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Cutting Edge: Nicastrin and Related Components of \( \gamma \)-Secretase Generate a Peptide Epitope Facilitating Immune Recognition of Intracellular Mycobacteria, through MHC Class II-Dependent Priming of T Cells

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Bacillus Calmette-Guérin (BCG), the antituberculosis vaccine, localizes within immature phagosomes of macrophages and dendritic cells (APCs), and avoids lysosomal degradation. BCG-derived antigenic peptides are thus inefficiently processed by APCs, and we investigated alternate mechanisms of Ag processing. Proteomics identified that BCG phagosomes are enriched for nicastrin, APH, and presenilin components of \( \gamma \)-secretase, a multimeric protease. Using an in vitro Ag presentation assay and BCG-infected APCs, we found \( \gamma \)-secretase components to cleave BCG-derived Ag85B to produce a peptide epitope, which, in turn, primed IL-2 release from Ag85B-specific T cell hybridoma. siRNA knockdown or chemical inhibition of \( \gamma \)-secretase components using L685458 decreased the ability of BCG or Mycobacterium tuberculosis-infected APCs to present Ag85B. In addition, L685458 inhibition of \( \gamma \)-secretase led to a decreased ability of BCG-dendritic cells to immunize mice and induce Ag85B-specific CD4 T cells in vivo. Because BCG and M. tuberculosis sequester within APCs preventing immune recognition, \( \gamma \)-secretase components appear to fortuitously process the immunodominant Ag85B, facilitating immune recognition. The Journal of Immunology, 2011, 187: 5495–5499.

Tuberculosis is the leading cause of death from an infectious disease, and bacillus Calmette-Guérin (BCG) is the only approved vaccine against human tuberculosis. BCG gives variable protection against tuberculosis depending on geographic region, and this is likely due to the loss of some immunogenic Ags encoded by the RD1 region, strain variation, and population immunity factors. Th1 immunity defends against tuberculosis and is dependent on the ability of APCs to process peptides from mycobacteria through lysosome- or proteasome-mediated degradation, and epitope presentation through MHC class II (MHC-II) or I (MHC-I), respectively, to CD4 and CD8 T cells. However, BCG vaccine sequesters within immature phagosomes of APCs, which do not fuse with lysosomes (1). Thus, we proposed earlier that BCG-infected APCs would be deficient in presenting MHC-II–dependent peptides to T cells. Initially, we found that phagosomes of BCG had near-neutral pH because of the exclusion of vATPase enzyme; as a consequence, intraphagosomal cathepsin-D (Cat-D) protease was inactive (2). Using a T cell hybridoma specific for the major secreted Ag85B, we then reported that BCG-infected APCs showed decreased Ag presentation (2). Interestingly, wild type Mycobacterium tuberculosis H37Rv phagosomes that also have near-neutral pH again showed reduced Ag presentation compared with the attenuated mutant, M. tuberculosis H37Ra. The wild type M. tuberculosis is the first pathogen reported to evade phagosome–lysosome fusion in macrophages (3). It is now evident that M. tuberculosis has multiple immune evasion mechanisms, and BCG vaccine seems to have inherited similar properties from wild type Mycobacterium bovis, the pathogen of bovine tuberculosis (4).

Tuberculosis is an important disease for which efficient vaccination strategies are required. We therefore investigated alternative mechanisms of Ag processing within macrophages that could lead to immune recognition of mycobacteria. BCG and M. tuberculosis phagosomes purified from macrophages were subjected to proteomic analysis that identified proteins relatively enriched on their phagosomes (5). We found that nicastrin, a component of the multimeric membrane-bound \( \gamma \)-secretase, was enriched on the phagosomes of the wild type M. tuberculosis, and to a lesser extent on BCG vaccine phagosomes. Because \( \gamma \)-secretase has been implicated in proteolysis and thought to recycle phagosomal proteins, we hypothesized..."
that it could cleave mycobacterial phagosome-derived proteins. We demonstrate in this article that nicastrin, presenilin, and anterior pharynx defective-1 (APH) components of γ-secretase generate a peptide epitope from BCG phagosome-derived Ag85B, which is subsequently presented to the T cells. Because this occurred in naive macrophages, we propose that the APCs have a novel, innate mechanism to produce antigenic peptides, which may fortuitously facilitate recognition of immune-evasive mycobacteria.

