 7 days before being used as targets in standard $CD4^+$ T cell recognition assays (12).

Ag transfer experiments

In cell mixing experiments, donor and recipient cell lines were seeded as a 1:1 mixture at low initial cell densities and then grown in coculture for up to 12 days without further feeding; cells were then harvested, washed, and used as targets in IFN-γ ELISAs as above. In other experiments, conditioned medium was harvested from donor line cultures (3 days postsubculture), centrifuged at 2000 rpm for 5 min, and filtered through a 0.2-μm membrane. Recipient cells were then grown in this conditioned medium, where necessary refeeding every 3 days, before washing and immediately testing as targets as above. In later experiments of this type, recipient cells were exposed overnight to concentrated conditioned medium prepared as above from donor lines growing in serum-free AIM-V lymphocyte medium but then concentrated with a Centricon centrifugal device (Amicon) with a 10-kDa molecular mass cutoff. Cells were then washed and used as targets with or without prepulsing for 1 h with 5 μM epitope peptide as above. In some cases, recipient cells were first prefixed by 1 min exposure to 0.05% glutaraldehyde (as above), then washed before exposure to concentrated conditioned medium or, as a positive control, to an EBNA2 protein preparation. In other cases, recipient cells were pre-exposed for 2 h to a range of concentrations of the cathepsin inhibitors E-64 or leupeptin (Sigma-Aldrich), then exposed overnight to concentrated medium in the continued presence of the inhibitors, the recipient cells were fixed as above, washed, and used as targets in T cell assays. In a final series of experiments, concentrated conditioned medium was fractionated on a Sephadex G-75 column (Amersham Biosciences) pre-equilibrated with PBS. Fractions from the column were mixed with an equal volume of 2× DMEM (Invitrogen Life Technologies) containing 20% FCS; recipient cells were then exposed to these fractions, washed and tested as targets in T cell assays. Purified BSA and cytochrome *c* (CytC) were separated under the same conditions to serve as m.w. markers; in addition, the 20-mer PRS epitope peptide and a fluorescent 9-mer marker peptide were separated under the same conditions and detected in fractions by T cell assays and fluorometry, respectively.

Results

$CD4^+$ T cell clones do not recognize cognate Ag overexpressed endogenously in LCL cells from vaccinia virus vectors

In a first series of experiments, we looked for evidence that endogenously expressed EBNA Ags could directly access the HLA class II presentation pathway in LCL cells by overexpressing individual Ags from the vaccinia virus vector MVA. Thus, we generated a panel of MVA recombinants encoding EBV B95.8 strain EBNA1 (GAr deleted), EBNA2, EBNA3A, and EBNA3C in their native, nuclear-localizing, form. As positive controls, MVA recombinants were also constructed to express these same proteins fused to LAMP or Ii-targeting sequences that deliver the Ag directly to endosomes/lysosomes and therefore into the HLA class II-processing pathway. Fig. 1, *right panels*, shows Western blots where protein extracts made from LCL cells 18 h postinfection with the relevant MVA recombinants were probed with EBNA-specific Abs. Note that the MVA vectors significantly increase the level of Ag above that already being expressed from the resident EBV genome. The MVA-coded EBNA1 protein runs at ~50 kDa, as expected for the GAr-deleted form, while the LAMP-targeted form runs at a slightly higher m.w.; both are significantly smaller than the EBV-coded native EBNA1 protein containing the GAr domain. The MVA-coded EBNA2, EBNA3A, and EBNA3C are expressed as proteins of the expected size and the Ii-chain-targeted forms are slightly larger.

We then used such LCL cells infected with appropriate MVA-EBNA constructs (or with the MVA-pSC11 control virus) as targets for $CD4^+$ T cells clones specific for each of the different EBNA-derived T cell epitopes shown in Table I, assaying T cell recognition by IFN-γ release. Note that although this panel of T cell clones includes some that are capable of directly recognizing the autologous B95.8 virus-transformed LCL, in almost every case, the baseline level of unmanipulated LCL recognition is sufficiently low as to allow any incremental recognition on MVA-infected targets to be easily detected. The exceptions are clones specific for the EBNA2-derived PRS epitope, which show the highest baseline recognition of B95.8 virus-transformed LCLs; in this case, we used target LCLs transformed with the EBV strain Ag876 where multiple sequence changes in the PRS epitope reduce baseline recognition to zero (9). Representative results are shown in Fig. 1 using $CD4^+$ T cell clones to two epitopes in EBNA1 (PQC and NPK), two in EBNA2 (PAQ and PRS), two in EBNA3A (EDL and GPW), and two in EBNA3C (SDD and PHD). These include clones that either fail to see the unmanipulated LCL (e.g., PQC) and others that naturally exhibit low (e.g., GPW), moderate (e.g., SDD) or high levels (e.g., PRS) of LCL recognition. A consistent pattern of results was obtained throughout. All clones failed to show any increased recognition of LCL cells overexpressing the native form of the Ag. By contrast cells expressing the HLA class II-targeted form were clearly recognized, up to levels that in many cases approached the optimal level seen using the same LCLs exogenously loaded with peptide. To ensure that the target cells expressing MVA-encoded native Ag could indeed be recognized by T cells, we included in the same experiments $CD8^+$ T cell clones specific for defined epitopes in the EBNA1, 2, 3A, and 3C proteins. Where an LCL with the appropriate class I- and class II-restricting alleles was available, we used exactly the same target cells in both the CD4 and CD8 assays; otherwise, we used a different LCL for the CD8 assay but infected this in parallel with the CD4 assay target. Although $CD8^+$ effectors will invariably recognize the unmanipulated LCL target in such assays, the level of recognition seen by IFN-γ release is again such as to allow any incremental increase to be observed. Importantly, all of the $CD8^+$ T cell clones showed significantly increased recognition of LCLs overexpressing the relevant native EBNA protein from the MVA vector.

