Mechanisms of TNF induction by heat-killed Staphylococcus aureus differ upon the origin of mononuclear phagocytes

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Kapetanovic R, Parlato L, Fitting C, Quesniaux V, Cavaillon JM, Adib-Conquy M. Mechanisms of TNF induction by heat-killed Staphylococcus aureus differ upon the origin of mononuclear phagocytes. Am J Physiol Cell Physiol 300: C850–C859, 2011. First published January 5, 2011; doi:10.1152/ajpcell.00187.2010.—Mononuclear phagocytes are among the first immune cells activated after pathogens invasion. Although they all derive from the same progenitor in the bone marrow, their characteristics differ on the compartment from which they are derived. In this work, we investigated the contribution of phagocytosis for tumor necrosis factor (TNF) production by murine mononuclear phagocytes (monocytes, peritoneal and alveolar macrophages) in response to heat-killed Staphylococcus aureus (HKSA). Mononuclear phagocytes behaved differently, depending on their compartment of residence. Indeed, when bacterial uptake or phagosome maturation was blocked, activation through membrane receptors was sufficient for a maximal production of TNF and interleukin-10 by peritoneal macrophages. In contrast, monocytes, and to a lesser extent alveolar macrophages, required phagocytosis for optimal cytokine production. While investigating the different actors of signaling, we found that p38 kinase and phosphatidylinositol 3-kinase were playing an important role in HKSA phagocytosis and TNF production. Furthermore, blocking the α6β1 integrin significantly decreased TNF production in response to HKSA in all three cell types. Finally, using mononuclear phagocytes from NOD2 knock-out mice, we observed that TNF production in response to HKSA was dependent on NOD2 for monocytes and peritoneal macrophages. In conclusion, we demonstrate that the mechanisms of activation leading to TNF production in response to HKSA are specific for each mononuclear phagocyte population and involve different recognition processes and signaling pathways. The influence of the compartments on cell properties and behavior should be taken into account, to better understand cell physiology and host-pathogen interaction, and to define efficient strategies to fight infection.

Macrophage; monocyte; phagocytosis; cytokine; kinases

SKIN AND MUCOSAL EPITHELIAL cells in the airways and the gut, protected by mucus, are the first lines of defense. If pathogens succeed in getting through these physical barriers, the innate immune system goes into action to eradicate or at least to contain the pathogen (21). Professional mononuclear phagocytes play a key role in the innate immune system. They are derived from the same progenitor in the bone marrow that gives rise to monocytes in the blood, which then migrate and differentiate in tissues, such as lungs (alveolar macrophages) and the peritoneal cavity (peritoneal macrophages) (14). These cells can recognize different pathogens and induce an inflammatory response, leading to the production of proinflammatory cytokines, such as tumor necrosis factor (TNF), a key cytokine for fighting infection. Staphylococcus aureus, a Gram-positive bacterium of the Micrococccaceae family, is frequently found in infected patients. It often colonizes the lungs of cystic fibrosis patients during childhood (34), it causes endocarditis (36) and osteomyelitis, and is one of the most common organisms isolated from patients in intensive care units. According to a European study, 68% of the patients had pneumonia, and 30% of the infections were caused by S. aureus, including 14% of methicillin-resistant S. aureus (45).

Many receptors have been shown to play a role in the detection of microbial products. Toll-like receptors (TLRs) detect highly conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns, since they are also found in nonpathogenic microorganisms (18, 23, 27). TLR2, in association with TLR6, can detect triacylated lipoproteins and lipoteichoic acid, which are present on the S. aureus cell wall (25, 41). S. aureus is also composed of 50% peptidoglycan (PGN) by weight. PGN recognition is still highly debated, nourished by contradicting results incriminating TLR2 or intracellular molecules (5, 10, 44). The sensing of PGN from Gram-positive bacteria by NOD2 induces the activation of NF-κB transcription factor through receptor-interacting protein-2 (19, 43). Other receptors on the membrane interact with S. aureus and lead to cellular activation. The scavenger receptor MARCO (macrophage receptor with collagenous structure) (4), the integrin αβ (39) and CD36 (40) have been shown to play a role in the response to S. aureus or its uptake. However, the relative role of these receptors has not been clearly established, and the link between S. aureus internalization and the induction of proinflammatory signals still needs to be deciphered.

