Bm-CPI-2, a cystatin homolog secreted by the filarial parasite *Brugia malayi*, inhibits class II MHC-restricted antigen processing

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While interference with the class I MHC pathway by pathogen-encoded gene products, especially those of viruses, has been well documented, few examples of specific interference with the MHC class II pathway have been reported. Potential targets for such interference are the proteases that remove the invariant chain chaperone and generate antigenic peptides. Indeed, recent studies indicate that immature dendritic cells express cystatin C to modulate cysteine protease activity and the expression of class II MHC molecules [1]. Here, we show that *Bm*-CPI-2, a recently discovered cystatin homolog produced by the filarial nematode parasite *Brugia malayi* (W. F. Gregory et al., submitted), inhibits multiple cysteine protease activities found in the endosomes/lysosomes of human B lymphocyte lines. CPI-2 blocked the hydrolysis of synthetic substrates favored by two different families of lysosomal cysteine proteases and blocked the in vitro processing of the tetanus toxin antigen by purified lysosome fractions. Moreover, CPI-2 substantially inhibited the presentation of selected T cell epitopes from tetanus toxin by living antigen-presenting cells. Our studies provide the first example of a product from a eukaryotic parasite that can directly interfere with antigen presentation, which, in turn, may suggest how filarial parasites might inactivate the host immune response to a helminth invader.

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Results and discussion

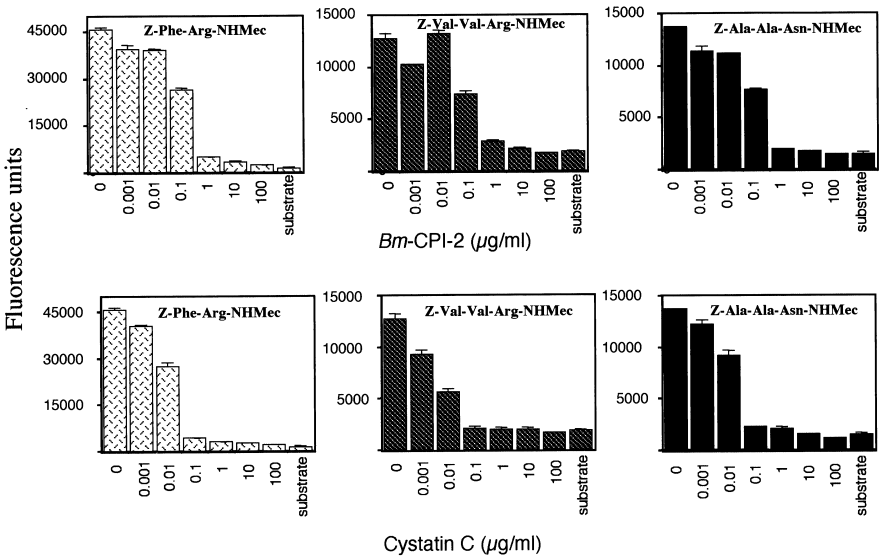
Two *B. malayi* cystatin-like genes that are homologous to type 2 cystatins were recently identified. Their sequence, homology to mammalian cystatins, and developmental expression pattern will be fully described elsewhere (W. F. Gregory et al., submitted). *Bm*-CPI-1 and *Bm*-CPI-2 (hereafter referred to as CPI-1 and CPI-2) both have the conserved Q-x-V-x-G and PW motifs that are found in all type 2 cystatins and are known to form part of the inhibitory site that is directed at the papain-like, or C1, family of cysteine proteases [2]. Interestingly, CPI-2 also has a conserved SND motif that was recently shown to constitute a distinct second inhibitory site that is specific for the C13 family of cysteine proteases, which includes asparaginyl endopeptidase (AEP), or legumain [3]. In addition, *Bm*-CPI-2 is expressed and secreted during parasitic stages that mature in the mammalian host and, therefore, has the potential to interfere with host cysteine protease activities. We therefore decided to focus on the CPI-2 protein.

We first tested the capacity of CPI-2 to block protease activities present in the endosomes and lysosomes of human antigen-presenting cells (APC). A preliminary analysis showed that recombinant CPI-2 inhibited the cysteine protease papain (W. F. Gregory et al.), raising the possibility that lysosomal cysteine proteases might be targets of these parasite gene products.

