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Lymphocryptovirus Infection of Nonhuman Primate B Cells Converts Destructive into Productive Processing of the Pathogenic CD8 T Cell Epitope in Myelin Oligodendrocyte Glycoprotein

S. Anwar Jagessar,*†‡ Inge R. Holtman,* Elena Morandi,* Nicole Heijmans,* Jon D. Laman,§ Bruno Gran,§ Bart W. Faber,‖ Sander I. van Kasteren,# Bart J. L. Eggen,§ and Bert A. ‘t Hart*†‡§

EBV is the major infectious environmental risk factor for multiple sclerosis (MS), but the underlying mechanisms remain obscure. Patient studies do not allow manipulation in vivo. We used the experimental autoimmune encephalomyelitis (EAE) models in the common marmoset and rhesus monkey to model the association of EBV and MS. We report that B cells infected with EBV-related lymphocryptovirus (LCV) are requisite APCs for MHC-E–restricted autoaggressive effector memory CTLs specific for the immunodominant epitope 40–48 of myelin oligodendrocyte glycoprotein (MOG). These T cells drive the EAE pathogenesis to irreversible neurologic deficit. The aim of this study was to determine why LCV infection is important for this pathogenic role of B cells. Transcriptome comparison of LCV-infected B cells and CD20+ spleen cells from rhesus monkeys shows increased expression of genes encoding elements of the Ag cross-presentation machinery (i.e., of proteasome maturation protein and immunoproteasome subunits) and enhanced expression of MHC-E and of costimulatory molecules (CD70 and CD80, but not CD86). It was also shown that altered expression of endolysosomal proteases (cathepsins) mitigates the fast endolysosomal degradation of the MOG40–48 core epitope. Finally, LCV infection also induced expression of LC3-II+ cytosolic structures resembling autophagosomes, which seem to form an intracellular compartment where the MOG40–48 epitope is protected against proteolytic degradation by the endolysosomal serine protease cathepsin G. In conclusion, LCV infection induces a variety of changes in B cells that underlies the conversion of destructive processing of the immunodominant MOG40–48 epitope into productive processing and cross-presentation to strongly autoaggressive CTLs. The Journal of Immunology, 2016, 197: 000–000.
neurologic deficits. The latter pathway involves MHC class I/Caja-E–restricted CD8+CD56+ autoreactive CTLs specific for myelin oligodendrocyte glycoprotein (MOG). A similar subset of HLA-E–restricted CTLs was identified recently in MS lesions (5), albeit with specificity for myelin basic protein.

In marmoset EAE, T cell autoimmunity against MOG is indispensible for the induction of chronic inflammation (6). The two immunodominant T cell epitopes are localized in the extracellular domain: residues 24–36, which activate Caja-DRB*W1201–restricted CD4+ Th1 cells, and residues 40–48, which activate Caja-E–restricted autoaggressive CD8+CD56+ CTLs (7–9). Intriguingly, when marmosets were immunized with recombinant human MOG (rhMOG), representing the full-length extracellular domain (1–125), formulated with IFA, we observed only T cell activation against the MOG24–36 epitope (10). However, in marmosets immunized with synthetic MOG34–56 in IFA, we observed activation of CTLs recognizing the MOG40–48 epitope associated with full-blown clinical EAE (11). These observations led us to hypothesize that the strongly pathogenic MOG40–48 epitope is destroyed during processing.

In our search for an APC type that has the capacity to cross-present the exogenous MOG epitope via Caja-E to the autoreactive CTLs, we found evidence for a crucial pathogenic role for B cells naturally infected with the EBV-related lymphocryptovirus (LCV) CalHV3 (12). We observed that the opposite clinical effect in relapsing-remitting MS of an anti-CD20 Ab (ofatumumab) and atacicept, a soluble antagonist of the B cell cytokines BAFF and APRIL (not effective), could be replicated in marmoset EAE (13, 14). The second issue is the observation that LCV in-
from Peptide 2.0 (Chantilly, VA) and Pepscan (Lelystad, the Netherlands). Peptides were derived from the MOG sequence of marmosets (Cj-MOGs) and from the MOG sequence of rhesus monkeys (Mm-MOGs). Sequences were downloaded from the National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/protein). Note that the MOG34–56 peptides from marmoset and rhesus monkey differ at only one position: a Ser or Pro residue on position 42, respectively. Modifications included substitution of the positively charged Arg residues on positions 41 and 46 (Argβ- and/or Argγ-) for uncharged Cit.

Magnetic bead assay for proteolytic degradation of MOG peptides

Streptavidin-labeled magnetic beads (Dynabeads; Invitrogen, Carlsbad, CA) were incubated with biotin/FITC-labeled peptides (peptides B1–B8), according to the manufacturer’s instructions. Briefly, after a 2-h incubation at room temperature, beads were precipitated with a strong magnet to collect the bound peptides. After two washings of the peptide-coated beads with 160 mM Tris-HCl + 1.6 M NaCl (pH 7.4), they were incubated for 24 h with 40 μl of cell lysates (0.5 mg/ml), 40 μl of CatG (0.25 mM/μl), or 50 μl of cathepsin H (CatH) (10 ng/μl). After another precipitation step with the magnet, 50 μl of supernatants was collected into a black 96-well plate (Greiner Bio-One, Frickenhausen, Germany). The FITC signal present in the supernatants was measured with a VICTOR Multilabel Counter model 1420 (PerkinElmer, Waltham, MA) to quantify the amount of peptide released from the beads as a measure of proteolysis.