Clarifying the role of surface sensing vs. phagocytosis in cell activation may provide insights into the interaction between immune cells and this bacterium during infection. Indeed, we previously showed that phagocytosis was dispensable for maximal production of TNF and interleukin (IL)-10 by murine peritoneal macrophages in response to heat-killed S. aureus (HKSA), as TLR2 on the cell surface could also detect the bacterium (22). In contrast, the activation of human monocytes by HKSA and TNF production were dependent on phagocytosis (1). Thus, in this study, we investigated whether phagocytosis was a required step for TNF production in other mouse mononuclear phagocytes, such as monocytes and alveolar macrophages, and if the signaling pathways differed in these various cell types.
Furthermore, in our previous work, the transfection of a macrophage cell line with a dominant-negative form of NOD2 pointed out a possible role of this molecule in the recognition of HKSA (22). Thus we evaluated the role of NOD2 in vitro in the three types of mononuclear phagocytes by comparing cells from wild-type and NOD2 knockout mice. We also investigated the potential role of TLR7, recently shown to contribute to S. aureus recognition by plasmacytoid dendritic cells (32).

Our work highlights the differences among three types of mononuclear phagocytes in terms of recognition of HKSA and signaling pathways involved in TNF production.

**MATERIALS AND METHODS**

**Mice.** We used 8- to 12-wk-old male C57BL/6 (Janvier, Le Genest-St.-Isle, France), Nod2+/− mice (from the animal facility of the Institut Pasteur), and Tlr7+/− mice (from the Transgenose Institute animal breeding facility, Orleans, France) with a C57BL/6 background. All of the mice spent at least 1 wk in the same room before experimentation. All animal care and experimentation were conducted in accordance with the Institut Pasteur guidelines.

Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture to perform experiments on live mice, in appliance of the French and European regulations on care and protection of the Laboratory Animals. Protocols were approved by the veterinary staff of the Institut Pasteur animal facility. Protocols were performed in compliance with the National Institutes of Health Animal Welfare Insurance no. A5476-01, issued on 02/07/2007.

**Monocyte and macrophage isolation.** To obtain elicited peritoneal macrophages, mice were injected intraperitoneally, 4 days before experiments, with 2 ml of thioglycollate broth (Bio-Rad, Marnes-la-Coquette, France). Mice were euthanized with 300 µl of pentobarbital (20%) intramuscularly, and exudate cells were isolated from the peritoneal cavity by washing with ice-cold RPMI 1640 (Glutamax; Lonza, Basel, Switzerland). Cells were counted and plated at 1 × 10⁶ cells/ml, incubated for 1 h at 37°C under an atmosphere containing 5% CO₂, and washed with RPMI to remove nonadherent cells.

Adherent cells among peritoneal cells were considered as peritoneal macrophages. To isolate monocytes, mice were anesthetized as above, and blood was taken by intracardiac puncture with a 1-ml syringe containing 100 µl of heparin (100 UI/ml; Sanofi-Synthelabo, Le Plessis Robinson, France). Blood cells were separated on lymphocyte-Mammal (Cedarlane, Hornby, Canada), and peripheral blood mononuclear cells (PBMC) were isolated after centrifugation (at 850 g at 4°C for 20 min). PBMC were counted and plated at 1 × 10⁶ cells/ml, incubated for 1 h at 37°C with 5% CO₂, and washed with RPMI to remove nonadherent cells. Adherent cells in the PBMC population were considered as monocytes. Alveolar macrophages were isolated by intratracheal washes, consisting of 10 washes with 1 ml of saline per mouse. Cells were counted and plated at 1 × 10⁵ cells/ml, incubated for 1 h at 37°C under an atmosphere containing 5% CO₂, and washed with RPMI to remove nonadherent cells. Adherent cells in the PBMC population were considered as monocytes. Alveolar macrophages were isolated by intratracheal washes, consisting of 10 washes with 1 ml of saline per mouse. Cells were counted and plated at 1 × 10⁵ cells/ml, incubated for 1 h at 37°C under an atmosphere containing 5% CO₂, and washed with RPMI to remove nonadherent cells.

Adherent cells among bronchoalveolar lavage cells were considered as alveolar macrophages. For all three compartments, the purity of adherent cells was checked by F4/80 staining using flow cytometry. All of the cells were cultured in RPMI in the presence of 1% heat-inactivated fetal calf serum (FCS; PAA Laboratories, Pasching, Austria).