We utilized three different fluorogenic substrates that were preferentially cleaved by cathepsin S, cathepsins B/L, and asparaginyl endopeptidase, respectively (Figure 1), and tested the ability of cystatin C and recombinant CPI-2 to inhibit hydrolysis of these substrates over a wide concentration range. As expected, cystatin C was able to block cleavage of all three fluorogenic substrates in the nanogram/milliliter range (Figure 1). CPI-2 also inhibited cleavage of all three substrates, including the substrate specfically cleaved by AEP. Thus, CPI-2 is able to inhibit the representatives of two distinct families of cysteine proteases, the C1 papain-like family and the C13 AEP/legumain family.

We next tested the capacity of CPI-2 to block antigen processing in vitro. Recent studies demonstrate that the processing of the tetanus toxin C fragment (TTCF) antigen requires the activity of AEP to initiate processing [5, 6]. To test the effect of CPI-2 on TT processing, we set

Figure 1



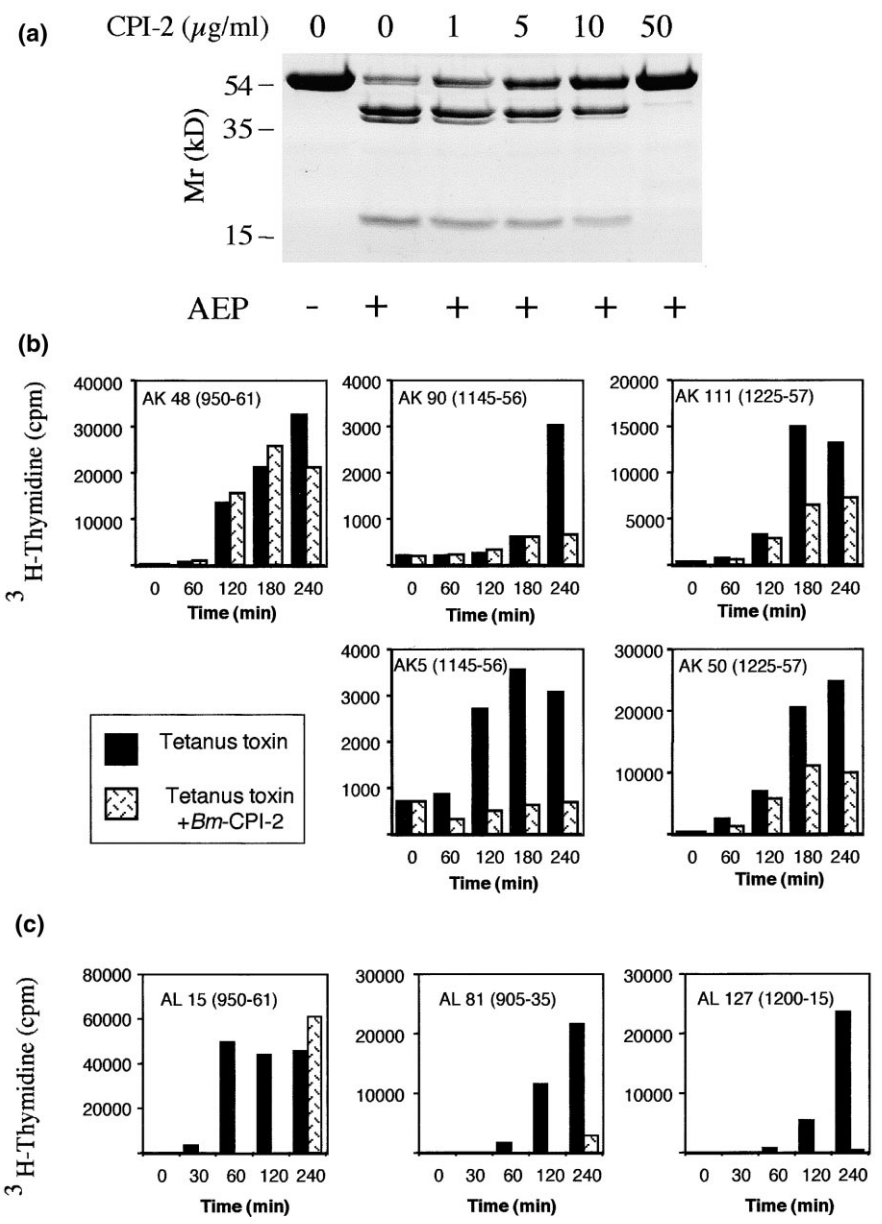
B. malayi CPI-2 inhibits multiple lysosomal cysteine proteases. The protease substrates (40 μM) shown, favored by cathepsin B/L (stippled bars), cathepsin S (gray hatched bars), and AEP (black bars), were incubated with 1 μg lysosomal protein in the presence of increasing concentrations of recombinant CPI-2 or human cystatin C (Calbiochem), essentially as described [5]. The buffer contained 50 mM citrate (pH 5.5), 1 mM EDTA, 5 mM DTT, and 0.1% CHAPS (AEP) or Brij 35 (Cathepsin S, B, and L). Release of 7-amido-4-methyl coumarin (NHmec) was measured after 30 min at 460 nm on a Cytofluor 4000 Fluorimeter (Perseptive Biosystems). CPI-2 was expressed as a hexa-histidine-tagged product in the pET-29T vector (Novagen) as described (W. F. Gregory et al., submitted, [4]). Enriched lysosomal fractions were prepared from EBV-transformed B cell lines as described [5]. More than five experiments were performed, and the result of one experiment is shown and expressed as the mean of duplicate points.

