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TAM receptors are dispensable in the phagocytosis and killing of bacteria ☆

By Julie C. Williams [a], Robin R. Craven [b], H. Shelton Earp [c], Tom H. Kawula [b], Glenn K. Matsushima [a,b,d,*]

[a] Curriculum in Oral Biology, UNC Neuroscience Center CB#7250, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[b] Department of Microbiology and Immunology CB#7290, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[c] Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[d] Program for Molecular Biology & Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

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ABSTRACT

Many receptors that are employed for the engulfment of apoptotic cells are also used for the recognition and phagocytosis of bacteria. Tyro3, Axl, and Mertk (TAM) are important in the phagocytosis of apoptotic cells by macrophages. Animals lacking these receptors are hypersensitive to bacterial products. In this report, we examine whether the TAM receptors are involved in the phagocytosis of bacteria. We found that macrophages lacking Mertk, Axl, Tyro3 or all three receptors were equally efficient in the phagocytosis of Gram-negative *E. coli*. Similarly, the phagocytosis of *E. coli* and Gram-positive *S. aureus* bioparticles by macrophages lacking TAM receptors was equal to wild-type. In addition, we found that Mertk did not play a role in killing of extracellular *E. coli* or the replication status of intracellular *Francisella tularensis*. Thus, while TAM receptors may regulate signal transduction to bacterial components, they are not essential for the phagocytosis and killing of bacteria.

1. Introduction

During the course of a bacterial infection, innate immune cells such as neutrophils and macrophages sense bacteria and bacterial products via pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) or peptidoglycan. PAMPs are recognized by several pattern recognition receptors on innate immune cells, including the Toll-like receptors (TLR) [1]. Following recognition, the resident cells release pro-inflammatory cytokines and chemokines to recruit neutrophils and additional macrophages to the lesion. These responding neutrophils and monocytes arrive equipped as professional phagocytes to quickly ingest and kill the invading bacteria. This rapid immune response and phagocytosis of bacteria is critical to the resolution of a bacterial infection.

Many other receptors are implicated in the recognition and phagocytosis of bacteria including: Scavenger receptor A, Mannose receptor, TLRs, integrins, as well as Fc receptors and complement receptors [2,3]. Upon binding of bacteria, these receptors signal a rearrangement of the actin cytoskeleton that is mediated by the Rho family of GTPases, Rac, Rho, and Cdc42, ultimately leading to engulfment of the bacteria [4]. One indication of the importance of these molecules is evident in that some bacteria attempt to evade phagocytosis by producing molecules that modulate the signaling to and from the Rho GTPases [5,6].

Phagocytosis is not only important for controlling infection, it is also key in down-regulating inflammation and limiting tissue damage by returning the infected area to proper homeostasis. This is partly accomplished by the phagocytosis of apoptotic cells. As bacteria grow and/or infect cells, these cells often succumb to the infection and die, either indirectly by toxic bacterial by-products or directly by intracellular pathogens. For example, neutrophils have been shown to become apoptotic after treatment with *E. coli* [7]. Apoptosis is a form of cell death that has been well characterized and includes exposure of phosphatidylserine from the inner membrane to the outer surface of the dying cell. Subsequently, these apoptotic cells are cleared from the infected area typically by macrophages. Interestingly, many of the same receptors that facilitate phagocytosis of bacteria are also important for the phagocytosis of apoptotic cells including CD14, Scavenger receptor A, Fc Receptor, complement, and integrins [8,9].

The Tyro3/Axl/Mertk (TAM) receptors have been shown to be required for efficient phagocytosis of apoptotic cells [10,11]. All three receptors are expressed by macrophage and dendritic cells (DC), however, Mertk is also expressed by NK cells, but is not expressed on neutrophils or non-malignant lymphocytes [11–13]. These proteins are receptor tyrosine kinases that are similar in structure and function. Each family member has two Ig-like and two fibronectin III-like extracellular domains, as well as a characteristic KWAIAES sequence in the intracellular domain [12,14]. In macrophages, Mertk is essential for rapid ingestion of apoptotic

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* Corresponding author. Address: UNC Neuroscience Center CB#7250, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA. Fax: +1 919 966 1050.
E-mail address: gkmats@med.unc.edu (G.K. Matsushima).

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cells [10]; though Axl and Tyro3 are partly involved [11]. In contrast, Axl and Tyro3 appear to function in DC and Mertk has no role in phagocytosis although the process is much slower [11,15]. Knock-out mice have been generated for all three family members [16,17]. The inability to properly clear apoptotic cells in these knock-out mice results in the development of a lupus-like autoimmunity characterized by autoantibody production as well as splenomegaly [10,15,18]. Gas6, a ligand for the TAM family members, binds these receptors with a relative affinity of Axl > Tyro3 >> Mertk [19]. Gas6 is produced by several cell types including macrophages, DC and apoptotic thymocytes [11,20]. Another ligand for the family member Tyro3 is the serum protein, Protein S [21]. Both Gas6 and Protein S bind phosphatidylserine on apoptotic cells and it is through this molecular bridging interaction that the TAM family members bind apoptotic cells [22].

In addition, to their role in phagocytosis of apoptotic cells, the TAM family members have also been shown to have a role in the innate immune response. Mice lacking functional Mertk (*mertk*$^{-/-}$) are hypersensitive to Gram-negative bacterial LPS and are susceptible to endotoxic shock that is mediated by prolonged NF-κB activity resulting in over production of TNFα [16]. This regulation of NF-κB by Mertk shown in macrophages has been corroborated in DC and the phagocytosis of apoptotic cells by DC appears to be important for self tolerance in a model of diabetes [23]. Furthermore, knock-out mice lacking all three TAM family members (TAM$^{-/-}$) mice also displayed an increase in serum TNFα levels following LPS injection [18] and DC from TAM$^{-/-}$ mice have been shown to have hyper-immune responses to TLR ligands [24]. Interestingly Axl and Interferon receptor appear to cooperate to modulate STAT, inhibiting the innate immune response to TLR mediated activation by the induction of SOCS1 [24]. However in these studies, the response to whole bacteria and importantly, phagocytosis of bacteria was not thoroughly examined.