**Blocking antibodies, PAMPs, and inhibitors.** Adherent cells were preincubated 30 min with inhibitors of phagocytosis (cytochalasin D, 3 µM, BioMol, Exeter, UK), of phagosomal acidification (chloroquine, 10, 20, and 50 µM, Sigma, St Louis, MO), of Rho-activated kinases (ROCK1/2), which are downstream Rho-GTPase (hydroxyfasudil, 20 µM, Merck Chemicals, Nottingham, UK), of Rac1 (NSC23766, 100 µM, Merck Chemicals), of p38 (SB-203580, 10 µM, Merck Chemicals), of ERK (PD-98059 10 µM, BioMol), or of phosphatidylinositol 3-kinase (PI3K) (LY-294002, 10 µM, BioMol). They were then stimulated with different PAMPs, such as LPS (100 ng/ml from Escherichia coli, Alexis Biochemicals, Lausen, Switzerland), a modified muramyl dipeptide (N-acetylmuramyl-Ala-α-isoglutaminyl-Ne-stearoyl-Lys, 14 µM, Sigma), loxoribine (100 µM/ml, Invivogen), HKSA (Cowan I, 10 µg/ml equivalent to 10⁶ bacteria/ml, Merck Chemicals), or E. coli (BL21 strain, 10⁸ bacteria/ml). In some experiments, blocking antibodies were used. These antibodies were preincubated with the cells for 1 h at 37°C in an atmosphere containing 5% CO₂ before stimulation. The antibodies targeted αβ1 (25 µg/ml; MAB1984, Millipore, Billerica, MA), MARCO (HM1068; HyCult Biotechnology, Uden, the Netherlands), scavenger receptor class A (SR-A) (HM1061; HyCult Biotechnology), and CD36 (10 µg/ml; MAB1258, Millipore). The absence of endotoxin contamination of these antibodies was verified by the Limulus amoebocyte lysate assay (LAL, QCL-1000, Lonza).

**ELISA.** TNF and IL-10 were measured in culture supernatants by ELISA, as specified by the manufacturer (Duoset, R&D systems, Minneapolis, MN).

**Surface TLR2, TLR6, and αβ1 expression measured by flow cytometry.** One million cells were incubated 30 min on ice with a rat anti-mouse TLR2 antibody (10 µg/ml, R&D Systems), a rat anti-mouse TLR6 antibody (2.5 µg/ml, Lifespan Biosciences), or a rat anti- mouse αβ1 antibody (1:100, Millipore), coupled to FITC, or with an IgG2b isotype control coupled to FITC (10 µg/ml, Beckton Dickinson, Franklin Lakes, NJ) in PBS supplemented with 0.5% FCS. The cells were then washed and resuspended in 500 µl of PBS-0.5% FCS. Data were acquired on 30,000 cells for peritoneal macrophages and 10,000 cells for other cells using a FACScan and CellQuest software (Beckton Dickinson).

**Phagocytosis assay.** Cells were plated as described before in RPMI containing 1% FCS in 24-well plates. After 1 h of adherence, cells were left untreated or pretreated 30 min with inhibitors, before stimulation with 10 µg/ml heat-killed Alexa 488-labeled S. aureus (Invitrogen, Cergy Pontoise, France). After 6 h, the cells were washed on ice with PBS-0.5% FCS containing 1.3 mM EDTA. Finally, 500 µl of PBS-0.5% FCS were added in the wells, and cells were detached from plates using a scraper. Data were acquired on 30,000 peritoneal macrophages and 10,000 monocytes and alveolar macrophages using a FACScan and CellQuest software (Beckton Dickinson). One plate of cells was cultured at 37°C and another at 4°C, to correct mean fluorescence intensity (MFI) due to adhesion of bacteria to cell surface (4°C) from the total MFI (adhesion and phagocytosis, 37°C). The calculated MFI reflected the phagocytosis of HKSA.

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was done with the Graph Pad Prism software using Mann-Whitney, Wilcoxon signed-rank test or ANOVA. A P value <0.05 was considered to be significant.

