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Distinct Protease Requirements for Antigen Presentation In Vitro and In Vivo

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Asparagine endopeptidase (AEP) or legumain is a potentially important Ag-processing enzyme that introduces limited cleavages that trigger unfolding and class II MHC binding of different Ag substrates. AEP is necessary and sufficient for optimal processing and presentation of the tetanus toxin C fragment (TTCF) Ag in vitro, but its importance has not been tested in vivo. Surprisingly, virtually normal T cell and Ab responses to TTCF were mounted in AEP-deficient mice when examined 10 d after immunization. This was the case when TTCF was emulsified with CFA, adsorbed onto alum, or expressed within live *Salmonella typhimurium*. In addition, the dominant Ab and T cell determinants recognized in TTCF were essentially unchanged in AEP-deficient mice. These data are explained, at least in part, by the much lower levels of AEP expressed in primary murine APCs compared with immortalized B cell lines. Even so, the initial in vivo kinetics of TTCF presentation were slower in AEP-deficient mice and, as expected, boosting AEP levels in primary APCs enhanced and accelerated TTCF processing and presentation in vitro. Thus, AEP remains the protease of choice for TTCF processing; however, in its absence, other enzymes can substitute to enable slower, but equally robust, adaptive immune responses. Moreover, clear relationships between Ags and processing proteases identified from short-term in vitro processing and presentation studies do not necessarily predict an absolute in vivo dependency on those processing enzymes, not least because they may be expressed at strikingly different levels in vitro versus in vivo. ***The Journal of Immunology*****, 2010, 184: 2423–2431.**

The proteolytic enzymes found in the endosomes and lysosomes of APCs perform two well-established roles in the class II MHC pathway: initiation of the removal of the invariant chain chaperone from class II MHC molecules and the generation of suitable peptides for class II MHC binding (1–3). However, the contribution played by individual enzymes, particularly in the context of Ag processing, is less clear. Several studies compared Ag presentation by wild-type and protease-deficient APCs in vitro. The loss of specific enzymes sometimes compromised Ag presentation, whereas in other cases, Ag presentation actually improved in protease-null APCs. For example, cathepsin S was shown to be required in vitro for the presentation of two H-2b–restricted epitopes in hen egg lysozyme (HEL) (4), and the loss of cathepsin L compromised the selection of CD4 T cells in the thymus, most likely because of an altered display of positively selecting peptides (5). In contrast, the elimination of cathepsin B did not affect the presentation of epitopes from OVA or HEL (6), and cathepsin L-deficient splenocytes presented a range of Ags normally (7). Positive and negative contributions have been described for other enzymes, including the aspartyl proteases cathepsins D and E and asparagine endopeptidase (AEP). Presentation of myoglobin (8), OVA (6), and the (161–175) epitope of the MAGE-3 tumor Ag (9) was more efficient in dendritic cells (DCs) or splenocytes treated with an aspartyl protease inhibitor or lacking cathepsin D, suggesting that destructive processing by this enzyme can occur. In contrast, an aspartyl protease inhibitor suppressed the presentation of OVA, an effect attributed to the blockade of cathepsin E (10).

AEP is an unusually specific cysteine protease homologous to the plant vacuole enzyme legumain (11). It cleaves after some, but not all, asparagine and, occasionally, aspartic acid residues. It emerged as the dominant Ag-processing enzyme when the tetanus toxin C fragment (TTCF; residues 865–1315 of tetanus toxin) Ag was exposed to lysosomal fractions purified from EBV-transformed human B cells (12, 13). Mutagenesis of the three principle AEP cleavage sites in TTCF or suppression of AEP activity inhibited TTCF presentation to T cells in vitro (12–15). AEP is also one of several enzymes able to initiate the processing of the invariant chain (18, 15). In contrast, presentation of the DR2-restricted 85–99 epitope of myelin basic protein was compromised by an AEP cleavage after Asn94 (16, 17). Thus, like cathepsin D, AEP may mediate destructive as well as productive processing events.

The relationship between AEP and the TTCF Ag is one of the clearest examples of an apparent nonredundant role for a specific protease in Ag processing and presentation in vitro. However, this dependency has not been tested in vivo. Studies on the tetanus toxin Ag yielded much basic information about the class II MHC pathway and how serological memory is maintained in humans (19–21). Tetanus toxin is also extensively used as a source of T cell epitopes in various vaccine systems (22). Therefore, understanding its processing requirements in vivo is highly desirable. More generally, evidence that individual enzymes make nonredundant contributions to Ag presentation might boost efforts to manipulate vaccine processing so that presentation in vivo is optimal. However, very few studies have compared the immune response to an exogenous Ag in wild-type and protease-deficient mice.

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Abbreviations used in this paper: AEP, asparagine endopeptidase; AMC, 7-amino-4-methyl coumarin; BMDC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; DC, dendritic cell; HEL, hen egg lysozyme; SDC, spleen-derived dendritic cell; TTCF, tetanus toxin C fragment.

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In this study, we immunized mice lacking AEP along with their wild-type littermates. We used the TTCF Ag, whose processing, as noted above, is dominated by AEP in vitro when lysosome fractions from B cell lines are used. Surprisingly, the absence of AEP made little difference to the final anti-tetanus immune response, although the initial T cell response was accelerated by the presence of AEP. The low levels of AEP found in primary murine APCs account for the low impact of its ablation and require that other proteases are able to contribute to TTCF processing in vivo. Our data also underscore the fact that the protease content of different APC types is highly variant when compared in vitro versus in vivo.

Materials and Methods

Mice

Mice were bred and maintained under specific-pathogen-free conditions. For generation of AEP knockouts, part of exon 3 and part of intron 2 of the murine AEP gene were deleted by homologous recombination in HM1 mouse embryonic stem cells with a targeting vector comprising a neomycine resistance cassette that is flanked by translation stop codons in all six reading frames (Supplemental Fig. 1*A*). G418-resistant HM1 cell clones were screened by Southern blot analysis of genomic DNA, which was digested with BamH I or BglII and hybridized with the external or internal probes, respectively (Suppl. Fig. 1*B*, 1*C*). Mutated embryonic stem cells were microinjected into blastocysts of C57BL/6N females. The resulting chimeras were used to generate heterozygous mutant offspring against the C57BL/6N genetic background. Mice were extensively backcrossed onto C57BL/6 (≥10 generations).

