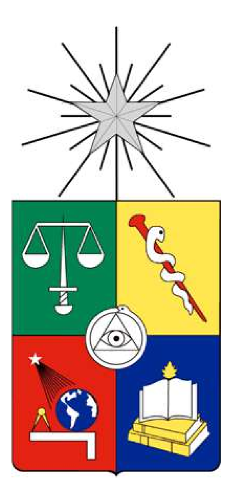

Mechanisms underlying hemocyanin antigen processing and presentation through MHC-I and MHC-II dependent pathways

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ABSTRACT

Mollusk hemocyanins are oligomeric glycoproteins with complex quaternary structures and heterogeneous glycosylations. They are widely used in biomedicine as adjuvants/immunomodulators because they bias towards Th1 immunity when inoculated in mammals. Structural features of hemocyanins support these effects; nevertheless, the underlying mechanisms are not entirely understood. Antigen-presenting cells (APCs) bind hemocyanins through mannose-binding C-type lectin receptors and Toll-like receptors. After clathrin-mediated internalization, hemocyanins are processed and presented to T lymphocytes. Antigen presentation of exogenous proteins commonly occurs through MHC-II to CD4+ T lymphocytes, although mannose-recognizing immune receptors may promote cross-presentation, where exogenous antigens bind MHC-I to stimulate CD8+ T lymphocytes. Cross-presentation is essential for antitumor responses, and it would partially explain hemocyanin-induced effects. Hence, we hypothesize that hemocyanins undergo both MHC-I and MHC-II dependent antigen presentation by APCs.

Using J774.2 murine macrophages as APCs, and *Fissurella latimarginata* hemocyanin (FLH), our results show that these glycoproteins are presented through MHC-II pathways. Indeed, pharmacological inhibitors of MHC-II pathway decreased FLH-induced cytokine secretion by macrophages, assessed by ELISA. Interestingly, inhibitors of the vacuolar MHC-I pathway had comparable effects, suggesting cross-presentation. Immunoblot suggested different FLH proteolysis patterns in macrophages treated with different inhibitors of both MHC-I and MHC-II pathways. Flow cytometry experiments showed an FLH-dependent upregulation of MHC-I, MHC-II and CD86, and pharmacological inhibitors of both MHC-I and MHC-II pathways impaired these effects. Overall, these results suggest that FLH, a model mannose-rich protein, undergoes cross-presentation, partially explaining its immunomodulatory effects.