Materials and Methods

Ag presentation

C57BL/6 mice bone marrow-derived primary macrophages and dendritic cells (DCs) were cultured and infected with BCG (Pasteur) or wild type M. tuberculosis (both from ATCC) as previously described (6, 7). They were overlaid with BB7 T cells (1 APC:20 T cells) specific for Ag85B (kind gift of Dr. Harding, Case Western Reserve University), and IL-2 secreted in response to Ag was quantitated using ELISA. Blockade of γ-secretase was carried out using specific small interfering RNA (siRNA) sets (Supplemental Fig. 1) (Santa Cruz Biotechnology and Invitrogen) against nicastrin, APH, and presenilin. siRNA knockdown was carried out for 4 h followed by resting cells for 18 h before experimentation. siRNAs were a mix of two or three duplexes, and the controls were scrambled siRNA supplied also by the manufacturer (SCB). Transfection was done using the transfectamine kit supplied by SCB (sc-29528). The effect of siRNA or L685458 on the surface expression of γ-secretase components in the membrane fractions, compared with knockdown with scrambled sequence.

Immunofluorescence analysis for γ-secretase

APCs were infected with gfpBCG or gfpM. tuberculosis (H37Rv), washed, fixed, counterstained, and scored for immunofluorescence colocalization with primary Abs and isotypes for each of the γ-secretase components as per published procedures (2, 9, 10) (Supplemental Fig. 2). Peptide VANNTRLWVYCGNGT was used to spike treated or untreated APCs, to measure direct presentation by APCs to BB7 T cells (7).

Phagosome fractionation and Ag presentation

To assess organelle (phagosome)-mediated Ag presentation versus APC-mediated presentation after L685458 treatment, BCG phagosomes or inert latex beads (3-μM diameter) phagocytosed into APCs were purified using sucrose gradients as per previously standardized methods and estimated for protein content (8). Pellets of phagosomes estimated to contain 5 μg/ml protein were gently sonicated to disperse, checked by microscopy, mixed with BB7 T cells (10^6 T cells per 5 μg pellet of phagosomes in triplicate wells per experiment), and supernatants collected after 4 h were estimated for IL-2.

Membrane or cytosol localization for γ-secretase

Macrophages were treated or not with L685458 in 24-well plates, infected with BCG, and washed. Monolayers were washed with PBS, extracted with 0.05% digitonin, 0.5% sucrose in PBS-EDTA buffer for 15 min for cytosol fraction, which was then ultracentrifuged and estimated for protein. The monolayer was washed with cold 0.05% digitonin, 0.5% sucrose in PBS-EDTA three times, and the membrane fraction was solubilized with 0.1% Triton X-100 in PBS for 15 min, estimated for protein, and stored until Western blot analysis. Phagosomes localize into the membrane fractions in primary APCs (9).

Mouse experiments

BCG-infected DCs were used as vaccines per protocols described previously (10). Bone marrow-derived cells were cultured in DMEM with GM-CSF (10 ng/ml; Invitrogen) for 7 d, and column-purified DCs (CD11c beads from Miltenyi) were infected before or after treatment with L685458 (15 and 100 μM/10^6 DCs; 4 h) with BCG. The DCs were washed, and 2 × 10^6 DCs/mouse were injected i.p. into C57BL/6 mice (4 mice/group; 2 experiments). Untreated DCs injected into mice were negative controls. Viability of DCs before and after treatment was confirmed to be >90% using LDH release assay. After 2 wk, spleens were removed and IFN-γ CD4 T cells were detected using flow cytometry for intracellular cytokines, and by ELISPOT analysis with Ag85B restimulation (10). All animal procedures were conducted under approved institutional protocols.