Animal experimentation was approved by the University of Dundee Animal Ethics Committee and was done under United Kingdom Home Office Project Licenses PPL60/3109 and PPL60/3851.

Immunizations

Littermate or age-matched AEP knockout and wild-type control mice were bred by heterozygote intercrossing and were used in immunization experiments at 6–15 wk of age. Mice were immunized s.c. with 50 μg rTTCF emulsified in 100 μl CFA. Ten days later, draining lymph node cells were collected and cocultured in triplicate with graded doses of TTCF Ag or TTCF peptides at 50,000–100,000 cells in flat-bottom 96-well plates. Proliferation was assessed 72 h later by [^{3}H]thymidine incorporation. Background TTCF-specific proliferation (from mice immunized with CFA alone) was consistently <10%. In some experiments, TTCF was adsorbed onto alum (Sigma-Aldrich, St. Louis, MO), and mice were immunized s.c. via the upper foot surface. A total of 10^4 CFU *Salmonella typhimurium* BRD745, which express TTCF (a kind gift from N. Fairweather) were administered i.v., and sera were collected 21 d later.

ELISAs

For quantitation of TTCF-specific serum Ab titers, 96-well flat-bottom plates (Nunc Immunosorb, Thermo Fisher Scientific, Rochester, NY) were coated overnight with 2 μg/ml TTCF in phosphate buffer (pH 9.2). After extensive washing, plates were blocked with 1% BSA prior to the addition of 10-fold dilutions of sera in triplicate. Abs were detected using peroxidase-conjugated goat-anti mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with tetramethylbenzidine substrate (BD Biosciences, San Jose, CA). To examine epitope preference, we used a panel of TTCF-specific mAbs generated from immunization of a C57BL/6 mouse that, therefore, approximated the wild-type B cell response. Sera were titrated onto TTCF-coated plates as before and, after washing, individual biotinylated mAbs were added at a constant concentration of 5 μg/ml. MAb binding was detected using streptavidin-HRP as before, and competition was expressed as the percentage of specific mAb binding that could be inhibited at each serum concentration.

Ag presentation

DCs were expanded in vitro from bone marrow or spleen cells cultured in the presence of 10 ng/ml GM-CSF without or with 1 ng/ml TGF-β, respectively, as described previously (23, 24). Kinetic assays were performed by pulsing DCs with 100 μg TTCF for different periods of time or by pulsing with graded doses of TTCF for 2 h. In either case, DCs were washed in cold medium at the end of each pulse, lightly fixed in 0.05% glutaraldehyde, and cocultured at 50,000–100,000 DCs per well with an equal number of T cell hybridoma cells in flat-bottom plates for 24 h. IL-2 was measured in cell-free supernatants by ELISA (BD OptEIA, BD Biosciences). For detection of the presentation of TTCF acquired in vivo by local DCs, draining lymph nodes were collected into ice-cold medium 8 h after s.c immunization with 50 μg TTCF/CFA and disrupted by passage through 70-μm mesh. CD11c$^+$ cells were purified by positive selection (MACS, Miltenyi Biotec, Auburn, CA) and divided between two groups: one was fixed immediately, and the other was left unfixed. Five thousand to 10,000 DCs from each group were cocultured, in duplicate, with 50,000 T cell hybridomas for 24 h before collection of supernatant. H-2^b–restricted T cell hybridomas used throughout were 2F2 (which recognizes TTCF 900–916), 1H3 (TTCF 950–966), 3A4 (TTCF 1145–1161), and 5A1 (TTCF 1225–1241). Residue numbers are those in the complete tetanus toxin sequence (25).

TTCF digestions

Lysosomes were enriched from the postnuclear supernatants of spleen DCs or B cell lines by Percoll density gradient fractionation, essentially as described previously (26). Lysosomal fractions were identified according to β-hexosaminidase activity. TTCF (4–10 μg) was incubated with 0.2–5 μg lysosomal protein in 0.2 M acetate buffer (pH 4.5) containing 5 mM DTT for 3–24 h at 37°C, and digestion products were separated on 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Leupeptin, E64d, CA-074, and Pepstatin A were used at 100 μM and were obtained from Sigma-Aldrich. PMSF (50 μM) and Cathepsin G Inhibitor 1 (20 μM) were both from Calbiochem (San Diego, CA). MVO26630 (20 μM) and PTL16643 (100 μM) were a generous gift from Medivir U.K.

Protease activity assays

Lysosomes (2 μg) or, where indicated, whole-cell lysates (10 μg) were incubated in 200 μl assay buffer (20 mM citric acid, 60 mM Na_2HPO_4, 1 mM EDTA, 0.1% CHAPS, and 1 mM DTT [pH 5.5]) containing 100 μM protease substrate at 37°C for 1 h. The protease substrate Z-Ala-Ala-Asn-7-amino-4-methyl coumarin (AMC) was used to assay for AEP activity, Z-Arg-Arg-AMC was used for cathepsin B, Z-Phe-Arg-AMC was used for cathepsin B/cathepsin L, and Z-Val-Val-Arg-AMC was used for cathepsin S (Bachem, Bubendorf, Switzerland). AMC, released by substrate cleavage, was quantified by measuring at 490 nm in a fluorescence plate reader (Fluostar Optima, BMG Labtech, Aylesbury, U.K.), and activities were calculated as the rate of change of fluorescence, in arbitrary units, per microgram of protein.

Immunoblotting

Cells were lysed in assay buffer as above. Postnuclear lysates were separated by SDS-PAGE and transferred to Hybond-P for immunoblotting. Rat anti-CatL and rabbit anti-CatS were purchased from Abcam (Cambridge, U.K.), goat anti-CatB was purchased from R&D Systems (Minneapolis, MN), and anti-CatH was a gift from Dr. T. Zavasnik-Bergant. AEP was detected with affinity-purified sheep antisera, as described previously (27). Peroxidase-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories.

Results

Quantitatively similar immune responses to TTCF in wild-type and AEP-null mice

AEP-null mice were described by Shirahama-Noda et al. (28). We used an independent line generated by the strategy outlined in Supplemental Fig. 1. As previously reported, mice lacking AEP were viable and outwardly healthy, although they did not gain weight at the same rate as wild-type littermates.

