Inhibition of Invariant Chain Processing, Antigen-Induced Proliferative Responses, and the Development of Collagen-Induced Arthritis and Experimental Autoimmune Encephalomyelitis by a Small Molecule Cysteine Protease Inhibitor


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Inhibition of Invariant Chain Processing, Antigen-Induced Proliferative Responses, and the Development of Collagen-Induced Arthritis and Experimental Autoimmune Encephalomyelitis by a Small Molecule Cysteine Protease Inhibitor


Members of the papain family of cysteine proteases (cathepsins) mediate late stage processing of MHC class II-bound invariant chain (II), enabling dissociation of Ii, and binding of antigenic peptide to class II molecules. Recognition of cell surface class II/Ag complexes by CD4+ T cells then leads to T cell activation. Herein, we demonstrate that a pan-active cathepsin inhibitor, SB-331750, attenuated the processing of whole cell Ii p10 to CLIP by Raji cells, and DBA/1, SJL/J, and C57BL/6 splenocytes. In Raji cells and C57BL/6 splenocytes, SB-331750 inhibited class II-associated Ii processing and reduced surface class II/CLIP expression, whereas in SB-331750-treated DBA/1 and SJL/J splenocytes, class II-associated Ii processing intermediates were undetectable. Incubation of lymph node cells/splenocytes from collagen-primed DBA/1 mice and myelin basic protein-primed SJL/J mice with Ag in the presence of SB-331750 resulted in concentration-dependent inhibition of Ag-induced proliferation. In vivo administration of SB-331750 to DBA/1, SJL/J, and C57BL/6 mice inhibited splenocyte processing of whole cell Ii p10 to CLIP. Prophylactic administration of SB-331750 to collagen-immunized/boosted DBA/1 mice delayed the onset and reduced the severity of collagen-induced arthritis (CIA), and reduced paw tissue levels of IL-1β and TNF-α. Similarly, treatment of myelin basic protein-primed SJL/J lymph node cells with SB-331750 delayed the onset and reduced the severity of adoptively transferred experimental autoimmune encephalomyelitis (EAE). Therapeutic administration of SB-331750 reduced the severity of mild/moderate CIA and EAE. These results indicate that pharmacological inhibition of cathepsins attenuates CIA and EAE, potentially via inhibition of Ii processing, and subsequent Ag-induced T cell activation. The Journal of Immunology, 2008, 180: 7989–8003.

One of the central occurrences in the generation of a cell-mediated immune response is the recognition of antigenic peptide by CD4+ T cells (1). When the antigenic peptide recognized by the CD4+ T cell is derived from an invading (foreign) organism, such as a bacterium, the ensuing immune response results in elimination of the deleterious organism and, thus, is beneficial to the host. In contrast, when the antigenic peptide is derived from host (autologous) tissue, the subsequent immune response results in destruction of self-tissue or autoimmunity. Examples of autoimmune responses include the joint inflammation and bone/cartilage destruction that occur in rheumatoid arthritis (RA),6 the demyelination of the CNS that is observed in multiple sclerosis, the intestinal lesions that occur in inflammatory bowel disease, and the pancreatic β cell destruction that results in type 1 diabetes.

In order for a peripheral CD4+ T cell to recognize an antigenic peptide, that peptide must be bound to an MHC class II molecule expressed on the cell surface of professional APC (macrophages, dendritic cells, and B cells). The binding of antigenic peptide to the Ag binding groove of an MHC class II molecule occurs intracellularly, within the endosomes of APC. Before the intracellular binding of antigenic peptide to the class II molecule, the class II molecule is bound by a protein called the invariant chain (Ii). This binding occurs in the endoplasmic reticulum, after which the Ii/class II complex is

6 Abbreviations used in this paper: RA, rheumatoid arthritis; cTEC, cortical thymic epithelial cells; CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; DPBS, Dulbecco’s PBS; MBP, myelin basic protein; LNC, lymph node cells; AUC, area under the curve.
transported through the golgi apparatus to the endosome. In the endosome, class II-bound Ig undergoes sequential proteolytic cleavages at the C terminus, generating II p22 (LIP, 22 kDa) and II p10 (SLIP, 10 kDa). The final N-terminal proteolytic cleavage of II generates CLIP (3 kDa), which consists of II residues 81–104. CLIP, which binds to the Ag binding groove of the class II molecule, is removed by a class II-like molecule, DM (HLA-DM/H-2M), enabling antigenic peptide to bind (2–4). If the final proteolytic cleavage of II does not occur, II p10 rather than CLIP remains bound to the class II molecule. Although HLA-DM/H-2M can interact with class II-II p10 complexes, its preferred substrate is class II-CLIP (5). Thus, in many cases, inhibition of CLIP generation prevents antigenic peptide from binding to the class II molecule and being presented to CD4+ T cells.

Studies using targeted gene deletion and/or cytochrome protease inhibitors have demonstrated a critical role for cathepsin S in the final proteolytic cleavage of II in murine B cells and dendritic cells, while cathepsins S, L, and F may collectively fulfill this function in murine macrophages (6–9). In the mouse, cortical thymic epithelial cells (cTEC), nonprofessional APC that mediate positive selection of CD4+ T cells, lack cathepsin S expression and use cathepsin L to mediate the final stage of II degradation (10), while in human cTEC, this process has been attributed to cathepsin V (11) and/or cathepsin S (12). In contrast to murine cTEC, intestinal epithelial cells in the mouse have been shown to use cathepsin S for late stage II processing (13). Thus, the requirement for a specific lysosomal cytochrome protease in order for the final stage of II processing and subsequent Ag presentation to occur depends, at least in part, on the APC type. In addition, the antigenic protein and MHC class II allele involved in the interaction influence the degree to which Ag presentation is dependent on cytochrome protease-mediated late stage II degradation (7). This suggests the potential for inhibitors of cytochrome proteases, such as cathepsins S, L, F, and V, to prevent the presentation of self peptides to autoreactive CD4+ T cells and, thus, inhibit autoimmunity, without resulting in general unresponsiveness of CD4+ T cells to foreign Ags.

The importance of the interaction between self peptide/MHC class II complexes and CD4+ T cells in the generation of a number of autoimmune responses is underscored by the requirement for CD4+ T cells to generate and/or transfer disease, and the association between increased disease susceptibility and the expression of particular MHC class II alleles. Collagen-induced arthritis (CIA) is a disease characterized by chronic inflammation of the joint, leading to progressive destruction of cartilage and bone, and, thus, is a model that resembles many aspects of RA (14). Like RA, the development of CIA in rodents is dependent on the presence of CD4+ T cells (15, 16) and the expression of specific MHC class II alleles (17, 18). In a second model of autoimmunity, experimental autoimmune encephalomyelitis (EAE), inflammation of the CNS results in demyelination, leading to paralysis. Thus, EAE is a relevant model of multiple sclerosis (19, 20). CD4+ T cells play a pivotal role in EAE development, as evidenced by the findings that CD4-specific Abs prevent EAE development, or reverse established disease, and that CD4+ T cells transfer disease to naive recipients (21). Like CIA, the development of EAE is linked to the expression of particular MHC class II alleles (22, 23).

Based on the pivotal role of cytochrome proteases, such as cathepsins S, L, F, and V, in the final stage of Ii degradation and subsequent presentation of antigenic peptide to CD4+ T cells, we initiated studies to determine the effect of a pan-active cathepsin inhibitor, SB-331750, in two models of Ag-dependent, CD4+ T cell-mediated autoimmunity: murine CIA and EAE.