Results

γ-Secretase components are enriched on mycobacterial phagosomes

Our proteomic study showed enrichment of nicastrin on wild type M. tuberculosis phagosomes, whereas others showed similar enrichment on BCG phagosomes (5, 11). Using immunofluorescence analysis, we identified the three γ-secretase components, relative to BCG phagosomes within APCs. Using in situ colocalization with deconvolution microscopy, we found Abs to nicastrin, APH, and presenilin to stain the phagosomes, with minimal speckled stain around the perinuclear zone (Fig. 1A, 1D). The positive control Ab LAMP1

![FIGURE 1](image-url)
stained most BCG phagosomes, whereas the isotype control did not stain a significant number of phagosomes (Fig. 1B). This observation was consistent with the Western blot profiles of BCG-infected primary macrophages, where the three components of γ-secretase were enriched on membrane fractions compared with cytosol (Fig. 1C, left panel). Membrane fractions of siRNA (SCB)-treated, BCG-infected macrophages were similarly tested using Western blotting. Specific siRNA knockdown reduced the levels of γ-secretase components compared with scrambled control sequence (Fig. 1C, right panel). In addition, BCG phagosomes purified from macrophages using sucrose gradients were found to have enzymatically active γ-secretase. They cleaved the amyloid precursor protein into an 87-kDa product (Fig. 1D). Similar studies were performed using DCs with identical results (not shown).

γ-secretase cleaves phagosome-derived Ag85B and generates peptide epitopes for Ag presentation

The proteolytic activity of γ-secretase is known and was found enriched on inert latex bead phagosomes of macrophages. It was proposed that γ-secretase could be involved in recycling and remodeling of phagosome proteins (12). Interestingly, both BCG vaccine and wild type M. tuberculosis secrete Ag85B and other immunogenic proteins that accumulate within the phagosomes (13–15). Furthermore, Ag85B has a major T cell epitope and is a powerful immunogen for mice and humans, either vaccinated with BCG or infected with M. tuberculosis (14). MHC-II–dependent presentation of a peptide epitope of Ag85B from mycobacteria-infected APCs has been shown to induce IL-2 secretion from Ag85B–specific T cell hybridoma in several studies (2, 6, 7). We therefore hypothesized that mycobacterial phagosome-derived Ag85B could be accessed and cleaved by γ-secretase components, and this event was measurable in vitro.

Because a specific chemical inhibitor for γ-secretase (L685458) has been used to block nicastrin activity, we initially tested the effect of L685458 on Ag presentation by APCs infected with the BCG vaccine. Fig. 2 shows that L685458 had a significant, dose-dependent, inhibitory effect on Ag presentation by both macrophages and DCs. Flow cytometry showed that L685458 did not affect the surface expression of MHC-II, CD80, or CD86 (Supplemental Fig. 2). Furthermore, when the Ag85B peptide was added to such cells, direct peptide presentation was comparable.

We previously reported that phagosomal Ag85B production could occur because of the action of Cat-D and pepstatin: an inhibitor of cathepsin proteases inhibited Ag presentation. To further determine whether both Cat-D and γ-secretase contributed to Ag85B epitope production, we blocked APCs with pepstatin to inhibit Cat-D and L685458 to inhibit γ-secretase. Fig. 2 indicates that each agent inhibited peptide presentation and their combination had an additive effect. Pepstatin either alone or in combination did not affect the surface expression of MHC-II, CD80, or CD86 (data not shown). We therefore propose that Cat-D, an intraphagosomal, acid pH-dependent protease, and membrane-bound γ-secretase, which works best at neutral pH, both produce Ag85B-derived peptide in and around the phagosomes.

siRNA blockade of γ-secretase affects Ag production in macrophages and DCs

Nicastrin and presenilin have been reported to possess protease activity on their own (16). To further evaluate whether γ-secretase components vary in their protease activity, we conducted siRNA knockdown experiments. APCs were treated or not with siRNA against nicastrin, APH, and presenilin, followed by BCG or M. tuberculosis infection and Ag presentation. Fig. 3A (BCG) and Fig. 3B (wild type M. tuberculosis) show that siRNA knockdown of γ-secretase components affected Ag presentation by APCs infected with BCG and M. tuberculosis. Control scrambled siRNA had no significant effect on Ag presentation. siRNA probes from two different sources had comparable effects on Ag presentation (Fig. 3, Supplemental Fig. 3). Furthermore, BCG phagosomes but not inert latex beads purified from L685458-treated or untreated macrophages were able to present Ag85B to BB7 T cells. L685458 significantly suppressed Ag presentation (Fig. 3C).