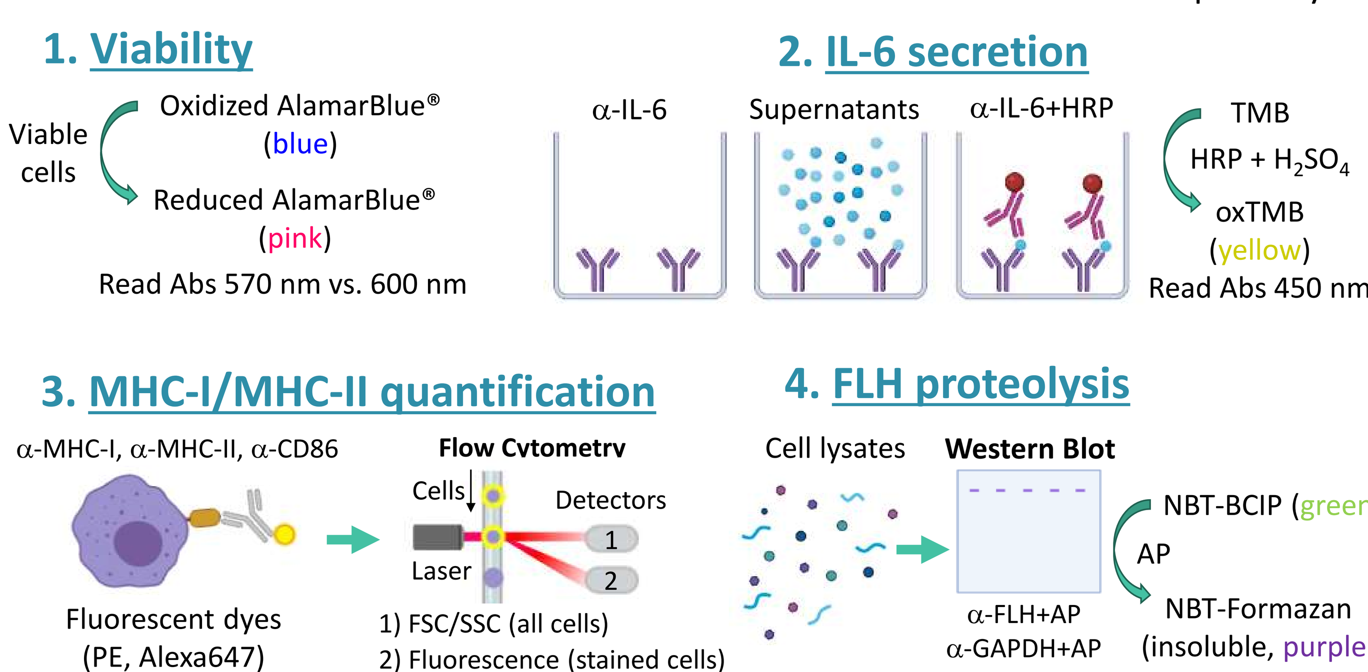
INTRODUCTION

Hemocyanins are widely used as adjuvants and immunomodulators, because they promote Th1 immune responses in mammals; however, the mechanisms underlying these effects are not entirely understood. As exogenous antigens, hemocyanins are partly internalized by antigen presenting cells (APCs) through clathrin-mediated endocytosis, being recognized as "highly mannosylated molecular pattern molecules" through innate immune receptors including MR, DC-SIGN, and TLR4[1-5]. These receptors shape different effector immune responses, such as the generation of proinflammatory mediators and the subcellular destination of antigens. Internalized antigens are commonly localized into late endocytic compartments and lysosomes for presentation onto MHC class II molecules to stimulate CD4+ T lymphocytes. Alternatively, these antigens could undergo cross-presentation onto MHC-I molecules to stimulate CD8+ T lymphocytes[6-11]. However, whether hemocyanins promote immunomodulatory effects depending on MHC-I and/or MHC-II pathways has not been entirely characterized. Our hypothesis is that "**hemocyanins are presented in MHC-II molecules, and cross-presented in MHC-I, thus initiating the specific adaptive immune response**".

In this context, we aim to characterize the antigen presentation pathways underlying hemocyanin immunomodulatory effects. For MHC-II, these molecules bind to peptides derived from exogenous antigens degraded in the endocytic pathway. For MHC-I, antigens can be cross-presented through two routes: cytosolic, which is dependent on proteasomal activity and TAP, or vacuolar, which is relies on phagosomal degradation of antigens. Here we explored these two pathways in APCs, using a representative hemocyanin (FLH from *Fissurella latimarginata*), and pharmacologic inhibitors, which act at different levels (**Figure 1, Table 1**)[6-11].

METHODS

➢ **Cell culture:** J774.2 macrophage cell line.



CONCLUSIONS

1. The antigenic presentation pathways for FLH have been preliminary studied in J774.2 macrophages using different inhibitors of intracellular antigen trafficking.
2. FLH is antigenically processed in APCs through MHC-I and/or MHC-II pathway because Brefeldin A, a specific inhibitor of exocytosis of proteins, significantly decreased all the markers (MHC-I, MHC-II, CD86, and IL-6).
3. Interestingly, the results obtained with epoxomicin, a proteasome inhibitor, and bafilomycin, an inhibitor of lysosomal acidification, show that FLH would also experience cross-presentation of antigens through MHC-I, either by the vacuolar or cytosolic route.
4. These findings allow us to better understand the immunomodulatory effects of these glycosylated antigens on the immune system of mammals.