Mice backcrossed for ≥10 generations were immunized with 50 μg TTCF in CFA. After 10 d, T cell responses in the draining lymph nodes were measured in vitro using graded doses of the TTCF Ag or fixed concentrations of known immunodominant TTCF peptides. As shown in Fig. 1*A*, we observed an essentially identical recall response to TTCF in lymph nodes from wild-type and AEP-null mice. In some experiments, there seemed to be some shortfall in the recall response in some AEP-null mice, but this effect did not reach statistical significance. Similarly, we saw no difference in the serum titers of TTCF-specific Abs generated in AEP wild-type and null mice (Fig. 1*B*). To be certain that an AEP-dependent response was not due to the level of Ag used, we also immunized mice with 2 μg of TTCF, but again we saw equivalent T cell and Ab responses in wild-type and AEP-null animals (Supplemental Fig. 2).

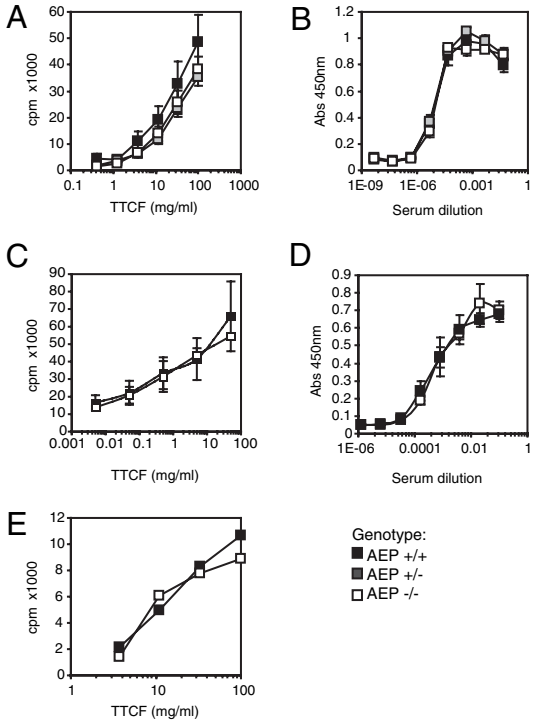

FIGURE 1. AEP-null, heterozygous, or wild-type mice were immunized with 50 μg TTCF emulsified in CFA (*A*, *B*), 2 μg TTCF adsorbed onto alum (*C*), or 10^4 CFU *S. typhimurium* BRD745 (*D*). Draining lymph node cells were collected after 10 d and cultured in the presence of graded doses of TTCF for an additional 3 d before assessment of proliferation by [^{3}H]thymidine incorporation (*A*, *C*). TTCF-specific IgG titers in the serum were measured by ELISA (*B*, *D*). Means ± SEM of at least four mice per group are shown. Data are from one of four experiments that gave similar results. *E*, CD4$^+$ T cells were purified from pooled lymph node cells and cultured with irradiated LB27.4 cells and graded doses of TTCF for 3 d. Proliferation was assessed by [^{3}H]thymidine incorporation.

We were concerned that the emulsification of TTCF in CFA might have partially denatured the protein, thus reducing the dependence on AEP for initial unlocking cleavages. To address this, we immunized mice with two additional forms of the TTCF Ag: TTCF adsorbed onto alum and TTCF expressed within live *S. typhimurium*. In both cases, we first established the minimum Ag dose needed to elicit an immune response in wild-type mice and then compared the response in AEP-null mice. We obtained efficient induction of T cell responses in draining lymph nodes in mice immunized with 2 μg of TTCF adsorbed to alum (Fig. 1*C*) and of serum Abs when mice were immunized with *S. typhimurium*/TTCF (Fig. 1*D*). However, AEP-null mice were able to respond with comparable efficiency to both forms of TTCF, even at this low Ag dose.

TTCF presentation in vitro is known to be diminished when AEP is inhibited in human EBV B cells (12, 15, 29). Because our lymph node recall assay depended upon processing and presentation of the TTCF Ag by endogenous APCs in vitro, we were concerned that the differential expression of AEP between wild-type and null mice might complicate interpretation of these data. To eliminate any confounding influence of altered TTCF processing in vitro and enable direct comparison of the in vivo T cell responses under conditions that were identical for wild-type and AEP-null mice, we purified CD4$^+$ T cells from draining lymph nodes and tested their capacity to proliferate when cocultured with wild-type APCs (LB27.4) pulsed with graded doses of the TTCF Ag. Under these conditions, proliferation of T cells from in vivo immunized wild-type and AEP-null mice was again comparable (Fig. 1*E*).

Qualitatively similar immune responses to TTCF in wild-type and AEP-null mice

We next considered the possibility that immunodominance of various T and B determinants within TTCF might be altered in the absence of AEP, without affecting the overall magnitude of the response. To establish whether the lack of AEP processing resulted in any new immunodominant T cell responses, we isolated lymph node T cells 10 d after immunization with TTCF/CFA and tested their reactivity with a nested set of 88 17mer peptides that spanned the entire TTCF sequence. As shown in Fig. 2*A*, T cell responses were remarkably similar, regardless of the AEP status of the mice, with the same four previously described immunodominant peptides (30) yielding the strongest responses in both groups. Similar data were obtained when mice were challenged with *S. typhimurium*-TTCF (data not shown).

AEP was first identified in human B cell lines; therefore, it seemed possible that processing of TTCF in B cells and, hence, Ab responses, might be dependent on AEP in vivo. Therefore, we tested the possibility that the loss of AEP-processing activity, although not affecting the aggregate Ab response, might nonetheless affect the Ab specificities obtained. We used the TTCF-expressing *S. typhimurium* as a source of Ag for these experiments. Twenty-one days after immunization, sera from AEP-sufficient and AEP-null mice showed essentially identical titers of anti-TTCF Abs, in agreement with the results obtained with CFA- and alum-adjuvanted TTCF (data not shown). To test the quality of the Ab response, we measured the capacity of the sera to compete for TTCF binding with various human and murine monoclonal anti-

