Neutrophils acROSs the enemy lines

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In this issue of *Immunity*, (Warnatsch et al., 2017) describe how neutrophils measure their microbial opponents by differential shuttling of reactive oxygen species (ROS), a process that determines their recruitment and distribution, and ultimately the strength of anti-microbial responses.

Survival of multicellular organisms in hostile environments demand constant battles between the host's immune system and breaching microbes. Because these encounters occur in host tissues, damage to the battlefield needs to be minimized. Recruitment of neutrophils, the leukocytes that most efficiently kill pathogens through a plethora of germicidal weapons, is critical to contain most pathogens. However, because their biological weaponry causes collateral tissue damage (Nicolas-Avila et al., 2017), mechanisms must exist that adjust the strength of a response to the specific features of the invader. Thus, like human wars, immunity must set strategies that preserve resources and prevent damage. Some strategies, like those regulating when and where to attack, are well understood. In contrast, strategies that measure the opponent's size and decide how many immune soldiers are to be deployed remain, paradoxically, poorly defined. Earlier studies by the Papayannopoulos group identified the C-type lectin receptor (CLR) Dectin-1 and regulated release of neutrophil extracellular traps (NETs) as one way to tailor responses to microbe size (Branzk et al., 2014).

What Papayannopoulos and colleagues propose in their new study (Warnatsch et al., 2017) is that pathogen recognition triggers either extracellular or intracellular reactive oxygen species (ROS) production, depending on the size of the microbe (Figure). When neutrophils encountered a relatively large pathogen, such as hyphae of *Candida albicans*, ROS were released into the extracellular milieu by an NADPH oxidase located in their plasma membrane, as this embraced a structure too large to be phagocytosed. Pumping out ROS not only contributed to killing *Candida*, it also prevented oxidation and degradation of the p50 subunit of NF-κB. The authors showed that this was important to allow interleukin-1β (IL-1β) transcription and processing by the inflammasome, with subsequent production of chemokines. In the context of pulmonary infection, this NFκB-mediated production of IL-1β contributed to massive recruitment of neutrophils, which formed clusters around hyphal foci in the lung that eventually resolved the infection. The importance of size in triggering this type of response was elegantly demonstrated by using a mutant strain of *Candida* that is unable to form hyphae (yeast-locked or YL *Candida*). Phagocytosis of these smaller variants of *Candida* instead triggered intracellular

formation of ROS, within the phagosome. Because NADPH oxidase-derived ROS stayed within the cell, they caused p50 oxidation and its subsequent degradation, inflammasome inhibition, and reduced production of IL1β. Consequently, pulmonary infection with the YL strain induced the recruitment of fewer neutrophils, which in this case were dispersedly distributed throughout the pulmonary tissue. The number of recruited neutrophils appeared to be essential as the authors showed that the leukocyte-to-yeast stoichiometry needed to kill the pathogen varied by up to one hundred-fold depending on the microbe's size. Consequently, pulmonary infection of *Cybb*$^{-/-}$ mice, which displayed defective ROS formation, triggered an exaggerated response against the YL strain, to the amounts of the hyphae-forming *Candida* variant. These series of elegant experiments convincingly demonstrated that ROS localization tailors the response against large or small yeast. Importantly, the authors found consistent results using other infectious agents, such as *Aspergillus fumigatus* (large) or *Streptococcus pneumoniae* (small). Experiments using large or small inert beads coated with the yeast wall component β-glucan further demonstrated that size, not the molecular makeup of the microbe, dictates the response of neutrophils. These data provide compelling evidence that the opponent's size, measured by the cell's capacity to engulf it or not, regulates immune responses via ROS.

By definition, phagocytes are specialized in phagocytosis such that when these cells encounter foreign particles they will attempt to engulf and eliminate them. One can envision, however, that evolution has provided additional, refined alternate solutions when the phagocytic process is frustrated; for instance, when the target material is too large. Indeed, this process of "frustrated phagocytosis" was proposed to elicit inflammatory responses (Rosas et al., 2008). Mechanistically, this process was modelled in response to β-glucans of different sizes and revealed that large particles generate a phagocytic "synapse" from which the inhibitory CD45 and CD148 phosphatases are excluded, allowing the initiation of pro-inflammatory signaling (Goodridge et al., 2011). Warnatsch et al. now suggest that the higher IL-1β production observed in neutrophils in response to large particles cannot be considered a frustrated event because downstream signaling is not significantly affected (Branzk et al., 2014). However, the inhibitory effect of small yeast on inflammasome activation and enhanced cytokine production when phagocytosis is not possible raises new questions: Is the response triggered by large particles dependent on phosphatase exclusion from the synapse? Could the small YL mutant induce increased IL-1β production by inhibiting such phosphatases? Regardless, the evident impact of phagocytosis over cytokine production prompts the redefinition of this process from a purely scavenging mechanism, to one that regulates downstream immune signaling.