RESULTS

➢ **MHC-I and MHC-II dependent pathways.**

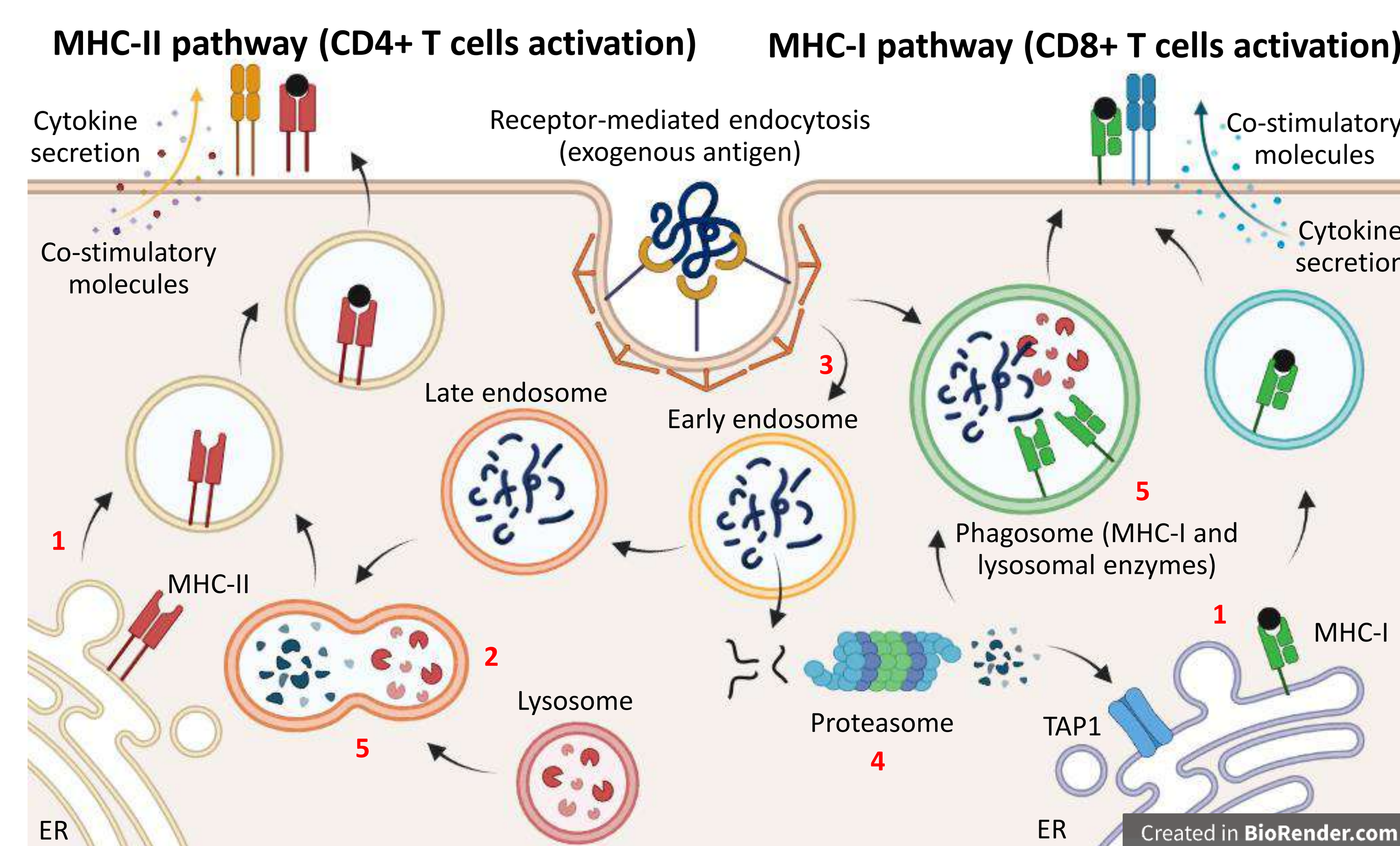


Table 1. Summary of pharmacological inhibitors and their targets on MHC-I and/or MHC-II dependent pathways.