To cover the possibility that endogenous Ag processing via the HLA class II pathway may take longer to detect, we extended the timeframe of the experiment from 18 to 42 h by delaying the addition of T cells until 24 h postinfection of the targets and then measuring IFN-γ release over the next 18 h of coculture. Fig. 2 shows the results from one representative experiment, in this case using $CD4^+$ T cells against the EBNA2-derived PRS epitope. Now, we did detect some low level IFN-γ release from T cells exposed to the MVA-EBNA2-infected HLA-matched target cells; this clearly reflected specific recognition because similarly infected HLA-mismatched LCL targets remained negative. However, this delayed presentation of the PRS epitope appeared to be occurring via Ag release from infected cells followed by subsequent uptake and processing in neighboring cells within the culture. Thus, if uninfected cells of the HLA-matched Ag876 LCL were added to the MVA-EBNA2-infected mismatched LCL targets for the last 24 h of the experiment (including the 18-h T cell assay period), we observed similar low level recognition by $CD4^+$ T cells. Indeed, we even observed a lower but still significant

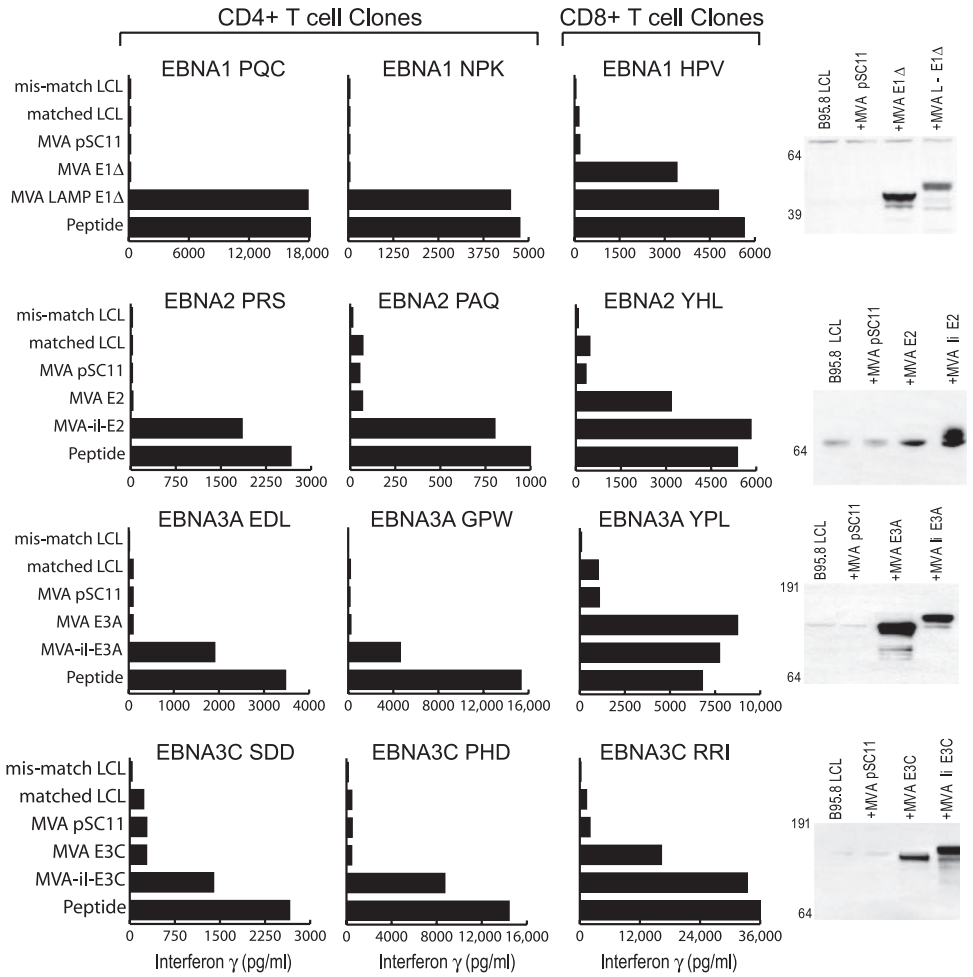

FIGURE 1. Assays of $CD4^+$ and $CD8^+$ T cell recognition of appropriately HLA-matched target LCLs either unmanipulated, or pre-exposed for 1 h either to MVA recombinant viruses (moi 10) or to 5 μM epitope peptide and washed, then T were cells added immediately for an 18-h period before supernatant medium was harvested and assayed for IFN-γ release. An unmanipulated HLA-mismatched LCL provided a negative control target in each assay. EBNA1-specific $CD4^+$ (PQC and NPK) and $CD8^+$ (HPV) clones were assayed using MVAs expressing GAr-deleted EBNA1 (MVA-E1Δ) or LAMP-targeted GAr-deleted EBNA1 (MVA L-E1Δ). EBNA2-specific CD4 (PRS and PAQ) and CD8 (YHL) clones were assayed using MVAs expressing native EBNA2 (MVA-E2) or invariant chain-targeted EBNA2 (MVA Ii E2). EBNA3A-specific CD4 (EDL and GPW) and CD8 (YPL) clones were assayed using MVAs expressing native EBNA3A (MVA-E3A) or Ii chain-targeted EBNA3A (MVA Ii E3A). EBNA3C-specific CD4 (SDD and PHD) and CD8 (RRI) clones were likewise assayed using MVAs expressing native EBNA3C (MVA E3C) or invariant chain-targeted EBNA3C (MVA Ii E3C). In each case, the MVA pSC11 recombinant virus was used in parallel with the above as a control. All assays involving the above clones were conducted on B95.8 strain EBV-transformed LCLs except for assays using the EBNA2-specific $CD4^+$ PRS T cells where assays were conducted on Ag876 strain EBV-transformed LCLs. Results of $CD4^+$ T cell assays (*left panels*) are expressed as IFN-γ release in picograms per milliliter. *Right column*, Immunoblotting results of protein extracts from the relevant target LCLs either uninfected or following infection with the indicated MVAs as above. Immunoblots were developed with MAbs to EBNA1, EBNA3A, EBNA3C, or EBNA2 as appropriate and the position of m.w. markers indicated alongside the image.

level of delayed presentation in parallel mixtures in which the HLA-mismatched LCL had either not been infected or had been infected with the control MVA-pSC11 vector. Note that in every case, delayed recognition of this type was always lower than that seen against an LCL target naturally expressing both the B95.8 EBNA2 protein and the correct HLA-restricting allele (Fig. 2, *bottom panel*).

$CD4^+$ T cell recognition of LCL targets following inhibition of autophagy

The next set of experiments sought to determine what role autophagy might play in the delivery of naturally expressed (rather than MVA-expressed) target Ags into the HLA class II-processing pathway. In this study, we used two sets of $CD4^+$ T cell clones, specific for the PRS epitope in EBNA2 and for the SDD epitope from EBNA3C, that gave the highest levels of unmanipulated LCL recognition and treated their LCL target cells with 3-MA, a known inhibitor of autophagy (19) to look for inhibition of Ag presentation. Two types of preliminary experiment were conducted as controls. First, we measured the stability of preformed PRS/DR52b and SDD/DQ5 complexes on target cell surfaces by pulsing a limited dilution of synthetic peptide onto the surface of either epitope-negative Ag876 LCL cells in the case of PRS or of Ag-negative BL cells in the case of SDD, then assaying the level of $CD4^+$ T cell recognition of the target cells after 1–7 days of chase in normal medium. As shown in Fig. 3*A*, the PRS epitope complex has a half-life of around 1.3 days while the SDD epitope complex has an even shorter half-life of <1 day. Thus, it is clear that if autophagy inhibition experiments are conducted over 2 or more days, then the natural turnover of pre-existing complexes from the LCL surface would allow any block in the supply of new complexes to be detected. A second control experiment sought to confirm that 3-MA could indeed inhibit autophagy in the particular LCLs used in our experiments. For this purpose, we used as an indicator the NeoR-GFP fusion protein known to be a target of autophagy (18).