Protease inhibitors

To gain insight into the mechanism of proteolysis by cells, we tested the effect of specific inhibitors of endosomal/lysosomal proteases, including pepstatin A (0.7 mg/ml), specific for aspartyl proteases (cathepsin D [CatD] and cathepsin E); cystatin C (0.7 mg/ml), specific for cysteine cathepsins (32). CatG activity was blocked with 0.1 mM Z-glycyl-leucyl-phenyl-chloromethyl ketone (CMK) (33). The inhibitor E64 (N-[V-L-3-trans-carboxyirane-2-carbonyl]-t-leucyl-argmine) (Sigma-Aldrich) is a cysteine peptidase inhibitor with relative selectivity for cysteine cathepsins (34, 35). We used 10 μM E64 to block cysteine cathepsin (CatH) activity.

In all experiments, the cell lysates were incubated for 30 min at 37˚C with inhibitors at the indicated concentrations prior to the addition of rhMOG or MOG peptide.

Electrophoresis-based assay for proteolytic degradation of MOG peptides

rhMOG protein (2 μg) or MOG peptides (10 μg) were incubated with cell lysates in 50 mM citrate buffer + 5 mM DTT + 0.1% Triton X-100 (pH 5) in a total volume of 15 μl in Eppendorf tubes. The tubes were incubated for 3, 24, or 48 h at 37˚C on an Innova 2000 platform shaker set at 40 rpm. After incubation, 6.25 μl of NuPAGE LDS sample buffer, containing 2.5 μl NuPAGE Sample Reducing Agent (Invitrogen) and 1.25 μl dH2O, was added to each sample. Subsequently, 18 μl of each sample was loaded onto a 4–12% Bis-Tris gradient gel (Invitrogen). After running for 60–90 min at 135 V, the gels were stained for 3–4 h with Simply Blue Safe Stain (Invitrogen) and destained with dH2O overnight to visualize the protein/peptide bands. A scan of the gel, which was taken with the ChemiDoc MP Imaging System and Image Lab software (Bio-Rad, Veenendaal, the Netherlands), was used for densitometric analysis. Data in the bar graphs are expressed as percentage of degradation relative to incubations containing rhMOG or MOG peptide only, which were run on the same gel. Calculations were corrected by subtracting the background staining and were normalized by measured area (mm²).

**CatH activity assay**

CatH activity in MNC lysates was determined with a commercially available assay (BioVision, Milpitas, CA). Briefly, 50 μl of MNC lysate (200 μg protein/ml) was incubated with CH reaction buffer and CH R-ACF substrate for 24 h at 37˚C. Color reaction was measured with the VICTOR Multilabel Counter model 1420 (PerkinElmer) at excitation 405 nm/emission 535 nm. Data are expressed as arbitrary counts. As positive control, 100 ng of active human CatH (BioVision) was used.

**CatG activity assay**

CatG activity was measured in 50 μl of 200 μg/ml MNC cell lysates in a total volume of 90 μl of 160 mM Tris-HCl + 1.6 M NaCl + 5 mM DTT (pH 7.4) and 10 μl of 200 μM Z-Gly-Gly-Arg-AMC substrate (Bachem, Bubendorf, Switzerland). The total mixture was incubated for 24 h at 37˚C, and fluorescence was measured with the VICTOR Multilabel Counter model 1420 (PerkinElmer) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. As positive control, 5 μM/ml human CatG (Sigma-Aldrich) was used.

**Quantitative PCR**

Total RNA was extracted from PBMCs and spleen MNCs, and cDNA was synthesized for quantitative PCR (qPCR) using primer–probe combinations (Table II) according to the Universal Probe Library (Roche, Indianapolis, IN), as previously described (24). Transcript levels were normalized against the reference gene Abelson (ABL). Only cathepsin mRNA transcripts were analyzed for which reliable primer–probe combinations could be designed.

**Western blotting for LC3**

BLCs and splenocytes (10⁶ cells) from C. jacchus and M. mulatta were incubated overnight at 37˚C with 500 nM bafilomycin (Sigma-Aldrich) to prevent maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. Cells were harvested in Eppendorf tubes and, after a short spin (10,000 rpm), cell pellets were resuspended in 40 μl of culture media (RPMI 1640 without FCS) and frozen at −80˚C for ≥3 h. After thawing of cell suspensions, 2 μl of 500 U/ml Benzonase Nuclease (Merck Millipore, Amsterdam, the Netherlands) was added to degrade all forms of DNA and RNA, followed by sonication in a sonic water bath (6 × 30 s with 30-s intervals) while cooling on ice. Six microliters of each sample was solubilized in 6 μl Laemmli sample buffer containing 0.3 μl of 50 mM 2-ME (Bio-Rad), and 10 μl was loaded on a mini PROTEAN TGX stain-free 4–20% gel (Bio-Rad), which was run for 35 min at 200 V. As a positive control, 10 ng of active human CatH (BioVision) was added.
control, LC3-I and LC3-II were loaded onto the gel, which were provided together with the anti-LC3 Ab (nanoTools, Teningen, Germany). The Bio-Rad Trans-Blot Turbo Transfer System was used for blotting the gels onto a 0.2-μm polyvinylidene difluoride membrane (predefined program: Low MW). Samples were immunoblotted with mouse anti-LC3 (clone 5F10; nanoTools; dilution 1:200) and with goat anti-GAPDH as loading control (Novus, Abingdon, U.K.; dilution 1:100,000) overnight at 4˚C. Rabbit anti-mouse–HRP (Jackson ImmunoResearch, Newmarket, U.K.; dilution 1:100,000) and mouse anti-goat–HRP (Abcam, Cambridge, U.K.; dilution 1:1,000) were used as secondary Abs. Blots were incubated for 1 h at room temperature and developed using Clarity Western ECL substrate (Bio-Rad). For immunofluorescence staining, cells were washed with PBS, and 2% paraformaldehyde (PFA) in PBS was added for 1 h.固定后的细胞用0.2% Triton X-100 in PBS通透化。After four final wash steps with PBS, coverslips were mounted with the antifade solution Citifluor AF-1 and observed with a Nikon Microphot-FXA microscope (Tokyo, Japan) with a 40× lens and a digital camera (Nikon FX-35DX).

