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Tet38 Efflux Pump Affects *Staphylococcus aureus* Internalization by Epithelial Cells through Interaction with CD36 and Contributes to Bacterial Escape from Acidic and Nonacidic Phagolysosomes

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ABSTRACT We previously reported that the Tet38 efflux pump is involved in internalization of *Staphylococcus aureus* by A549 lung epithelial cells. A lack of *tet38* reduced bacterial uptake by A549 cells to 36% of that of the parental strain RN6390. Using invasion assays coupled with confocal microscopy imaging, we studied the host cell receptor(s) responsible for bacterial uptake via interaction with Tet38. We also assessed the ability of *S. aureus* to survive following alkalinization of the phagolysosomes by chloroquine. Antibody to the scavenger receptor CD36 reduced the internalization of *S. aureus* RN6390 by A549 cells, but the dependence on CD36 was reduced in QT7 *tet38*, suggesting that an interaction between Tet38 and CD36 contributed to *S. aureus* internalization. Following fusion of the *S. aureus*-associated endosomes with lysosomes, alkalinization of the acidic environment with chloroquine led to a rapid increase in the number of *S. aureus* RN6390 bacteria in the cytosol, followed by a decrease shortly thereafter. This effect of chloroquine was not seen in the absence of intact Tet38 in mutant QT7. These data taken together suggest that Tet38 plays a role both in bacterial internalization via interaction with CD36 and in bacterial escape from the phagolysosomes.

KEYWORDS CD36, *S. aureus*, endosomes, internalization, survival

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Staphylococcus aureus is a versatile bacterium capable of causing acute and chronic infections in humans and animals due to its arsenal of virulence factors and its ability to acquire multiple drug resistance phenotypes (1–3). Chronic infections caused by *S. aureus*, such as osteomyelitis, are difficult to treat, and it and other staphylococcal infections are notable for their propensity to recur after treatment (4–6). This persistence may be due in part to the ability of *S. aureus* to survive in and adapt to the host intracellular environment, enabling escape from the effect of antibiotic treatment and the host immune response (7–10). Although *S. aureus* is not a traditional intracellular pathogen, many studies have demonstrated that it can invade and survive within nonprofessional phagocytic host cells, such as epithelial and endothelial cells (9, 11). In the case of *Listeria monocytogenes*, the internalization process starts with a molecular interaction between a bacterial surface protein and a host cell receptor, which leads to a series of signal inductions across the cell membrane, resulting in the rearrangement of the host cell cytoskeleton and finally internalization of the bacteria (12). *S. aureus* expresses a number of extracellular matrix proteins, termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including fibronectin-binding proteins (FnBPs), which bind the heat shock protein Hsp60 of the host cell, the iron-regulated surface determinant B (IsdB), which interacts with host cell integrins, and

lipoteichoic acids (LTAs), which are recognized by the Toll-like receptor TLR2/TLR6 dimers (13–15). The host cell receptor CD36 is a membrane glycoprotein of the class B scavenger family that interacts with Toll-like receptors TLR2 and TLR6 acting as a facilitator in the recognition of diacylglyceride components of bacteria. CD36 is also a long-chain fatty acid transporter present on the surface of epithelial and endothelial cells, as well as in intracellular compartments such as endosomes (16, 17). In a recent study of myocardial fatty acid uptake, Glatz et al. demonstrated that CD36 is able to translocate between the endosomes and the sarcolemma, enabling the transport of fatty acids to different intracellular locations and thereby playing an important role in the coordination of cardiac fatty acid uptake to meet myocardial energy needs (18). As a scavenger receptor, this protein can also recognize and internalize apoptotic cells, pathogenic fungi, and bacteria such as *Escherichia coli*, mycobacteria, and *S. aureus* (17–19). CD36 was reported as a phagocytic receptor for *S. aureus* that internalized this bacterium together with its LTA via the COOH-terminal cytoplasmic portion of CD36 (20).

Tet38 is an *S. aureus* efflux pump that can extrude both tetracycline and unsaturated free fatty acids, such as palmitoleic acid and undecanoic acid (21, 22). Tet38 plays an important role in bacterial colonization and internalization, but the mechanism of this involvement has not been explored. After internalization and fusion with lysosomes, depending on the cell lines, *S. aureus* can replicate rapidly and escape from the phagolysosome or persist for a time and escape later. In both circumstances, *S. aureus* produces alpha-toxin that induces cell apoptosis (23, 24). Recent studies by Leimer et al. showed that acidic pH induced nonstable *S. aureus* small-colony variants (SCVs) and nonreplicating persister cells that were localized to the phagolysosome. These SCVs were eliminated after alkalinization of the acidic milieu of the phagolysosome with chloroquine or other lysomotropic alkalinizing agents (25). Chloroquine diffuses freely and rapidly across cell membranes and accumulates in lysosomes in its unprotonated form. In the acidic environment of lysosomes (pH 4.5), chloroquine becomes protonated and is trapped in the acidic compartment (26).

In the present study, we evaluated the role of Tet38 in adherence, internalization, and intracellular trafficking in epithelial cells. We found that in the absence of Tet38 there was loss of the dependence of internalization on CD36, suggesting an interaction between Tet38 and CD36. Following the fusion of the *S. aureus*-associated endosomes with lysosomes, alkalinization of the acidic environment with chloroquine led to a rapid increase in the number of bacteria in the cytosol followed by a decrease shortly thereafter. This effect of chloroquine was also dependent on intact Tet38.

