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Research Paper

Staphylococcus aureus Promotes Smed-PGRP-2/Smed-setd8-1 Methyltransferase Signalling in Planarian Neoblasts to Sensitize Anti-bacterial Gene Responses During Re-infection

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ABSTRACT

Little is known about how organisms exposed to recurrent infections adapt their innate immune responses. Here, we report that planarians display a form of instructed immunity to primo-infection by *Staphylococcus aureus* that consists of a transient state of heightened resistance to re-infection that persists for approximately 30 days after primo-infection. We established the involvement of stem cell-like neoblasts in this instructed immunity using the complementary approaches of RNA-interference-mediated cell depletion and tissue grafting-mediated gain of function. Mechanistically, primo-infection leads to expression of the peptidoglycan receptor *Smed-PGRP-2*, which in turn promotes *Smed-setd8-1* histone methyltransferase expression and increases levels of lysine methylation in neoblasts. Depletion of neoblasts did not affect *S. aureus* clearance in primo-infection but, in re-infection, abrogated the heightened elimination of bacteria and reduced *Smed-PGRP-2* and *Smed-setd8-1* expression. *Smed-PGRP-2* and *Smed-setd8-1* sensitize animals to heightened expression of *Smed-p38 MAPK* and *Smed-morn2*, which are downstream components of anti-bacterial responses. Our study reveals a central role of neoblasts in innate immunity against *S. aureus* to establish a resistance state facilitating *Smed-sted8-1*-dependent expression of anti-bacterial genes during re-infection.

1. Introduction

Staphylococcus aureus persistently colonizes the skin and mucosa of 20% of the human population and is a major cause of severe infections (Lowy, 1998; Edwards and Massey, 2011). There has been a dramatic increase in antibiotic-resistant *S. aureus* linked to chronic infections, emphasizing the importance of characterizing the molecular basis of the immune mechanisms involved in re-infection. Vertebrate immunity relies on a first-line innate immune defence of low specificity that induces parallel highly specific adaptive responses that persist over long periods of time as a type of immune memory (Delves et al., 2006; Murphy et al., 2014; Moret and Siva-Jothy, 2003). Vertebrates, such as mice, that lack B and T cells are protected against secondary infections after primo-infection or vaccination (Bistoni et al., 1986, 1988). A few studies have revealed the existence of different forms of innate immune memory in vertebrates and invertebrates, defined as heightened innate immune responses, against previously encountered pathogens (Netea et al., 2016; Milutinovic and Kurtz, 2016). This so-called trained immunity has attracted much attention given its potential as a strategy to promote immune resistance against pathogens. To date, several ways have been defined to use the trained immunity concept as novel therapeutic approach to fight infectious diseases. Vaccines which combine adaptative immune memory and trained immunity, or the use of inducer of trained immunity as vaccine adjuvant (Netea et al., 2016).

Trained immunity in vertebrates involves epigenetic reprogramming through histone post-translational modifications, particularly by histone methyltransferases, that enhance the expression of antimicrobial genes during re-infection (Netea et al., 2016; Pereira et al., 2016). Interestingly, most studies in this field have been performed

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in vitro with differentiated immune cells such as monocytes, macrophages or natural killer cells (Netea et al., 2016; Pereira et al., 2016). However, these cell types are already trained for immune function. Investigating the details of innate immune memory in undifferentiated cell types, such as stem cell-like neoblasts, is more challenging. Planarians are a classic model system for the study of adult wound healing and tissue regeneration (Reddien, 2013; Elliott and Sanchez Alvarado, 2013). These free-living members of the phylum Platyhelminthes contain a persistent pool of adult pluripotent stem cells, termed neoblasts, that are capable of producing all cell types and regenerating all types of tissues (Wagner et al., 2011). The ablation of neoblasts via irradiation or specific RNA-based depletion of essential gene products compromises the regenerative capacity of these animals (Reddien et al., 2005b).

Planarians represent a remarkable system with an unmatched capacity to fight infectious agents, including *S. aureus*, indicating the presence of remarkably efficient but uncharacterized innate immunity (Abnave et al., 2014). Notably, several new components of the innate immune system that are conserved in humans and absent from Ecdysozoa (e.g., flies and nematodes) were discovered by studying this model organism (Abnave et al., 2014). The varied abilities of planarians underscore the value of studying microbial defences in this model organism to identify components that are common between innate immunity and regeneration processes.

2. Material and Methods

2.1. Planarians

Schmidtea mediterranea (asexual clonal line ClW4) were maintained at 20 °C in static culture in autoclaved water as previously described (Cebria and Newmark, 2005). The water was changed every two days, and the planarians were maintained without antibiotics. The animals were fed once per week with homogenized calf liver and were starved for at least 1 week prior to experiments.

2.2. Bacterial Strains

Staphylococcus aureus (ATCC25923) was grown on blood agar plates (BioMerieux SA, Fr). *Legionella pneumophila* (ATCC33152) was grown on buffered charcoal yeast extract (BCYE) agar (Oxoid). *Mycobacterium avium* (BAA-535) was grown on 7H10 agar (Becton Dickinson).