**Results**

**Comparative RNA sequencing of LCV-infected and noninfected B cells**

We performed RNA sequencing to examine changes in molecular pathways induced in *M. mulatta* B cells upon immortalization by experimental infection with the LCV HV papio. The analysis included four samples: HV papio-immortalized Mm-BLCs, CD20⁺ B cells isolated from rhesus monkey PBMCs (Mm-PBMCs), the residual CD20⁻ PBMC fraction, and unsorted Mm-PBMCs. Three main analyses were performed: principal component analysis (PCA) to determine the similarity between the biological replicates for each condition and the differences among conditions; differential gene-expression analysis to identify gene differences among conditions; and Ingenuity Pathway Analysis to identify pathways and gene categories that are overrepresented in the differential gene-expression analyses (Fig. 1).

PCA (Fig. 1A) shows close clustering of the expression profiles of the three biological replicates within each condition, indicating that they are highly similar, as was expected. The figure also shows that the first principal component clearly distinguishes Mm-BLCs from the other samples. The primary aim of the differential gene-expression analysis was to identify genes that were differentially expressed between HV papio-infected and noninfected *M. mulatta* B cells. The most relevant comparison for the current study is Mm-BLCs versus CD20⁺ Mm-PBMCs (Fig. 1B). In total, 3108 (1055 + 2053) genes were expressed at a significantly higher level in Mm-BLCs compared with CD20⁺ Mm-PBMCs compared with CD20⁺ *M. mulatta* B cells, and 2721 (1087 + 1634) genes were expressed at a significantly lower level (false discovery rate, p < 0.01; logFC > 1). Furthermore, we observed a strikingly high overlap between Mm-BLCs and the CD20⁺ Mm-PBMC fraction (2053 and 1634 up- and downregulated genes in common, respectively).

We were particularly interested in genes related to the Ag processing and presentation pathways, including the vacuolar route via endolysosomes and the cytosolic route via the proteasome (17). Using Gene Ontology categories, all genes that were differentially expressed between Mm-BLCs and CD20⁺ Mm-PBMCs were depicted as a heat map (Fig. 1C). Individual genes with their fold change (BLC versus CD20⁺ BLC versus CD20⁺, and CD20⁺ versus CD20⁺) are listed in Supplemental Table I.

Gene candidates of interest are CIITA and NLRC5, the master regulators of MHC class II and MHC class I expression, respectively (36, 37), which are both downregulated in HV papio-infected *M. mulatta* B cells. Several costimulatory molecules were upregulated, such as CD80, TNFSF4 (Ox40L), CD70, and Fas ligand. Others were downregulated, such as CD40, CD86, and PDCD1 (PD1). Moreover, a number of cathepsins were differentially expressed.

### Table II. Primers with corresponding probes used for qPCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>3′-5′ Primer</th>
<th>3′-5′ Primer</th>
<th>Probe</th>
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<td>Marmoset</td>
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<td>GGTGAGTTTCCAGAACAAAGAG</td>
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</tr>
<tr>
<td></td>
<td>CatB</td>
<td>GGGACGCTTCCTCGGTGTTAG</td>
<td>CTCGCGAAGTCAGTGGTCG</td>
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<td>CatC</td>
<td>CGATGTGAACACTGTCGGTTA</td>
<td>AATTGCGAAAGCTCATTACG</td>
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<tr>
<td></td>
<td>CatD</td>
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<td>GTCACAGTGCTGTCAGAA</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>CatF</td>
<td>CCGAAGAAGTTGTACGTTCA</td>
<td>CGGTTTATAAGTTAAGAATGTTCC</td>
<td>135</td>
</tr>
<tr>
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<tr>
<td></td>
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<td>CatK</td>
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<td>63</td>
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<tr>
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<tr>
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<td>PAD4</td>
<td>TACATCTACCGGTCTCCAGT</td>
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<td>Rhesus monkey</td>
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<td>ATGGCAAGCAGGACCATGAT</td>
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<tr>
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<tr>
<td></td>
<td>CatH</td>
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<tr>
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<td>CatK</td>
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<td></td>
<td>CatL2</td>
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<td></td>
<td>CatO</td>
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<td></td>
<td>PAD4</td>
<td>TACATCTACCGGTCTCCAGT</td>
<td>CCAACTGTCTGAGAACAACA</td>
<td>22</td>
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</tbody>
</table>
Lastly, a plethora of proteasome-related genes were consistently upregulated in HV papio–infected M. mulatta B cells. Taken together, the RNA-sequencing data indicate that HV papio infection of M. mulatta B cells induces a unique gene-transcription profile that clearly differs from noninfected CD20+ M. mulatta B cells. Differentially transcribed genes include those associated with Ag presentation and with proteasome and endolysosome functions; however, the most significantly upregulated pathways relate to enrichment of cell cycle and cholesterol biosynthesis.