**RESULTS**

The importance of phagocytosis in TNF and IL-10 production is different in the three mononuclear phagocyte populations. In a previous study, our laboratory found that elicited mouse peritoneal macrophages produced TNF and IL-10 in response to HKSA via two distinct pathways: TLR2 and after phagocytosis (22). Peritoneal macrophages were activated via TLR2 located on their membrane, but, when this receptor was absent (TLR2-deficient macrophages), optimal cytokine production was still obtained after phagocytosis of the bacteria. The present study was made to determine whether this was also true for mononuclear phagocytes from two other compartments. Thus we measured TNF and IL-10 production by elicited peritoneal macrophages, blood monocytes, and alveolar macrophages isolated from C57BL/6 mice after stimulation...
with 10 µg/ml of HKSA, corresponding to 10⁷ bacteria/ml, in the presence or absence of cytochalasin D to prevent phagocytosis. As Gram-negative bacteria activate monocytes/macrophages through TLR4 and induce TNF production in a phagocytosis-independent way (1), we also stimulated these cells with an equivalent concentration of E. coli to verify that cytochalasin D was not toxic. A cell viability test [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] further supported that conclusion (Supplemental Fig. S1; Supplemental Material for this article is available online at the Journal website). We confirmed our previous results, showing that phagocytosis was not mandatory in the TNF production by HKSA-stimulated elicited peritoneal macrophages (Fig. 1A). In contrast, monocytes (Fig. 1B) and alveolar macrophages (Fig. 1C) needed the contribution of phagocytosis for optimal TNF production. In monocytes from C57BL/6 mice, 55% of TNF production was phagocytosis dependent (714 ± 113 pg/ml with vs. 1,590 ± 196 pg/ml without cytochalasin D). For alveolar macrophages, 35% of TNF production was phagocytosis dependent (559 ± 71 pg/ml with vs. 853 ± 66 pg/ml without cytochalasin D). TNF production in response to heat-killed E. coli, unlike HKSA, was not inhibited by cytochalasin D, independent of the phagocyte cell type.

Similar results were observed with IL-10. The impact of phagocytosis was not visible for peritoneal macrophages (Fig. 1D), whereas IL-10 production by monocytes (Fig. 1E) appeared to be very much dependent on phagocytosis (28 ± 5 pg/ml with vs. 188 ± 25 pg/ml without cytochalasin D). The production of IL-10, like that of TNF, after stimulation with E. coli, was independent of phagocytosis. No IL-10 was detected in the supernatants from alveolar macrophages (data not shown), in accordance with a previous report (35).

Heterogeneity of mononuclear cells has previously been reported. For example, Taylor and Gordon (42) described two different populations among the blood monocytes: one giving rise to resident macrophages, and the other to inflammatory ones. Therefore, we compared resident and elicited (inflammatory) macrophages from the peritoneal cavity. The results for resident macrophages were comparable to those of elicited ones. After HKSA stimulation, a similar TNF production was obtained, even when phagocytosis was blocked (Supplemental Fig. S2 in online supplement). The only difference between resident and elicited macrophages was the higher amount of TNF in culture supernatants of the elicited ones. It can be noted that TNF levels for resident peritoneal macrophages were in the same range as those obtained for monocytes and alveolar macrophages. Nevertheless, because thioglycollate elicitation is a convenient way to obtain a larger number of cells per mouse, the rest of the study was performed with elicited peritoneal macrophages.