RESULTS

Interplay of host cell CD36 and the *S. aureus* Tet38 efflux pump contributes to efficient internalization of *S. aureus* by A549 cells. As we reported previously (22), Tet38 contributes to internalization of *S. aureus* by A549 cells. QT7 *tet38* was internalized 6-fold less in A549 and human microvascular endothelial cells (HMECs) than the parent strain RN6390. The membrane-associated host cell receptor CD36 is a transporter of long-chain fatty acids and is also known to contribute to *S. aureus* invasion of host cells (18, 27). To determine if CD36 is the host cell ligand with which Tet38 interacts in the internalization process, we tested the effect of anti-CD36 antibody on internalization of RN6390 and QT7. We treated A549 epithelial cell monolayers with anti-CD36 antibody (50 nM) for 30 min prior to infection by RN6390 or QT7. After 1 h of contact, the monolayers were washed and incubated with assay medium containing gentamicin and lysostaphin to eliminate extracellular bacteria. All values (in percentages) were based on the value of the total internalization (100%) of RN6390 by A549. In the absence of anti-CD36 antibody, the amount of QT7 (*tet38* mutant) internalized was 38% of the amount of internalized RN6390 (Fig. 1A). These data were similar to those reported in our previous study (22). After the monolayers were preincubated with anti-CD36 antibody at 50 nM, the amount of internalized wild-type RN6390 was reduced to 56% of the amount in the assay without CD36 antibody preincubation. In

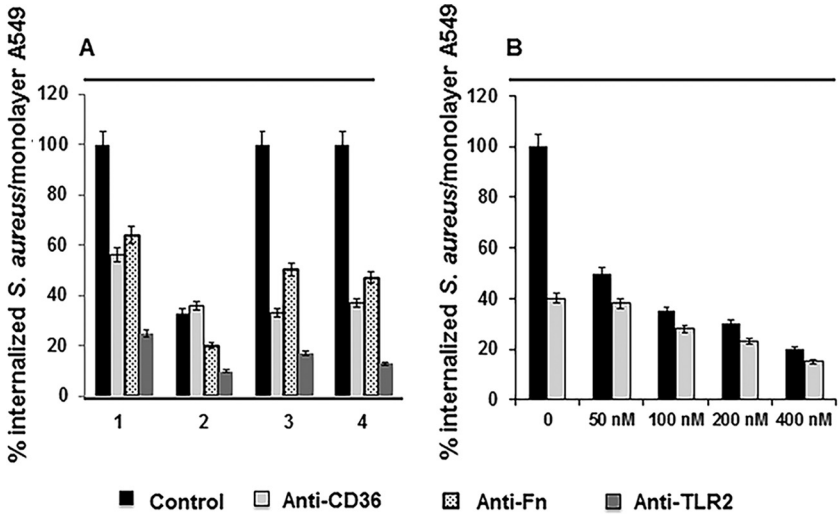

FIG 1 Effect of anti-CD36, anti-Fn (fibronectin), and anti-TLR2 (Toll-like receptor 2) antibodies on internalization of *S. aureus* RN6390 and QT7 by A549 cells. (A) Invasion assays using *S. aureus* RN6390, QT7, and the *tet38*-complementing transformants, with anti-CD36, anti-Fn, and anti-TLR2 antibodies at 50 nM. Lane 1, RN6390; lane 2, QT7 (*tet38* mutant); lane 3, RN6390(pLZ113-*tet38*); lane 4, QT7(pLZ113-*tet38*). (B) Invasion assays using *S. aureus* RN6390 (black bars) and QT7 (gray bars) with anti-CD36 antibody at increasing concentrations from 0 nM to 400 nM. The A549 cell monolayers were preincubated for 30 min with antibody diluted to 50 nM (A) or the indicated concentrations (B). The number of bacteria per monolayer was determined following a standard internalization assay with an MOI of 100:1. The results are presented as a percentage of the CFU count/monolayer of the treated assay versus that of the nontreated assay, with the RN6390 CFU count/monolayer as the reference point for all assays (100%). All values are means ± standard deviations of three independent experiments. The differences in the numbers of bacteria recovered in each assay with and without treatment with antibodies are statistically significant as determined by Student's *t* test ($P < 0.05$).

contrast, there was little or no reduction in internalization of QT7 (36% versus 38%) after CD36 antibody preincubation. To compare the effect of CD36 with that of a known host cell ligand, fibronectin, we also carried out the internalization assays after pretreatment with anti-fibronectin (anti-Fn) antibody. After preincubation with 50 nM antibody, RN6390 internalization decreased to 64%, and QT7 showed an additional decrease from 36% to 20% of the RN6390 level, representing a similar relative reduction to 55% of its value in the absence of anti-CD36. Thus, the effect of anti-CD36 antibody but not anti-fibronectin antibody on *S. aureus* internalization was reduced in the absence of Tet38.

Since the Toll-like receptor TLR2 was shown to form a complex with CD36, we carried out a similar invasion assay using anti-TLR2 antibody to assess the effect of this receptor on *S. aureus* internalization by A549 in the absence of *tet38*. After preincubation with 50 nM antibody, RN6390 internalization decreased to 25%, and QT7 showed an additional decrease from 36% to 10% of the RN6390 level, representing a relative reduction to 28% of its value in the absence of anti-CD36. These data indicated that TLR2 had its own bacterial target and also confirmed that the effect of anti-CD36 antibody was reduced in the absence of Tet38 (Fig. 1A).

Dose titrations of anti-CD36 antibody from 50 nM to 400 nM revealed that the progressively higher concentrations of anti-CD36 antibody had increasing effects on *S. aureus* internalization for both RN6390 and QT7 *tet38* although the effect on QT7 remained attenuated relative to that on RN6390, with a reduction of 2.7-fold (15% versus 40%) for QT7 and 5-fold (20% versus 100%) for RN6390 in comparison to the results with no anti-CD36 antibody and those with the highest concentration, 400 nM (Fig. 1B). In addition, in internalization assays with Jurkat cells, which lack CD36, there was no difference between internalization of RN6390 and QT7. Both strains were internalized into Jurkat cells at 50% efficiency compared to that of RN6390 in A549 cells. Internalization values remained unchanged when anti-CD36 antibody was added to the

Jurkat cell monolayer for 30 min prior to infection by either bacterial strain. In contrast, following preincubation with anti-Fn antibody, both RN6390 and QT7 showed similar levels of reduction from 50% (no preincubation) to 36% internalization into Jurkat cells (data not shown). Thus, these data suggest that an interaction between Tet38 and CD36 contributes to the internalization of *S. aureus* in A549 epithelial cells. Since CD36 forms complexes with other host cell receptors, such as TLR2/TLR6, it is possible that the interactions with Tet38 also involve other proteins, interactions that could be disrupted at the highest concentrations of anti-CD36.

Dose titrations of anti-Fn antibody from 50 nM to 400 nM confirmed that the roles of fibronectin-binding proteins in *S. aureus* internalization were similar in the presence or absence of Tet38, with anti-Fn providing a reduction of 64% to 25% for RN6390 and of 20% to 10% for QT7 at the highest antibody concentration (400 nM) (data not shown).