**Materials and Methods**

**Inhibitors**

5-(2-Morpholin-4-ylethoxy)benzoofuran-2-carboxylic Acid ((S)-3-Methyl-1-[(S)-3-oxo-[1-[(3-pyridin-2-ylphenyl)-acetyl]azepan-4-yl-carbamoyl]buto]amide (SB-331750) and N-[5-(2-phenylthio)-3-phenylsulfonylala- lyl]-4-methyl-2R-piperazinyl carbobinolavonolame (APC3328) were synthesized at GlaxoSmithKline by the Department of Medicinal Chemistry, Infectious, Musculoskeletal, and Proliferative Diseases Center of Excellence for Drug Discovery, as previously described (24–26). Leupentin was purchased from Sigma-Aldrich.

**In vitro enzyme assays**

Cathepsin activity assays were performed in microtiter plates using fluorogenic substrates at concentrations equal to their Km in assay buffers optimized for each enzyme. The human and mouse cathepsin S assays consisted of 1.25 nM human and mouse cathepsin S, respectively, 30 μM Z-Phe-Arg-AMC in 50 mM MES, 10 mM t-cysteine, 5 mM EDTA, and 0.75 nM CHAPS (pH 6.5). The human cathepsin K assay consisted of 0.1 μM human cathepsin K, 30 μM Z-Lys-Gln-Lys-Leu-Arg-AMC in 100 mM sodium chloride, 20 mM t-cysteine, and 1 mM CHAPS (pH 5.5). The human cathepsin C assay consisted of 0.010 nM human cathepsin C, 5 μM (H-Gly-Arg)2-R110 in 50 mM sodium acetate, 30 mM NaCl, 1 mM DTT, 1 mM EDTA, and 1.25 nM CHAPS (pH 5.5). The human cathepsin K assay consisted of 0.1 nM human cathepsin K, 30 μM Z-Lys-Gln-Lys-Leu-Arg-AMC in 100 mM sodium chloride, 20 mM t-cysteine, and 1 mM CHAPS (pH 5.5). The human cathepsin C assay consisted of 0.010 nM human cathepsin C, 5 μM (H-Gly-Arg)2-R110 in 50 mM sodium acetate, 30 mM NaCl, 1 mM DTT, 1 mM EDTA, and 1.25 nM CHAPS (pH 5.5). The mouse cathepsin L assay consisted of 100 pM mouse cathepsin L, 5 μM Z-Phe-Arg-AMC in 100 mM sodium acetate, 20 mM L-lysine, and 5 mM EDTA (pH 5.5). The mouse cathepsin K assay consisted of 1 nM mouse cathepsin K, 20 μM Z-F-R- AFC in 100 mM sodium chloride, 4 mM t-cysteine, 5 mM EDTA, and 1 mM CHAPS (pH 5.5). The mouse cathepsin C assay consisted of 0.015 nM mouse cathepsin C, 5 μM (H-Gly-Arg)2-R110 in 50 mM sodium carbonate, 30 mM NaCl, 1 mM DTT, 1 mM EDTA, and 1.25 mM CHAPS (pH 5.5). All reactions were initiated by the addition of enzyme-containing assay buffer to the substrate and inhibitor sample and were read after 1 h of incubation at 25°C. Hydrolysis products of the fluorogenic peptide substrates were measured using an Analyst AD microtiter plate reader (Molecular Devices) under the following conditions: for AMC substrates, excitation at 360 nm and fluorescence emission at 460 nm using a 400 nm dichroic filter; for AFC substrates, excitation at 405 nm and fluorescence emission at 530 nm using a 505 nm dichroic filter; and for R110 substrates, excitation at 485 nm and fluorescence emission at 535 nm using a 505 nm dichroic filter. Compound IC50 values were determined from dose-response curves, which were performed using a starting compound concentration of 25 μM that was serially diluted 3-fold over 11 repetitions using the following equation:

\[
y = \min(1 + 10^{(x/C_{50}^-)}^{-1})
\]

In the equation above, x is the log concentration of the test compound, y is the fluorescence response, \( C_{50}^- \) is the minimum response plateau, and \( C_{max}^- \) is the maximum response plateau. \( C_{max}^- \) was calculated by dividing the IC50 values by two, as the assays were run at substrate concentrations equal to Km, and the inhibition was assumed to be competitive.

**Mice**

DBA/1 mice were obtained from Harlan U.K., and SJL/J mice were obtained from Harlan France S.A.R.L. or from Taconic. C57BL/6 (B6) mice were obtained from The Jackson Laboratory. Cathepsin K−/− mice and K−/− littermate controls were bred at the GlaxoSmithKline animal facility. Generation of these mice has been previously described (27). All mice were maintained on a 12 h:12 h light:dark cycle and fed standard rodent chow and water ad libitum. All procedures performed on animals were reviewed and approved by GlaxoSmithKline Institutional Animal Care and Use Committee.

**Mouse splenocyte culture**

Mice were euthanized by CO2 asphyxiation and each spleen placed in 3 ml of sterile HBSS (Mediatech) containing 1 mM EGTA. Spleens were mechanically disrupted with forceps and pipetting before being passed through sterile 70-μm filters. Cells were centrifuged at 600 × g for 5 min, and RBC were lysed by resuspension of the pellet in 1 M-M-Lyse buffer (R&D Systems) at 2 ml/spleen for 10 min followed by addition of 1× wash buffer (R&D Systems) at 8 ml/spleen. Cells were centrifuged as above,
washed in 10 ml of medium (RPMI 1640 containing 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1 mM of sodium pyruvate, 90%, and 10% heat inactivated FBS (HyClone), and counted. Cells were cultured at 1 × 10^5 cells/ml of medium at 37°C in 5% CO_2 on a shaking platform for 4–5 h. Where indicated, cultures were treated with SB-331750, leupeptin, APC328, or vehicle (0.1% DMSO). Following incubation cells were examined for toxicity by trypan blue exclusion and then processed for immunoprecipitation/Western blot analysis or for flow cytometry.

Raji cell culture

The human B cell line, Raji, was purchased from American Type Culture Collection. Cells were cultured at 5 × 10^5 cells/ml of medium (RPMI 1640 containing 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1 mM sodium pyruvate, 90%, and 10% heat inactivated FBS) at 37°C in 5% CO_2 for 24 h. Where indicated, cultures were treated with SB-331750, leupeptin, or vehicle (0.1% DMSO), and then processed for immunoprecipitation/Western blot analysis or for flow cytometry.

Assessment of intracellular Ii processing intermediates

An aliquot of each spleenocyte culture described above was centrifuged at 600 × g for 5 min, the pellets washed with 10 ml of cold Dulbecco’s PBS (DPBS), and each pellet resuspended in ice cold lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, and 1 protease inhibitor minitab (Roche)) at 5–8.5 × 10^5 cells/ml and incubated on a tumbler for 30 min at 4°C. The lysates were then clarified at 12,000 g and the supernatants collected and stored at −80°C. A protein assay was performed on each sample using the Bio-Rad protein assay (Bio-Rad).