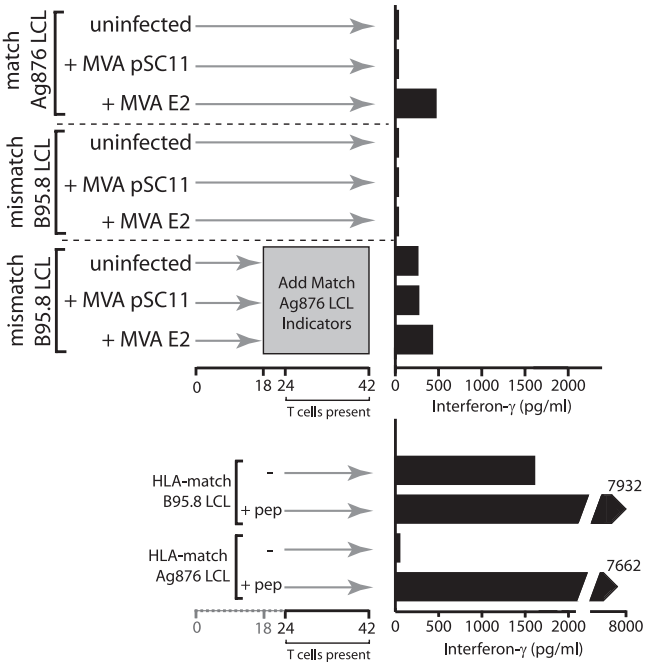

FIGURE 2. Results of T cell recognition assays conducted over a longer (42 h) time frame using an EBNA2 PRS epitope-specific CD4+ T cell clone and MVA-infected target cells. As indicated in the figure (*upper left quadrant*) at time 0 h an HLA-matched Ag876-transformed (epitope-negative) LCL or an HLA-mismatched B95.8-transformed (epitope-positive) LCL were exposed for 1 h either to MVA-E2 or to MVA-pSC11 as a control, or left uninfected. At time 18 h, some cells from the three types of HLA-mismatched B95.8-transformed targets were mixed with an equal number of uninfected indicator cells of the HLA-matched Ag876-transformed LCL (gray box). At time 24 h, T cells were added to all nine sets of target cells above, supernatant harvested 18 h later at time 42 h and assayed for IFN-γ release. Additional control targets in this experiment (*lower left quadrant*) were cells of an HLA-matched B95.8-transformed LCL and of a HLA-matched Ag876-transformed LCL that at time 24 h had been exposed for 1 h to 5 μM epitope peptide (+pep) or to an equivalent concentration of DMSO solvent as a control (−), then washed and T cells immediately added and the assay conducted in parallel with those above. Results (*right quadrant*) are expressed as IFN-γ release in picograms per milliliter.

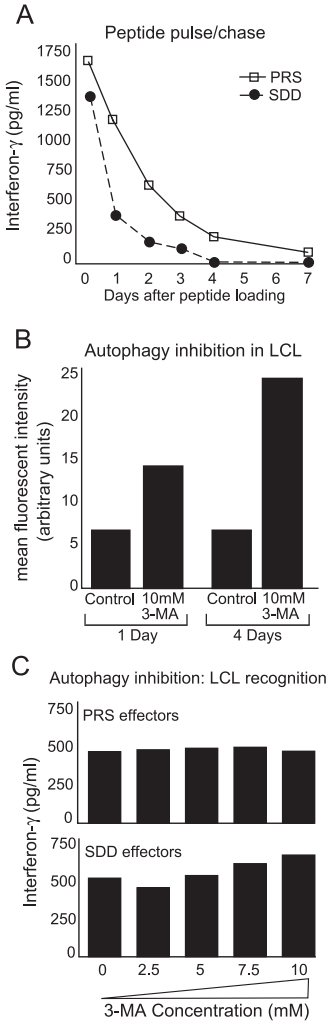

FIGURE 3. Results of assays investigating the potential role of autophagy in the processing of endogenously expressed EBNA2 and 3C. *A*, Determination of the half-life of HLA-DR52b-restricted PRS (□) and HLA-DQ5-restricted SDD (●) epitopes on the surface of B cell lines. For up to 7 days before the start of the experiment, cells were loaded with 1×10^{-7} M peptide for exactly 1 h, then washed and incubated at 37°C. All of these targets, and cells peptide pulsed on the day of the assay (day 0), were harvested and again washed then equal numbers incubated for 18 h with 1000 epitope-specific CD4+ T cells. T cell stimulation was determined by measuring by ELISA the IFN-γ released into the culture supernatants (expressed as picograms per milliliter). *B*, Confirmation in LCL cells of autophagy inhibition by 3-MA. LCL cells nucleofected with a NeoR-GFP-expressing plasmid were incubated for 1 or 4 days in the absence (control) or presence of 10 mM 3-MA. The cells were then analyzed for NeoR-GFP levels by flow cytometry. Results are expressed as the mean fluorescent intensity of live cells in the cultures. *C*, Measuring the effect of autophagy inhibition upon levels of cell surface EBNA-derived epitope peptides as detected by CD4+ T cell clones. HLA-DR52b- or -DQ5-positive LCL cells were incubated for 3 days in the presence of the indicated concentrations of 3-MA. Cells were then fixed with glutaraldehyde and equal numbers of cells used as targets for CD4+ T cell clones (1000 T cells/well) specific for either the PRS (EBNA2) or SDD (EBNA3C) epitopes. T cell stimulation was determined by measuring IFN-γ release as described above.