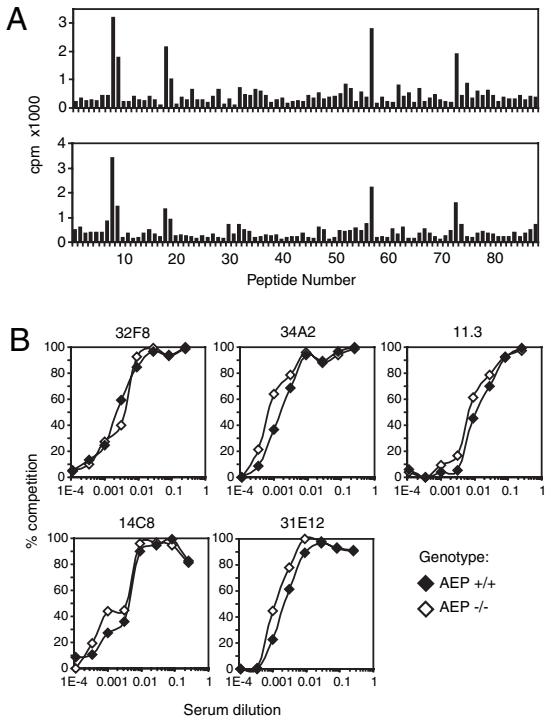

FIGURE 2. *A*, Lymph node cells from AEP wild-type (top) or null (bottom) mice were collected 10 d after immunization with 50 μg TTCF in CFA and stimulated with a panel of 88 overlapping 17mer peptides spanning the entire TTCF sequence. Proliferation was measured by [^{3}H]thymidine incorporation after 3 d. *B*, Sera were collected from mice 21 d after immunization with 10^4 CFU *S. typhimurium*-TTCF and tested for their ability to inhibit binding of various TTCF-specific mAbs by ELISA. Data are expressed as percentage inhibition.

TTCF Abs that recognized different epitopes within the TTCF Ag. Five anti-TTCF mAbs were equally well displaced by sera from TTCF-immunized wild-type and AEP-null mice, indicating that Ab specificities similar to those previously obtained had developed in wild-type and AEP-null mice (Fig. 2*B*).

We also compared T cell and Ab responses to three additional Ags. Similar to TTCF, responses to RNase A, OVA, and BSA were equivalent in wild-type and AEP-null mice (Supplemental Fig. 2). Thus, contrary to our expectations, neither the magnitude nor the quality of primary T and B cell responses to TTCF or other Ags tested were adversely affected by the absence of AEP in vivo.

Primary murine APCs express low levels of AEP compared with human B cell lines

The above results were surprising because, at least in vitro, the processing of the TTCF Ag by lysosomes from human EBV-transformed cell lines is dominated by AEP. Moreover, we and other investigators showed that presentation of TTCF epitopes was slowed or diminished when AEP or its action on the TTCF Ag was blocked (12, 14, 15, 29). We considered various reasons for this anomaly, including the possibility that AEP levels in primary murine APCs were lower than in transformed human B cell lines. Differences in protease content between different APC types have been reported, including the apparent absence of AEP from primary human B cells (31) and from ex vivo peripheral blood-derived DCs (32). We exposed TTCF to lysosomal fractions purified on density gradients from murine spleen-derived DCs (SDCs) or from immortalized human and murine B cells. We performed these digests at pH 4.5, which is optimal for AEP activity, although not necessarily for other enzymes. As shown in Fig. 3*A*, TTCF was digested into a set of distinct fragments when lysosomes from human EBV-B cells (PALA) and from the murine B cell line LB27.4 were used, as seen previously (12). As expected, lysosomes isolated from cultured SDCs from AEP-null mice failed to digest TTCF in this short-term assay. However, the same amount of lysosomes from wild-type DCs, although able to digest TTCF, did so much less efficiently than those from either of the B cell lines (Fig. 3*A*). When we measured AEP activity directly using the fluorogenic substrate Z-Ala-Ala-Asn-NHMec, we found that murine APCs contained much less activity (~7%) than either of the B cell lines tested (Fig. 3*B*). This shortfall in AEP activity was mirrored by a substantially lower amount of AEP protein present in DC lysosomes (Fig. 3*C*). Moreover, the relative lack of AEP was not due to a general deficiency in the proteolytic capacity of the DCs, because several other lysosomal proteases were present and active at considerably higher levels in DCs from AEP wild-type and null mice (Fig. 3*B*, 3*C*). The amount of AEP present, although low, was clearly sufficient for at least one of its known physiological functions (28), because the two chain forms of cathepsin H and cathepsin L were present in the wild-type, but not in AEP-null, lysosomes (Fig. 3*C*). We also prepared postnuclear lysates from isolated primary murine B cells and measured their TTCF-processing and AEP activity alongside equal amounts of lysate from bone marrow-derived macrophages (BMMs) and bone marrow-derived DCs (BMDCs). TTCF processing activity was very modest in the B cell lysates: less than that in the BMM and BMDC lysates and far less than that seen even with 20-fold less lysate from the LB27.4 murine B cell line (Supplemental Fig. 3*A*). Consistent with this, AEP levels in murine B cell lysates were lower than those seen in BMMs or BMDCs (Supplemental Fig. 3*B*), a result that mirrors the very low levels of AEP seen in primary human B cells (31). Thus, TTCF was remarkably resistant to digestion by lysosomes from murine APCs as a result of their low levels of AEP activity. Despite this, it was still a good immunogen in vivo.

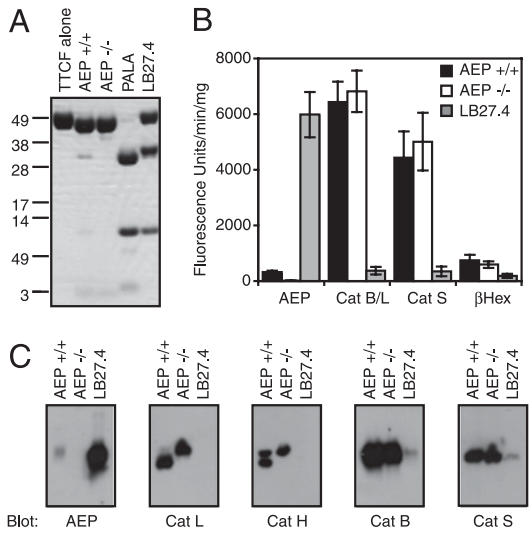

FIGURE 3. *A*, TTCF (5 μg) was incubated with lysosomal membrane fractions from $AEP^{+/+}$ or $AEP^{-/-}$ SDC or immortalized human (PALA) or murine (LB27.4) B cell lines at pH 4.5, 37°C for 4 h before separation by SDS-PAGE. *B*, The activities of various proteases in lysosomes from AEP wild-type or null SDC or LB27.4 mice were measured using fluorescent substrates. Means ± SEM from three (LB27.4) or four lysosome preps are shown. *C*, 10 μg lysosomes were separated and immunoblotted for the indicated proteases.