2.3. Worm Feeding With Bacterial Pathogens

For all experiments, planarians were infected with 1×10^9 CFU of *S. aureus*, *L. pneumophila*, or *M. avium* as previously described. The planarians were fed with the bacterial pathogens using a protocol adapted from a dsRNA feeding method (Reddien et al., 2005a; Abnave et al., 2014). Briefly, bacterial pellets containing 1×10^9 CFU were suspended in 30 μL of homogenized calf liver, mixed with 15 μL of 2% ultra-low-gelling-temperature agar and 0.7 μL of red food colouring, and allowed to solidify on ice. Room temperature (RT) solidified food was fed to the planarians. After 6 h (defined as day 0, primo-infection) of feeding, the planarians were washed extensively and used for experiments. Thirty days later, the planarians that underwent primo-infection were re-infected with the same bacterial species at the same concentrations used for the primo-infection and then processed as described above. For some experiments, planarians were treated 24 h prior to infection with 1 mM 5′-deoxy-5′-(methylthio) adenosine (MTA, Sigma). The MTA was maintained throughout the duration of the experiments.

2.4. CFU Counting

Planarians were collected and homogenized in 20 μL of phosphate-buffered saline. The lysate was passed 5 times through a sterile syringe with a 29 G needle to disrupt planarian tissue clumps, and CFUs were counted after plating 10-μL serial dilutions onto agar plates containing appropriate bacterial growth media.

2.5. Gene Prediction and Phylogenetic Tree Construction

Smed-PGRP-2, a homologue of *hs-PGLYRP-2*, was identified using the SmedGD genome database (http://smedgd.neuro.utah.edu/, parameters e-value 1e-5) (Robb et al., 2015) with the sequence Smed Unigene SMU15000352 (e-value 8e-53). A phylogenetic tree for PGRP-2 was constructed as follows. From the inferred Smed-PGRP-2 sequence, a dataset of putative homologous sequences was built a BLAST (Altschul et al., 1997) search of the NCBI non-redundant (NR) database. Raw data were manually filtered to eliminate potentially non-homologous sequences that would disturb alignments. Alignments were generated using MUSCLE and refined manually (Edgar, 2004). We used the maximum likelihood method in PhyML to perform phylogenetic reconstruction (Guindon and Gascuel, 2003). Branch statistical supports were estimated using the Shimodaira-Hasegawa-like test (SH) (Anisimova and Gascuel, 2006).

2.6. Cloning

To generate an RNAi library, cDNA from *S. mediterranea* was amplified via PCR using primers designed with Primer3 (http://primer3.sourceforge.net/). The primers contained an attB recombination sequence (CATTACCATCCCG). The obtained PCR products were cloned into *E. coli* strain HT115 as described elsewhere (Timmons et al., 2001; Newmark et al., 2003; Reddien et al., 2005b). Targeted transcript sequences were extracted between the 3′ end of the 5′ primer and the 5′ end of the 3′ primer and used for cloning. The extracted sequences were then cut into 21-mers using a sliding window of 1 nucleotide, and all possible RNAi sequences were generated. Each putative RNAi was aligned to the planarian transcriptome using BLAST (Altschul et al., 1990) with a word size of 21; only perfect matches were considered. For each transcript for which an RNAi was designed, the theoretical target accuracy was calculated based on the number of RNAi sequences that matched the target divided by the total number of generated RNAi sequences. The number of theoretical off-target events was equal to 0, thus giving a target accuracy of 100%, which strongly suggests a gene-specific effect but does not exclude the possibility of an off-target effect.

2.7. Delivery of dsRNA and RT-qPCR

dsRNAs were delivered as previously described (Newmark et al., 2003; Reddien et al., 2005a). Briefly, worms were fed three times over the course of six days with a planarian artificial food mixture containing homogenized liver, dsRNA-expressing *E. coli* cells, ultra-low gelling temperature agarose, and food colouring. The quality of gene silencing in naive worms was validated by performing real-time RT-qPCR three days after the last RNAi treatment. When gene silencing occurred before infection, naive worms were silenced as described above and then infected with bacteria three days after the last RNAi treatment. When gene silencing occurred after primo-infection, instructed worms were silenced fifteen days after primo infections as described above and then re-infected with bacteria nine days after the last RNAi treatment. Gene expression in animals was measured by performing real-time RT-qPCR as described previously by (Abnave et al., 2014; Forsthoefel et al., 2012). Briefly, total RNA samples (one animal per sample) were prepared using TRIzol according to the manufacturer's instructions (Invitrogen). The following primers were used for RT-qPCR: *Smed-Morn2* (CGTCAAGGGAAAGGTATTAGCG, GTCGCCTTCATATTTTGCACCA), *Smed-p38 MAPK* (GCGAGGCAGACAGATGAAGA, GCGTGTAAACAATTCGGCCA), *Smed-setd8-1* (CAAGCAAGATCCCAGCAAAGG, GGTTCAGTAGACGCCCCAAT), *Smed-PGRP-2* (CACGGAAAGAATGGGGAGCT, TGCTTTGGTTCATAATGAGGCC),

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