up TT digestions in vitro using either purified B cell lysosomes or purified pig kidney AEP as a source of proteases. As shown in Figure 2a, the inclusion of increasing amounts of CPI-2 clearly inhibited the processing of the TTCF antigen. Similar results were obtained when lysosomal extracts were used instead of purified AEP. Thus, CPI-2 is able to block cysteine protease action on both artificial substrates and on authentic protein substrates.

These results raised the possibility that CPI-2 might be able to interfere with TTCF processing in living cells. To test this, we examined the kinetics of tetanus toxin presentation, in the presence and absence of the CPI-2 protein, to specific T cell clones generated from two different donors. EBV-transformed B cell lines established from each donor were pulsed with TTCF antigen and fixed at different times prior to coculture with autologous T cell clones. To test the effect of CPI-2, the antigen presenting EBV-transformed B cells were preincubated with 200 μg/ml CPI-2 prior to antigen pulsing. Relatively high concentrations of the parasite cystatin were used because uptake of proteins by fluid phase pinocytosis in B cells is inefficient relative to other APC types. As shown in Figure 2b, the presentation to T cell clone AK 48 (specific for TT residues 950–961) was essentially unaffected by the presence of CPI-2. However, the presentation to clones AK 111 and AK 50 (both specific for 1235–1246) was clearly slower in the presence of CPI-2, while presentation to clones AK 5 and AK 90 (specific for 1145–1156) was barely detectable even after 4 hr of antigen pulsing (Figure 2b). To verify these results, we also tested a smaller panel of T cell clones from a second donor. As shown in Figure 2c, a similar pattern emerged in that presentation to some clones, for example, AL 81 and AL 127, were dramatically inhibited in the presence of CPI-2, while presentation to others, for example, AL 15, was barely affected. (Note that in this series of experiments, the CPI-2 inhibitor was only tested at the 4 hr time point). Thus, CPI-2 induced a dramatic, but selective, effect on the presentation of T cell epitopes in the tetanus toxin antigen. The presentation of four different epitopes recognized by T cells from two different donors was inhibited, whereas the presentation of epitope 950–961 to clones from both donors remained intact. This striking result rules out the possibility that CPI-2 exerts some nonspecific effect on APC or T cell function.

CPI-2 appears to inhibit a broad range of lysosomal cysteine proteases in lysosomal extracts from APC that are required for both antigen and invariant chain (Ii) processing. We directly tested the possibility that CPI-2 could affect Ii processing by including CPI-2 in both in vitro digestions of biosynthetically labeled class II/Ii complexes and during biosynthetic pulse/chase labeling experiments. We also tested leupeptin and cystatin C as controls. As shown in Figure 3a, the p31 isoform of Ii was efficiently digested by crude lysosomal extracts, and this could be significantly, though not completely, inhibited by the presence of leupeptin, cystatin C, or recombinant CPI-2. Because Iip31 was difficult to resolve from the class II MHC α chain, we quantitated the relative amount of the Iip22 processing intermediate. In the presence of leupeptin, cystatin C, or CPI-2, we observed a 2.3-, 2.6-, or 4.6-fold increase in Iip22 (Figure 3a), demonstrating