Transcription profiles and activity of cathepsin in APC subsets

Cathepsins are proteases of the endolysosomal pathway that are centrally involved in the processing of protein Ags (38). We analyzed mRNA transcript profiles for a number of cathepsins in total PBMCs or SCs, the CD20+ and CD20− SC subfractions, and BLCs generated from marmosets and rhesus monkeys. Because we were unable to isolate sufficient numbers of CD20+ M. mulatta B cells, differentially transcribed genes include those associated with Ag presentation and with proteasome and endolysosome functions; however, the most significantly upregulated pathways relate to enrichment of cell cycle and cholesterol biosynthesis.

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Proteolytic degradation of rhMOG in SC lysates from marmosets and rhesus monkeys

We analyzed the degradation of rhMOG in lysates from Cj-SCs and EBV–B95-8–induced Cj-BLCs essentially mirrored those obtained in the corresponding fractions from rhesus monkeys, with the exception of CatD and cathepsins K and O (Fig. 2B), we assume that values obtained for the other cathepsins in CD20+ and CD20− SC fractions from rhesus monkeys may be tentatively extrapolated to marmosets.
compared with Cj-SCs. The observation that degradation is slower in Mm-SCs than in Cj-SCs, with the persistence of intermediate degradation products being observed in Mm-SCs, indicates that processing of rhMOG may be different between rhesus monkeys and marmosets.

To test whether B cell proteases contribute to the proteolytic degradation of rhMOG, we incubated the protein with lysates from the CD20\(^{−}\) and CD20\(^{+}\) fractions of Mm-SCs for 3, 24, and 48 h. Fig. 3D–F show that rhMOG was rapidly and completely degraded in lysates from the CD20\(^{−}\) fraction of Mm-SCs, whereas only moderate degradation could be detected in the CD20\(^{+}\) fraction.

Regarding the prominent role of LCV-infected B cells as APCs in the nonhuman primate EAE models (12), we also tested proteolytic degradation of rhMOG in lysates of BLCs. BLCs were generated by infecting marmoset and rhesus monkey MNCs with the relevant LCV (i.e., human EBV–B95-8 and HV papio, respectively). Fig. 3G–I demonstrate that rhMOG is minimally degraded in lysates of rhesus monkey or marmoset BLCs, although some degradation products could be distinguished as smaller.

**FIGURE 2.** qPCR of cathepsin transcripts in MNC subsets from rhesus monkey and marmosets. Total RNA was extracted from rhesus monkey (A) and marmoset (B) PBMCs and spleen MNCs. Also, CD20\(^{−}\) and CD20\(^{+}\) fractions were isolated from rhesus monkey samples. cDNA was synthesized for qPCR using primer–probe combinations (Table II). Transcript levels were normalized against ABL. Data are presented as mean ± SEM.
bands in the Cj-BLC incubate at 48 h. These data indicate that, although B cells are capable of proteolytic degradation of rhMOG, this activity is nearly completely abolished by infection with LCV.

Delineating the roles of CatG and CatH

Data reported by Burster et al. (39) show a central role for CatG in the processing of the myelin Ag myelin basic protein in human B cells. However, the qPCR data in Fig. 2A show high levels of CatG mRNA in CD20− SCs, whereas it was undetectable in CD20+ SCs. Because the absence of (active) CatG protein may well explain the different degree of rhMOG degradation in both fractions, we examined the effect of the specific CatG inhibitor CMK. Because the qPCR data in Fig. 2 show that CatH mRNA expression in B cells is downregulated after LCV infection, we also included CatH in this analysis, a protease with aminopeptidase and endopeptidase activity that can also cleave peptides at Arg residues (40, 41). Because a specific inhibitor of CatH is not available, we used the covalent cysteine cathepsin inhibitor E64. Note that E64 is not specific for CatH; at the concentrations used, it also inhibits the other cysteine cathepsins.

First, we tested the level of CatG and CatH activity in the different cell lysate preparations using protease-specific assay kits; for validation purposes, the inhibitors CMK and E64 were included. The data in Fig. 4A show that considerable CatG activity was detected in all analyzed cell preparations and that activity could be inhibited to background level with CMK; E64 had no effect on CatG activity (data not shown). The highest CatG activity was observed in BLCs from marmosets and rhesus monkeys and in CD20− Mm-SCs. All assayed cell preparations also tested positively for CatH activity, which could be inhibited with the nonspecific cysteine cathepsin inhibitor E64; CMK had no effect in the CatH assay (data not shown). The highest CatH activity was detected in the lysate of unfractionated Cj-SCs; it was approximately twice as high as the activity in Mm-SCs.

