A novel toxin for M. tuberculosis

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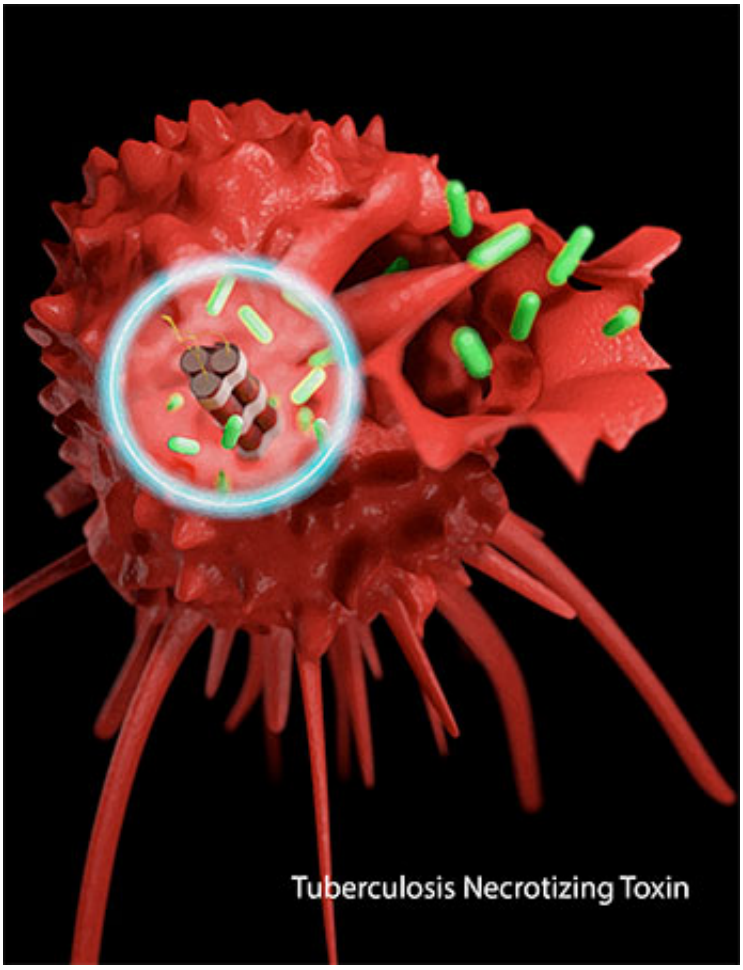

In this illustration, the Tuberculosis Necrotizing Toxin — symbolized by the explosives — has caused necrotic cell death and release of the tuberculosis bacteria from the macrophage. Credit: Mathew Schwartz (Advanced Institutes of Convergence Technology, SNU)

Despite 132 years of study, no toxin had ever been found for the deadly pathogen Mycobacterium tuberculosis, which infects 9 million people a year and kills more than 1 million.

Now, Michael Niederweis, Ph.D., professor of microbiology at the University of Alabama at Birmingham, and colleagues have described the first known toxin of this pathogenic bacterium. This toxin—Tuberculosis Necrotizing Toxin, or TNT—is the founding member of a novel class of previously unrecognized toxins present in more than 600 bacterial and fungal species, as determined by protein sequence similarity. Before the Niederweis discovery, those toxins were identified only as the "Domain of Unknown Function 4237."

Bacteria with those newly recognized toxins include Yersinia pestis, the pathogen that caused the bubonic plague known as the Black Death in Medieval Europe, and Listeria monocytogenes, one of the most virulent and deadly food-borne infections and the cause of Blue Bell Creameries recalls this year.

The lack of an identified toxin in M. tuberculosis had contrasted with nearly all other pathogenic bacteria whose toxins contribute to illness or death.

M. tuberculosis is notable for its survival inside macrophages, the immune cells that ingest and destroy infectious bacteria. The newly identified TNT, Niederweis says, plays a key role to induce necrotic death of the infected macrophage. Thus, TNT enables the M. tuberculosis bacteria to escape from the macrophage and disseminate to other host cells in a person infected with tuberculosis, thus contributing to the survival of M. tuberculosis and spreading the disease.

"The battle between M. tuberculosis and the human immune system to control the fate of infected macrophages is critical in determining the

outcome of the infection," Niederweis wrote in the TNT paper. "The control of host cell death is of utmost importance for the survival, escape and dissemination of M. tuberculosis."

The paper, "The tuberculosis necrotizing toxin kills macrophages by hydrolyzing NAD," was published online Aug. 3 in *Nature Structural & Molecular Biology*.