The *tet38*-overexpressor RN6390(pLZ113-*tet38*) showed a similar level of internalization in A549 cells as that of RN6390 alone, suggesting that expression of *tet38* in RN6390 may be a maximal level for interaction with CD36 in A549 cells. Invasion assays carried out using the *tet38*-complementing strain QT7(pLZ113-*tet38*) yielded the same level of internalization as that of RN6390 (100%) in the control assay. Figure 1A represents the internalization data of all *S. aureus* strains as percentages of the internalization of RN6390.

Effect of Tet38 on interaction of *S. aureus* with host cell membranes. To identify further the events occurring at the *S. aureus*-A549 cell membrane interaction, we carried out confocal microscopy imaging to visualize the association between bacteria and host cell membranes. We stained the A549 cells with the dye FM4-64, which labels the plasma membrane (red) and various organelles except the nucleus and used yellow fluorescent protein (YFP)-labeled RN6390 and QT7 for the infection (Fig. 2). *S. aureus* was added to A549 cells for 2 h, followed by incubation of the monolayers with assay medium containing gentamicin and lysostaphin for 60 min to eliminate remaining extracellular bacteria. Under these conditions, bacteria not associated with cell membrane structures fluoresce green, and those colocalized with the cell membrane (endosomes) fluoresce yellow. We enumerated green and yellow bacteria in 10 contiguous confocally imaged fields, each with an average of six A549 cells. Overall, as expected, there were fewer A549-associated bacteria (yellow) for QT7 (34%) than A549-associated bacteria (yellow) for RN6390 (considered as 100%) (Fig. 3). Preincubation of A549 cells with anti-CD36 antibody resulted in a 2-fold reduction (100% to 50%) in cell-associated RN6390 but no reduction in cell-associated QT7 (1.03-fold, or 34% to 33%). In contrast, anti-fibronectin antibody resulted in similar reductions for cell-associated RN6390 and QT7 (1.7-fold, or 100% to 58%, and 1.4-fold, or 34% to 25%, respectively). There was little or no effect of either antibody on the number of remaining non-cell-membrane-associated bacteria (green). Thus, internalization of *S. aureus* in A549 cell membranes is also affected by blocking CD36 in a Tet38-dependent manner (Fig. 3).

Involvement of Tet38 in *S. aureus* trafficking within A549 cells. We next examined the trafficking of internalized *S. aureus* with phagolysosomes, which are formed by the fusion of *S. aureus*-associated endosomes with the lysosomes of the A549 host cell. For these experiments, we extended the period of incubation of A549 cells after internalization of *S. aureus* out to 4 days, stained the lysosomes of A549 using the LysoTracker dye DND99 (red), and used the YFP-expressed RN6390 and QT7 (green) to localize cells after internalization. We observed the colocalization of the internalized bacteria/lysosomes (yellow) over time using confocal microscopy (Fig. 4). Both free RN6390 and bacteria associated with acidic lysosomes increased over 3 days and then decreased, with somewhat fewer phagolysosome-associated bacteria than free bacteria throughout that time period (Fig. 5A). In contrast, for QT7, there were 2- to 3-fold more cells associated with the phagolysosome than free cells on days 1 and 2, followed by

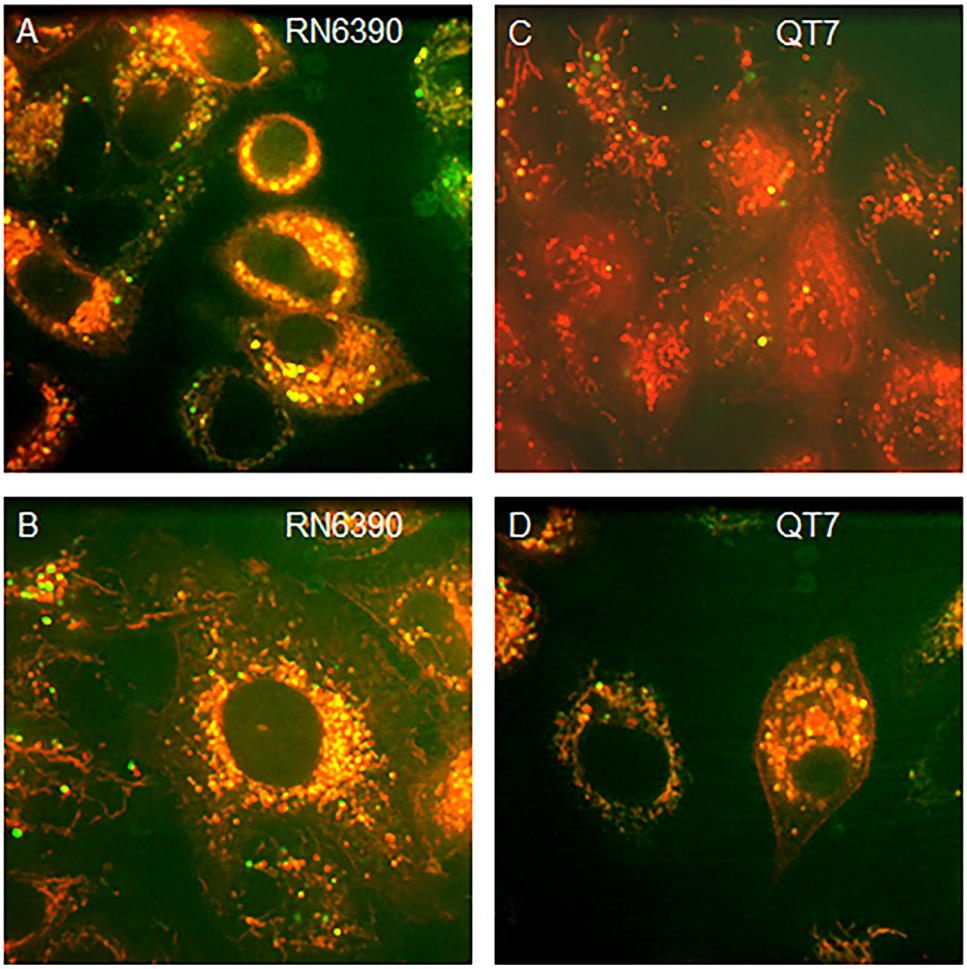

FIG 2 Confocal microscopy of *S. aureus* RN6390(pAH16) and QT7(pAH16) after 2 h of internalization following a 30-min treatment with lysostaphin and gentamicin in DMEM. A549 lung epithelial cells were stained with FM4-64 (red). The bacteria expressed YFP and were viewed as green fluorescence. Colocalization of *S. aureus* and A549 was viewed as yellow fluorescence. Representative fields are shown.

decreases in phagolysosome-associated cells without an increase of free cells and with similar numbers of free and phagolysosome-associated cells by day 4 (Fig. 5B).