L685458 treatment of DCs affects subsequent in vivo immunogenicity

We previously demonstrated that DCs infected with BCG vaccine elicit immune responses in mice after vaccination, which are protective against subsequent challenge with virulent tuberculosis (10). Furthermore, Ag85B protein or its DNA vaccine offers significant protection against tuberculosis in mice. These two concepts provided a model to examine the effect of γ-secretase–mediated Ag85B–epitope production on the immunogenicity of BCG-infected DCs. DCs were treated or not with L685458, infected with BCG, and injected into mice. Two weeks later, splenic T cells were assayed for Ag85B-specific responses of T cells secreting IFN-γ, using flow cytometric analysis and ELISPOT analysis of spleens. Fig. 4A illustrates that DCs infected with BCG elicited markedly better IFN-γ–CD4 T cell response in mouse spleens compared with L685458-treated DCs with BCG. Similarly, ELISPOT analysis of corresponding spleens showed that L685458-treated DCs induced lesser numbers of Ag85B-specific CD4 T cells (Fig. 4B). These data strongly suggest that γ-secretase plays a significant role in generating a peptide epitope from phagosome-associated Ag85B, and this event helps immune recognition of mycobacteria, which are otherwise sequestered within macrophages and DCs.
Discussion

Because wild type *M. tuberculosis* and BCG vaccine sequester within immature phagosomes of APCs avoiding lysosomal degradation, mechanisms of peptide-mediated priming of T cells have been thought to be suboptimal during tuberculosis or vaccination. Sequestration enables *M. tuberculosis* to be immune-evasive, whereas BCG would become less immunogenic (17). Until recently, however, the mechanistic basis of immune sequestration remained unclear. Significantly, two studies proposed a new concept that phagosomes themselves are capable of assembling peptide epitopes into MHC-II and MHC-I for eventual presentation to T cells. Harding’s group demonstrated that the immunodominant, secreted Ag85B is cleaved and presented by mycobacterial phagosomes (6). However, mycobacterial phagosomes from human macrophages were also found capable of presenting peptides through MHC-I (18, 19).

Because Ag85B is by itself highly immunogenic and used as a vaccine, we addressed the mechanisms of in situ epitope generation using BCG-infected macrophages and Ag85B-specific T cell hybridoma (20, 21). Although several proteases were found at trace levels, Cat-D was found markedly enriched in BCG phagosomes. Using siRNA knockdown studies, we then demonstrated that Cat-D–mediated proteolysis leads to Ag85B-derived peptide epitope, which, in turn, primes BB7 T cells. However, the efficacy of Cat-D, an acid pH-dependent aspartic acid protease was reduced because of the near-neutral pH of the BCG phagosome lumen. We also reported that BCG excluded the acidifying enzyme, vacuolar proton ATPase relative to *M. tuberculosis* H37Ra, and the phagosomes of the latter had more active Cat-D. As a consequence, macrophages infected with *M. tuberculosis* H37Ra were better able to activate BB7 T cells to secrete IL-2.

We therefore concluded that intraphagosome generation of Ag85B epitope is regulated by the pH. This is consistent with the known mechanism of peptide production in lysosomes where the acidifying enzyme vATPase reduces the pH to activate several proteases. Unexpectedly, the proteomics of BCG phagosomes revealed the presence of γ-secretase, and in this study, we demonstrated that phagosome-associated Ag85B is also processed by γ-secretase. Our studies therefore propose a novel observation that mycobacterial phagosomes have at least two mechanisms of peptide generation, one of which is acid pH dependent and the other is a membrane-associated, acid pH-independent process.

This model appears consistent with the known cell biology of BCG vaccine and *M. tuberculosis* phagosomes. Although BCG and *M. tuberculosis* pathogen have adapted to survive in...
an intracellular niche avoiding the lysosome, the host seems to have adapted to deliver proteases into phagosomes to facilitate immune recognition. Although the characterization of membrane-bound γ-secretase has been difficult, Jutras et al. proposed that it may perform a physiological function of recycling phagosome proteins (12, 16). We propose that during this natural recycling process, γ-secretase fortuitously produces peptide epitopes from bacteria enclosed within phagosomes. There is an intriguing possibility that γ-secretase may also produce shorter peptide fragments, and downstream events may channel them toward the CD8 pathway. It is conceivable that it may also cleave proteins from other intraphagosomal pathogens.