How did this toxin evade discovery for more than a century? First, it is produced in vitro only in very small quantities—the Niederweis lab could detect it only in a cell culture filtrate that was concentrated 1,000-fold, equivalent to concentrating a gallon of milk to about one-third of a teaspoon. Second, the toxin is deadly only when it is inside the host-cell cytosol; if the toxin is in the bloodstream or is added to the culture medium of in vitro host cells, it has no effect. Third, the toxin has no similarities to any other known toxin.

Niederweis discovered TNT while searching for something completely different. He was hunting for outer-membrane proteins that can act as a door to let nutrients outside the bacteria pass through the extremely impermeable, outer-membrane barrier of M. tuberculosis. The Niederweis group thought they had found such a porin protein; but it had an unusual property—the end portion of the protein broke off after the pore formed in the outer membrane, and that end portion was extremely toxic, both in simple prokaryotic cells like bacteria and in the more complex eukaryotic cells of yeast, mammals and fish. In a paper published in *Proceedings of the National Academy of Sciences* in 2014, Niederweis said this discovery "challenges the paradigm that M. tuberculosis is one of few bacterial pathogens that does not produce toxins."

The current paper fully establishes this new paradigm by identifying the mechanism of TNT-induced necrotic cell death at the functional and

structural levels. Like an optical illusion where at first one sees a vase, and it then appears to be two faces peering at each other, Niederweis initially he believed he had found an outer-membrane porin that lets nutrients in and carried an artefact. Now he sees the pore part of that protein as a bacterial autotransporter (similar to those seen in other bacteria) that exports its TNT protein cargo to the outside of the outer membrane. After that export is done, the transporter pore remains in the outer membrane.

The similarity of the tnt gene to DNA sequences in more than 600 other bacterial and fungal species will enable research on how this novel class of toxins may function in other pathogens, especially in microorganisms that depend on induction of necrosis to survive or spread.

In a laborious search for the molecular function of TNT, Jim Sun, Ph.D., a postdoc in the Niederweis lab, found that TNT hydrolyzes the essential co-enzyme nicotinamide adenine dinucleotide (NAD+). This explains why it kills every type of cell it is cloned into, because NAD+ is necessary for the cell's normal metabolism. Researchers were able to clone the TNT gene only by placing it next to an inducible promoter that tightly represses transcription until induced. The TNT enzyme hydrolyzes NAD+ inside of cells and in vitro. It is blocked by antibodies against TNT, and specific TNT point mutations that eliminate all enzymatic activity.

That noncatalytic TNT mutant is not able to kill macrophages, showing that the hydrolase activity is required for TNT-induced cell death.

If TNT were produced inside M. tuberculosis, it would kill the cell. Niederweis and colleagues found that M. tuberculosis, similar to the bacterial pathogen Streptococcus pyogenes, produces an antitoxin to its toxin. The TNT antitoxin binds to the toxin and blocks its hydrolase activity, thus making it harmless inside the bacteria. The researchers

have named the antitoxin immunity factor for TNT (IFT).

Cloning the genes for both TNT and IFT into E. coli, where IFT protects the bacteria from death, allowed the researchers to produce milligram quantities of TNT and IFT. In a collaborative effort, Gino Cingolani, Ph.D., a professor from the Thomas Jefferson University, produced crystals of the purified protein complex and determined its molecular structure to an astonishing resolution of 1.1 Å in a matter of weeks. The TNT molecule is shaped like a grasping hand, with fingers on one side and an extended thumb on the other. The IFT fits into the TNT like a ball held in a hand.

When pathogenic M. tuberculosis grows inside a macrophage phagosome, the TNT rapidly gains access to the cytosol of the infected macrophage and hydrolyzes NAD+, depleting that essential co-factor. This initiates necrotic cell death through downstream signals that are not yet characterized.

Curiously, a literature search revealed that an uncharacterized, heat-stable NAD+-glycohydrolase activity in M. tuberculosis cell extracts had been described half a century ago, as well as an uncharacterized heat-labile inhibitor of that hydrolase activity. Several biochemical characteristics of TNT and IFT found by the Niederweis lab match those of the uncharacterized proteins described in the reports from the 1960s.

However, the lack of the modern equipment and antibodies of today, and the very low levels of TNT present in M. tuberculosis, prevented those researchers from finding the toxin.

More information: "The tuberculosis necrotizing toxin kills macrophages by hydrolyzing NAD." *Nature Structural & Molecular Biology* (2015) DOI: 10.1038/nsmb.3064

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