Effect of chloroquine on the survival and localization of *S. aureus* in A549 cells. Chloroquine is taken up by A549 and other epithelial cells and is concentrated in the phagolysosome, causing an increase in its pH (25). To assess the effect of phagolysosomal pH on the localization patterns of RN6390 and QT7, tracking experiments were repeated in the presence of chloroquine (20 μM) in a manner similar to that previously reported (13). Chloroquine had similarly limited effects on growth of both RN6390 and QT7 *in vitro*, and the two strains did not differ in their inabilities to grow *in vitro* at pH 4.5 (Fig. 6A), the pH of the phagolysosome (26). Within A549 cells, however, survival of RN6390 was increased over 2-fold in the presence of chloroquine, with no apparent enhancement of QT7 growth (Table 1; Fig. 6B). Thus, Tet38 appears to enable increased intracellular growth upon increase of pH in the phagolysosome.

We next evaluated the effect of chloroquine on intracellular trafficking of RN6390 and QT7 (Fig. 5). For RN6390 chloroquine resulted in a decrease in phagolysosome-associated bacteria and a substantial increase in free bacteria, reaching a peak of approximately 2-fold at day 3. In contrast, chloroquine had little effect on QT7 intracellular localization, with no change in free bacteria and a slight decrease in phagolysosome-associated bacteria. Thus, Tet38 enables escape from or avoidance of the phagolysosome associated with increased phagolysosomal pH, an effect that may

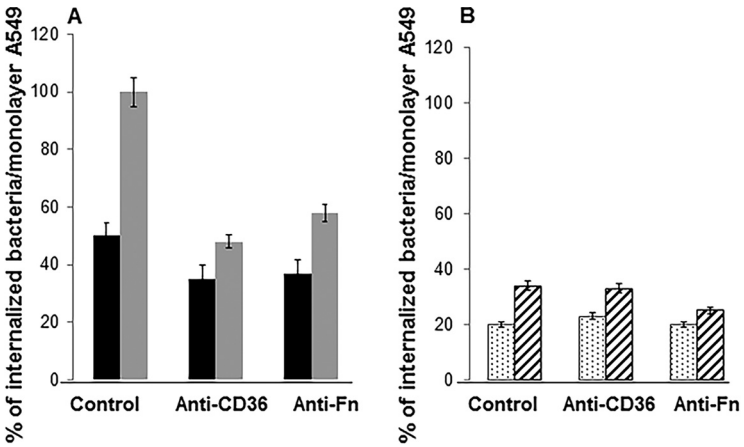

FIG 3 Quantification of the association of internalized *S. aureus* RN6390 (A) and QT7 (*tet38*) (B) and the A549 cell membrane using confocal microscopy. A549 cells were stained with the dye FM4-64. YFP-expressing bacteria (green) and host cells (red) were in contact for 2 h, followed by an additional 30 min in medium containing gentamicin and lysostaphin. We counted the numbers of green fluorescent bacteria (nonassociated bacteria) and yellow fluorescent bacteria (associated bacteria) from 60 A549 cells of 10 fields. The percentage of internalized bacteria was calculated based on the total number of green or yellow fluorescent bacteria counted from treated assays versus that of nontreated assays. The total number of RN6390 bacteria in nontreated assays was used as the reference point (100%). The assays were done in triplicate. The differences in the numbers of bacteria recovered in each assay with and without treatment with antibodies are statistically significant as determined by Student's *t* test ($P < 0.05$). Black bars in panel A represent nonassociated green cells, and gray bars represent yellow cells associated with A549 membrane. Dotted bars in panel B represent nonassociated green cells, and striped bars represent yellow cells associated with A549 membrane.

underlie the increased intracellular survival seen with RN6390 in the presence of chloroquine.

DISCUSSION

We previously demonstrated that *S. aureus* RN6390 internalized efficiently into the A549 lung epithelial cells (22) and that this ability was reduced in the absence of Tet38, a proton antiporter efflux pump that is capable of transporting tetracycline and certain antibacterial fatty acids (palmitoleic acid and undecanoic acid) but whose specific role in bacterial invasion and intracellular survival is not well understood (21). We thus undertook to evaluate directly the role of Tet38 in *S. aureus* in adherence and internalization for epithelial cells and its effect on trafficking within the host cells.

A number of *S. aureus* surface proteins are known to mediate adherence to host cells, including fibronectin-binding proteins A and B (FnbAB), elastin-binding proteins A and B (EbhAB), extracellular adhesion protein (Eap), protein A, autolysin (Atl), and other proteins collectively referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These proteins recognize and adhere to the extracellular matrix components of the host cells, such as fibronectin, fibrinogen, $\alpha_5\beta_1$ integrins, heat shock proteins Hsp60 and Hsp70, and various other receptors (28–31). Among host cell receptors, CD36 plays a major role in *S. aureus* internalization. CD36 is a member of the scavenger receptor family and is involved in the uptake of long-chain fatty acids by myocardial cells. CD36 receptors regularly cycle between the myocardial cell surface pools and endosomes (18). In certain cell lines such as the HeLa cells, overexpression of CD36 leads to an increase in *S. aureus* uptake. This capacity to facilitate bacterial uptake was demonstrated by lysosomal colocalization of *E. coli* K-12 and CD36 protein within CD36-overexpressing HeLa cells (17). In addition, there is a reduction in *E. coli* and *S. aureus* internalization and survival in CD36-deficient phagocytes and in mice deficient in class B scavenger receptors, such as CD36 and SR-BI II (16). CD36 is thought to form a cluster complex with Toll-like receptors TLR2 and TLR6 in response to diacylated lipopeptides. As a component of the host innate immunity, TLR2 recognizes teichoic acids exposed on the surface of the *S. aureus* cell wall. Furthermore,