To confirm the involvement of CatG and CatH in the degradation of rhMOG, we tested the effect of E64 and CMK on rhMOG degradation in lysates of CD20+, CD20−, and unfractionated Mm-SCs. Fig. 4B and 4C show that the degradation of rhMOG in lysates of nonfractionated Mm-SCs or CD20− Mm-SCs was only partially inhibited by CMK or E64, whereas the degradation of rhMOG in the CD20+ Mm-SC lysate was reduced to background levels. Interestingly, Fig. 4D and 4E show that the degradation of rhMOG in the lysate of unfractionated Cj-SCs was almost completely suppressed by CMK and E64.

In conclusion, the data indicate an active role for CatG and probably for CatH in the proteolytic degradation of rhMOG.

Degradation of MOG35−51 in cell lysates of rhesus monkeys and marmosets

The specificity profile of CatG from rhesus monkeys and marmosets differs. Although CatG from both species has chymotryptic
activity, cleaving at aromatic residues (Trp, Phe, Tyr), rhesus monkey and human CatG also have trypsin activity and can cleave after Lys and Arg residues (42).

The pathogenically dominant MOG epitope 40–48 (YRRPPFSRVV) contains four potential CatG cleavage sites: the two Arg residues at position 41 and 46 and the aromatic residues Tyr (Y40) and Phe (F44) (Fig. 5). This was confirmed when we incubated the longer peptide MOG35–51 with human CatG and analyzed the generated fragments with mass spectrometry (data not shown). Therefore, we reasoned that CatG destroys the pathogenically highly important MOG40–48 epitope during processing in B cells.

For the initial analyses of MOG peptide proteolysis we used magnetic beads coated with MOG35–51 peptides. Alignment of the peptide sequences from a variety of species ranging from the naked mole rat to human shows the high conservation of this sequence; peptide sequences from a variety of species ranging from the naked mole rat to human shows the high conservation of this sequence; however, in this case, the inhibitor CMK was only partially effective. The inhibitor E64 had no effect in this assay, indicating that CatG is the principal protease for degradation of the peptide.

In the next set of experiments, degradation of the MOG peptide was confirmed on an SDS-PAGE gel. Fig. 7A and 7B show that about 80% of the Cj-MOG35–51 and Mm-MOG35–51 peptides was degraded in lysates of total Mm-SCs and of the CD20+ and CD20− fractions. The inhibitors CMK and E64 were not equally effective. The CatG inhibitor CMK reduced degradation of both peptides in BLC lysates. Fig. 6B shows that the two MOG peptides (Cj-MOG35–51 and Mm-MOG35–51) are more or less equally degraded in Mm-BLC lysate and that degradation is completely suppressed by the inhibitor CMK. Also, the two peptides are equally degraded in lysates of marmoset Cj-BLCs; however, in this case, the inhibitor CMK was only partially effective. The inhibitor E64 had no effect in this assay, indicating that CatG is the principal protease for degradation of the peptide.

We used the same assay for testing the proteolytic degradation of the two MOG peptides in BLC lysates. Fig. 6B shows that the two MOG peptides (Cj-MOG35–51 and Mm-MOG35–51) are more or less equally degraded in Mm-BLC lysate and that degradation is completely suppressed by the inhibitor CMK. Also, the two peptides are equally degraded in lysates of marmoset Cj-BLCs; however, in this case, the inhibitor CMK was only partially effective. The inhibitor E64 had no effect in this assay, indicating that CatG is the principal protease for degradation of the peptide.
CMK than was degradation of the Mm-MOG35–51 peptide, whereas the cysteine cathepsin inhibitor E64 was about equally effective. Finally, we also tested the proteolytic degradation of the two peptides in lysates of LCV-infected B cells from the two species. Fig. 7D and 7E show that the degradation of both peptides is about equally effective in Mm-BLCs and Cj-BLCs and that the inhibitors CMK and E64 were about equally effective.

In conclusion, the presence of a Ser or Pro residue on position 42 has no clear influence on the sensitivity of MOG35–51 peptide to proteolytic degradation in B cell lysates. Both assays show a clear role for CatG in the degradation of the peptide.

Degradation of citrullinated MOG35–51 peptides in cell lysates

Because CatG is prominently involved in the degradation of rhMOG in B cells and has a preference for Arg residues at the cleavage site, we hypothesized that substitution of the Arg41 and/or Arg46 residues by neutrally charged Cit might affect the degradation of the peptides in BLC lysates. Fig. 8A shows a major effect of Arg substitution by Cit on the degradation of the MOG peptides, although the effect differed remarkably between the Cj-MOG35–51 and Mm-MOG35–51 peptides. Replacement of the Arg41 or Arg46 residue or both for Cit did not alter the sensitivity of the Mm-MOG35–51 peptide to proteolytic degradation in B cell lysates. Both assays show a clear role for CatG in the degradation of the peptide.

FIGURE 5. Alignment of recombinant MOG sequences. Recombinant MOG sequences encoding from 1 to 125 were aligned for the following species: human, rhesus monkey, marmoset, mouse, rat, and naked mole rat. The pathogenically relevant T cell epitope in the rhMOG sequence MOG24–36 (MHC class II/Caja-DRB1*W1201–binding Th1 epitope) is single underlined, whereas the one in MOG40–48 (MHC class I/Caja-E–binding NK-CTL epitope) is double underlined. The two epitopes are located in close proximity within the highly conserved sequence 21–58. The only difference between the sequence in mouse/marmoset/rat versus rhesus monkey/human MOG is a substitution of proline (P) by a serine (S) at position 42 (indicated in green). The Arg (R) residues, which can be citrullinated by posttranslational modification, are indicated in red. The pound sign (#) marks the only N-glycosylation site at the asparagine (N) at position 31.