Wild-type and AEP-null mice respond equally well to an AEP-resistant mutant of TTCF

We reported earlier that a form of TTCF, in which three of the major AEP cleavage sites were eliminated (TTCF-TM), was processed and presented much less efficiently, at least in vitro (14). If in vivo responses are AEP independent, as the above results suggest, it would be predicted that TTCF and TTCF-TM should be equally immunogenic. We first confirmed that TTCF-TM was AEP resistant. As shown in Fig. 4*A*, digestions with postnuclear lysates from wild-type BMDCs produced a characteristic AEP cleavage pattern when TTCF was used as a substrate but not when TTCF-TM was used. We then immunized wild-type and AEP-null mice with TTCF or TTCF-TM and, as before, compared T cell and Ab responses. As shown in Fig. 4*B*, the recall T cell proliferative response to TTCF (Fig. 4*B*) or TTCF-TM (data not shown) was essentially identical, irrespective of the immunogen. Similarly, Ab responses to both forms of the Ag were identical (Fig. 4*C*). This result confirms that adaptive murine anti-TTCF responses are not compromised by the absence of AEP action on this Ag substrate.

Exogenous boosting of AEP activity restores AEP dependency of TTCF presentation

If the discrepancy between B cell lines and primary murine APCs, with regard to the dependency of TTCF processing on AEP, is due to the low levels of AEP expressed by murine APCs, then elevation of AEP levels in the latter cells should restore AEP-dependent TTCF processing. To test this idea, we boosted the levels of AEP in SDCs by feeding purified AEP precursor to the intact cells. AEP is initially made as an inactive 56-kDa proform that can be taken up by endocytosis and activated autocatalytically (27). Whole-cell lysates from wild-type and AEP-null DCs acquired approximately five times as much AEP following pro-AEP feeding (Fig. 5*A*) and showed much-improved TTCF processing ability (Fig. 5*B*). AEP-boosted APCs were given graded doses of TTCF for 2 h, fixed, and cocultured with TTCF-specific hybridomas 3A4 (Fig. 5*C*) and 5A1 (Fig. 5*D*). Wild-type SDCs induced slightly stronger T cell responses compared with AEP-null cells. However, following

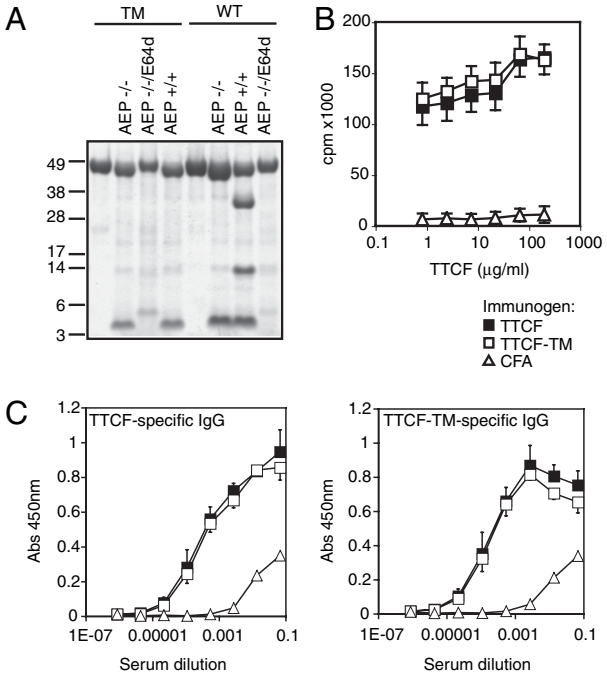

FIGURE 4. *A*, Five micrograms of TTCF or TTCF-TM was incubated with 0.5 μg postnuclear lysates from $AEP^{+/+}$ or $AEP^{-/-}$ BMDCs plus, where indicated, 100 μM E64d for 24 h before separation by SDS-PAGE. *B* and *C*, AEP wild-type mice were immunized with 2 μg TTCF or TTCF-TM emulsified in CFA. Draining lymph node cells were collected after 10 d and cultured in the presence of graded doses of TTCF for an additional 3 d before assessment of proliferation by [^{3}H]thymidine incorporation (*B*). TTCF- and TTCF-TM–specific IgG titers in the serum were measured by ELISA (*C*). Means ± SEM of at least four mice per group are shown.

AEP boosting, both types of DCs stimulated substantially better T cell responses, confirming the ability of this enzyme to efficiently release T cell epitopes from TTCF (Fig. 5*C*, 5*D*).

Other cysteine proteases can initiate TTCF processing

We obtained more efficient TTCF digestion by wild-type DCs when we increased the amount of lysosomes or extended the digestion time to 24 h. Under these conditions, we were able to confirm that the presence of AEP, even at comparatively low levels relative to B cell lines, still conferred a substantial advantage to DC lysosomes to digest TTCF (Fig. 6). The pattern of TTCF processing by wild-type APCs was similar, but not identical, to that produced by lysosomes from AEP-rich B cell lines, indicating the involvement of proteases other than AEP (Fig. 6*B*). Indeed, we could now detect some processing by AEP-null lysosomes, confirming that other proteases were capable of initiating TTCF cleavage in the absence of AEP. Consistent with this, AEP-null lysosomes and wild-type lysosomes treated with an AEP inhibitor gave an identical pattern of TTCF digestion (Fig. 6*A* and data not shown).

We determined the N-terminal sequences of the most abundant TTCF fragments produced by extended digestions with AEP wild-type or null lysosomes (Supplemental Fig. 4). As expected, most fragments from wild-type digests were produced by cleavage after Asn or Asp at or near sites previously identified as AEP targets (12, 33). At the low pH typically found in lysosomal compartments, AEP can efficiently cleave after Asp residues (27), and it was formally possible that in digestions by wild-type DC lysosomes, AEP might be responsible for a cleavage after Asp 872. However, this cleavage was also detected when AEP-null lysosomes were used, suggesting that other lysosomal proteases could cleave at this site. In addition, several novel digestion fragments were identified that were produced by cleavage after residues Gly 970 and Gly 1062. The sequence context of each of these is similar to the preferred cleavage sites for the cathepsins S, L, and H (35). Both of these residues are located in external loops of the TTCF protein, and either might be