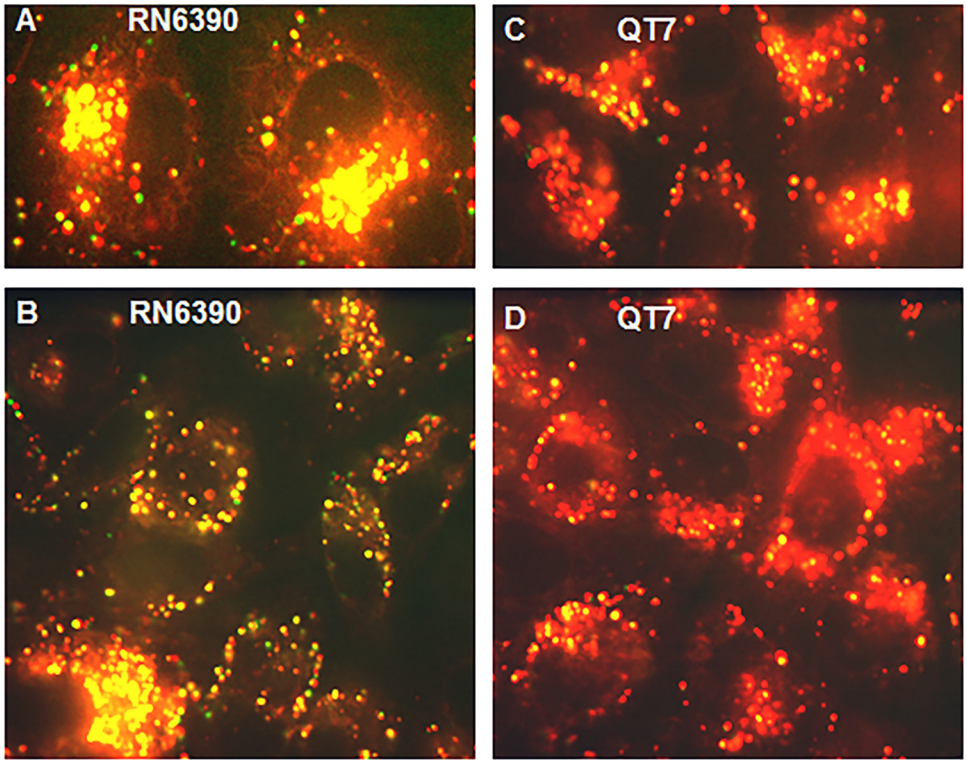

FIG 4 Confocal microscopy of *S. aureus* RN6390(pAH16) and QT7(pAH16) at 4 h after addition of lysostaphin and gentamicin in DMEM. The lysosomes of the lung epithelial cells A549 were stained with LysoTracker DND99 (red). The bacteria expressed YFP and were viewed as green cells. Colocalization of *S. aureus* and A549 was viewed as yellow cells. Representative fields are shown.

CD36 was reported as a selective and nonredundant sensor of microbial diacylglycerides that signal via the TLR2/TLR6 heterodimer (32). Thus, we tested the hypothesis that Tet38 is a bacterial protein that interacts with CD36 in mediating internalization of *S. aureus* into epithelial cells. Tet38 is an efflux pump located in the cell membrane of *S. aureus*. It is predicted from hydropathy plots to have only small extracellular domains. Thus, to be able to interact with a host cell component such as CD36, Tet38 may need an intermediate partner such as the LTA to establish the contact. The hypothesis regarding the connection between Tet38 and LTA is under investigation. Our findings document that anti-CD36 antibody specifically reduces internalization of *S. aureus* in A549 cells and that the effect of antibody is either eliminated or attenuated in an otherwise isogenic *tet38* mutant. Such attenuation in a *tet38* mutant is not seen using antibody to fibronectin, a known target of *S. aureus* fibronectin-binding protein, or antibody to TLR2, supporting the specificity of the interaction of Tet38 with CD36. In addition, the reduced *S. aureus* internalization seen in Jurkat cells, which lack CD36, is not dependent on the presence of Tet38. Our data thus strongly suggest an interaction between Tet38 and CD36, probably in an indirect manner due to the location of Tet38 within the bacterial membrane. Because high concentrations of anti-CD36 antibody can somewhat reduce internalization further in Tet38-deficient cells, it is possible that CD36 also has additional bacterial ligands or that high antibody concentrations can affect other coreceptor complex members such as TLR2 and its binding to lipoteichoic acids. Internalization assays using anti-TLR2 antibody showed that the absence of Tet38 in mutant QT7 did not affect the ability of TLR2 to interact with other bacterial components, suggesting that TLR2 has a specific staphylococcal ligand(s) that is different from Tet38. Additional binding studies are ongoing.

Endosome-internalized *S. aureus* bacteria are known to traffic to the phagolysosome, which maintains a growth-inhibitory acid pH (26). Since the *tet38* mutant fails to replicate within A549 cells and since replication of wild-type cells is delayed (which is

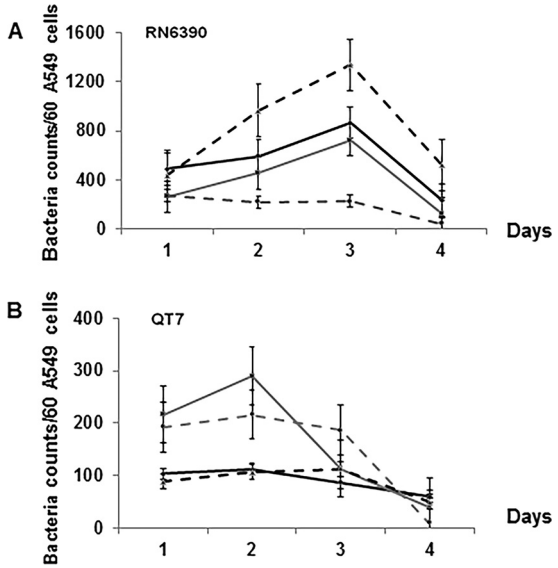

FIG 5 Quantification of the association between internalized *S. aureus* strains RN6390 and QT7 (*tet38*) and A549 phagolysosomes using confocal microscopy. A549 cells were stained with the dye LysoTracker DND99. YFP-expressing bacteria (green) and lysosomes (red) were in contact for 4 h following an additional 30 min in medium containing gentamicin and lysostaphin. The assays were carried out in the presence or absence of chloroquine (20 μM). We counted the numbers of green cells (nonassociated bacteria) and yellow cells (associated bacteria) from 60 A549 cells of 10 images (number of CFU/60 A549 cells). The assays were done in triplicate. The differences in RN6390 and QT7 bacterial cells treated or not treated with chloroquine are statistically significant as determined by Student's *t* test ($P < 0.05$). In both panels, the symbols are as follows: black line, green cells, nonassociated with lysosomes, without chloroquine; dashed black line, green cells, nonassociated with lysosomes, plus chloroquine; gray line, yellow cells, associated with lysosomes, without chloroquine; dashed gray line, yellow cells, associated with lysosomes, plus chloroquine.