![Fig. 1. TNF (A, B, and C) and interleukin (IL)-10 (D and E) production by elicited peritoneal macrophages (A and D), monocytes (B and E), and alveolar macrophages (C) from C57BL/6 mice. Cells were stimulated for 24 h with heat-killed Staphylococcus aureus (HKSA; 10 µg/ml, equivalent to 10⁷ bacteria/ml; shaded bars) or heat-killed E. coli (10⁷ bacteria/ml; solid bars) in the absence or the presence (hatched bars) of cytochalasin D (3 µM), an inhibitor of phagocytosis. Results represent the means ± SE of a minimum of 5 independent experiments. Each experiment included 10 mice. *P < 0.01 (Wilcoxon test).](http://ajpcell.physiology.org/doi/abs/10.1152/ajpcell.00177.2010)
Phagosome maturation is necessary for TNF production after *S. aureus* uptake. Cytochalasin D was used to block phagocytosis (7, 12). However, this drug inhibits actin polymerization and, therefore, may also impact other actin-dependent processes. To confirm the involvement of phagocytosis, we used chloroquine, a drug that increases phagosome pH and blocks phagosome maturation and its fusion with lysosomes (30). Cells were isolated and incubated with various doses of chloroquine (10, 20, and 50 μM) and then stimulated with 10 μg/ml of HKSA for 24 h. As shown in Fig. 2, chloroquine had no effect on TNF production by peritoneal macrophages from C57BL/6 mice (Fig. 2A). In contrast, chloroquine inhibited, in a dose-dependent manner, TNF production by monocytes and alveolar macrophages (Fig. 2, B and C, respectively). These data concur with those from experiments done with cytochalasin D and show differences of impact of phagocytosis in the three cell populations. Similarly to cytochalasin D, we checked the absence of toxicity for chloroquine by a cell viability test using annexin V and propidium iodide (Supplemental Fig. S1). Cells were stimulated for 24 h, and no effect on TNF production by peritoneal macrophages from C57BL/6 mice (Fig. 2, B and C, respectively). These data concur with those from experiments done with cytochalasin D and show differences of impact of phagocytosis in the three cell populations. Similarly to cytochalasin D, we checked the absence of toxicity for chloroquine by a cell viability test using annexin V and propidium iodide (Supplemental Fig. S1).

**Fig. 2.** TNF production by peritoneal macrophages (A), monocytes (B), and alveolar macrophages (C) from C57BL/6 mice. Cells were stimulated for 24 h with HKSA (10 μg/ml) in the absence or presence of chloroquine (CQ; 10, 20, or 50 μM). Control values, obtained with unstimulated cells, were close to the detection limits (data not shown). Results represent the means ± SE of a minimum of 5 independent experiments. Each experiment included 10 mice. ND, no drug. *P < 0.05 (Wilcoxon test).

αβ1 contributes to TNF production in response to HKSA in all mononuclear phagocytes. We then tried to identify the surface receptor contributing to phagocytosis and TNF production after stimulation with HKSA. Blocking antibodies against MARCO, SR-A, or CD36 did not inhibit TNF production in response to HKSA (Supplemental Fig. S3, online supplement). We also investigated the role of the integrin αβ1, a transmembrane molecule that interacts with fibrinogen and forms a bridge between the cell and the fibrinogen-binding protein of *S. aureus* (39). The three cell populations were confirmed to express αβ1 on their surface by flow cytometry (Fig. 4A). The cells were cultured and stimulated with HKSA, with or without pretreatment with a blocking antibody targeting αβ1. As shown in Fig. 4B, blocking αβ1-integrin partially, but significantly, inhibited TNF production in all three types of phagocytes in response to HKSA.

p38 and PI3K are important for HKSA phagocytosis in all cell types, whereas the role of Rho and Rac1 varies on the subset of mononuclear cell. By using specific inhibitors, we blocked the MAPK p38 (SB-203580) or ERK (PD-98059), as well as other proteins known to play a role in the signaling cascade leading to TNF production, such as PI3K (LY-294002). We also evaluated the contribution of Rho and Rac1, two G proteins involved in the phagocytosis process. In addition, Rac1 is known to interact with TLR2 and contribute to the signaling through this receptor (3). For Rho-GTPase, we used hydroxylfasudil, an inhibitor of the downstream ROCK1/2. We used a pharmacological approach to study the signaling pathways involved in TNF production in each phagocyte population. Nevertheless, we always used the various inhibitors in the range of a specific activity, but without any toxicity. The optimal concentration for each inhibitory drug was chosen according to the literature (17, 26, 29, 37). We also used our own experience for ERK, p38, and PI3K inhibitors (2). These inhibitors were used at 10 μM, a concentration giving a maximal specific inhibition but without interfering with other...
kinases. Tested up to 50 µM, LY-294002 had no effect on PKC, PKA, MAPK, or tyrosine kinases (46). Similarly, SB-203580 was tested up to 100 µM without affecting JNK, ERK, or PKA (8). Furthermore, for SB-203580, the inhibitory effect on p38 phosphorylation was confirmed by Western blot (Supplemental Fig. S4, online supplement). To measure the effect of these inhibitors on the phagocytic process of HKSA by the three cell populations, we first determined the optimal incubation time to be 6 h (data not shown). Cytochalasin D was a positive control of phagocytosis inhibition, for which a representative result is shown in Fig. 5A. Indeed, in all cells, cytochalasin D efficiently inhibited HKSA phagocytosis (Fig. 5B). To evaluate the phagocytosis, we subtracted the MFI at 4°C (only binding, no phagocytosis) from the MFI at 37°C (phagocytosis and binding). The inhibition of ROCK1/2 had a slight but significant effect on the phagocytosis inhibition, for which a representative result is shown in Fig. 5A. Indeed, in all cells, cytochalasin D efficiently inhibited HKSA phagocytosis (Fig. 5B). To evaluate the phagocytosis, we subtracted the MFI at 4°C (only binding, no phagocytosis) from the MFI at 37°C (phagocytosis and binding). The inhibition of ROCK1/2 had a slight but significant effect on the phagocytosis of HKSA by peritoneal and alveolar macrophages. On the other hand, the inhibition of Rac1 decreased the phagocytosis of HKSA by peritoneal and alveolar macrophages, but not by monocytes. This suggests that Rac1 has a distinct role in monocytes and macrophages. The inhibition of p38 (SB-203580) or of PI3K (LY-944002) decreased the phagocytosis of HKSA in all three phagocyte populations. Finally, the inhibition of ERK (PD-98059) had no impact on phagocytosis. This result is in accordance with TNF production shown in Fig. 6 and suggests that ERK does not play a role in the response to HKSA.