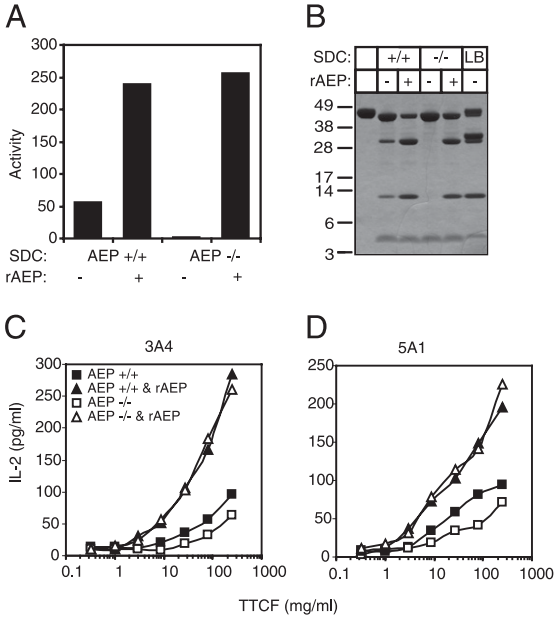

FIGURE 5. SDCs from AEP wild-type or null mice were incubated in the presence of 20 μg/ml of rAEP (56 kDa) or left untreated. *A*, After 2 h, postnuclear lysates were prepared, and AEP activity was measured using a fluorescent substrate. *B*, Five micrograms TTCF was digested for 4 h by 500 ng of each SDC lysate or, for comparison, LB27.4 lysate. *C* and *D*, Untreated and AEP-boosted SDCs were pulsed with titrated doses of TTCF for 3 h, washed, fixed, and cocultured for an additional 18 h with the T cell hybridomas indicated. IL-2 release was measured in the cell-free supernatant by ELISA.

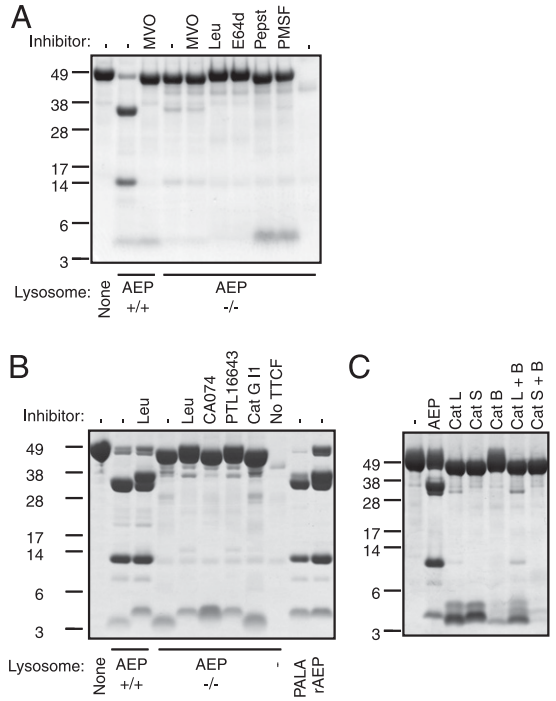

FIGURE 6. *A* and *B*, TTCF digests by lysosomes from $AEP^{+/+}$ or $AEP^{-/-}$ SDCs were performed in the presence of various protease inhibitors. All digestions were done at pH 4.5, 37°C for 24 h with 5–10 μg TTCF and 1 μg lysosomes. *C*, TTCF (10 μg) was incubated with 5 ng of AEP, Cat B, Cat L, Cat S, or combinations thereof for 4 h.

sufficient to enable partial unfolding during lysosomal processing in null APCs in lieu of AEP-mediated proteolysis.

We sought to determine which enzymes contributed to TTCF processing by AEP-deficient lysosomes by including various class-specific protease inhibitors in the digestion reactions. In contrast to the AEP inhibitor MVO, neither pepstatin nor PMSF blocked digestion of intact TTCF, although both inhibitors seemed to stabilize a 5-kDa processing product (Fig 6*A*). In contrast, leupeptin and E64d clearly arrested the digestion of intact TTCF (Fig. 6*A*, 6*B* and data not shown), demonstrating a role for one or more papain-like cathepsins in TTCF processing. Cathepsins S, L, H, B, and C, among others, are inhibited by leupeptin, although as exopeptidases, cathepsins C and H were unlikely to be the primary candidates. Use of the more selective inhibitors CA074 and PTL16643, which inhibit cathepsin B and cathepsin S, respectively, indicated a role for both enzymes in TTCF processing. For example, suppression of cathepsin S activity slowed the initial truncation of the TTCF Ag to the ~45-kDa species seen following digestion with wild-type or AEP-null lysosomes (Fig. 6*B*). We confirmed the ability of these cysteine proteases to contribute to TTCF processing by in vitro digestions with recombinant cathepsins B, L, or S or combinations thereof (Fig. 6*C*). All three enzymes were able to liberate a series of low m.w. fragments, and cathepsin L additionally generated two fragments of similar size to those produced by AEP alone.

Taken together, these digestions revealed significant differences between digestion of the same Ag substrate by immortalized B cell lines and cultured murine DCs. Murine DCs use AEP and leupeptin/E64-sensitive proteases, whereas immortalized lines use primarily AEP. The contribution of the leupeptin/E64-sensitive enzymes is evident in digestions of TTCF by wild-type and AEP-null DC lysosomes. Inclusion of leupeptin (or E64d) with wild-type DC lysosomes restored the digestion pattern to that seen when lysosomes from B cell lines or rAEP were used (Fig. 6*B*). Thus, the dependence of B cell lysosomes upon AEP for TTCF digestion is governed by two factors: the comparatively high expression of AEP in B cell lines relative to DCs and the comparatively low expression of papain-like proteases. Murine DCs, in which the relative amounts of these two protease types are reversed, are more flexible in their ability to degrade TTCF, and this is reflected in their reduced dependency upon AEP for processing and presentation in vitro and in vivo.

AEP increases the rate of TTCF processing and presentation by professional APCs in vivo

The above data showed that TTCF can still be processed in the absence of AEP, albeit more slowly. This suggested that the kinetics of presentation of TTCF might differ between AEP-sufficient and -null APCs. We tested this possibility in vitro and in vivo.