Alternatively, an aliquot of each Raji cell culture described above was centrifuged at 400 × g for 5 min, the pellets washed with 10 ml of cold DPBS, and each pellet resuspended in ice cold lysis buffer at 15–30 × 10^6 cells/ml. Suspensions were incubated on a tumbler for 30 min at 4°C, the lysates clarified at 12,000 × g, and the supernatants collected and stored at −80°C. A protein assay was performed on each sample using the Bio-Rad protein assay.

For assessment of Ii processing intermediates in whole cell lysates, an equal amount of protein from each sample (2 μg for C57BL/6, DBA/1, and SJL/J samples, and 2–20 μg for Raji samples) was diluted in an equal volume of loading buffer (62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 4% SDS, 0.01% bromphenol blue, and 5% 2-ME), and the samples placed in boiling water for 5 min. Samples were then loaded onto an 18 or 4–20% Tris-HCl mini gel (Bio-Rad). To assess class II-associated Ii processing, membranes were transferred to 96-well round-bottom plates and Abs added at the following amounts/10^5 cells: 0.5 μg of PE anti-mouse CD19 (1D3) or 0.5 μg of the isotype control PE rat IgG2a (R35–95), purchased from BD Pharmingen; 20 μl (4 μg) of FITC anti-I-IA^b/CLIP (15G4) or 20 μl of the isotype control FITC mouse IgG2a (C1130–84), purchased from BD Pharmingen; 1 μg of FITC anti-H-2K^d/2 (2G9) or 1 μg of the isotype control FITC rat IgG2a (R35–95), purchased from BD Pharmingen; and 0.5 μg of Alexa Fluor 488 anti-I-IA^b/mAb (KH116) or 0.5 μg of the isotype control Alexa Fluor 488 mouse IgG2b, purchased from BioLegend. Samples were plated on an orbital shaker for 30 min at 4°C, and then 100 μl of Ab diluent added and the cells pelleted as described above. Samples were washed twice with 200 μl of Ab diluent and then resuspended in 500 μl of Ab diluent for flow cytometric analysis. Before analysis, 20 μl of Vio- Probe (BD Pharmingen) was added to allow exclusion of dead cells from the analysis.

Alternatively, an aliquot of each Raji cell culture described above was centrifuged at 400 × g for 5 min, the pellets washed with 3 ml of cold Ab diluent (DPBS, 1% FBS, and 0.1% azide), and each pellet resuspended at 10 × 10^6 cells/ml in cold Ab diluent containing 10 μg/ml of an anti-mouse CD16/ CD32 mAb (2.4G2; BD Pharmingen) to block FcR binding. Following incubation for 5 min at 4°C, 100 μl aliquots of each sample (1 × 10^5 cells) were transferred to 96-well round-bottom plates and Ab added at the following amounts/10^5 cells: 0.5 μg of PE anti-mouse CD19 (1D3) or 0.5 μg of the isotype control PE rat IgG2a (R35–95), purchased from BD Pharmingen; 20 μl (4 μg) of FITC anti-I-IA^b/CLIP (15G4) or 20 μl of the isotype control FITC mouse IgG2a (C1130–84), purchased from BD Pharmingen; 1 μg of FITC anti-H-2K^d/2 (2G9) or 1 μg of the isotype control FITC rat IgG2a (R35–95), purchased from BD Pharmingen; and 0.5 μg of Alexa Fluor 488 anti-I-IA^b/mAb (KH116) or 0.5 μg of the isotype control Alexa Fluor 488 mouse IgG2b, purchased from BioLegend. Samples were plated on an orbital shaker for 30 min at 4°C, and then 100 μl of Ab diluent added and the cells pelleted as described above. Samples were washed twice with 200 μl of Ab diluent and then resuspended in 500 μl of Ab diluent for flow cytometric analysis. Before analysis propidium iodide (Invitrogen) was added at 20 μg/ml, to allow exclusion of dead cells from the analysis.

Events were collected on an LSR or FACSDiva flow cytometer (BD Pharmingen) using CellQuest or FACSDiva software, respectively, with individual unlabeled and one-color samples being prepared to set color compensation. All data were analyzed using CellQuest software (the FACSDiva-generated data was converted before analysis) gating on live cells, and using the geometric mean fluorescence 1 content as a direct assessment of class II/CLIP or total class II expression. The percent inhibition values of class II/CLIP or total class II expression by SB-331750 or vehicle (0.1% DMSO)-treated cells, were calculated following subtraction of the geometric mean fluorescence 1 content obtained with relevant isotype control mAb.

Induction and assessment of CIA

On day 0, 12–24 wk-old male DBA/1 mice were immunized intradermally at the base of the tail with a total of 100 μl of IFA (Sigma-Aldrich) containing 200 μg of bovine type II collagen (Elastin Products) and 250 μg of Mycobacterium tuberculosis H37Ra (Difco Laboratories). On day 21, mice were boosted intradermally with 100 μl of PBS containing 200 μg of bovine type II collagen. For prophylactic studies, SB-331750 (50 mg/kg) or vehicle (200 μl PBS) was administered s.c., twice a day (b.i.d.), from days 1 to 40. For therapeutic studies, commencement of SB-331750 or vehicle, as described above, was initiated (day 1 of treatment) once an animal exhibited a clinical score of “1” or greater for 2 consecutive days (days 0 and 1 of treatment). Mice were scored daily for clinical symptoms of disease
using a micrometer caliper to measure paw thickness. Each paw was assigned a score ranging from 0 to 4 based on the following criteria: 0, asymptomatic (paw thickness = 1.8–1.9 mm and no swollen digits); 1, paw thickness = 1.8–1.9 mm and one or more swollen digits; 2, paw thickness = 2.0–2.5 mm and one or more swollen digits; 3, paw thickness = 2.6–3.0 mm and one or more swollen digits; and 4, paw thickness = 3.0+ mm and one or more swollen digits. For each animal, the clinical score for all paws was summed, giving a potential score of 0–16.

In addition to the mice that were scored throughout the experiment (n = 12 per treatment group), on days 28 and 41, mice (n = 6 per treatment group per time point) were removed from the experiment and used to measure paw tissue cytokine levels. These mice were scored on a daily basis until their removal from the study and the data integrated into the analysis using the log rank test.

Quantitative analysis of blood SB-331750 levels
Analysis of mouse blood samples (25 μl of mouse blood diluted with 25 μl of water) for SB-331750 was performed using liquid chromatography/tandem mass spectrometric detection. Analytical standards were prepared, and the compound was isolated from the samples and standards by protein precipitation with acetonitrile containing a mass spectral internal standard and the resulting mixture was vortex-mixed followed by centrifugation. The resulting supernatant was subjected to chromatographic separation on a Luna C18 column under isocratic conditions (30/70 - 10 mM ammonium acetate and acetonitrile mobile phases at a 300 g/ml flow rate). The eluted flows into a SGE API365 triple-quadrupole mass spectrometer (Applied Biosystems) using positive-ion electrospray multiple-reaction monitoring set to monitor for the (M+H) plus precursor to produce ion transition.

Data were reported as quantitative concentrations as determined by standard calibration curve analysis, using linear fitting of a 1/x-weighted plot of the SB-331750/internal standard peak area ratios vs SB-331750 concentration, with a lower limit of quantification of 10 ng/ml.