Role of p38, PI3K, and Rac1 in HKSA-induced TNF production, depending on the cell type. We investigated the difference in signaling pathways leading to TNF production in the three types of mononuclear phagocytes. The inhibitor of ROCK1/2 had no effect on TNF production, by all three cell types (Fig. 6). The inhibition of Rac1 had no effect on the peritoneal population, but, interestingly, it had an amplificatory effect on TNF production by monocytes and alveolar macrophages, two cell types requiring phagocytosis for optimal TNF production. Regarding p38 and PI3K, TNF production was significantly decreased in all three populations, except for PI3K in the alveolar macrophages. Finally, no role was found for ERK in TNF production in the three cell populations.

NOD2 contributes to TNF production after HKSA stimulation. In our laboratory’s previous study, the transfection of a plasmid coding for a dominant-negative form of NOD2 into the murine macrophage cell line RAW 264.7 caused an inhibition of NF-κB activation after HKSA stimulation (22). We asked whether NOD2 was also playing a role in primary cells and if mononuclear phagocytes from wild-type and nod2−/− mice had a distinct pattern in TNF production. Figure 7, top left, shows the control experiments, where peritoneal macrophages were stimulated for 24 h with a TLR4 agonist (LPS), or a NOD2 agonist (muramyl dipeptide) alone or in synergy with low amounts of LPS. The response to LPS was similar in all type of mice, whereas TNF production in response to muramyl
Dipeptide + LPS was abolished, in macrophages devoid of NOD2 receptors. The absence of this receptor had a different impact on TNF production in response to HKSA, depending on the cellular origin. For peritoneal macrophages (Fig. 7, top right), TNF production was reduced in the absence of NOD2, even if the reduction was not very important. For monocytes (Fig. 7, bottom left), TNF production was significantly reduced in the absence of NOD2. Similar results were found when using live S. aureus instead of HKSA (data not shown). Finally, we checked a possible role of TLR7 in intracellular detection of S. aureus, using macrophages from Tlr7 knockout mice. Indeed, a recent study suggested that S. aureus induced IFN-α production by plasmacytoid dendritic cells through a TLR7-dependent pathway (32). We found that, when TLR7 was absent and TLR2 was concomitantly blocked with an antibody, TNF production was not altered (Supplemental Fig. S5, online supplement). Altogether, these results suggest that NOD2 in addition to TLR2 contributes to TNF production by mouse monocytes and peritoneal macrophages after internalization of HKSA.