Figure 2

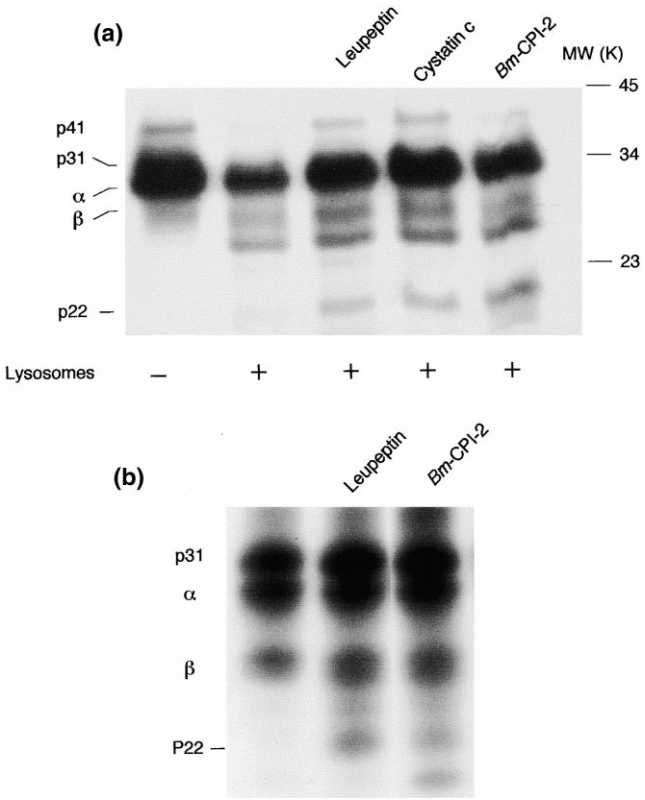


(a) CPI-2 inhibits the processing and presentation of the tetanus toxin antigen. A histidine-tagged domain of the tetanus toxin antigen (Mr 47 kDa) was prepared as described [22] and incubated for 1 hr with 5 μg/ml of purified pig kidney legumain/AEP [23] in the absence or presence of different concentrations of CPI-2 as indicated, using the same buffer as in the legend of Figure 1. Samples were electrophoresed on 15% Tris-Tricine gels and stained with Coomassie blue. **(b)** CPI-2 inhibits the presentation of multiple T cell epitopes by human B cell lines. EBV-transformed B cell lines from donor AK were used as antigen-presenting cells (APC) and incubated with 50 μg/ml TTCF in the presence (stippled bars) or absence (black bars) of 200 μg/ml CPI-2 for various times at 37°C. The cells were then harvested, washed, and fixed for 45 s in 0.05% glutaraldehyde prior to coculture with T cell clones as described [5]. After 48 hr, T cell proliferation was measured after pulsing with 1 μCi [^{3}H]thymidine (Amersham) and harvested 18 hr later onto nitrocellulose filters for scintillation counting. This experiment is representative of five other experiments that were performed. **(c)** Similar to (b), but using B and T cells from donor AL, where CPI-2 was tested only at the 4 hr time point. More details on the T cell clones that were used can be found in [5] and [11]. Note that the epitopes recognized by clones 50, 81, and 111 have not been precisely mapped, but the epitope for clone 81 lies between residues 905 and 937, and the epitope for clone 50 and 111 lie in the region 1225–1257.

that in vitro CPI-2 can inhibit proteases that degrade the Iip22 intermediate (as well as those that degrade the Iip31 substrate). In living cells, inclusion of leupeptin during the pulse/chase labeling of human class II MHC molecules induces the accumulation of a 22 kDa fragment (LIP) of the invariant chain [7, 8] (Figure 3b). As shown in Figure 3b, CPI-2 also induced the appearance of a similarly sized Ii fragment and, apparently, a smaller (~18 kDa) fragment. These data indicate that CPI-2 may inhibit both antigen and invariant chain processing.