Paw tissue cytokine analysis
Mice were removed on days 28 (n = 6) and 41 (n = 6) of the CIA study, as well as those scored through day 50 (n = 12), were euthanized on the indicated day by CO2 asphyxiation. The four paws from each mouse were collected and the four paws from each mouse were combined randomly within each treatment group, resulting in an n = 3, 3, and 6 for days 28, 41, and 50, respectively. The tissue was weighed, flash frozen in liquid nitrogen, ground using a mortar and pestle, and then homogenized for 1–2 min with a Polytron homogenizer. Cytokine levels were analyzed using mouse IL-1 and the resulting supernatants frozen at 80°C until use. Cytokine levels were analyzed using mouse IL-1β, TNF-α, and IFN-γ ELISA kits purchased from R&D Systems. Assays were performed according to the manufacturer’s instructions.

Induction and assessment of EAE
For induction of EAE by adoptive transfer, on day 0, 12–16-wk-old female SJL/J mice were immunized s.c. at the base of the tail with a total of 100 μl of IFA (Sigma-Aldrich) containing 400 μg of bovine myelin basic protein (MBP) (Sigma-Aldrich) and 50 μg of M. tuberculosis H37Rv (Difco Laboratories). On day 7, mice were boosted s.c. in the flank with 100 μl of IFA containing 400 μg of bovine MBP and 50 μg of M. tuberculosis H37Rv. On day 17 postimmunization, inguinal lymph nodes were collected and single-cell suspensions prepared as described below. A total of 2 × 10^6 cells/ml containing 50 μg/ml bovine MBP in a volume of 70 μl of RPMI 1640 containing 10% FBS and gentamicin (100 μg/ml), in the presence of water) for SB-331750 was performed using liquid chromatography/tandem mass spectrometric detection. Analytical standards were prepared, and the compound was isolated from the samples and standards by protein precipitation with acetonitrile containing a mass spectral internal standard and the resulting mixture was vortex-mixed followed by centrifugation. The resulting supernatant was subjected to chromatographic separation on a Luna C18 column under isocratic conditions (30/70 - 10 mM ammonium acetate and acetonitrile mobile phases at a 300 g/ml flow rate). The eluted flows into a SGE API365 triple-quadrupole mass spectrometer (Applied Biosystems) using positive-ion electrospray multiple-reaction monitoring set to monitor for the (M+H) plus precursor to produce ion transition.

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Cultures were harvested using a Packard Filtermate 196 (Packard Instrument), and radioactivity was quantified using a Packard TopCount liquid scintillation counter.

For measurement of anti-CD3 mAb-induced proliferation, splenocytes from naive DBA/1 and SJL/J mice were prepared as described above. A total of 2 × 10^6 cells/ml containing 100 μg/ml of RPMI 1640 containing 10% FBS, penicillin (100 units/ml)-streptomycin (100 μg/ml), and gentamicin (50 μg/ml), in the absence or presence of 5 μg/ml of plate-coated anti-mouse CD3 mAb (clone 500A2; Caltag Laboratories), and to the anti-CD3 mAb-stimulated splenocytes cultures, SB-331750 was added to a final concentration of 0 (0.1% DMSO vehicle), 0.3, 1, 3, or 10 μM. Following incubation for 72 h at 37°C in 5% CO2, 10 μl of 100 μM BrdU labeling solution (BrdU Cell Proliferation ELISA; Roche Diagnostic Systems) was added to each well, and, after an additional 24 h of incubation, cultures were processed according to the manufacturer’s instructions and chemiluminescence measured using a Wallac Envision 2102 Multilabel Reader.

Statistical analysis
Statistical differences in cytokine levels were determined using the two-tailed Student’s t test. Analysis of CIA and EAE disease severity was performed by calculating, for each animal within a treatment group, the area under the curve (AUC) using the trapezoidal rule, and then using either the Student’s t test (for uncensored data), or the log rank test, which is a nonparametric test that allows for right censored observations (i.e., for any animal removed before the study’s completion, the animal’s complete AUC is considered to be at least as large as the partial AUC exhibited). In cases where scores for some days were missing in the interior of the animal’s time profile, score values were interpolated over time. For CIA and EAE studies, day of disease onset was defined as the first of two consecutive days on which an animal exhibited a clinical score > 0. Analysis of disease onset data was performed using the log rank test. Values of p < 0.05 were considered statistically significant.

Results
Structure and characteristics of SB-331750
SB-331750, 5-(2-Morpholin-4-ylethoxy)benzofuran-2-carboxylic Acid ((S)-3-Methyl-1-(S)-3-oxo-1-[2-(3-pyridin-2-ylphenyl)- acetyl]azepan-4-ylcarbamoyl)butyl)amide, is a pan-active inhibitor of the papain family of cysteine proteases, exhibiting potent activities for human cathepsins S, L, F, V and K, and weak activity for human cathepsin C. Similarly, SB-331750 is a potent inhibitor of mouse cathepsins S, L, and K, but not mouse cathepsin C (Fig.
In contrast, cathepsin K shown) and p31 isoforms of Ii, but no Ii degradation intermediates. Treated in the presence of DMSO expressed both the p41 (data not

proteases); cathepsin D, pepsin, and

follows: trypsin, chymotrypsin, cathepsin G, and elastase (serine

teases); matrix metalloproteinase-1, -2, -3, -7, -9, angiotensin-con-

verting enzyme, and neutral endopeptidase (metallo proteases); and caspase-3 and -4 (caspase family of cysteine proteases).

In vitro treatment with SB-331750 results in comparable

inhibition of li processing by cathepsin K −/− vs cathepsin K +/+ splenocytes

Murine cathepsins S, L, and F have been implicated in the final

proteolytic cleavage of li (6–10, 13), whereas cathepsin K, to our

knowledge, has not. To demonstrate the cell-based inhibition of li

processing by SB-331750, and to determine whether the activity

exhibited by SB-331750 for mouse cathepsin K (Fig. 1) contrib-

utes to the compound’s inhibition of li processing, cathepsin K +/+ and K −/− splenocytes were incubated for 18 h in the absence (Un-

treated) or presence of vehicle (0.1% DMSO), SB-331750 (5 µM), or the broad-spectrum cysteine protease inhibitors, leupeptin (200 µg/ml) or APC3328 (5 µM). Cell lysates were then subjected to SDS-PAGE and Western blot analysis using the anti-mouse li mAb, In-1. Untreated cathepsin K +/+ splenocytes and those cultured in the presence of DMSO expressed both the p41 (data not shown) and p31 isoforms of li, but no li degradation intermediates. In contrast, cathepsin K −/− splenocytes treated with leupeptin, APC3328, or SB-331750 exhibited prominent accumulation of the CLIP precursor li p10 with APC3328 inducing weak accumulation of the li p10 precursor li p22 as well (Fig. 2A). Under the same experimental conditions, cathepsin K −/− splenocytes (Fig. 2B) exhibited li processing intermediates comparable to those exhibited by cathepsin K +/+ splenocytes. In additional studies, the accumulation of li processing intermediates was found to occur rapidly, such that treatment of cells for 4–5 h resulted in consistent and quantifiable accumulation of li p10 (Fig. 3). In the experiment depicted in Fig. 2, and in those subsequently described, viability of mouse splenocytes following 18 h or 4–5 h of treatment with vehicle or inhibitor was confirmed by trypan blue exclusion. These results demonstrate the cell-based inhibition of late stage li pro-

cessing by SB-331750 and indicate that cathepsin K does not play a role in li processing in these cells.