possibly related to the time of the bacteria's release from the phagolysosome), we postulated that Tet38 may also affect intracellular trafficking. Using fluorescently labeled *S. aureus* and a lysosome-specific dye with confocal microscopy, we could localize intracellular bacteria within and outside the phagolysosome over the course of

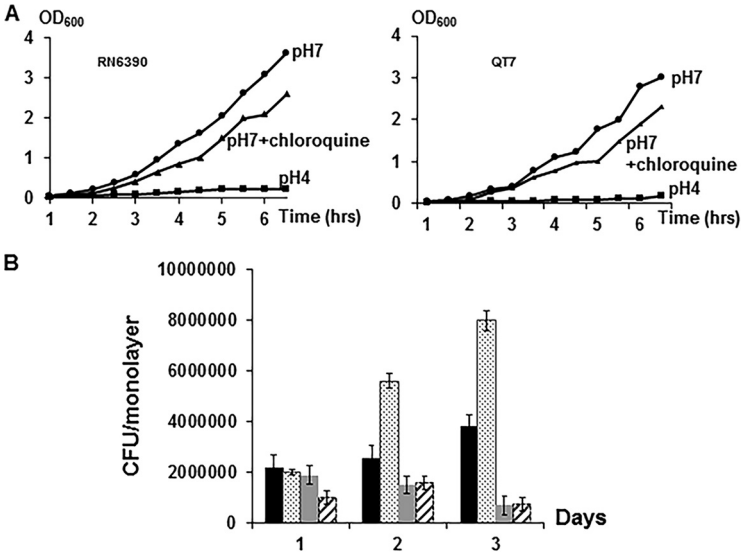

FIG 6 Growth curves of *S. aureus* in different media. (A) Growth *in vitro*. (B) Internalization of *S. aureus* in A549 cells. The effect of chloroquine treatment is shown. Black bars, RN6390; gray bars, QT7 (*tet38*); dotted bars, RN6390 with chloroquine; striped bars, QT7 (*tet38*) with chloroquine. The assays were done in triplicate. The differences in CFU of RN6390 and QT7 per monolayer recovered in each assay with and without treatment with chloroquine are statistically significant as determined by Student's *t* test ($P < 0.05$).

TABLE 1 Internalization of *S. aureus* by A549 cells

Time point (day)	*S. aureus* internalization by strain and condition (CFU/monolayer)[a]			
	RN6390		QT7	
	− Chloroquine	+ Chloroquine	− Chloroquine	+ Chloroquine
1	$(2.2 \pm 0.005) \times 10^6$	$(2.0 \pm 0.001) \times 10^6$	$(1.9 \pm 0.001) \times 10^6$	$(1.0 \pm 0.001) \times 10^6$
2	$(2.6 \pm 0.003) \times 10^6$	$(4.6 \pm 0.002) \times 10^6$	$(1.5 \pm 0.005) \times 10^6$	$(1.2 \pm 0.002) \times 10^6$
3	$(3.8 \pm 0.005) \times 10^6$	$(8.0 \pm 0.001) \times 10^6$	$(7.0 \pm 0.002) \times 10^5$	$(7.5 \pm 0.002) \times 10^5$
4	$(7.2 \pm 0.02) \times 10^3$	$(5.6 \pm 0.01) \times 10^3$	$(2.1 \pm 0.02) \times 10^3$	$(9.5 \pm 0.2) \times 10^2$
5	300 ± 10	250 ± 20	90 ± 5	50 ± 2
6	150 ± 5	100 ± 10	25 ± 5	10 ± 1

[a]Experiments were done in triplicate and with three separate biological samples with (+) and without (−) chloroquine. The differences in RN6390 and QT7 bacterial cells treated or not treated with chloroquine are statistically significant as determined by a Student's *t* test ($P < 0.05$).

several days. Intracellular *tet38* mutant bacteria remained dominantly associated with the phagolysosome over 2 days, with a progressive decrease in numbers thereafter (Fig. 5), which temporally correlated with a failure to replicate, followed by a reduction in viable cells (Fig. 6B). In contrast, wild-type intracellular bacteria with intact Tet38 consistently had greater numbers of cells not associated with the phagolysosome than associated with it, correlating with increasing numbers of viable cells over time. These findings suggest that in the absence of Tet38, bacteria become trapped in the phagolysosome, and viable cells are thereby reduced.

Acidification of the phagolysosome is thought to be an important component of the ability of host cells to control survival of intracellular bacteria (25). It has been shown that acidification of the phagosomes was dependent on the movement of protons mediated by a vacuolar-type H^+-ATPase across the phagosomal membrane. This acidification could be selectively blocked by bafilomycin A1, a macrolide antibiotic, or by the antimalaria drug chloroquine (25, 33). Chloroquine diffuses into cells and concentrates in phagolysosomes, raising their pH (26). We determined the role of acid pH in the differences between wild-type and *tet38* mutant intracellular bacteria by assessing the effect of chloroquine on cell viability and trafficking in A549 cells. As seen in other studies, reduced acidification of the phagolysosome with chloroquine increased both viability and the escape of wild-type cells from the phagolysosome (25). In contrast, reduced acidification of the phagolysosome had little or no effect on the viability of the intracellular *tet38* mutant or its remaining dominantly associated with the phagolysosome. Thus, Tet38 enables further escape from the phagolysosome when acidification is decreased. The mechanism of this effect is uncertain. Tet38 is a member of the major facilitator superfamily (MFS) which includes proton antiporters and is capable of extruding tetracycline and certain fatty acids (22). If Tet38 played a role in exchanging efflux substrates such as antibacterial fatty acids for uptake of protons, higher external proton concentrations (acidity in phagolysosome versus bacterial cell) would be expected to reduce its efflux activity and presumably also reduce its ability to protect against antibacterial fatty acids or other unknown substrates. In this case, Tet38 would not be beneficial to the bacterial cell in this acidic environment, leading to downregulation of the transcription of *tet38* within the initial hours (2 h) of internalization by epithelial cells, followed by an increase of transcription at a later time (6 h) (11, 34). Although the findings (Fig. 5) might be interpreted as evidence that Tet38 removes chloroquine from the phagolysosome, this hypothesis is unlikely since if there is export of chloroquine by Tet38 in *S. aureus* cells, it would be transported from the bacterial cytoplasm into the phagolysosome. Furthermore, although CD36 is known to traffic in endosomes, internalization experiments argue that CD36 and Tet38 interact at the neutral pH in the uptake experiments and would not support a CD36-mediated model for endosomal release of *S. aureus* when the pH of the phagolysosome is raised. The role of Tet38 in extruding various substrates, including certain unsaturated fatty acids, as well as its location in the cell membrane suggests that Tet38 might also participate in maintaining the staphylococcal membrane fluidity and composition. This