DISCUSSION

Compartmentalization of the immune response is an emerging concept. Indeed, an increasing number of studies underline the differences that exist in host response, depending on the site of infection. S. aureus is a threat for immuno-compromised patients, and its increasing resistance to antibiotics becomes an important public health issue. Thus understanding how phagocytes from different compartments detect this bacterium and mount an inflammatory response is essential. In this work, we point out the important roles of phagocytosis in cytokine production in response to S. aureus. Our laboratory previously showed, for peritoneal macrophages, that detection through TLR2 and phagocytosis was redundant for TNF and IL-10 production, although the response of peritoneal macrophages to S. aureus was totally MyD88 dependent (22). This observation was true for both elicited and nonelicited peritoneal...
In contrast, we now report that phagocytic engulfment is necessary for a maximum TNF or IL-10 production for monocytes, and to a lesser extent alveolar macrophages. In our work, the contribution of bacterial uptake in cytokine production was assessed using cytochalasin D and chloroquine. We can exclude any toxicity as viability of the cells was checked, and that no effect on cytokine production was seen in response to *E. coli*. Thus phagocytosis, as well as maturation and acidification of the phagosome, are critical steps in the response of monocytes and alveolar macrophages to *S. aureus* in terms of TNF and IL-10 production. In contrast, phagocytosis was not mandatory for optimal TNF production by peritoneal macrophages. Our findings on peritoneal macrophages are supported by similar results recently published with other microorganisms, such as *Streptococcus pyogenes* and *Borrelia burgdorferi* (16, 38). Furthermore, the redundancy between TLRs and bacterial uptake for cytokine production can be extended to bone marrow-derived macrophages, as these mononuclear phagocytes were used in these later studies.

Fig. 5. Phagocytosis of HKSA by peritoneal macrophages, monocytes, and alveolar macrophages derived from C57BL/6 mice. Cells were incubated for 6 h with 10 μg/ml of Alexa 488-labeled *S. aureus*, in the absence or presence of cytochalasin D (3 μM), inhibitors of Rho-activated kinases (ROCK1/2; 20 μM), Rac1 (100 μM), p38 (SB-203580; 10 μM), ERK (PD-98059; 10 μM), or phosphatidylinositol 3-kinase (PI3K) (LY-294002; 10 μM). Inhibitors were added to culture media 30 min before incubation with the labeled bacteria. A: a representative flow cytometry result showing peritoneal macrophages pretreated with cytochalasin D (cyto. D) or not (no drug) and incubated with Alexa-488-*S. aureus* at 37 or 4°C. NS, macrophages without any stimulation. B: results represent the means ± SE of 5 independent experiments, normalized to the mean fluorescence intensity (MFI) of cells without drugs. Each experiment included 10 mice. *P < 0.05 (Wilcoxon test).

Fig. 6. TNF production by peritoneal macrophages (A), monocytes (B), and alveolar macrophages (C) from C57BL/6 mice. TNF production is expressed as percentage of the control value (without inhibitor). Cells were stimulated for 24 h with HKSA (10 μg/ml, equivalent to 10⁷ bacteria/ml) in the absence (solid bars) or the presence of inhibitors of ROCK1/2 (20 μM), Rac1 (100 μM), p38 (SB-203580; 10 μM), ERK (PD-98059; 10 μM), or PI3K (LY-294002; 10 μM). Inhibitors were added to culture media 30 min before stimulation. Results represent the means ± SE of a minimum of 5 independent experiments. Each experiment included 10 mice. #P < 0.01 (Wilcoxon test).
A shortcut is often made by comparing human monocytes to mouse macrophages or macrophage cell lines. Our laboratory already observed that part of TNF and IL-10 production by human monocytes was dependent on phagocytosis of \textit{S. aureus} (1), and this result stands in contrast to our laboratory’s previous observation with mouse peritoneal macrophages (22). We now show that mouse monocyte reactivity is indeed very close to that of human monocytes. Thus one has to be cautious when comparing inflammatory responses of mouse macrophages and human monocytes.