![Figure 1](image1.png)

**FIGURE 1.** The chemical structure and selectivity profile of SB-331750, 5-(2-Morpholin-4-ylethoxy)benzofuran-2-carboxylic Acid ((S)-3-Methyl-1-((S)-3-oxo-1-[(2-(3-pyridin-2-ylphenyl)-acetyl]azepan-4-ylcarbamoyl)butyl)amide. ND = Not determined.

![Figure 2](image2.png)

**FIGURE 2.** In vitro treatment with SB-331750 or broad-spectrum cysteine protease inhibitors results in inhibition of li processing by cathepsin K −/− splenocytes comparable to that exhibited by cathepsin K +/+ splenocytes. Splenocytes from cathepsin K +/+ (A) or K −/− (B) mice were cultured for 18 h in the absence or presence of vehicle (0.1% DMSO), leupeptin (200 µg/ml), APC3328 (5 µM), or SB-331750 (5 µM). Splenocytes were lysed, and the resulting lysates subjected to SDS-PAGE on an 18% Tris-HCl gel, followed by Western blot analysis using the mAb In-1. The results are representative of two independent experiments.

![Figure 3](image3.png)

**FIGURE 3.** In vitro treatment with SB-331750 induces concentration-
dependent accumulation of li p10 in DBA/1, SJL/J, and C57BL/6 splenocytes. Splenocytes from DBA/1, SJL/J, and C57BL/6 mice were cultured for 4.5 h in the presence of SB-331750 at the indicated concentrations. Following lysis the cells were subjected to SDS-PAGE on an 18% Tris-HCl gel, and then Western blot analysis using the mAb In-1. The results are representative of four independent experiments.
FIGURE 4. In vitro treatment with SB-331750 or leupeptin induces accumulation of MHC class II-associated Ii processing intermediates, and decreases cell surface expression of class II/CLIP complexes, by Raji cells. Raji cells were cultured for 24 h in the presence of vehicle (0.1% DMSO), SB-331750 (10 μM), or leupeptin (200 μg/ml), and cells lysed for SDS-PAGE/Western blot analysis, or intact cells subjected to flow cytometric analysis. Nonimmunoprecipitated cell lysates were then subjected to SDS-PAGE on an 18 or 4–20% Tris-HCl gel, and then Western blot analysis using the anti-human Ii mAb PIN.1. The results are representative of two independent experiments. Intact Raji cells were stained with the FITC anti-human HLA-DR mAb G46-6 or with control mouse IgG (C), or with control mouse IgG2a (not shown) (D), or with the FITC anti-HLA-DR mAb G46-6 or with control FITC mouse IgG2a (not shown) (E), and subjected to flow cytometric analysis. Dead cells were excluded using propidium iodide. The results are representative of three to four independent experiments.

In vitro treatment with SB-331750 inhibits processing of MHC class II-associated Ii, and decreases cell surface expression of class II/CLIP complexes, by Raji cells and C57BL/6 splenocytes

It has been demonstrated that within the cell, Ii is present in large excess relative to class II molecules. In the absence of association with class II molecules, this pool of Ii is localized primarily in the endoplasmic reticulum (31). To determine whether the SB-331750-mediated inhibition of late stage Ii processing observed in whole cell lysates is representative of the compound’s activity on class II-associated Ii, human Raji cells were cultured for 24 h with vehicle (0.1% DMSO), SB-331750 (10 μM), or leupeptin (200 μg/ml), and an aliquot of the cell lysates from each treatment was immunoprecipitated using the mouse anti-HLA-DR mAb, TAL 1B5, or control mouse IgG (Fig. 4). Ig H and L chains were detected due to recognition of the mouse immunoprecipitating Abs by the HRP-conjugated goat anti-mouse IgG mAb used as the detection reagent. To determine whether the SB-331750- and leupeptin-mediated inhibition of class II-associated Ii processing results in modulation of class II/CLIP complexes on the cell surface, DMSO-, SB-331750-, or leupeptin-treated Raji cells were analyzed by flow cytometry using the mAb CerCLIP. As shown in Fig. 4D, cells treated with SB-331750 or leupeptin exhibited decreased class II-associated CLIP expression compared with cells treated with DMSO. In three to four experiments, SB-331750 and leupeptin induced mean decreases in class II-associated CLIP expression of 65.4% (range, 62.1–71.3%) and 74.4% (range, 72.6–76.1%), respectively. Total class II expression on the cell surface, detected by the FITC anti-HLA-DR mAb G46-6, was decreased modestly, but detectably, by SB-331750 (mean reduction of 17.3%; range, 11.2–23.3%) and leupeptin (mean reduction of 29.8%; range, 22.1–34.2%) (Fig. 4E). Treatment of Raji cells with SB-331750 at 30 μM induced mean decreases of 71.4% (range, 70.3–73.1%) and 23.5% (range, 17.9–29.0%) in class II-associated CLIP expression and total class II expression, respectively (data not shown).

Similar results were obtained following culture of C57BL/6 splenocytes for 4 h with vehicle (0.1% DMSO), SB-331750 (10 μM), or leupeptin (200 μg/ml). The prominent accumulation of Ii (Fig. 3), whereas untreated and vehicle (0.1% DMSO)-treated splenocytes from each strain exhibited no accumulation of Ii p10 (data not shown). Interestingly, the pattern of Ii p10-fragment accumulation differed among the strains, suggesting heterogeneity in the proteolytic events leading to Ii p10 generation. These data indicate that SB-331750 inhibits the final stage of Ii processing in genetically distinct strains of mice, allowing for investigation of the effects of inhibition of CLIP generation in multiple models of autoimmune disease.
p10 and weak accumulation of li p22 observed in SB-331750- or leupeptin-treated nonimmunoprecipitated whole cell lysates (Fig. 5A) was also seen following immunoprecipitation of whole cell lysates with the anti-I-A^b mAb AF6–120.1 (Fig. 5, B and C), but not with control mouse IgG2a (C), or with control PE rat IgG2a, to allow gating on CD19^+ cells. In addition, splenocytes were stained with the FITC anti-I-A^b/CLIP mAb 15G4 or with control FITC mouse IgG1 (not shown) (D), or with the FITC anti-H-2^d/H-2^k mAb 2G9 or with control FITC rat IgG2a (not shown) (E), and subjected to flow cytometric analysis. Dead cells were excluded using 7-amino-actinomycin D. The results are representative of three independent experiments. Anti-I-A^b mAb, AF6–120.1 (Fig. 5, A and lane 1 of B), and cell lysates immunoprecipitated with the anti-I-A^b mAb, AF6–120.1 (B and C), or with control mouse IgG2a (C), were subjected to SDS-PAGE/Western blot analysis, or intact cells subjected to flow cytometric analysis. Nonimmunoprecipitated cell lysates (A and lane 1 of B), and cell lysates immunoprecipitated with the anti-I-A^b mAb, AF6–120.1 (B and C), or with control mouse IgG2a (C), were subjected to SDS-PAGE on an 18% Tris-HCl gel, and then Western blot analysis using the anti-mouse li mAb In-1. The results are representative of two pooled mice/experiment in three independent experiments. Intact C57BL/6 splenocytes were stained with the PE anti-mouse CD19 mAb 1D3 or with control PE rat IgG2a, to allow gating on CD19^+ cells. In addition, splenocytes were stained with the FITC anti-I-A^b/CLIP mAb 15G4 or with control FITC mouse IgG1 (not shown) (D), or with the FITC anti-H-2^d/H-2^k mAb 2G9 or with control FITC rat IgG2a (not shown) (E), and subjected to flow cytometric analysis. Dead cells were excluded using 7-amino-actinomycin D. The results are representative of three independent experiments.