Inhibitor	Pathway	Target	Observed effects
Brefeldin A[6,7] 1	MHC-I MHC-II	ER-Golgi traffic	↓ IL-6 secretion ↓ MHC-I, MHC-II, CD86
Leupeptin[8] 2	MHC-II / Vacuolar MHC-I	Cathepsin B (lysosomal aspartil-protease)	↓ IL-6 secretion ↑ FLH fragments >250 kDa ↓ MHC-II and CD86
Simvastatin[9] 3	MHC-II / Vacuolar MHC-I	GTPases (endosomal traffic)	↓ IL-6 secretion ↑ FLH fragments >250 kDa ↓ MHC-II
Epoxomycin[10] 4	Cytosolic MHC-I	Proteasome	↓ IL-6 secretion ↓ FLH fragments ~30-40 kDa ↓ MHC-II, MHC-I, CD86
Bafilomycin[11] 5	MHC-II/ Vacuolar MHC-I	Endosome-lysosome fussion	↓ IL-6 secretion ↓ FLH fragments ~30-40 kDa ↓ MHC-II, MHC-I, CD86

Figure 1. MHC-I and MHC-II dependent pathways. The targets of each inhibitor are indicated in red numbers (figure and table).

➢ **Pharmacological inhibitors of the MHC-I and MHC-II pathways decreased FLH-dependent IL-6 secretion in macrophages.**

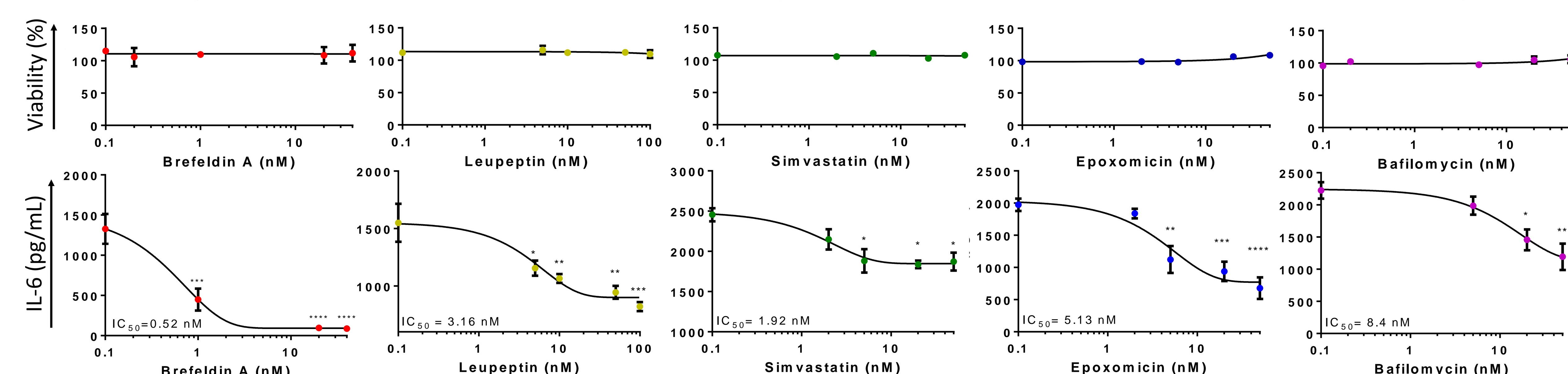


Figure 2. Effect of MHC-I and MHC-II pathway inhibitors on viability and IL-6 secretion in macrophages. J774.2 macrophages were treated with increasing concentrations of pharmacological inhibitors (0-100 nM) for 30 minutes, and then stimulated with FLH (0.5 mg/mL) for 24 hours. For cell viability assays (upper panels), cells were treated with AlamarBlue® 10% for 3 hours, and then absorbance was measured at 570 and 600 nm. For cytokine quantification (lower panels), cell culture supernatants were collected and analyzed using BD OptEIA kits, according to manufacturer's instructions. Data as mean ± SEM of 3 independent experiments. Statistical analyses were performed by one-way ANOVA, where $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