Why does the presentation of some epitopes appear to be unaffected by CPI-2 uptake? One possibility is that CPI-2-sensitive and -insensitive T cell epitopes require processing in different compartments of the class II MHC system and utilize either invariant chain–associated or recycling populations of MHC molecules (reviewed in [9]). Consistent with this possibility, clones from the AL panel showed differential requirements for presentation on newly synthesized versus recycling class II MHC [10] and differential sensitivity to the *Helicobacter pylori* toxin Vac A [11]. Whereas presentation to clone AL 81 was relatively slow and sensitive to cycloheximide, clone AL 220 (which, like AL15, recognizes the epitope 950–961) was triggered rapidly and in a cycloheximide-insensitive manner [10]. Moreover, the latter clone was insensitive

Figure 3



CPI-2 partially blocks the processing of the Ii chain in vitro and in vivo. **(a)** EBV cells (Pala, 10^7 cells) were pulse labeled with 0.5 mCi ^{35}S-Translabel (Amersham, Pharmacia Biotech) for 20 min and lysed. Ii chain/ class II complexes were immunoprecipited with antibody VICγ1 directed against the cytoplasmic tail of the Ii chain, essentially as described [24]. The complexes were then digested in vitro with 1 μg enriched lysosomal fractions in the absence (lane 2) or presence of different inhibitors, leupeptin (10 μg/ml, lane 3), cystatin C (100 μg/ml, lane 4), and CPI-2 (100 μg/ml, lane 5). The samples were boiled for 5 min before analysis on a 14% Tris-Tricine gel. The amount of Ii p22 was quantitated by NIH image software. **(b)** The same APC were incubated for 20 min in the absence (lane 1) or presence of leupeptin (2 mM, lane 2) or CPI-2 (200 μg/ml, lane 3), pulse labeled with 0.5 mCi/ml ^{35}S-Translabel for 20 min, and then chased for 2 hr in the continuous presence of the inhibitors. MHC class II molecules were immunoprecipited with the class II β chain–specific antibody DA6 321 and analyzed on a 12% Tris-Tricine gel as described [24].

to the effects of Helicobacter pylori toxin Vac A, which interferes with TT processing in late compartments, whereas other clones, including AL 81, were sensitive [11]. Our data are therefore consistent with the hypothesis that CPI-2 inhibits presentation on newly synthesized, but not necessarily on recycling, class II MHC.

Two other parasite-encoded cystatins have been described [12, 13]. A cystatin homolog from the rodent filarial parasite *Acanthocheilonema viteae* was shown to directly downregulate T cell responses induced by mitogens, CD3 ligation, or specific antigen [13]. Since CPI-2 was not present during the coculture of antigen-pulsed APC with T cells in our experiments, a direct effect on T cell proliferation is unlikely to be the basis of the suppression we observed.

CPI-2 is one of the most abundant transcripts made by infective *B. malayi* larvae, constituting 0.6% of ESTs sequenced to date [14], and its production and secretion during the mammalian stages of the parasite life cycle point to a role in maintaining the parasite within the host enviroment. Adult filarial worms establish themselves in lymphatic vessels afferent to the lymph nodes in which the specific immune response would be initiated. Thus, CPI-2 could gain ready access to dendritic cells and other APC populations in lymphoid tissue, leading to the impairment or ablation of antiparasite immune responses. Although direct evidence for this hypothesis is not yet available, two observations from in vivo infections can be recalled. First, in dogs infected in the footpad with *Brugia pahangi* (a sibling species of *B. malayi*), the draining lymph node cells are far less responsive to parasite antigen than PBL (peripheral blood lymphocytes), indicating a local suppression of immune capacity [15]; and, second, in serological studies of infected humans, a relative loss of antibody reactivity to proteins >30 kDa has been observed, suggesting a diminution of antigen-processing capacity in the presence of heavy filarial infection [16]. However, it will be important to measure CPI-2 levels in infected lymphatic tissues.

Viral pathogens, encoding no more than 200 genes, display a remarkable array of immune evasion devices [17–19], aimed particularly at the MHC class I pathway. Filarial nematodes, with a gene complement of 15,000 or more [20], are likely to have evolved immune evasion strategies no less complex but orientated toward the class II pathway, as befits their extracellular niche. Although there are now examples of both viral [21] and bacterial [11] pathogens targeting the class II pathway, our studies provide the first example of a product from a eukaryotic parasite that may interfere with efficient generation and loading of class II MHC-restricted T cell epitopes, potentially hampering the host immune response to a helminth invader.