**Absence of detectable MHC class II-associated li processing intermediates in SB-331750-treated DBA/1 and SJL/J splenocytes**

Similar to the results obtained with C57BL/6 splenocytes, following in vitro culture of DBA/1 (Fig. 6A) and SJL/J splenocytes (Fig. 7A) for 4 h with SB-331750 (10 μM) or leupeptin (200 μg/ml), nonimmunoprecipitated whole cell lysates exhibited marked accumulation of li p10 and weak accumulation of li p22, compared with nonimmunoprecipitated whole cell lysates of relevant vehicle (0.1% DMSO)-treated splenocytes. Interestingly, li p10 accumulation was not detected following immunoprecipitation of DBA/1 (Fig. 6B) and SJL/J (Fig. 7B) lysates with the anti-I-A^d/I-A^e mAb KH116. In the DBA/1 immunoprecipitates, the identity of the species that migrated coincident with li p22 is unknown, although its appearance following immunoprecipitation with control mouse IgG as well as with KH116, from lysates of both vehicle-treated and inhibitor-treated cells (Fig. 6B), suggests that this band is nonspecific and does not represent a class II-associated li processing intermediate. To confirm that the lack of detectable class II-associated li p10 in these strains was not unique to use of KH116 as the immunoprecipitating mAb, DBA/1 whole cell lysates were immunoprecipitated with the anti-H-2^d/H-2^k mAb 2G9 and similar results were obtained (data not shown). Levels of surface class II/CLIP on CD19^+ and CD19^- splenocytes from DBA/1 and SJL/J mice, detected by the mAb 15G4, were not elevated above levels.
detected by an isotype control mAb (data not shown), although interpretation of this result is confounded by the fact that we cannot distinguish between absence of surface class II/CLIP expression, and lack of cross-reactivity of 15G4 with the H-2q and H-2s haplotypes. CD19 B cells from DBA/1 mice expressed high levels of total class II molecules on the cell surface (detected by the mAb 2G9), which were decreased by an average of 46.7% (range, 44.7–50.5%, n = 3 mice) as a result of treatment with SB-331750, and by an average of 33.0% (range, 32.7–33.3%, n = 3 mice) following treatment with leupeptin (Fig. 6C). Expression of total class II molecules on the surface CD19 B cells was low, and was decreased by an average of 14.9% (range, 11.0–18.7%, n = 3 mice) and 15.3% (range, 3.6–21.2%, n = 3 mice), following treatment with SB-331750 and leupeptin, respectively (data not shown). High and low levels of total MHC class II (detected by the mAb KH116) were also observed on CD19 (Fig. 7C) and CD19 (data not shown) SJL/J splenocytes, respectively. SB-331750 induced mean decreases of 18.6% (range, 17.5–19.4%, n = 3 mice) and 4.9% (range, 0.43–10.7%, n = 3 mice), on CD19 and CD19 splenocytes, respectively, and leupeptin induced mean decreases of 18.0% (range, 16.1–19.9%, n = 2 mice) and 10.9% (range, 6.0–15.8%, n = 2 mice), respectively, compared with DMSO-treated CD19 and CD19 splenocytes, respectively.

These data suggest that in two autoimmune-prone strains of mice, DBA/1 and SJL/J, II processing intermediates may readily dissociate from MHC class II molecules. Interestingly, similar to the inhibitor-mediated modulation of total class II expression on the surface of Raji cells and C57BL/6 splenocytes, SB-331750 treatment resulted in varying degrees of reduction of surface class II expression by DBA/1 and SJL/J splenocytes.

In vitro treatment with SB-331750 inhibits Ag-induced proliferation by LNC/splenocytes from collagen-sensitized DBA/1 mice and MBP-sensitized SJL/J mice

To confirm that inhibition of late stage II processing by SB-331750 results in the reduction of Ag-induced cellular responsiveness,
LNC/splenocytes from collagen-immunized/boosted DBA/1 mice, and from MBP-immunized/boosted SJL/J mice, were collected 24 and 17 days, respectively, postimmunization and stimulated with the relevant immunizing Ag. Culture of collagen-primed DBA/1 cells (Fig. 8A), and of MBP-primed SJL/J cells (Fig. 8B), with collagen and MBP, respectively, resulted in robust proliferative responses, each of which was inhibited in a concentration-dependent manner by SB-331750. This inhibition was not observed in DBA/1 (Fig. 8C) and SJL/J (Fig. 8D) splenocytes induced to proliferate via a mechanism not dependent on presentation of Ag, i.e., stimulation with anti-CD3 mAb. A similar lack of inhibition was observed when proliferation was induced by mitogen (data not shown). Viability of cells following culture with vehicle or SB-331750 was confirmed by trypan blue exclusion. These results demonstrate that in conjunction with its ability to inhibit late stage Ii processing in DBA/1 and SJL/J APC, in vitro treatment with SB-331750 prevents T cell proliferation in response to Ags that induce autoimmune syndromes in these strains of mice.

In vivo administration of SB-331750 inhibits Ii processing by DBA/1, SJL/J, and C57BL/6 splenocytes

To demonstrate the inhibition of Ii processing by SB-331750 in vivo, DBA/1, SJL/J, and C57BL/6 mice were administered SB-331750 (50 mg/kg) or vehicle (PBS) s.c., b.i.d. on days 1–3, and 90 min after a single administration of SB-331750 or vehicle on day 4, spleens were collected and lysed. The resulting whole cell lysates were then subjected to SDS-PAGE and Western blot analysis using the mAb In-1 to assess Ii p10 accumulation. As shown in Fig. 9, DBA/1, SJL/J, and C57BL/6 mice treated with SB-331750 exhibited enhanced accumulation of Ii p10 compared with DBA/1, SJL/J, and C57BL/6 mice, respectively, treated with vehicle, demonstrating that in vivo administration of SB-331750 results in the inhibition of processing of Ii p10 to CLIP.

In vivo prophylactic administration of SB-331750 decreases the severity and delays the onset of CIA, and reduces levels of tissue proinflammatory cytokines, in DBA/1 mice

To determine whether the inhibition of Ii processing and collagen-induced T cell proliferation by SB-331750 is sufficient to prevent the development of CIA, collagen-immunized and boosted DBA/1 mice (24 wk of age at the time of immunization) were administered SB-331750 (50 mg/kg) or vehicle (PBS) s.c., b.i.d. from days 1 to 40. In a previous pharmacokinetic study, blood concentrations of SB-331750 were measured in samples from three DBA/1 mice that were administered SB-331750 (50 mg/kg) s.c., b.i.d. on day 0, and once on day 1. Immediately before compound administration on day 1, blood
delayed as a result of treatment with SB-331750 (PBS). In addition, the time of onset of disease was significantly
331750 (PBS) were collected. Splenocytes were lysed, and the resulting lysates subjected
to SDS-PAGE on an 18% Tris-HCl gel, followed by Western blot analysis
were representative of two independent
experiments. Using the mAb In-1. The results are representative of two independent

FIGURE 9. In vivo prophylactic administration of SB-331750 induces accumulation
of Ii p10 in DBA/1, SJL/J, and C57BL/6 splenocytes. DBA/1, SJL/J, and
C57BL/6 mice were administered vehicle (PBS) (n = 2 mice/strain) or
SB-331750 (n = 2 mice/strain) at 50 mg/kg s.c., b.i.d on days 1–3, and 90
min after a single administration of vehicle or SB-331750 on day 4, spleens
were collected. Splenocytes were lysed, and the resulting lysates subjected
to SDS-PAGE on an 18% Tris-HCl gel, followed by Western blot analysis
using the mAb In-1. The results are representative of two independent experiments.