➢ **FLH upregulated MHC-I, MHC-II, and CD86 in macrophages.**

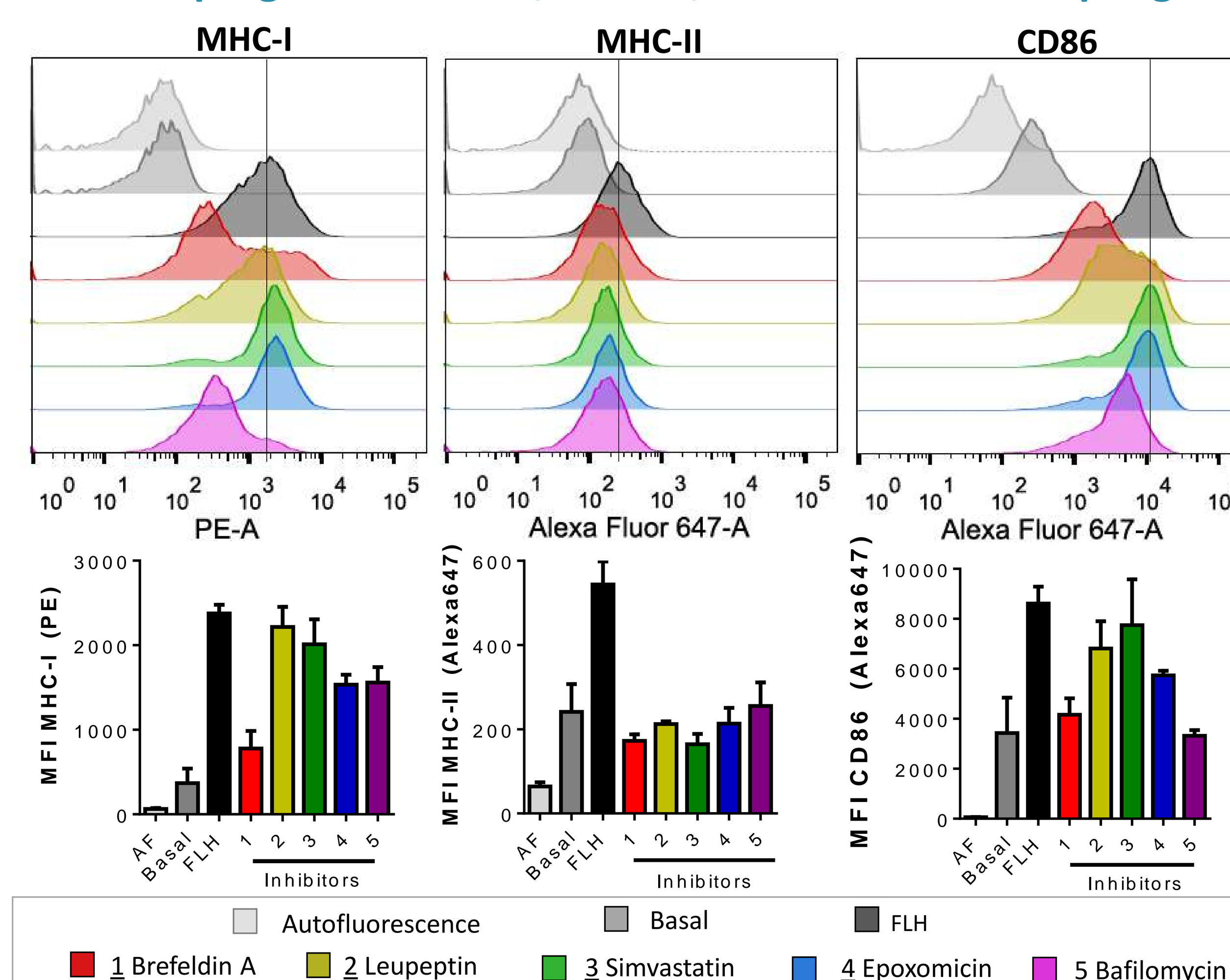


Figure 3. MHC-I, MHC-II, and CD86 upregulation induced by FLH in macrophages. J774.2 cells were treated with pharmacological inhibitors (concentration = IC_{50}) for 30 minutes, and then stimulated with FLH (0.5 mg/mL) for 24 hours. Then, cells were harvested and incubated with monoclonal antibodies (α-MHC-I-PE, α-MHC-II-Alexa647, α-CD86-Alexa647) for 1 hour. Cells were fixed and analyzed by flow cytometry. Upper panels: Representative histograms. Lower panel: Data as mean ± SEM of 2 independent experiments. Statistical analyses were not performed because N=2.