FIGURE 6. Degradation of bead-coated Mm-MOG35–51 and Cj-MOG35–51. Peptides that were labeled at the N terminus with biotin and at the C-terminal end with FITC were coated on magnetic beads via streptavidin. The peptide-coupled beads were incubated for 24 h at 37˚C in lysates of CD20+ (upper panel) and CD20− (lower panel) SC fractions from rhesus monkeys (A) or Mm-BLCs (upper panel) and Cj-BLCs (lower panel) (B). The indicated inhibitors were tested at the optimal concentration. Depicted is the release of FITC in arbitrary units (AU) from the peptide into the supernatant, as a measure of proteolytic activity.
degradation, whereas the Arg46Cit substitution reduced the sensitivity of the Cj-MOG35–51 peptide to degradation. It can also be seen that the CatG inhibitor CMK had less effect than the CatH inhibitor E64 on the proteolytic degradation of the Mm-MOG34–56 Cit41 or Mm-MOG34–56 Cit46 modifications in the total spleen or CD20+ SC lysate. The two inhibitors were equally effective in the CD20+ SC lysate. Similar effects were observed with the lysate of Cj-SCs (Fig. 8B).

We then tested the degradation of MOG35–51 peptides in lysates of LCV-infected B cells from rhesus monkeys (Mm-BLCs) or marmosets (Cj-BLCs), with or without inhibition of CatG (CMK) or CatH (E64). The results in Fig. 8C show that substitution of the Arg46 residue for Cit made the Cj-MOG35–51 peptide resistant to degradation, whereas the Arg41 for Cit substitution had no detectable effect. The Arg46 for Cit substitution had no effect on the proteolytic degradation of the Mm-MOG35–51 peptide.

In conclusion, the combination of the Arg46 to Cit substitution with the presence of a Ser42 residue made the complete MOG35–51 peptide resistant against proteolytic degradation in all tested lysates.

Autophagy induction in LCV-infected B cells

Citrullination of antigenic peptides is a physiological process that can take place in autophagosomes of APCs (43). If citrullination of the encephalitogenic MOG34–56 peptide played any role in the conversion of destructive processing to productive processing of the MOG40–48 epitope in LCV-infected B cells, we would expect to observe induction of autophagy and concomitant induction of PAD enzymes by the virus.

In LCV-infected and uninfected cells we analyzed the expression of two PAD genes that are involved in the citrullination of Ag: PAD2 and PAD4. Fig. 9A and 9B show that PAD2 and PAD4, respectively, are highly expressed in Cj-BLCs compared with SCs and fractionated SCs. Interestingly, the PAD2 and PAD4 transcript levels were lower in Mm-BLCs than in Cj-BLCs.

Regarding the relevance of autophagy for cross-presentation by DCs (20), the capacity of the autoantigen to associate with autophagosomes may be a rate-limiting step. The microtubule-associated LC3, and, in particular, the phosphatidylethanolamine-linked isoform LC3-II, is an accepted phagophore/autophagosome marker. LC3-II expressed on the inner membrane of the autophagosomes serves as a docking molecule for cargo binding. We noticed that the Arg46 residue is located in a sequence that is a putative LIR motif: [W/Y/F]xx[L/I/V], where x can be any amino acid (21). The motif expressed in the Cj-MOG40–48 epitope is YRSFSRV (21).

We reasoned that if autophagy is involved in autoantigen cross-presentation by LCV-infected B cells, infection with the virus should induce the expression of autophagosomes. This was investigated...
FIGURE 8. Citrullination prevents degradation of MOG peptides. MOG35–51 peptides derived from the marmoset and rhesus monkey sequence were synthesized; Arg at positions 41 and/or 46 were replaced by Cit. The peptides were incubated for 24 h at a fixed concentration (10 μg/ml) with total SC lysate, CD20+ and CD20− spleen fractions from rhesus monkeys (A), marmoset total SCs (B), or Mm-BLC lysates and Cj-BLC lysates (C). All lysates were incubated in the presence or absence of the inhibitors CMK and E64. The graphs represent the percentage degradation of the indicated peptide. Data shown are a representative of at least three replicates. SDS-PAGE gels are shown in Supplemental Fig. 1.

at the protein and cellular levels. On Western blot, the expression of LC3-I and LC3-II was determined. Fig. 9C and 9D reveal high expression of LC3-II (±14 kDa) in Cj-BLCs. The cell fractions derived from the spleen and Mm-BLCs showed only a marginal expression of LC3-II compared with Cj-BLCs. A similar pattern was observed for LC3-I, although in Mm-BLC, the expression of LC3-I is slightly
higher than LC3-II. The expression of LC3-I was slightly higher than LC3-II in Mm-BLCs. Protein expression of PAD2, PAD4, and LC3 was confirmed on Mm-BLCs and Cj-BLCs with immunofluorescence staining (Fig. 9E). This staining was also performed on total Mm-SCs, CD20⁺, CD20⁻, and total Cj-SCs. As expected, these cells were smaller than BLCs and it seemed that relatively fewer PAD2⁺, PAD4⁺, and LC3⁺ cells were observed compared with BLCs.