As shown in Fig. 7*A*, wild-type DCs and LB27.4 cells rapidly processed and presented TTCF in vitro, whereas AEP-null DCs displayed slower kinetics but still reached a similar maximum after 8 h. Wild-type DCs preincubated with the AEP inhibitor MVO26630 (29) performed similarly to AEP-null DCs. In contrast, TTCF presentation by LB27.4 cells was severely diminished by inclusion of the AEP inhibitor to a greater extent than even the AEP-null DCs. Thus, AEP is dispensable for TTCF presentation by murine DCs, but presentation is accelerated in its presence. In contrast, murine and human B cell lines, which express much more AEP, and rather less cathepsins B, L and S, are more dependent on the enzyme for optimum TTCF presentation.

Finally, we asked whether the kinetic advantage conferred by AEP in vitro was also detectable in vivo. We immunized AEP wild-type or null mice with TTCF and purified DCs from the draining

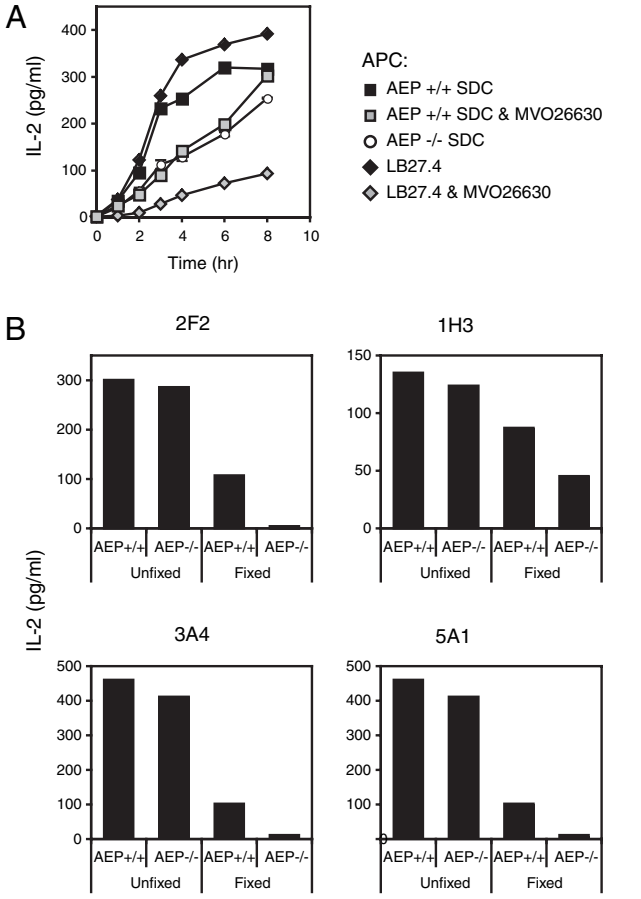

FIGURE 7. *A*, AEP$^{+/+}$ SDCs, AEP$^{-/-}$ SDCs, or LB27.4 cells were preincubated with 40 μM MVO26630 or DMSO control for 2 h prior to the addition of 100 μg/ml TTCF. Cells were fixed at various times and cocultured with the hybridoma 2F2 for 24 h. IL-2 was measured by ELISA of cell-free supernatants. One of three experiments that produced similar results is shown. *B*, CD11c^{+} cells were enriched from draining lymph node cells of AEP wild-type or null mice 8 h after immunization with 50 μg TTCF in CFA and fixed or left untreated. Ten thousand cells were cocultured with 50,000 of the T cell hybridoma for 24 h, after which IL-2 was measured in the cell-free supernatant. One of two experiments that produced similar results is shown.

lymph nodes of AEP wild-type or null mice 8 h later. Equal numbers of DCs, either fixed or unfixed, were cocultured with four T cell hybridomas. As shown in Fig. 7*B*, unfixed DCs from AEP wild-type or null mice were able to fully process TTCF Ag acquired in vivo and presented similar levels of four different TTCF epitopes. However, presentation of the same epitopes by the fixed wild-type DCs, which provides a "snapshot" of the extent of TTCF processing and MHC class II loading at the time of collection, was markedly improved compared with the AEP-null DCs (Fig. 7*B*). Thus, the presence of AEP, even at low levels, increases the rate of TTCF presentation by professional APCs in vivo, but its absence does not translate to a shortfall in adaptive immunity.

Discussion

Whether specific proteases are required for processing and presentation of specific T cell epitopes is still not clear. This is an important issue, because clear evidence that individual enzymes control the T cell response to protein Ags would spur efforts to engineer vaccines so that they are better tuned to the processing system they encounter. For example, appropriate processing sites

might be incorporated by mutagenesis to improve the presentation of protective epitopes; conversely, unfavorable sites could be eliminated. However, few studies have tested this idea in vivo, particularly with Ags that are known to be preferential substrates for a given enzyme. Most studies have focused on mice lacking cathepsin S. One study found impaired IgG responses to OVA and HEL in cathepsin S-null mice (35), but this was attributed primarily to defective invariant chain processing in the H-2b haplotype, rather than to any alteration in Ag processing. Analysis of a separate line of cathepsin S-deficient H-2b and H-2q mice noted blunted Ag presentation that was likely due to diminished Ag processing as well as invariant chain processing (36). Interestingly, perhaps the clearest evidence for a role for cathepsin S was established in the case of Ag processing for TAP-independent cross-presentation on class I MHC molecules (37). One recent study found that mice selectively lacking cathepsin D in their hematopoietic system had apparently normal responses to hapten-globulin and sheep RBC Ags (38).

Studies on the tetanus toxin Ag have contributed significantly to our understanding of various aspects of the adaptive immune response, including collaboration between T and B cells (39), Ag processing and presentation (19, 20), and immunogenicity and immunological memory in humans (21). In addition, tetanus toxoid is frequently used in conjugate vaccines as a source of T cell epitopes (22). Our earlier investigations into the processing of the C-fragment domain of tetanus toxoid identified an asparagine-specific endopeptidase (AEP) that dominated the degradation of TTCF during in vitro digestions with lysosomes from EBV-B cells. Subsequent studies by us and other investigators confirmed its importance as the rate-limiting enzyme of TTCF processing and presentation by immortalized APCs in vitro (12, 14, 15). Preventing the action of AEP, either by chemical inhibition or by mutagenesis of even a single AEP cleavage site, adversely affected TTCF presentation when EBV-transformed human B cells, human PBMCs, or murine B cell lines were used as APCs. This led to the idea that the introduction by AEP of a very limited number of cleavages mediated an essential unlocking step, triggering sufficient unfolding of TTCF to enable its binding to class II molecules and, hence, efficient presentation (40).