This protein was expressed by transient transfection in the relevant LCLs and its level monitored by flow cytometry after 1–4 days exposure of the transfected cells to 3-MA at concentrations up to 10 mM. As previously reported (18), 3-MA treatment led to a progressive accumulation of NeoR-GFP levels over the baseline levels seen in untreated cells. Using 10 mM 3-MA, increases were of the order of 2.5-fold within 1 day and reached 3.5-fold by day 4 (Fig. 3*B*); lower doses of 3-MA gave slightly lower effects (data not shown). We then conducted CD4+ T cell recognition experiments under these same conditions of autophagy inhibition. As shown by the representative results in Fig. 3*C*, 3-MA treatment had no detectable effect on the level of LCL recognition either by PRS-specific or by SDD-specific CD4+ T cell clones. We reproducibly observed this result in several independent experiments.

Intercellular Ag transfer in B cell cocultures can sensitize cells to CD4+ T cell recognition

As the above experiments gave no evidence for a direct intracellular route of EBNA entry into the HLA class II pathway, we examined the possible involvement of intercellular Ag transfer in EBNA processing. Thus, latently infected cells naturally expressing cognate Ag but lacking the relevant HLA-restricting allele (donor cells) were cocultivated for 3–7 days with equal numbers of latently infected cells lacking the cognate Ag but with the correct restricting allele (recipient cells). For the DR52b-restricted PRS