To investigate the signaling pathways involved after \textit{S. aureus} phagocytosis, we used various inhibitors. In all cases, we observed a contribution of p38 and PI3K on the phagocytosis itself, as well as on TNF production (although we noted that PI3K played a minor role for TNF production by alveolar macrophages). Our data demonstrate that p38 plays a key role in the \textit{S. aureus}-dependent TNF response and on the phagocytosis itself, which is in agreement with previous studies performed on macrophage cell lines RAW 264.7 (6) and THP-1 (31, 33), or more recently on primary peritoneal macrophages (20). ERK phosphorylation after stimulation of macrophages with \textit{S. aureus} has been demonstrated by other groups (31, 33). However, using a specific inhibitor, we showed that ERK activation was not required for TNF production in monocytes and macrophages from C57BL/6 mice. Similarly, Zhao et al. (49) showed that, in epithelial cells, TNF production after \textit{S. aureus} infection was not dependent on ERK, but needed p38 MAPK. The inhibition of Rac1 and that of ROCK1/2 (kinases acting downstream of Rho-GTPase) had no effect, or an amplificatory effect on TNF production by the cells, whereas an inhibition in phagocytosis was seen, depending on the phagocyte. Our results concur with another study showing an increased TNF production by the RAW 264.7 macrophage cell line in the presence of small GTPase inhibitors (28). The fact that partial Rho inhibition did not alter TNF production is in agreement with earlier results of Erwig et al.
Rac1 may downregulate a not yet identified signaling pathway.

The enhanced production of TNF in monocytes and alveolar macrophages in the presence of Rac1 inhibitor suggests that Rac1 may downregulate a not yet identified signaling pathway that leads to TNF production.

Interestingly, when we blocked the integrin α3β1, TNF production was decreased in all three cell types in response to S. aureus. We wondered whether this could be due to an effect on calcium flux through this receptor, as it was previously shown that α3β1 engagement in human embryonic kidney-293 cells with fibronectin or an antibody increased cytosolic calcium concentration (47).

Moreover, calcium mobilization is important for cytoskeleton mobility and phagocytosis. However, in contrast to ionomycin, our positive control, we found that HKSA was a poor inducer of calcium flux (data not shown). In addition, we found that the role of this integrin in TNF production was not due to an increase in bacterial uptake (data not shown). Our data are in accordance with other studies showing that α3β1 is rather involved in the binding of HKSA to the phagocyte and its sensing than in its phagocytosis (13), and that it may induce intracellular signaling via the focal adhesion kinase, which is downstream of α3β1 and interacts with MyD88 (48).

Nevertheless, we cannot exclude a lack of sensitivity of the technique used to measure the calcium flux in response to HKSA.

In a previous study, our laboratory observed that the transfection of a dominant-negative form of NOD2 into the macrophage cell line RAW 264.7 decreased NF-κB activation after stimulation with HKSA (22). We considered NOD2 to be a possible intracellular sensor for muropeptides, resulting from the degradation of the S. aureus PGN in the phagolysosome. To test this idea, we used monocytes and macrophages isolated from Nod2 knockout mice. Our results show that NOD2 does not contribute to TNF production by alveolar macrophages. Since phagocytosis inhibition also impacts TNF production by these cells, the existence of another intracellular sensor for HKSA in alveolar macrophages is most probable. In contrast, TNF production by monocytes and peritoneal macrophages is partially NOD2 dependent. In agreement with these data, a recent study also demonstrated an important role of NOD2 in the detection of S. aureus in an in vivo intraperitoneal infection model (9). The link between phagocytosis and NOD2 is not yet explained or even proven, and the fact that phagocytosis was not required for TNF production by peritoneal macrophages, while NOD2 appears to play a role, may suggest that PAMPs released by HKSA could contribute to its involvement.

In conclusion, this study underlines the fact that the role of phagocytosis and that of NOD2, in cytokine production in response to HKSA, depends on the compartment from which the macrophages are derived. Our work demonstrates the importance of p38 and PI3K signaling pathways for TNF production in response to S. aureus, but also highlights differences among the three types of mononuclear phagocytes (Fig. 8). Although these cells share the same progenitor, once they reach a specific compartment (blood, lung, peritoneal cavity), the mechanisms by which they are activated appear to be different. The heterogeneity of mononuclear phagocytes has recently been further emphasized by Gorgani et al. (15), who showed that the gene expression profiles of splenic macrophages, Kupffer cells, and resident peritoneal cells were completely different. The influence of the microenvironment (e.g., plasma proteins for monocytes, granulocyte/macrophage colony-stimulating factor within the lungs) plays a key role in modulating the intracellular machinery that allows phagocytes to be activated by HKSA. We believe that these differences are of interest for cell physiologists. The variations depending on the compartment may also be true for other cell types. These differences should be taken into account to better understand cell physiology and host-pathogen interaction, and to define efficient strategies to fight infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


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