Discussion

One of the unresolved mysteries in MS is its association with EBV infection (44). Mechanistic studies need to help explain how a virus that infects >90% of the adult human population can be the trigger of a relatively uncommon disease, such as MS, on the appropriate genetic susceptibility background. The observation that EBV is also significantly associated with the risk for systemic lupus erythematosus might suggest that the association is based on generic, in addition to disease-specific, mechanisms. To find an explanation, we used our EAE models in marmosets and rhesus monkeys, nonhuman species that are naturally infected with EBV-related LCV (23).

Collectively, previous data obtained in the marmoset model and replicated in part in rhesus monkeys (reviewed in Refs. 12, 45) show that LCV-infected B cells have a central pathogenic role in the activation of highly pathogenic MHC-E-restricted effector memory cytotoxic T cells that, upon activation with the MOG34–56 peptide (core epitope 40–48), can elicit MS-like pathology and disease. Notably, similar autoaggressive CTLs were found in MS lesions, where they seemed to be engaged in cytotoxic interactions with HLA-E⁺ oligodendrocytes (5). The concept that LCV-infected B cells are essential APCs in the immunopathogenic process is supported by the potent clinical effects of depleting anti-CD20 B cells are infected with the EBV-related LCV CalHV3 (12). As a basis for our study, we assumed that, analogous to the situation in human SCs, LCV infection might endow B cells with cross-presentation capacity when at least two requirements are fulfilled: activation of the cross-presentation machinery and prolongation of the half-life of the CTL epitope.

Activation of the cross-presentation machinery includes immunoproteasome induction for processing of the Ag and induction of costimulatory molecules. The RNA-sequencing data presented in this article show that LCV infection of rhesus monkey B cells induced expression of genes encoding several immunoproteasome elements, supporting the idea that functional immunoproteasomes are formed in LCV-infected B cells. In this study, LCV-infected B cells of rhesus monkeys were well able to induce Ag-specific T cell responses in vivo (18). Hence, we expected to find relevant expression of costimulatory molecules. The RNA-sequencing data show marked mRNA transcript upregulation for CD70 and CD80, whereas mRNAs encoding CD86 and CD40 were reduced. It is of note that CD80 binds preferentially with CTLA4, whereas CD86 is the preferred ligand of CD28 (49). This implies that LCV-infected B cells suppress the activation of naive T cells; however, the constitutively expressed CD70 molecules on mouse DCs relay strong in vivo activation signals to T cells via homodimeric CD27 receptors (50). We reported previously that CD27 is expressed by autoaggressive MOG34–56-specific CTLs induced in the marmoset EAE model (9). Ligand binding to CD27 complements CD28 in determining the size of CD8⁺ effector memory T cell populations (51). With regard to soluble costimulation factors, we observed that expression of IL-23 mRNA, but not IL-12 mRNA, is highly increased; this is compatible with the observation that autoaggressive CTLs express IL-17A as a signature cytokine (11).

Prolongation of the half-life of the CTL epitope

An important condition for Ag cross-presentation is that fast proteolytic degradation of the Ag in the endolysosomal compartment is suppressed to enable translocation to the MHC class I loading pathway. Indeed, prolongation of the Ag’s half-life favors cross-presentation (52). The data presented in this article indicate that, as a consequence of LCV infection, destructive processing of the epitope in B cells is converted into productive processing. To our knowledge, the term “destructive processing” was first coined by Manoury et al. (22) as a mechanism used by thymic APCs to prevent activation of autoreactive T cells.

A key observation is that activation of T cells against the CD4⁺ T epitope 24–36 is clearly detectable in marmosets immunized with rhMOG/IFA, whereas activation of T cells against the CTL epitope 40–48 was undetectable (10). Nevertheless, the induction of robust encephalitogenic CTL activation in marmosets immunized with MOG34–56/IFA showed that CTLs against the 40–48 epitope are present in the repertoire and can be activated (11). Because a core-pathogenic role for B cells as APCs could be demonstrated in the latter model, we hypothesized that the immunizing MOG34–56 peptide may be rescued from proteolytic degradation when the B cells are infected with the EBV-related LCV CalHV3 (12).

The current study shows that rhMOG is rapidly degraded in cell-free lysates of rhesus monkey and marmoset SCs. Although the strongest proteolytic activity was located in the CD20⁺ fraction, which contains the myeloid APCs, considerable activity was also found in the CD20⁻ fraction, which contains the B cells. The activity in BLCs was even lower than in untransformed B cells. Using a peptide inhibitor of defined specificity (CMK), we found a dominant role for the serine protease CatG in the proteolytic degradation of rhMOG. Although low transcript levels of CatG mRNA in B cells were measured with qPCR, the enzyme-activity assays show high activity in B cell lysates. Burster et al. (39) reported a similar discrepancy for CatG in the human system. As an explanation, it was proposed that B cells take up exogenous CatG (e.g., produced in myeloid APCs) (53), although this is not a satisfactory explanation for the high CatG activity observed in the LC20-transformed B cell lines. Because CatG is transcribed as proform, the activity could be due to variations in activation and deactivation of the bioactive enzyme (54). We also observed inhibition of rhMOG degradation by the cysteine protease inhibitor E64. It is unclear whether this effect can be attributed to the inhibition of CatH, because E64 is a general cysteine protease inhibitor that also inhibits CatC, which is the activator of serine cathepsins, including CatG. The two inhibitors were strongly effective in lysates of rhesus monkey SCs but were only moderately effective in lysates of marmoset SCs. Both inhibitors were also fully effective in the CD20⁺ B cell fraction of rhesus monkey SCs. The observed species differences might be attributed, in part, to the specificity difference of CatG from marmosets and
rhesus monkeys. Although CatG of marmosets has a mouse-like narrow specificity for aromatic (chymotryptic) cleavage sites (Trp, Phe, Tyr), a double mutation in the specificity-determining residues (Ala-189-Ser and Glu-226-Ala) broadens the activity of rhesus monkey and human CatG to basic (tryptic) cleavage sites (e.g., Arg and Lys) (42).