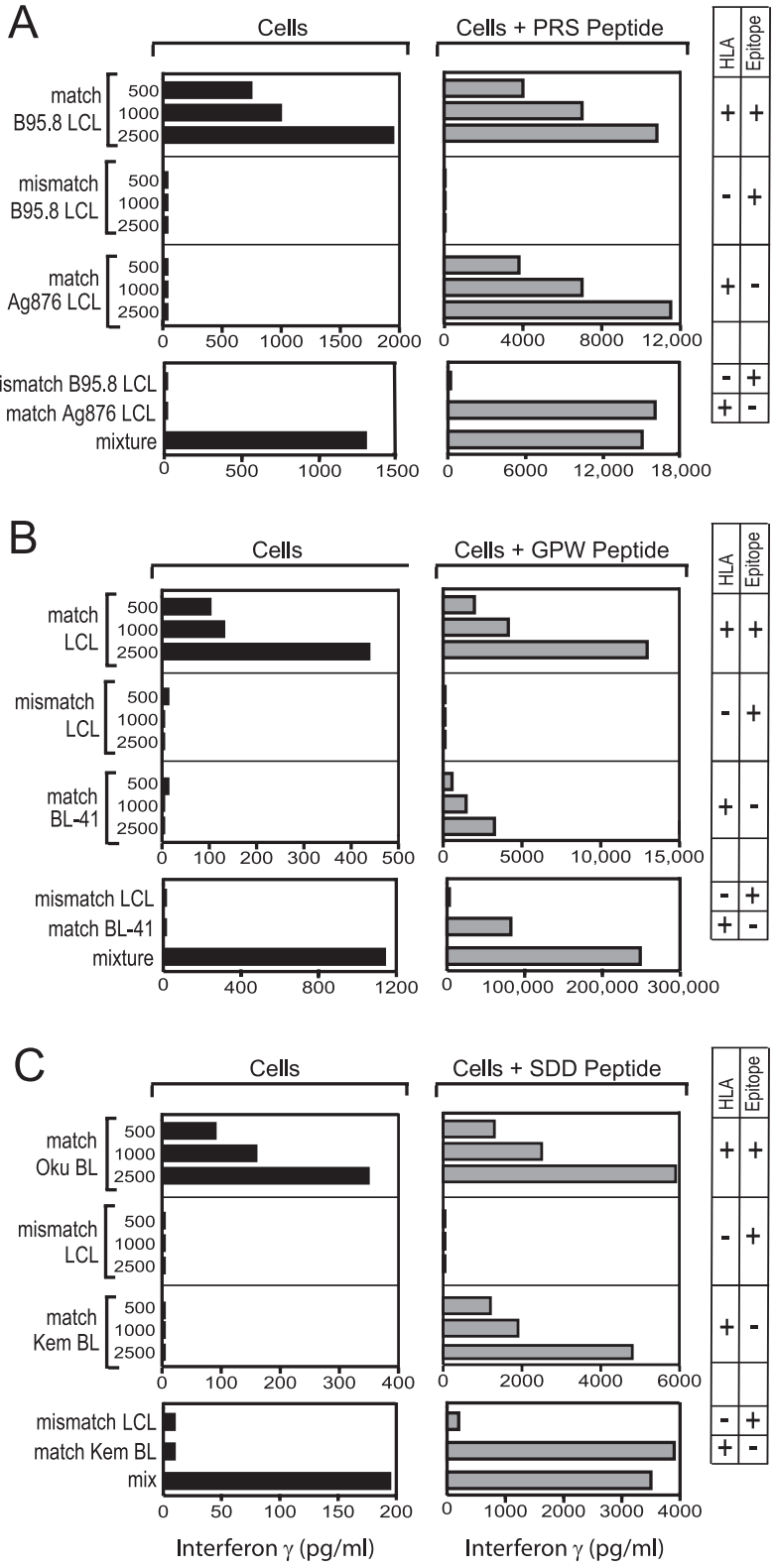

FIGURE 4. Results of CD4$^+$ T cell recognition assays involving target cell mixtures. *A*, *Upper panels*, Recognition by a CD4$^+$ T cell clone specific for the HLA-DR52b-restricted PRS (EBNA2) epitope assayed against a HLA-DR52b-positive B95.8 LCL (match B95.8), a HLA-DR52b-negative B95.8 LCL (mismatch B95.8), and a HLA-DR52b-positive Ag876 LCL (match Ag876). The clone was assayed at 500-2500 T cells/well against target cells (50,000 cells/well) either unmanipulated (■) or previously exposed to 5 μM epitope peptide and then washed before the assay (▨). *Lower panels*, Recognition by the same T cell clones against the HLA-DR52b-negative B95.8 LCL cultured alone (mismatch B95.8-donor cell line), the HLA-DR52b-positive Ag876 LCL cultured alone (match Ag876-recipient cell line), and against a 1:1 mixture of these two lines that had been cultured for 3 days before the assay. The clone was assayed at 2500 T cells/well against target cells (50,000 cells/well) either unmanipulated (■) or previously exposed to 5 μM epitope peptide and then washed before the assay (▨). *B*, *Upper panels*, Recognition by a CD4$^+$ T cell clone specific for the HLA-DR1-restricted GPW (EBNA3A) epitope assayed against an HLA-DR1-positive LCL line (match LCL), a HLA-DQ5-negative LCL line (mismatch LCL), and the HLA-DQ5-positive EBNA3C-negative BL-41 cell line (match BL-41). The clone was assayed at 500-2500 T cells/well against target cells (50,000 cells/well) either unmanipulated (▨) or previously exposed to 5 μM epitope peptide and then washed before the assay (▨). *Lower panels*, Recognition by the same CD4$^+$ T cell clone (10,000 T cells/well) against a HLA-DQ5-negative LCL cultured alone (mismatch LCL-donor cell line), the HLA-DQ5-positive EBNA3C-negative BL-41 line cultured alone (match BL41-recipient cell line), or against a 1:1 mixture of these two lines that had been cultured for 12 days before the assay (and fed with fresh cell medium as required). Targets were again assayed without (■) or with (▨) pre-exposure to the epitope peptide and washing immediately before the addition of T cells. All results are expressed as IFN-γ release in picograms per milliliter after overnight incubation of T cells and targets. *C*, *Upper panels*, Recognition by a CD4$^+$ T cell clone specific for the HLA-DQ5-restricted SDD (EBNA3C) epitope assayed against the HLA-DQ5-positive EBNA3C-positive Oku-BL cell line (match Oku-BL), a HLA-DQ5-negative EBNA3C-positive LCL line (mismatch LCL), and the HLA-DQ5-positive EBNA3C-negative Kem-BL cell line (match Kem BL). The clone was assayed at 500–2,500 T cells/well against target cells (50,000 cells/well) either unmanipulated (■) or previously exposed to 5 μM epitope peptide and then washed before the assay (▨). *Lower panels*, Recognition by the same CD4$^+$ T cell clone (2500 T cells/well) against a HLA-DQ5-negative EBNA3C-positive LCL cultured alone (mismatch LCL-donor cell line), the Kem-BL line cultured alone (match Kem BL-recipient cell line), or against a 1:1 mixture of these two lines that had been cultured for 3 days before the assay. Targets were again assayed without (■) or with (▨) pre-exposure to the epitope peptide and washing immediately before the addition of T cells. All results are expressed as IFN-γ release in picograms per milliliter after overnight incubation of T cells and targets.

epitope, as in the preceding experiments, we used a B95.8-transformed but HLA-mismatched line as the donor and an Ag876-transformed but HLA-matched line as the recipient. T cell assays were conducted on the donor-recipient coculture, on the donor and recipient lines cultured alone, and (as a positive control) on the relevant B95.8-transformed HLA-matched line; all targets were tested with and without pulsing with epitope peptide immediately before the T cell assay. Fig. 4*A* shows the results. As expected, there is good recognition of the HLA-matched B95.8-transformed LCL, levels of IFN-γ release increasing progressively with T cell input. Also as expected, there is no recognition of either the donor or recipient LCL, though the Ag876-transformed recipient line clearly did express the HLA DR52b-restricting allele as shown by its efficient recognition after exogenous peptide loading. The key result (Fig. 4*A*, *lower panel*, *left*) is that cocultivating the donor and recipient LCLs together for 3 days reproducibly led to significant

recognition at levels which approached those shown by the same CD4+ T cell clones tested against the B95.8-transformed HLA-matched positive control.

A similar approach was used to study presentation of the DR1-restricted GPW epitope from EBNA3A; clones to this epitope do recognize unmanipulated LCL targets but at much lower levels (see Table I). This epitope is antigenically conserved among EBV strains and thus no GPW epitope-negative LCL was available to be used as a recipient cell line. Instead, we used the HLA-DR1-expressing BL line BL41 derived from an EBV-negative Burkitt tumor and known to be capable of processing exogenous Ag as efficiently as LCL cells (34); this was cocultured with a B95.8-transformed but DR1-negative donor LCL. As shown in Fig. 4*B* (*lower panels*), this donor-recipient cell mixture again led to significant recognition after coculture; however, sensitization was not as strong as that illustrated earlier using PRS-specific clones and required a greater number of T cells in the assay. Again, donor and recipient lines alone were never recognized.

Fig. 4*C* shows the results from a third such experiment using CD4+ T cell clones against the HLA-DQ5-restricted SDD epitope in EBNA3C. The recipient cell line in this experiment was the DQ5-positive BL cell line Kem BL that lacks EBNA3C expression; the donor line was an HLA-mismatched LCL. Again, we observed CD4+ T cell recognition of the cell mixture but not of either line individually. Note that the positive control in this experiment was the DQ5-positive Oku-BL line which is unusual in expressing the EBNA3 proteins as well as EBNA1 (23). Interestingly, the SDD-specific clone recognized unmanipulated Oku-BL cells to levels similar to those described earlier for DQ5-positive LCLs; this supports the view that BL lines, though known to be deficient in the HLA class I presentation pathway (35, 36), are equivalent to LCLs in their susceptibility to EBNA-specific CD4+ T cell recognition providing the BL cells express the cognate Ag (10).

Detection of antigenic species in medium harvested from LCL cultures

In additional experiments, we used CD4+ T cell clones against the better recognized epitopes, PRS (EBNA2) and SDD (EBNA3C), to follow the kinetics of sensitization. As illustrated in Fig. 5*A* using PRS-specific CD4+ T cells, sensitization was not apparent immediately but was detectable within 24 h of the donor-recipient coculture; furthermore, levels of recognition exceeded 30% of that induced by a positive reference target (a HLA-DR52b positive B95.8 LCL) within 6 days and in another experiment exceeded 50% within 12 days. In the same experiment, we also tested these cell mixtures for recognition by T cells against CD8 epitopes (in this case, the AVF and IVT epitopes in EBNA3B) which like PRS is present in B95.8 but lost in the Ag876 virus strain. Interestingly, we never found sensitization to CD8+ T cell recognition either in this experiment (Fig. 5*A*) or in a number of other cocultivation experiments using various EBNA epitope-specific CD8+ clones (data not shown).

We then found that a degree of sensitization was detectable even without contact between donor and recipient cells. Thus, DR52b-positive Ag876-recipient LCLs could reproducibly be sensitized to PRS-specific T cell recognition simply by culturing in filtered conditioned medium harvested from donor LCL cultures. Fig. 5*B* presents results from two such experiments. In one, feeding over 5 days produced a steady rise in recognition of the recipient cells to almost 10% of that seen for the positive control target (the DR52b-positive B95.8 LCL). In another, recognition reached >15% of control values within 5 days and could be maintained at this level for up to 40 days by regular refeeding with donor-LCL conditioned medium; however, recognition was lost within 3 days of switching the recipient cells back to fresh medium. Again, there was never any evidence that exposure to conditioned medium could sensitize the cells to EBNA epitope-specific CD8+ T cell clones.