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References

1. Pierre P, Mellman I: **Development regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells.** *Cell* 1998, **93:**1135-1145.
2. Bode W, Engh R, Musil D, Thiele U, Huber R, Karshikov A, *et al.*: **The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases.** *EMBO J* 1988, **7:**2593-2599.

3. Alvarez-Fernandez M, Barrett AJ, Gerhartz B, Dando PM, Ni, J, Abrahamson M: **Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site**. *J Biol Chem* 1999, **272:**19195-19203.
4. Gregory WF, Blaxter ML, Maizels RM: **Differentially expressed, abundant *trans*-spliced cDNAs from larval *Brugia malayi.*** *Mol Biochem Parasitol* 1997, **87:**85-95.
5. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C: **An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation.** *Nature* 1998, **396:**695-699.
6. Antoniou AN, Blackwood SL, Mazzeo D, Watts C: **Control of antigen presentation by a single protease cleavage site.** *Immunity* 2000, **12:**391-398.
7. Blum JS, Cresswell P: **Role for intracellular proteases in the processing and transport of class II HLA antigens.** *Proc Natl Acad Sci USA* 1988, **85:**3975-3979.
8. Neefjes JJ, Ploegh HL: **Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS-resistant $\alpha\beta$ heterodimers in endosomes.** *EMBO J* 1992, **11:**411-416.
9. Watts C: **Capture and processing of exogenous antigens for presentation on MHC molecules.** *Annu Rev Immunol* 1997, **15:**821-850.
10. Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A: **Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells.** *Nature* 1997, **388:**782-787.
11. Molinari M, Salio M, Galli C, Norais N, Rappuoli R, Lanzavecchia A, *et al.*: **Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA**. *J Exp Med* 1998, **187:**135-140.
12. Lustigman S, Brotman B, Huima T, Prince AM, McKerrow JH: **Molecular cloning and characterization of onchocystatin, a cysteine proteinase inhibitor of *Onchocerca volvulus.*** *J Biol Chem* 1992, **267:**17339-17346.
13. Hartmann S, Kyewski B, Sonnenburg B, Lucius R: **A filarial cysteine protease inhibitor down-regulates T cell proliferation and enhances interleukin-10 production.** *Eur J Immunol* 1997, **27:**2253-2260.
14. Allen JE, Daub J, Guilliano D, McDonnell A, Lizotte-Waniewski M, Taylor D, *et al.*: **Analysis of genes expressed at the infective larval stage validate the utility of *Litomosoides sigmodontis* as a murine model for filarial vaccine development.** *Infect Immun* 2000, **68:**5454-5458.
15. Miller S, Schreuer D, Hammerberg B: **Inhibition of antigen-driven proliferative responses and enhancement of antibody production during infection with *Brugia pahangi.*** *J Immunol* 1991, **147:**1007-1013.
16. Kurniawan A, Sartono E, Partono F, Yazdanbakhsh M, Maizels RM**: Specificity of predominant IgG4 antibodies to adult and microfilarial stages of *Brugia malayi.*** *Parasite Immunol* 1998, **20:**155-162.
17. Ploegh HL: **Viral strategies of immune evasion**. *Science* 1998, **280:**248-253.
18. Alcami A, Koszinowski UH: **Viral mechanisms of immune evasion.** *Immunol Today* 2000, **21:**447-455.
19. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL: **Viral subversion of the immune system**. *Annu Rev Immunol* 2000, **18:**861-926.
20. The Filarial Genome Project: **Deep within the filarial genome: an update on progress in the Filarial Genome Project.** *Parasitol Today* 1999, **15:**219-224.
21. Miller DM, Rahill BM, Boss JM, Lairmore MD, Durbin JE, Waldman JW, *et al.*: **Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway.** *J Exp Med* 1998, **187:**675-683.
22. Hewitt EW, Truemann A, Morrice N, Tatnell PJ, Kay J, Watts C: **Natural processing sites for human cathepsin E and cathepsin D in tetanus toxin. Implications for T cell epitope generation.** *J Immunol* 1997, **159:**4693-4699.
23. Chen JM, Dando PM, Rawlings ND, Brown MA, Young NE, Stevens RA, *et al.*: **Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase**. *J Biol Chem* 1997, **272:**8090-8098.
24. Pond L, Watts C: **Functional early endosomes are required for maturation of major histocompatibility complex class II molecules in human B lymphoblastoid cells.** *J Biol Chem* 1999, **274:**18049-18054.