FIGURE 9. Expression of PAD and LC3 in LCV-infected B cells. Total RNA was extracted from different cell lysates to determine expression of mRNA encoding PAD2 (A) and PAD4 (B) by qPCR. Transcript levels were normalized against the reference gene ABL. Median of each cell population is shown. (C) Cell lysates were immune-blotted for LC3 and GAPDH as loading control. As positive controls, Abs against LC3-I and LC3-II were run along with the other samples. (D) The density of the LC3-I and LC3-II band in (C) is related to the GAPDH band. (E) Immunofluorescence staining was performed with rhesus monkey cells (left panel) and marmoset cells (right panel) with anti-PAD2, anti-PAD4, and anti-LC3. Original magnification ×400. *p < 0.05, **p < 0.001, Cj-BLC/Mm-BLC versus other cell subsets, Mann–Whitney U test.
To test the proteolytic degradation of the pathogenically dominant Mm-MOG34–56 peptide we used the shorter peptide MOG35–51 to avoid interference of the Arg residue at position 52, which is outside the epitope of interest (residues 40–48). The reported in vitro data show that native Mm-MOG35–51 peptide is rapidly degraded in cell-free lysates of noninfected and LCV-infected B cells of rhesus monkeys as well as in LCV-infected B cells of marmosets. Again, a dominant role of the endolysosomal protease CatG in the degradation of Mm-MOG35–51 was observed. Interestingly, substitution of the Pro residue for Ser and the Arg46 residues for Cit conferred complete resistance of the MOG35–51 peptide to proteolytic degradation in the rhesus monkey cell lysates. Substitution of the Arg41 residue for Cit had no detectable effect. How can this be explained?

The alignment of MOG extracellular domain sequences (Fig. 5) shows that, in a wide range of mammalian species, a Pro residue is present at position 42, whereas a Ser is present at this position only in rat, mouse, and marmoset MOG. Data obtained from mice show that the presence of a Pro or Ser residue on position 42 has a strong influence on the encephalitogenic potency of the MOG34–56 peptide (55). The Arg to Cit substitution is a physiologically relevant modification of antigenic peptides, mediated by the enzyme PAD (43), which can occur in stressed B cells, such as those infected by LCV. The investigators claim that citrullination by PAD occurs in autophagosomes. It is well-documented that LCV infection of B cells induces autophagy (56). Consistent with this notion, our RNA-sequencing data showed increased expression of autophagy-related genes, such as beclin1, ATG5, and ATG12, in LCV-infected B cells of rhesus monkeys. Moreover, we show that the autophagosomes marker LC3-II is increased in LCV-infected B cells of rhesus monkeys.

Autophagosomes are implicated in cross-presentation (20). On the basis of the data presented in this article, we propose that they are also engaged in the protection of MOG peptide against degradative processing. We assumed that, for association of the MOG35–51 peptide with LC3 molecules in the inner concave surface of growing autophagosomes, the peptide should contain a LIR motif. The consensus sequence of LIR motifs is [W/Y/F][x][L/I/V], where x can be any amino acid (21). In addition to this core motif, the presence of an acidic residue (E, D, S, T) close to the N-terminal site of the LIR motif is important. Using this algorithm, we scanned the rMOG sequences of marmoset and rhesus monkeys and found three potential LIR motifs: residues 3–6 (FRV), 44–47 (FSRV), and 118–122 (FYVV). The 44FSRV47 LIR motif is encompassed within the pathogenically relevant MOG40–48 epitope. In the marmoset MOG sequence, it is preceded by Ser42, which contains the favorable Ser42 residue for Cit interaction. Because the CatG cleavage site Arg46 is inside the LIR motif, we speculate that substitution of the Arg46 residue for Cit prevents cleavage of the LIR motif by CatG.

In conclusion, based on the results of this study we propose a physiological role for B cells in the protection against autoimmunity in MS. They take up autoantigens and neutralize them via degradative processing; in this way, activation of autoimmune CD8+ T cells is avoided. By infection of B cells with LCV, this protective mechanism is converted into productive processing of the autoantigen, which enables the activation of autoimmune T cells. The proposed underlying mechanism is that, as a result of several cell-biological changes induced in the B cells, in particular the formation of autophagosomes with which the encephalitogenic Ag may associate, the CD8+ T cell epitope is protected against degradation and remains available for cross-presentation.

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Disclosures

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