➢ **Inhibitors of both MHC-I and MHC-II pathways induced partial differences in FLH proteolysis.**

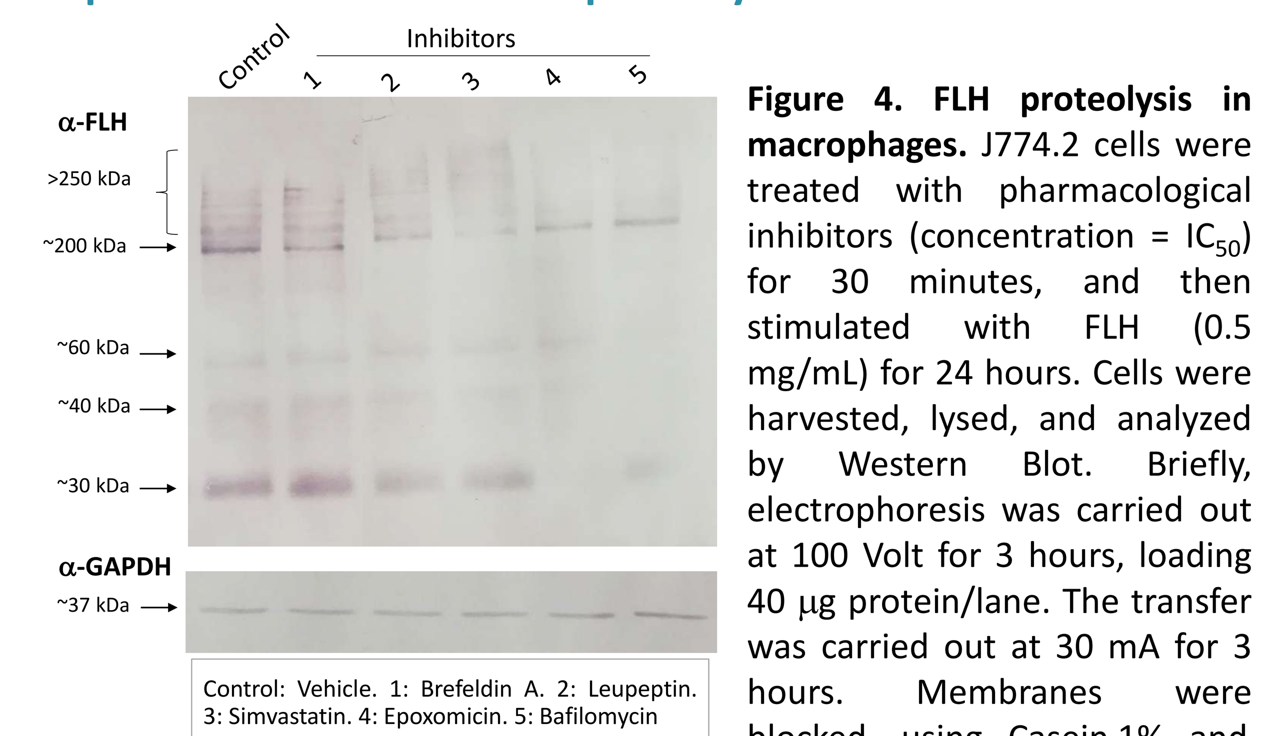


Figure 4. FLH proteolysis in macrophages. J774.2 cells were treated with pharmacological inhibitors (concentration = IC_{50}) for 30 minutes, and then stimulated with FLH (0.5 mg/mL) for 24 hours. Cells were harvested, lysed, and analyzed by Western Blot. Briefly, electrophoresis was carried out at 100 Volt for 3 hours, loading 40 µg protein/lane. The transfer was carried out at 30 mA for 3 hours. Membranes were blocked using Casein 1% and incubated with monoclonal antibodies α-FLH and α-GAPDH, and secondary α-mouse IgG coupled to alkaline phosphatase (AP). Bands were revealed using NBT-BCIP (cholorimetric). Representative result of 2 independent experiments.

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