TABLE 2 Bacterial strains, plasmids, and cell lines used in this study

Strain, plasmid, or cell line	Genotype or relevant characteristic(s)	Reference or source
S. aureus strains		
RN6390	8325-4 wild type	39
QT7	8325-4 *tet38*::*cat* or Δ*tet38*	40
RN6390(pLZ113-*tet38*)	*tet38* overexpressor	This study
RN6390(pAH16)		This study
QT7(pAH16)		This study
Plasmids		
pAH14	Plasmid for *sarA* P1-dependent YFP 10B expression, Erm^r	36
pAH16	pAH14 with *sod* ribosome binding site	36
pLZ113	*E. coli-S. aureus* shuttle vector pRB373 with *xyl/tet* promoter cloned into the multiple cloning site	41
Cell lines		
A549	Human lung adenocarcinoma	11
Jurkat	Leukemic T-cell lymphoblast	11

hypothesis would explain the perturbation that occurred in the absence of this transporter. Such a case was previously reported with the QacA efflux pump of *S. aureus*, where a *qacA*-bearing strain exhibited larger membrane fluidity than its parental strain while conferring resistance to a cationic antimicrobial polypeptide, tPMP-1 (35). Future work will be necessary to define other roles of Tet38 and the effects of pH on Tet38 function in the phagolysosome.

In summary, we have shown that interaction of Tet38 with CD36 contributes to the internalization of *S. aureus* into nonprofessional phagocytes such as A549 epithelial cells. In addition, Tet38 also affects trafficking to and from the phagolysosome, contributing to the ability of intracellular *S. aureus* to replicate and remain outside acidic, and strikingly outside nonacidic, phagolysosomes. These findings highlight the multiple functions of bacterial efflux pumps that extend beyond their ability to confer resistance to antimicrobials and host antibacterial substances to function in support of bacterial pathogenesis, thereby linking fitness and resistance capabilities.

MATERIALS AND METHODS

Cell lines, bacterial strains, and culture media. The bacterial strains, plasmids, and cell lines used in this study are listed in Table 2. Human lung adenocarcinoma A549 cells and Jurkat cells were purchased from ATCC (CCL-185) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) and RPMI medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine (Fisher Scientific, Waltham, MA). The medium is referred to as assay medium depending on the cell lines used in the assays. For confocal microscopy experiments, DMEM without phenol red and pyruvate was used. A549 cells were grown at 37°C in 5% CO_2. The *S. aureus* parental strain RN6390 was compared with the *tet38* isogenic mutant QT7 (*tet38*::*cat*) to assess their ability to survive inside epithelial cells following standard invasion assays. The growth curves of RN6390 and QT7 were carried out using Luria-Bertani (LB) broth with the pH adjusted to 4.5 or 7. Chloroquine, ampicillin, gentamicin, Triton-X, and lysostaphin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

***S. aureus* expressing YFP protein from a plasmid.** Plasmid pAH16 carries the *sarA* P1 promoter and the ribosome-binding site of the *sod* gene that drive expression of the YFP 10B fluorescent reporter protein. This plasmid confers resistance to erythromycin (10 μg/ml) and provides fluorescence in *S. aureus* transformants (36). RN6390 and QT7 were transformed with plasmid pAH16 by electroporation and plated on LB agar plates supplemented with 10 μg/ml erythromycin to select YFP-expressing transformants.

***S. aureus* growth in the presence of acid pH and chloroquine (*in vitro*).** *S. aureus* RN6390 and QT7 were prepared from overnight cultures and grown until the optical density at 600 nm (OD_{600}) reached 0.4. At this time point (time zero), two series of tubes (one series for RN6390 and one series for QT7) with 10 ml of bacterial culture in each tube were centrifuged to collect the bacterial pellets. Each pellet was resuspended into 10 ml of LB broth at pH 4.5 or at pH 7.0. To assess the effect of chloroquine on bacterial growth, we resuspended the bacterial pellet for each strain in 10 ml of LB broth at pH 7.0, supplemented with chloroquine at 20 μM. The cultures were then incubated under shaking at 37°C. Culture samples (1 ml) were taken hourly up to 6 h to measure the OD_{600} and for serial dilution and plating on LB agar plates.