Work from the authors' laboratory previously identified Dectin-1 in detection of pathogen size. In contrast to the current study, however, they found that Dectin-1-mediated phagocytosis of small microbes impaired NETosis by inhibiting the translocation of elastase into the nucleus (Branzk et al., 2014). Interestingly, all the infectious agents defined as "large" pathogens (*Candida albicans*, *Aspergillus fumigatus*, BCG) are recognized by this receptor (Schorey and Lawrence, 2008), and β-glucans derived from the wall of *Candida* yeast or

hyphae trigger distinct cytokine expression patterns in a Dectin-1-dependent manner (Lowman et al., 2014). Could this mean that Dectin-1 is a “master” size-sensing receptor? Is it really only size, or does size-sensing additionally rely on distinct signaling pathways? In addition to Dectin-1, it is possible that other CLR allow distinction of pathogen size, because Mrc-1 or DC-SIGN have been shown to recognize large pathogenic microbes (Geijtenbeek and Gringhuis, 2009). The authors find that differential formation of the NADPH complex in the phagosomal or plasma membranes, a process dependent on the site-specific cytoplasmic regulators $p40^{phox}$ and $p47^{phox}$, respectively, is the key step that dictates where ROS will be released (Ueyama et al., 2011). This prompts the question of what processes, such as physical stretching of the plasma membrane, triggering of specific signaling pathways or engagement of different CLR, underlies the location and activation of either of these proteins. This series of observations may incite new immunosuppressive therapies based on modulation of phagocytosis, for example in the context of hyper-inflammatory states such as those found in chronic granulomatous disease or inflammatory bowel disease.

The formation of large neutrophil clusters have been also found in the context of sterile inflammation. For example, neutrophil swarming within damaged tissues is instigated by scouting cells that amplify the response via the potent chemoattractant lipid leukotriene B4 (LTB4), a process proposed to allow massive concentration of neutrophils and sealing of the lesion (Lammermann et al., 2013). It would be important to define whether, and if so how, ROS-driven regulation and secondary amplification guided by LTB4 are mutually regulated and cooperate not only to initiate a response, but also to contain the affected site. In addition, it is conceivable that pathogenic microbes elicit their own defensive strategies by combining populations of small and large structures (e.g., by aggregation of small bugs), which the authors show attenuate the strength of the immune response. The current study thus provides new paradigms to understand how pathogens may evade the immune system, and could therefore prompt new therapies.

In summary, the Warnastch study identifies phagocytosis as a key regulatory event in sensing small versus large pathogens, and establishes the differential localization of ROS inside or outside neutrophils as a signaling hub that ultimately dictates the number of immune soldiers deployed to the battlefield. Immune evolution, it seems, would concur with legendary general Sun Tzu when advising on warfare: “It is the rule in war, if ten times the enemy's strength, surround them; … if equal, engage them”.

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References

Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., and Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol *15*, 1017-1025.

Geijtenbeek, T.B., and Gringhuis, S.I. (2009). Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol *9*, 465-479.

Goodridge, H.S., Reyes, C.N., Becker, C.A., Katsumoto, T.R., Ma, J., Wolf, A.J., Bose, N., Chan, A.S., Magee, A.S., Danielson, M.E.*, et al.* (2011). Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature *472*, 471-475.

Lammermann, T., Afonso, P.V., Angermann, B.R., Wang, J.M., Kastenmuller, W., Parent, C.A., and Germain, R.N. (2013). Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. Nature *498*, 371-375.

Lowman, D.W., Greene, R.R., Bearden, D.W., Kruppa, M.D., Pottier, M., Monteiro, M.A., Soldatov, D.V., Ensley, H.E., Cheng, S.C., Netea, M.G., and Williams, D.L. (2014). Novel structural features in Candida albicans hyphal glucan provide a basis for differential innate immune recognition of hyphae versus yeast. J Biol Chem *289*, 3432-3443.

Nicolas-Avila, J.A., Adrover, J.M., and Hidalgo, A. (2017). Neutrophils in Homeostasis, Immunity, and Cancer. Immunity *46*, 15-28.

Rosas, M., Liddiard, K., Kimberg, M., Faro-Trindade, I., McDonald, J.U., Williams, D.L., Brown, G.D., and Taylor, P.R. (2008). The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis. J Immunol *181*, 3549-3557.

Schorey, J.S., and Lawrence, C. (2008). The pattern recognition receptor Dectin-1: from fungi to mycobacteria. Curr Drug Targets *9*, 123-129.

Ueyama, T., Nakakita, J., Nakamura, T., Kobayashi, T., Kobayashi, T., Son, J., Sakuma, M., Sakaguchi, H., Leto, T.L., and Saito, N. (2011). Cooperation of p40(phox) with p47(phox) for Nox2-based NADPH oxidase activation during Fcgamma receptor (FcgammaR)-mediated phagocytosis: mechanism for acquisition of p40(phox) phosphatidylinositol 3-phosphate (PI(3)P) binding. J Biol Chem *286*, 40693-40705.

Warnatsch, A., Tsourouktsoglou, D., Branzk, N., Wang, Q., Reincke, S., Herbst, S., Gutierrez, M., and Papayannopoulos, V. (2017). Reactive oxygen species localization programs inflammation to clear microbes of different size. Immunity.

Figure legend

Pathogen size-sensing and responses by neutrophils. Phagocytosis of small pathogens (*Candida albicans* yeast, for instance) recruits the NADPH p67 subunit to the phagosome, which generate intracellular ROS. These ROS both oxidize the NF-kB p50 subunit leading to its ubiquitination and degradation, and inhibit inflammasome activation. This results in reduced IL-1β production and restricted neutrophil recruitment to infected tissue. In contrast, when neutrophils

encounter large pathogens (*Candida* hyphae), both p47 and p67 NADPH subunits are recruited to the neutrophil outer membrane, and release ROS to the extracellular milieu. This leads to robust NF-κB p50 activation and IL-1β production, which allow massive recruitment of neutrophils and formation of clusters around hyphae.