Data are expressed as mean

FIGURE 10. In vivo prophylactic administration of SB-331750 decreases the severity of CIA in the DBA/1 mouse
To determine whether SB-331750 is capable of modulating the severity of CIA when administered therapeutically, collagen-
immunized and boosted DBA/1 mice were injected with SB-
331750 (50 mg/kg) or vehicle (PBS) s.c., b.i.d. following the onset of clinical symptoms. We have observed that mice im-
munized at 12 wk of age exhibit moderate disease severity (Fig. 11A), whereas mice immunized at 24 wk of age develop severe disease (Fig. 11B). Under moderate disease conditions, SB-331750-treated mice exhibited a significantly decreased mean clinical score compared with vehicle (PBS)-treated mice
Under severe disease conditions, SB-331750-treated mice exhibited a trend toward decreased disease severity, although statistical significance was not achieved (Fig. 11B). Under the latter conditions, in addition to administration of SB-331750 from the onset of symptoms through completion of the study (days 1–24), mice were administered SB-331750 (50 mg/kg) s.c., b.i.d. on days 1–5, and then administered vehicle (PBS) s.c., b.i.d on days 6–24. During the course of drug and/or vehicle administration, mice were scored for clinical symptoms of disease. Data are expressed as mean ± SEM (n = 8–13 mice).

In vivo therapeutic administration of SB-331750 decreases the severity of EAE in the SJL/J mouse

To determine whether therapeutic administration of SB-331750 modulates the severity of actively-induced EAE, MBP-immunized SJL/J mice were injected with SB-331750 (50 mg/kg) or vehicle (PBS) s.c., b.i.d. following the onset of clinical symptoms, through day 14 of the study. Immunization of SJL/J mice with bovine MBP produced a mild, transient form of EAE, the severity of which was significantly reduced as a result of treatment with SB-331750 (p < 0.05) (Fig. 13). These results, together with those obtained in the CIA model, indicate that when administered in a clinically relevant dosing regimen, SB-331750 provides therapeutic benefit under mild/moderate autoimmune disease conditions.
Discussion

The studies described herein demonstrate that a small m.w., pan-active inhibitor of the papain family of cysteine proteases, SB-331750, attenuates late stage Ii processing by DBA/1 and SJL/J splenocytes, and Ag-induced T cell proliferation by collagen-primed DBA/1 and MBP-primed SJL/J cells. In vivo prophylactic administration of SB-331750 to collagen-immunized/boosted DBA/1 mice delayed the onset and reduced the severity of CIA, and resulted in decreased paw tissue levels of IL-1β, TNF-α, and IFN-γ. Similarly, treatment of MBP-primed SJL/J LNC with SB-331750 delayed the onset and reduced the severity of adoptively transferred EAE. When administered therapeutically, SB-331750 reduced the severity of mild/moderate CIA and EAE. That SB-331750 exerts its effects on CIA and EAE via inhibition of Ag presentation, and not an alternative mechanism, is suggested by the following: SB-331750 inhibited processing of whole cell Ii p10 to CLIP, the preferred substrate of the class II-associated Ii processing intermediates following treatment of APC with the broad-spectrum cysteine protease inhibitor morpholinurea-leucyl-homophenylalanyl-vinylsulfone-phenyl (LHVS) degrading enzyme (32, 33), TNF-α converting enzyme (34), and proteinase 3 (35), respectively, the inhibition of which could potentially account for the observed reductions in paw tissue IL-1β and TNF-α levels. This report of attenuated autoimmune disease development, specifically of murine CIA and EAE, as a result of deficient cysteine protease activity is consistent with the observations that cathepsin S-deficient mice exhibited reduced susceptibility to CIA (7) and myasthenia gravis (36), that the irreversible broad-spectrum protease inhibitor morpholinurea-leucyl-homophenylalanyl-vinylsulfone-phenyl (LHVS) decreases adjuvant-induced arthritis in the rat (37), and that a cathepsin S-selective inhibitor abrogated Sjögren syndrome in the mouse (38). This report is, to our knowledge, the first demonstration of attenuation of CIA as a result of treatment with a cysteine protease inhibitor and of modulation of EAE using either a pharmacologic or genetic approach to inhibit cysteine protease activity. Collectively, these observations suggest broad-spectrum and/or selective inhibition of members of the papain family of cysteine proteases as a potential therapeutic approach to the treatment of autoimmunity.

Similar to the inhibition of presentation of type II collagen to collagen-primed DBA/1 cells by SB-331750, Nakagawa et al. (7) reported a block in the presentation of the type II collagen epitope, 260–270, by macrophages and dendritic cells from cathepsin S-deficient DBA/1 mice. Both SB-331750-treated and cathepsin S-deficient DBA/1 mice exhibited delayed onset and reduced severity of CIA compared with relevant control mice. In addition, both cathepsin S deficiency and SB-331750 treatment resulted in the absence of detectable class II-associated Ii processing intermediates in DBA/1 (H-2q) splenocytes, but not in C57BL/6 (H-2b) splenocytes. In contrast to these similarities, cathepsin S deficiency induced minimal whole cell li fragment accumulation in DBA/1 splenocytes compared with the accumulation observed in C57BL/6 splenocytes (7), whereas SB-331750 treatment induced whole cell Ii fragment accumulation in both DBA/1 and C57BL/6 splenocytes. The lack of Ii fragment accumulation in the cathepsin S-deficient DBA/1 splenocytes, but not in cathepsin S-deficient C57BL/6 splenocytes, was suggested to result from efficient dissociation of Ii processing intermediates from I-Aa but not from I-Aq (7). If this is the case, the accumulation of whole cell Ii processing intermediates in SB-331750-treated DBA/1 splenocytes, but not in cathepsin S-deficient DBA/1 splenocytes, may be due to increased susceptibility of dissociated Ii fragments to degradation by cysteine proteases other than cathepsin S, the activities of which would be inhibited by SB-331750.

In addition to DBA/1 splenocytes, SJL/J splenocytes, but not Raji cells or C57BL/6 splenocytes, exhibited an absence of class II-associated Ii processing intermediates following treatment with SB-331750 or leupeptin. These results offer further support for the occurrence of efficient dissociation of Ii degradation intermediates from selected MHC haplotypes. Class II polymorphism has been shown to dramatically affect the affinity of the class II-CLIP (39, 40) and class II-Ii p10 (7, 41) interactions. Whether decreased stability of class II/Ii fragment complexes contributes to autoimmune susceptibility is unclear. Although multiple autoimmune-association MHC class II molecules have been shown to form low stability complexes with CLIP, including RA- (42), type 1 diabetes- (43, 44), and celiac disease- (45) associated allele products, expression of low affinity I-Aq/CLIP complexes was reported not to alter the peptide repertoire bound by class II molecules or increase the susceptibility of the mice to autoimmunity (46).