Internalization of *S. aureus* by A549 epithelial cells. The internalization assays were performed as previously described with some modifications (22). A549 cells were cultured in 5 ml of assay medium until they reached 90% confluence in a 25-ml tissue culture flask and were then seeded into 24-well plates (Costar) in assay medium and grown again to 90% confluence. The A549 cell concentration was adjusted to 10^4 cells/ml. *S. aureus* RN6390 or QT7 was prepared from overnight cultures and grown to an OD_{600} of 0.4. The bacteria were washed twice with 1× phosphate-buffered saline (PBS), and the bacterial pellet was resuspended in 10 ml of fresh assay medium to a concentration of 10^6 CFU/ml. For internalization assays and confocal microscopy imaging, A549 cells were infected with *S. aureus* at a multiplicity of infection (MOI) of 100 bacteria per epithelial cell (MOI of 100:1; 10^6 washed bacteria/10^4 A549 cells). The 24-well plates were centrifuged quickly for 1 min at 500 × *g* to allow bacterial adhesion to the cell monolayer. The bacterium-cell mixtures were incubated at 37°C in 5% CO_2 for 2 h, and then the infected monolayers were washed three times with 1× PBS to remove residual nonadherent bacteria. The washed monolayers were incubated for 30 min at 37°C in 5% CO_2 in assay medium with 200 μg/ml gentamicin and 20 μg/ml lysostaphin. Monolayers were again washed three times with 1× PBS, and the A549 epithelial cells were lysed with 200 μl of Triton X-100 (1×). Bacteria were diluted in PBS and plated on LB agar plates, and colony counts were performed to determine the number of viable intracellular bacteria. To assess the intracellular viability of *S. aureus* over time, the monolayers were incubated at 37°C in 5% CO_2 in assay medium with 200 μg/ml gentamicin and 20 μg/ml lysostaphin for an additional 60, 120, and 180 min prior to plating. The *tet38*-overexpressor RN6390(pLZ113-*tet38*) and the complemented mutant strain QT7(pLZ113-*tet38*) were used as additional controls for the effect of *tet38* on bacterial internalization.

Intracellular survival of *S. aureus* in the presence of chloroquine. The intracellular survival assay was based on the method of Leimer et al. (25). Gentamicin- and lysostaphin-containing medium was supplemented with chloroquine to a final concentration of 20 μM (G/L medium-chloroquine) and used in the survival assays. Gentamicin and lysostaphin medium (G/L medium) was used as a control. A549 cells were infected with *S. aureus* RN6390 or QT7 as described above at an MOI of 100. Bacterial internalization was carried out for 2 h, and the monolayers were washed three times with 1× PBS and then treated with G/L medium for 2 h to eliminate extracellular bacteria. Absence of bacteria at the surface of the monolayers was verified by plating of the assay medium on LB agar plates. The monolayers were again washed three times with 1× PBS, and new medium (G/L medium-chloroquine or G/L medium) as a control was added to the monolayers. The assays were carried out over 6 days, with fresh medium added to the infected monolayers daily. At each time point, the monolayers were washed with 1× PBS and lysed with 150 μl of Triton-X. Serial dilutions of the cell lysates were performed, followed by plating on LB agar plates. Integrity of the A549 cells was verified by microscopy with or without invasion by *S. aureus* RN6390 or QT7.

Confocal microscopy of intracellular *S. aureus*. A549 epithelial cells were cultivated in assay medium without phenol red (Fisher Scientific, Waltham, MA), seeded into an eight-chambered cover glass (250 μl per chamber), and incubated at 37°C and 5% CO_2 overnight. Following addition of the dye FM4-64 (which stains cell membranes and organelles) according to the manufacturer's recommendation (50 μM final concentration; 20× dilution from the stock solution of 1 mM) or of the red fluorescent dye LysoTracker DND99 (1 μM final concentration; 1,000× dilution from the stock solution of 1 mM) (Fisher Scientific, Waltham, MA), the cells were incubated for 30 min at 37°C and 5% CO_2 for the staining step. After staining, the A549 cells were washed twice with 1× PBS, and 250 μl of fresh medium without phenol red was added. RN6390 and QT7 transformed with the YFP fluorescent plasmid pAH16 were subcultured from an overnight culture and incubated until the OD_{600} reached 0.4. The bacterial cultures were adjusted to yield an MOI of 100 bacteria per A549 cell for the internalization assays. The assays were carried out as described above, and confocal imaging was done every hour following treatment of the infected monolayers with G/L medium up to 5 h. In assays performed with chloroquine-supplemented medium, the imaging was carried out for a period of 4 days to assess the survival of *S. aureus* after fusion of the bacterium-containing endosomes with lysosomes. A chambered cover glass was mounted onto a Nikon Ti-E inverted microscope fitted with a spinning disc confocal detection head (Yokogawa, Sugar Land, TX). A 4-W, continuous-wave laser (Coherent, Santa Clara, CA) or solid-state UV laser was used to produce excitation wavelengths of 405 nm or 647 nm. Cells and bacteria were imaged using a Nikon 100× objective with a high numerical aperture (NA) (1.49 NA, oil immersion objective; Nikon). Images were captured using an electron-multiplying charge-coupled device (EM-CCD) camera (C9100-13; Hamamatsu, Bridgewater, NJ) and analyzed using MetaMorph software (Molecular Devices, Downington, PA) (37).

Quantitation and localization of internalized bacteria. The bacterial internalization assays (*S. aureus* with A549 cells) for confocal microscopy imaging were carried out as described above. A549 cells were stained with LysoTracker DND99. Following 30 min of incubation in G/L assay medium at 37°C under 5% CO_2, the *S. aureus*-infected monolayers were washed three times with 1× PBS, and new G/L assay medium without phenol red was added to each chamber of the eight-chambered cover glass. The imaging was focused on various fields of *S. aureus*-internalized A549 cells, and an average of 10 images (10 fields) was captured for each *S. aureus* strain (RN6390 or QT7). The number of bacteria within six host cells in each of 10 successive fields was counted at each time point. The bacteria were represented as green dots (nonassociated with lysosomes) or yellow dots (associated with phagolysosomes). The bacterial counts are the average numbers taken from three separate experiments for each strain and condition. We counted the bacteria of 60 A549 cells in 10 confocal image fields. Each experiment was done in triplicate.

Internalization of *S. aureus* into A549 cells treated with anti-CD36, anti-Fn, and anti-TLR2 antibodies. The standard assay described above was carried out with some modifications. Prior to adding *S. aureus* to the monolayers, the host cells were preincubated at 37°C under 5% CO_2 for 30 min in assay medium containing 1% bovine serum albumin (BSA) plus anti-CD36, anti-Fn, or anti-TLR2 antibody (Life Technology, Grand Island, NY) at a final concentration of 50 nM. This assay was based on a prior study of the heat shock protein Hsp60 (13). The cell cultures, without washing, were infected with *S. aureus* RN6390 or QT7 according to the standard protocol. The number of intracellular bacteria was enumerated as above, and each assay was repeated three times. Internalization assays with or without added antibody were also carried out using Jurkat cells, a CD36-nonexpressing cell line, as a control (38). A mouse IgG nonspecific isotype control (Life Technology, Grand Island, NY) was used at the same concentration (50 nM) as the antibodies tested in the assays and served as a negative control for the tested antibodies.

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