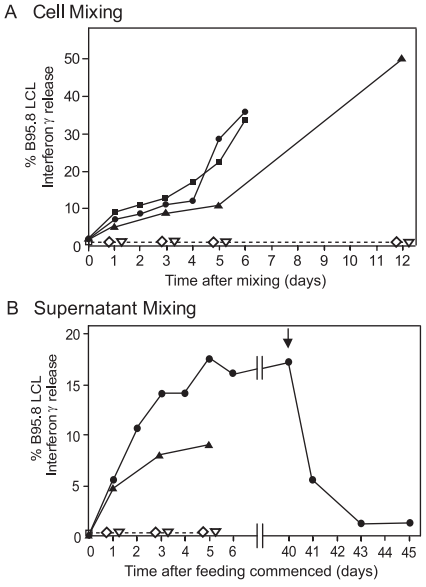

FIGURE 5. Results of T cell recognition assays involving target cell mixtures and target cells fed conditioned medium. All assays involved CD4+ T cell clones to the HLA-DR52b-restricted PRS (EBNA2) epitope or, where indicated, CD8+ T cell clones to the HLA-A11-restricted epitopes IVT or AVF (EBNA3B). *A*, Equal numbers of a HLA-A11/DR52b-positive Ag876 LCL and a HLA-A11/DR52b-negative B95.8 LCL were mixed and cocultured for the indicated lengths of time. The cell mixtures were harvested and tested in a single assay for CD4+ T cell recognition. Results of three independent experiments are shown (●, ■, and ▲), and in each case, the level of recognition of the mixture is expressed as a percentage of that seen in the same assay against a standard HLA-DR52b-positive B95.8 LCL target. The result shown for day 0 represents that seen when the two cell lines were mixed immediately before the T cell assay. One of the experiments (■) used an HLA-A11/DR52b-negative LCL established using a recombinant B95.8 strain EBV lacking the *BZLF1* gene and was unable to enter lytic cycle replication. Note that the individual cell lines from which the mixture was made were never recognized when tested alone as target cells (data not shown). Also shown is one representative result obtained when cell mixtures were tested for CD8+ T cell recognition by IVT (◇) or AVF (▽) specific clones. *B*, A HLA-A11/DR52b-positive Ag876 LCL was cultured for the indicated lengths of time in filtered cell-free medium conditioned by a HLA-A11/DR52b-negative B95.8 LCL before harvesting, washing and, for times up to day 6, testing in a single assay for CD4+ T cell recognition (● and ▲). Results of two independent experiments are shown and in each case the level of LCL recognition is expressed as a percentage of that seen in the same assay against a standard HLA-DR52b-positive B95.8-LCL target. The result shown for day 0 represents that seen when the cell line is pre-exposed for 1 h to the conditioned medium immediately before the T cell assay. Also shown are the results obtained when cells were tested for CD8+ T cell recognition by IVT (◇) or AVF (▽) -specific clones. Results are expressed as in Fig. 4*A*. Note that the target cell line cultured in unconditioned medium was never recognized at any time throughout the duration of these experiments (data not shown). In one experiment, the LCL culture was regularly refed with supernatant for 40 days and then the cells were tested for CD4+ T cell recognition immediately following washing (vertical arrow), or after a further 1, 3, and 5 days of culture in fresh medium.

Sensitization to CD4+ T cell recognition could not be ascribed to preformed peptide fragments binding directly to surface HLA class II molecules. Thus, exposing recipient cells to conditioned medium for 1 h at 37°C or for up to 72 h at 4°C immediately before