AEP-deficient mice were described previously and some aspects of their Ag-presenting function were investigated in vitro. Maehr et al. (41) reported no disturbance in the processing of the invariant chain in BMDCs or primary spleen cells from AEP-null mice, consistent with the finding that AEP was one of, but not the only enzyme able to initiate invariant chain processing (18). Processing and presentation of OVA and myelin oligodendrocyte glycoprotein by BMDCs in vitro was similarly unaffected (41). In contrast to the redundant initiation of invariant chain processing, a recent study reported that TLR9-signaled responses were dependent on AEP in vitro and in vivo, most likely as a result of TLR9 processing by AEP (42).

Our studies used a new line of AEP-deficient mice with an essentially similar phenotype to the previously published line. Because processing and presentation of TTCF is inhibited by ablation of AEP or its target sites in vitro, we anticipated that these mice would exhibit compromised immune responses to TTCF. Surprisingly, we found that immunity to TTCF is essentially unaffected in mice lacking AEP. Although we identified a delay in processing and presentation of several TTCF peptides in AEP-null mice, this was overcome over the longer timescale of the adaptive immune response and, ultimately, there was no measurable effect on the quality or magnitude of T or B cell immune responses.

This result was initially unexpected considering that lysosomal extracts from B cell lines seem to exclusively use AEP for efficient digestion of TTCF in vitro. However, we found that cultured murine DCs expressed far lower levels of AEP relative to B cell lines. In contrast, levels of leupeptin/E64d-sensitive enzymes were higher in murine DCs. In DCs, but not in B cell lines, the latter enzymes (most likely CatL and/or CatS) contributed, alongside AEP, to TTCF digestion. When we boosted the levels of AEP in murine DCs, a much clearer dependency on this enzyme was observed for rapid TTCF presentation in vitro. Even so, the low levels of AEP normally present in murine DCs still accelerated TTCF processing in vitro, because lysosomes from AEP-null DCs were extremely inefficient compared with wild-type DCs or either of the human and murine B cell lines tested.

It is not completely clear why these differences in in vitro processing did not affect in vivo immune responses. In vitro processing studies most likely do not precisely reproduce the microenvironments of endolysosomal-processing compartments, in which the effects of local concentrations of proteases or MHC-guided processing or unfolding of the native TTCF may compensate for the absence of AEP. In addition, the timescale of in vitro and in vivo Ag processing and presentation is normally very different. Indeed, when DCs were collected from draining lymph nodes and aldehyde fixed shortly after immunization (8 h), AEP-null DCs were consistently less stimulatory for T cells, a finding most likely explained by the significant shortfall in the TTCF-processing capacity of AEP-null lysosomes observed in vitro. Although slower presentation did not compromise the induction of T and B cell immune responses measured days later, it is possible that during a microbial infection, the kinetics of Ag presentation may be important for a favorable outcome. In the case of class I MHC-restricted responses, immunodominance is affected by presentation kinetics: when the presentation of a rapidly processed immunodominant epitope of a model Ag was delayed by a similar period of several hours only, the dominant response was directed instead to a second epitope whose presentation remained unaffected (43).

Although contraindicated by our earlier in vitro experiments, it was formally possible that elimination of AEP might actually enhance TTCF presentation, because several recent studies showed that increasing antigenic stability to proteases could increase immunogenicity (8, 44, 45). For example, mice immunized with different forms of the same Ag that were more or less resistant to lysosomal processing demonstrated substantially enhanced T and B cell immunity to the more stable form (45). This improvement was attributed to a delay in destructive processing, leading to increased availability of intact peptides for display by class II MHC and a consequent increase in presentation to T cells. Similarly, our own studies showed that although myoglobin was preferentially digested by the aspartyl proteases cathepsins D and E, presentation was actually enhanced in DCs lacking cathepsin D (8). Although the absence of AEP during lysosomal processing renders TTCF more resistant to degradation in vitro, we did not detect any enhancement in immunogenicity in mice lacking AEP. However, compared with other Ags, such as myoglobin and BSA, TTCF proved remarkably resistant to digestion by DC lysosomes, even when AEP was present (Supplemental Fig. 5 and data not shown). This property of TTCF may underlie its remarkable potency as an Ag. Therefore, it seems likely that despite the measurable effects of the absence of AEP upon TTCF processing in vitro, the stability of this Ag is sufficient that no additional immunological advantage is gained by delaying its processing further in vivo.

A recent study suggested that TTCF processing by PMA-activated human B cells is controlled by cathepsin E (46). Pepstatin A did not inhibit the initiation of TTCF processing by DC lysosomes, suggesting that the aspartyl proteases cathepsins E and D are unlikely to be major contributors to TTCF processing by

murine DCs. Nonetheless, this observation emphasizes the remarkable plasticity that exists among the proteolytic capacities of various APCs. Similarly, the considerable discrepancy between protease contents of immortalized B cells and DCs revealed in the current study is interesting. Other studies have likewise reported variations in proteolytic capacities of macrophages and DCs (47) between different DC subtypes (48, 49) and between cytokine-treated versus untreated cells (50, 51). Burster et al. (31, 32) showed that DCs and B cells isolated directly from human peripheral blood differ markedly in their protease content compared with their in vitro cultured counterparts. In particular, very little AEP was found in human B cells and DCs isolated directly ex vivo, which is in good agreement with our results for murine APCs. The existence of qualitative and quantitative differences between different APC populations, such as the specialized presenting cells of the thymus and the CNS, may result in altered processing kinetics and/or repertoires of MHC II-associated peptides in discrete immunological niches, with potential effects upon memory and autoimmunity.

In summary, our studies showed that although in vitro Ag processing studies can identify candidate processing enzymes, those enzymes may be present at very different levels in immortalized B cell lines compared with short-term DC cultures and APCs in vivo. Enzymes, such as AEP, that introduce primary unlocking cleavages, even when expressed at low levels, may nonetheless boost the kinetics of Ag presentation in vivo. In the future, studies that aim to identify relevant processing enzymes should use endosome/lysosome fractions isolated ideally from APC populations that are actually engaged in Ag presentation in vivo. Finally, our study shows that an Ag that is robust and protease resistant in vitro, can nonetheless be a good immunogen in vivo, a finding that is in good agreement with other recent results (8, 45).

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Disclosures

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