Previous studies that have investigated the involvement of TAM receptors in phagocytosis of bacteria have provided conflicting results [10,18]. Macrophages isolated from *mertk*$^{-/-}$ mice were shown to phagocytize the Gram-positive bacterium *L. monocytogenes* similarly to wild-type [10]. However, the TAM$^{-/-}$ macrophages displayed an increased ability to phagocytize Gram-negative *E. coli* [18].

Based on the importance of TLRs for phagocytosis, the ability of the Mertk to regulate LPS signaling, and the increase in phagocytosis of *E. coli* by the TAM$^{-/-}$ macrophages, we sought to determine whether Mertk regulated phagocytosis of the Gram-negative bacterium, *E. coli*. In this study we demonstrate, by multiple methods, that individually or collectively the TAM family members are not essential for phagocytosis of *E. coli* by thioglycollate-elicited macrophages. In addition, we show that individual TAM receptors are also not important for phagocytosis of bacteria by resident and bone marrow-derived macrophages. Complementary to the lack of a role in phagocytosis, we show that Mertk is not essential for killing of *E. coli* or modulation of growth of the intracellular pathogen *Francisella tularensis*.

2. Methods

2.1. Animals

Mice were housed in a specific pathogen-free facility and maintained according to the UNC-Chapel Hill Institutional Animal Use and Care Committee guidelines. *mertk*$^{-/-}$ mice were developed here at UNC. *mertk*$^{-/-}$ is the updated nomenclature which was described previously as *mer*^{kd} or *mertk*^{kd}[10,11]. Although the cytoplasmic kinase domain was targeted, the lack of protein expression makes these mice null for Mertk. *axl*$^{-/-}$, and *tyro3*$^{-/-}$ mice were kindly provided by Dr. Stephen P. Goff (Columbia University, New York) and Dr. Greg Lemke (Salk Institute for Biological Studies, San Diego) respectively. TAM$^{-/-}$ mice were generated by breeding of backcrossed single knock-outs. All genotypes were backcrossed at least 6 generations to the C57BL/6J background. Male mice 8-12 weeks old were used in all studies. Wild-type mice used were strain C57BL/6J.

2.2. Macrophages

Thioglycollate-elicited macrophages were obtained as previously described[10]. Briefly, 3 mL of 3% thioglycollate was injected intraperitoneally (I.P.) 3 days prior to harvest by peritoneal wash in PBS. Cells were washed 3 times in PBS and allowed to adhere for 3 hours in media (RPMI 1640 (Gibco) supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate, 2 x 10^{-5} 2-ME, 10 mM HEPES, 50 units penicillin G and 50 μg/mL streptomycin sulphate). Non-adherent cells were washed off and fresh media was added. In order to eliminate activity due to stimulation by thioglycollate, macrophages were allowed to rest at 37°C 5% CO_2 for seven days prior to experimental use.

Resident peritoneal macrophages were obtained by 60 seconds of gentle peritoneal lavage in Versene. Cells were removed, washed and allowed to adhere onto glass coverslips in 24 well plates or plastic tissue culture dishes. Non-adherent cells were washed off which resulted in a highly enriched population of resident macrophages.

Bone marrow-derived macrophages were obtained by harvesting bone marrow from mouse femurs. Cells were flushed from femurs, washed and incubated overnight in DMEM-H (Gibco) supplemented with 50 units penicillin G, 50 μg/mL streptomycin sulphate and 10 mM HEPES. Non-adherent cells were removed and plated in media containing 10 ng/mL recombinant mouse M-CSF (Peprotech). Fresh media was added on day 4. Macrophages were harvested on day 8 and re-plated at appropriate density for experimental use on day 9. Bone marrow-derived macrophages were confirmed to be 100% CD11b-positive and 100% CD11c-negative by flow cytometry (data not shown).

2.3. Phagocytosis Assays

Phagocytosis assays were preformed using a MOI of 10 *E. coli* 0111:B4 (ATCC) transformed with green fluorescent protein plasmid (pEGFP; Clontech). Green fluorescent protein (GFP)-containing *E. coli* (*E. coli* O111:B4-GFP) were added to antibiotic-free, serum-free media and aliquoted into wells containing macrophages on glass coverslips. Macrophages were allowed to phagocytize bacteria for the indicated time periods. Macrophages were then washed 5 times in PBS and 0.2% trypan blue was added to wells to quench fluorescence from extracellular bacteria. Cells were then fixed in 2% paraformaldehyde and coverslips were mounted on slides with Vectamount mounting media for fluorescence (Vector). Phagocytosis was determined by overlays of bright field and GFP images captured using an Olympus fluorescent microscope equipped with a DP70 digital camera and Image Pro Plus software (Opelco). At least 100 macrophages were counted per time point with triplicate samples per genotype.

Fluorimetric phagocytosis assays were also preformed. Briefly, cells were plated in 96-well plates and a MOI of 10 bacteria in serum-free and antibiotic-free media was added to each well. After indicated time periods, cells were washed as described above and trypan blue was added to quench any extracellular fluorescence. Plates were then read on a Fluoroskan Ascent FL fluorimeter (Thermo Labsystems) according to manufacturers instructions. *E. coli* and *S. aureus* Bioparticles used for fluorimetric assays were obtained from Molecular Probes. Normalized fluorescent intensity