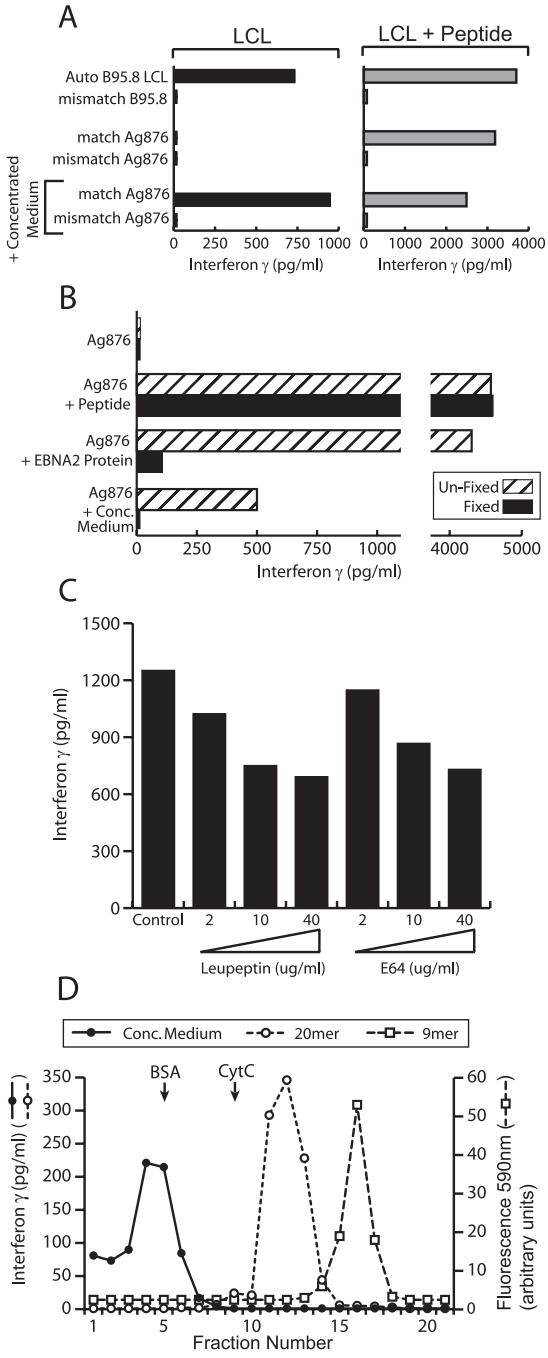

FIGURE 6. Results of assays using concentrated conditioned medium. All target cells were tested for recognition by $CD4^+$ T cell clones to the HLA-DR52b-restricted PRS (EBNA2) epitope. *A*, HLA-matched or mismatched Ag876 LCLs, either unmanipulated or exposed for 24 h to concentrated conditioned medium (harvested from a HLA-mismatched B95.8 LCL line) were washed and used as targets in a T cell recognition assay (■). Half of the cells were pulsed with 5 μM epitope peptide immediately before use as targets in the same assay (▨). Included as controls in the recognition assay (either unpulsed or pulsed with peptide as described above) are the autologous B95.8 LCL line and the same HLA-mismatched B95.8 LCL line that was used to prepare the conditioned medium. Results of the $CD4^+$ T cell recognition assay are expressed as IFN-γ release in picograms per milliliter. *B*, HLA-matched Ag876 LCL cells, either fixed lightly with glutaraldehyde or unfixed were exposed for 1 h to 5 μM epitope peptide, EBNA 2 protein or concentrated conditioned medium (harvested from a HLA-mismatched B95.8 LCL line). The cells were then washed and used as targets in a T cell recognition assay. Unmanipulated Ag876 LCL cells were not recognized by PRS-specific $CD4^+$ T cells. *C*, HLA-matched Ag876 LCL cells were pretreated for 2 h with the indicated concentrations of the cathepsin inhibitors leupeptin or E64 then exposed for 24 h to concentrated conditioned medium in the continued presence of the same concentration of inhibitor. Control cells were exposed in parallel to concentrated conditioned cell medium in the absence of inhibitors. All cells were then fixed with glutaraldehyde and tested as targets in a T cell recognition assay. *D*, Concentrated conditioned medium was fractionated by gel filtration chromatography, and the fractions tested for their ability to sensitize HLA-matched Ag876 cells to T cell recognition. The synthetic 20-mer PRS epitope peptide and a fluorescently labeled 9-mer peptide, separated under the same chromatographic conditions, were detected by T cell assay and fluorometry, respectively. Also indicated on the figure are the fractions in which the size standards BSA (66 kDa) and CytC (12 kDa) were eluted when separated under the same conditions.

the assay did not result in any recognition by $CD4^+$ T cell clones, whereas parallel treatments with culture medium spiked with a limiting dose of synthetic epitope peptide clearly were sensitizing (data not shown). It is also important to note that sensitization, either by the donor LCL or by its supernatant medium, did not depend upon the presence of a small number of cells in the donor LCL undergoing EBV lytic replication leading to cell death. Thus, Fig. 5 includes data from experiments using donor LCLs transformed with a B95.8 recombinant strain deleted for the *BZLF1* gene and therefore incapable of entering lytic cycle (22).

The efficiency of target cell sensitization could be increased by concentrating conditioned medium (in this case, from donor LCLs growing under serum-free conditions in AIM-V) in a Centricon centrifugal concentrator. Thus, recipient Ag876-transformed cells exposed for only 24 h to concentrated medium were then recognized by PRS-specific $CD4^+$ T cells at levels exceeding recognition of an unmanipulated HLA-matched B95.8 LCL tested in parallel (Fig. 6*A*). Subsequent experiments confirmed that sensitization by concentrated conditioned medium required active Ag uptake and processing. Thus, glutaraldehyde-fixed recipient cells could not be sensitized by using concentrated medium, nor could they present baculovirus-expressed EBNA2 protein provided as an exogenous Ag, whereas they were capable of binding and presenting synthetic epitope peptide (Fig. 6*B*). Furthermore, sensitization by the concentrated medium was decreased in a dose-dependent manner by the cathepsin inhibitors E64 and leupeptin (Fig. 6*C*), whereas in control experiments these inhibitors had no effect on recognition when epitope peptides were loaded onto the same LCL (data not shown). Equally efficient sensitization of recipient cells using concentrated medium was obtained in experiments using $CD4^+$ T cell clones to the EBNA 3A-derived epitope GPW and the EBNA3C-derived epitope SDD (data not shown).

Finally, to investigate the size of the antigenic species in concentrated culture medium, we fractionated such preparations by gel filtration chromatography on a Sephadex G-75 column (effective separation range 3–80 kDa) and then looked for the ability of fractions to sensitize recipient cells to recognition by PRS-specific $CD4^+$ T cells. Included as controls in these experiments were a fluorescent 9-mer peptide, detected by fluorometry, and the 20-mer PRS epitope peptide, detected by $CD4^+$ T cell recognition, both of which were subjected to the same chromatographic separation. As shown in Fig. 6*D*, these low m.w. peptides were detected in fractions 14–17 and 11–14, respectively. However, fractionation of the concentrated culture medium showed that all the antigenic species eluted in fraction 4–6, representing molecules similar to or larger than the 66-kDa BSA marker; no evidence of any small antigenic peptide species was ever observed.

Discussion

The CD4+ T cell response to EBV latent cycle proteins has attracted much interest because of its potential for direct recognition of latently infected cells. However, although a number of CD4+ T cell epitopes have been identified in latent cycle proteins, only a subset of these epitopes appear to be presented at sufficient levels on the surface of EBV-transformed LCL cells to allow direct recognition by in vitro-reactivated CD4+ T cell clones. That subset is not limited to epitopes derived from one particular Ag; thus, the literature contains examples of both well-presented and poorly presented epitopes derived from EBNA1 (8, 10–12, 37–39), from EBNA2 (9, 12, 40), from EBNA3A (12), and from EBNA3C (12, 41, 42). As a first step toward understanding these differences, here, we set out to study how endogenously expressed EBNA proteins are being processed for CD4+ T cell recognition in latently infected cells. From the literature, there are several potential routes whereby endogenously expressed Ags might directly enter the MHC class II pathway. Some of these appear to be restricted to membrane or secreted proteins which naturally intercept nascent MHC class II molecules in the endoplasmic reticulum (13–16). Others can involve cytoplasmic and even nuclear proteins being directly delivered to the endosomal MHC class II-loading compartment (18, 21, 43–46). This can occur by incorporation of cellular components into autophagosomes which then fuse to endosomal/lysosomal vesicles, a pathway induced under conditions of stress but active to some degree under conventional culture conditions (20), or by a specialized chaperone-mediated pathway transporting peptide fragments generated by the proteasome or other cytoplasmic proteinases (17, 45).