Consistent with previous reports using leupeptin (47, 48), we observed reduced cell surface expression of total class II molecules as a result of SB-331750 or leupeptin treatment. This decrease may reflect inhibition of the formation of mature class II/peptide complexes normally expressed on the cell surface, although measurement of SDS-stable (high-affinity peptide loaded) class II complexes would be needed to confirm this. Interestingly, the magnitude of the SB-331750- or leupeptin-induced decreases in surface class II levels did not appear to correlate with the presence of stable class II-associated Ii processing intermediates. All inhibitor-treated cells exhibited some degree of reduced surface class II expression, with both Raji cells and SJL/J splenocytes, in which stable class II-associated Ii intermediate complexes were present and absent, respectively, demonstrating small decreases, and C57BL/6 splenocytes and DBA/1 splenocytes, in which these complexes were present and absent, respectively, demonstrating more substantial decreases. Consistent with the proposed interpretation of these data given above, Villadangos et al. (41) reported that treatment of APC with the broad-spectrum cysteine protease inhibitor...
inhibitors, leupeptin or morpholinurea-leucyl-homophenylalanyl-vinylsulfone-phenyl (LHVS), prevented Ii degradation and formation of SDS-stable class II/peptide complexes in an MHC allele-dependent manner, with Ii p10 dissociating much more efficiently from I-A^d than from I-A^k, and the latter exhibiting twice the reduction in class II/peptide complexes as the former.

Based on the results described herein, a potential mechanism by which SB-331750 attenuates CIA and EAE is via the inhibition of late stage Ii processing, preventing the binding of collagen/MBP peptide to class II molecules, and the subsequent presentation of class II/peptide complexes to CD4^+ T cells. However, the absence of stable class II/Ii fragment complexes in SB-331750-treated DBA/1 and SJL/J splenocytes appears to argue against this mechanism. It is possible that this observation could be explained on the basis of the reduced affinity of Ii intermediates for class II molecules of the H-2^d and H-2^k haplotypes, coupled with the experimental manipulation necessary for detection of class II/Ii fragment complexes, facilitating enhanced dissociation of the complexes under in vitro or ex vivo conditions. The class II/Ii association has been shown to be susceptible to disruption by detergents used for cell solubilization, such as Triton X and Nonidet P-40 (49). Alternatively, a different mechanism may underlie the inhibition of CIA and EAE by SB-331750. Based on the lack of Ii fragment accumulation and the presence of mature SDS-stable I-A^d dimers exhibited by cathepsin S-deficient DBA/1 splenocytes, Nakagawa et al. (7) proposed that the role of cathepsin S in collagen presentation and induction of CIA may be one of direct or indirect involvement in processing of Ag, rather than Ii. Consistent with this hypothesis, cathepsin S has been shown to play an important function in the generation of a subset of antigenic epitopes derived from myoglobin, SA85 (50), and hen egg lysozyme (51), and in cell-free assays, was shown to hydrolyze collagen (52), and initiate the proteolytic processing of MBP resulting in the generation of the epitope MBP_111–129 (53). Conversely, the lack of acetylcholine receptor Ag-induced T cell proliferative and cytokine responses by Ag-primed cathepsin S-deficient LNC was demonstrated to be due not to defective processing of the acetylcholine receptor protein, but rather to Ii-dependent restriction of peptide loading, suggesting that inhibition of Ii processing accounts for the reduced susceptibility of cathepsin S-deficient mice to myasthenia gravis (36). Thus, the precise mechanism by which cysteine proteases modulate susceptibility to Ag-driven autoimmunity likely depends on multiple factors, including the antigenic epitope being processed and presented, the complement of active proteases present, and the haplotype of the class II molecule to which the antigenic epitope binds.

In conjunction with the inhibition of the clinical symptoms of CIA, administration of SB-331750 resulted in reduced paw tissue levels of IL-1β, TNF-α, and IFN-γ, proinflammatory cytokines associated with RA and CIA. It is widely accepted that synovial macrophages are the principal producers of IL-1β and TNF-α, while CD4^+ Th1 cells, a primary T cell subset in the RA joint, produce IFN-γ that likely promotes macrophage activation, resulting in the abundant secretion of IL-1β and TNF-α (54, 55). Thus, the inhibition of Ag (collagen) presentation resulting from SB-331750 administration may reduce the magnitude of the CD4^+ T cell-derived IFN-γ response, thereby decreasing macrophage activation and the subsequent production of IL-1β and TNF-α. Further inhibition of cytokine production may occur as the result of the attenuation of downstream proinflammatory cytokine cascades. In this regard, IL-1β has been shown to stimulate the production of IL-1β, TNF-α, and IFN-γ (56), and TNF-α to induce the production of TNF-α and IL-1β (57).

To date, evidence supporting a role for the papain family of cysteine proteases in the etiology of EAE has been indirect. It has been demonstrated that the development of EAE by C57BL/6 mice in response to the dominant encephalitogenic peptide, myelin oligodendrocyte glycoprotein (MOG)_{15–55}, requires Ag processing and presentation in the CNS (58, 59). Microglia, resident CNS APC, express active cathepsins S and L (60, 61) and have been shown to function efficiently in the presentation of myelin and other Ags, while the role of astrocytes in Ag presentation and T cell activation in the CNS remains controversial (62–65). The purported lack of efficient Ag-presenting capacity by astrocytes has been ascribed to decreased cathepsin S and L activities, and subsequent inhibition of Ii processing (61). As described herein, the results of treatment of MBP-primed LNC with the pan-active cathepsin inhibitor SB-331750 before transfer of the cells into naive recipients directly implicates one or more of these proteases in the development of EAE. In EAE induced by adoptive transfer, Ag-primed donor CD4^+ T cells collected from the periphery, subjected to an in vitro activation step, and transferred to the periphery of naive recipients migrate across the blood-brain barrier, recognize autoantigen in the CNS, and exert their effector function. The delayed onset and reduced severity of EAE exhibited by recipients of SB-331750-treated cells, compared with recipients of vehicle-treated cells, indicate that in this model, the compound attenuated effector cell migration and/or function, and did so through its action in the periphery. However, the efficacy achieved following therapeutic administration of SB-331750 in actively-induced EAE may be the result of the compound’s action in the periphery and/or the CNS. Thus, our observations, together with those of other investigators described above, suggest that cathepsins contribute to the etiology of EAE by modulating Ag presentation in the periphery and/or the CNS.

In summary, the studies presented in this paper demonstrate that a pan-active inhibitor of the papain family of cysteine proteases, SB-331750, attenuates late stage Ii processing by DBA/1 and SJL/J splenocytes, and subsequent Ag-induced T cell proliferation by collagen-primed DBA/1 and MBP-primed SJL/J cells, respectively. In conjunction with the inhibition of these processes, SB-331750 induces a delay in onset and decrease in severity in the corresponding models of experimentally-induced autoimmune disease, CIA in the DBA/1 mouse and EAE in the SJL/J mouse. These observations suggest that inhibition of Ag presentation and the resulting activation and expansion of Ag-specific T cells by inhibitors of the papain family of cysteine proteases offers a potential therapeutic approach to the treatment of human autoimmunity.

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Disclosures

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References