We therefore propose that APCs have an innate mechanism to generate immune signals from mycobacterial phagosomes that otherwise remain evasive. It is pertinent to note in this article that strategies to bypass immune sequestration have generally enhanced the immunogenicity of BCG vaccine. Thus, expression of a pH-independent, active Cat-S in BCG led to increased Ag85B presentation by macrophages infected with recombinant BCG–Cat-S (22). Expression of the recombinant pore-forming toxin listeriolysin in BCG enabled Ags to leak into cytosol across the phagosome membrane and enhanced the ability of recombinant BCG–LLO to induce CD8 T cells in mice (23). Finally, enhanced localization of BCG into autophagolysosomes through rapamycin treatment of APCs led to increased Ag85B presentation and enhanced immunogenicity in mice (10, 23, 24).

The enrichment of nicastrin on mycobacterial phagosomes was initially identified using proteomics of phagosomes (5, 11). These studies highlighted that the mycobacterial phagosomes are complex structures that contain several hundred proteins and the daunting task of functional characterization. This study illustrates the use of a system biology approach to dissect the function of a phagosome-associated protein. We anticipate that a similar dissection of other novel phagosome proteins will lead to a better understanding of mycobacterial immunogenicity.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Figure-S1:** siRNA sequences from San Cruz Biotechnology (left). Probes from Invitrogen were also tested for inhibition of antigen presentation and data are shown below, right.

- **Aph siRNA** is a pool of 2 different siRNA duplexes:
  - Sense: GGUUUGCGUUGUCCAUUUUt
  - Antisense: AAAUGGACACGCGCAAACGtt
  - Sense: CAUCAUAGUGUGUACUUt
  - Antisense: AGUUACACCACUAUGUAGGtt

- **Nicastrin siRNA (m)** is a pool of 3 different siRNA duplexes:
  - Sense: CGUAGUGGAGAAAGAAGAAtt
  - Antisense: UUCUUCUUUCUCCACUGCtt
  - Sense: CAACGGCUUGGCUUAAGAAtt
  - Antisense: UUCUUCUUUCUCCACUGCtt
  - Sense: GUGACCCCUUAUCUGACUAtt
  - Antisense: UAGUCAGAUAAGGGGUCACtt

- **Presenilin siRNA (m)** is a pool of 3 different siRNA duplexes:
  - Sense: GAGUGGAGCUAGAGAUAAGAAtt
  - Antisense: UCUAUCUCUACUGACUAtt
  - Sense: CCAGAAUGACAGCCAAGAAtt
  - Antisense: UUCUUCUUUCUCCACUGCtt
  - Sense: CUACGUGCCACUAUUGGtt
  - Antisense: UGAUUGGUUGGACACGUAAGt

The sequence of Control siRNA-A is:

- Sense: UUCUCCGAACGUGUCAGCUAtt
- Antisense: ACGUGACCCGUUGUGAGAAC ATT
Supplemental Figure-S2: siRNA or L685 treatment of macrophages does not affect the surface expression of molecules involved in antigen presentation. Macrophages were treated with siRNA vs. nicastrin (purple), APH (black) or presenilin (blue) and then infected with BCG. Histogram top row: After 24 hrs, macrophages were tested for receptors or their isotype using flow cytometry. DCs showed similar profiles (not shown). Histogram Bottom row: BCG alone (green), L685 at 15 (blue), 100 (black) and 500 μM (purple) doses tested against macrophages followed by BCG infection. Bar graph: Ag85B-peptide (sequence in Methods) was pulsed to triplicate wells of siRNA or L685 (100μM) treated macrophages or DCs in culture and evaluated for antigen presentation.

c) Antibodies used in this study. Antibody to Nicastrin (N-19) sc-14369; Presenilin-1 (N-19) sc1245; APH-1 (N-20) sc-30240; Lamp-1 (1D4B) sc-19992; Goat IgG Isotype (sc-3887); Rat IgG isotype sc-3883; Texas-red donkey anti-goat IgG conjugate- (sc-2033), Texas red goat anti-rat IgG (LAMP1)-(sc-2032). d) Antibodies used for flow cytometry staining were: MHC-II 12-5322; CD80 12-0801; CD86 12-0862 (all from Ebioscience); Isotype FITC Ebioscience- 11-4011-85.