Several mechanistic studies of endogenous Ag presentation to CD4+ T cells have successfully used vaccinia vectors to express the Ag within target cells, whether in murine models using a mouse B cell line (47) or in human systems using LCLs as the targets for vaccinia infection (44, 48–51). Therefore, we reasoned that if EBNAs did enjoy direct intracellular access to the HLA class II pathway in LCL cells, such access should be exaggerated if the native Ag is overexpressed in these cells from a vaccinia vector. This would therefore allow LCL recognition by CD4+ T cell clones to be increased over baseline levels or (for clones with no baseline recognition) to be revealed for the first time. Vaccinias expressing a class II-targeted form of the Ag were generated to serve as a positive control in such experiments. We deliberately used the MVA vaccinia strain as a vector for the relevant EBNA constructs because, unlike other strains, this does not encode a decoy IFN-γ receptor (52) and so does not introduce artifacts into IFN-γ release assays. Furthermore, MVA does not replicate in human cells and thus avoids potential complications from spread of the infection within the assay culture. The results were remarkably consistent for CD4+ T cell clones to all nine epitopes tested in standard assays. There was never any detectable recognition of the vector-expressed native Ag yet the corresponding class II-targeted protein was always well-recognized. By contrast, vector-expressed native Ag was efficiently processed for recognition by EBV-specific CD8+ T cell clones, often using the very same target cells as in the CD4+ T cell assays. Interestingly, in each case the HLA class II-targeted protein was also efficiently processed for recognition by CD8 T cells, an observation noted in other CD8+ T cell systems (30, 31). All these assays were conducted over an 18-h timeframe which in other studies using influenza matrix protein expressed from a vaccinia vector (48) was much longer than required to allow HLA class II presentation in LCL cells. However, to allow for the possibility that EBNAs can directly access both the HLA class I and class II pathways but at different rates, we allowed the vaccinia infection to proceed for 24 h before the 18 h T cell assay was initiated. Then, we did notice a low level of presentation to CD4+ T cells in cells expressing native Ag, but the inclusion of uninfected recipient LCL as an indicator showed that this reflected Ag release and reprocessing within the extended timeframe of the assay.

In view of recent reports identifying autophagy as an intracellular route for Ag feeding into the HLA class II pathway (21), we set up a new series of experiments using 3-MA to inhibit the autophagic process. Using as an indicator protein NeoR-GFP, a known substrate of autophagy (18), we confirmed earlier findings that treatment with 3-MA did inhibit autophagy in LCL cells (21) and that the blockade could be maintained for several days. We then used these same conditions to look for evidence of 3-MA-induced inhibition of LCL recognition by CD4+ T cell clones against the two epitopes, PRS from EBNA2 and SDD from EBNA3C, mediating the highest baseline levels of recognition. Note that these epitopes were shown to have half-lives of 1.3 and <1 day, respectively, on the cell surface, and so any blockade of de novo epitope supply to the HLA class II pathway would easily be detectable within the 3-day time scale of the experiment. In fact, there was never any effect of 3-MA treatment on LCL recognition by either PRS- or SDD-specific T cells.

Given these negative results, we turned to the possibility (first raised by the 42-h MVA infection experiments) that the EBNAs were accessing the HLA class II pathway by a slower and less direct route involving intercellular Ag transfer. This possibility was examined by coculturing HLA-matched recipient cells lacking the Ag or epitope in question with HLA-mismatched donor cells expressing the Ag at physiologic levels from the resident EBV genome. For all three epitopes tested (PRS, SDD and GPW), sensitivity to T cell recognition was slowly but cumulatively acquired by recipient cells with increasing time of coculture. This did not involve superinfection of the recipient with virus released from donor cells because donor LCLs carrying a replication-deficient EBV strain gave the same results. We infer that such intercellular transfer of antigenic species must be happening continuously in standard LCL cultures. Furthermore, this transfer did not require cell contact because recipient cells could be sensitized to CD4+ T cell recognition by exposure to cell free-conditioned medium. This effect was accelerated using concentrated medium and assays using this as an Ag source confirmed that sensitization required the active uptake and processing of antigenic species by recipient cells.

Several reports in other systems have looked for the presence of Ag transfer in cocultivation or conditioned medium feeding experiments and have failed to detect significant recognition (37, 43, 47, 48, 51). However, these very often involve overnight or 24-h incubation times. In our system, although we can see low level sensitization with conditioned medium within 24 h, the effect is more apparent at later times and in other systems may have been missed if assays were not conducted over longer periods. It was interesting that both the coculture and conditioned medium protocols reproducibly sensitized cells to recognition by EBNA epitope-specific CD4+ but not CD8+ T cell clones. This cannot be ascribed to differential sensitivity because CD8+ T cells are consistently the more avid, often detecting peptide in the 10^{-9} to 10^{-11} M range (24, 25, 29) compared with the 10^{-7} to 10^{-9} M range typically shown by the present CD4+ T cells (12). We infer that, although LCL cells are capable of processing exogenously acquired Ag via the HLA class I pathway if Ag is provided at high concentrations experimentally (25), the levels of exogenous Ag available within LCL cultures are only ever sufficient to charge the HLA class II pathway. Indeed from recent work, this also seems to

be true of exogenously acquired EBV lytic cycle Ags. Thus, coculture between appropriate mixtures of semipermissive HLA-mismatched (donor) and nonpermissive HLA-matched (recipient) LCL never led to recognition by lytic epitope-specific $CD8^+$ T cells (53). By contrast, the recognition of semipermissive LCL cells by virus-structural Ag-specific $CD4^+$ T cells was found to be dependent upon the intercellular transfer of virions within the culture (54). Furthermore, a similar example of LCL recognition by $CD4^+$ T cell clones against the nonstructural lytic cycle protein BHRF1 has been ascribed to slow charging of the HLA class II pathway by Ag released from lytically infected cells (55).

The identity of the latent cycle antigenic species being transferred in the present work remains to be determined. Gel filtration chromatography demonstrated that the molecular mass of the sensitizing species exceeded 66 kDa in the case of EBNA2 (an 85-kDa protein in its native form) and was clearly much larger than the 20-mer epitope peptide run under the same conditions. These findings are consistent with the transfer of intact EBNA2 Ag but also with several other possibilities, for example the transfer of Ag or antigenic fragments either complexed with other proteins or even as components of exosomes, structures known to be shed in abundance from LCL cells (56). Whatever the nature of the transferred Ag, the levels of sensitization achieved in cell mixing and conditioned medium feeding experiments identify intercellular Ag transfer as the major route whereby at least three EBNA proteins, EBNA2, 3A, and 3C, gain access to the HLA class II pathway in LCL cells. Our results provide no evidence for the existence of a second pathway providing direct intracellular access. If such a pathway does exist, it must be a minor contributor to the presentation of EBNA2-, 3A-, and 3C-derived epitopes on the LCL surface and, moreover, must involve a mechanism that is not operational in MVA-infected cells. We can make no definitive statement regarding EBNA1 Ag processing in the type of cell mixing and conditioned medium feeding experiments described here because sufficiently sensitive $CD4^+$ T cell clones were not available. Recently, it has been reported by one group that EBNA1 is processed intracellularly by an autophagosomal pathway in LCL cells (21). In contrast, another group who first described autophagosome-mediated processing of an indicator cytoplasmic Ag in LCL cells (18) could find no evidence for endogenous EBNA1 presentation by this or any other route (11). Further work will be needed to determine whether EBNA1 is somehow different from the other EBNAs in its intracellular processing for $CD4^+$ T cell recognition.

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Disclosures

The authors have no financial conflict of interest.

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