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Human Leukocytes Adhere to, Penetrate, and Respond to *Staphylococcus aureus* Biofilms

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***Staphylococcus aureus* is a common pathogen responsible for nosocomial and community infections. It readily colonizes indwelling catheters, forming microbiotic communities termed biofilms. *S. aureus* bacteria in biofilms are protected from killing by antibiotics and the body's immune system. For years, one mechanism behind biofilm resistance to attack from the immune system's sentinel leukocytes has been conceptualized as a deficiency in the ability of the leukocytes to penetrate the biofilm. We demonstrate here that under conditions mimicking physiological shear, leukocytes attach, penetrate, and produce cytokines in response to maturing and fully matured *S. aureus* biofilm.**

Bacterial biofilms are mechanisms used by bacteria that cause chronic infections in humans (5). They are associated with a number of diseases including cystic fibrosis (24), endocarditis (3), osteomyelitis (23), and various nosocomial diseases related to central venous catheters (19), urinary catheters (20), prosthetic heart valves (12), and orthopedic devices (10). Biofilms are well suited for resistance to antibiotics and evasion of the immune system's defenses, and the biofilm mode of growth may be a specific form of microbial development (18, 21, 27).

The bacterial pathogen *Staphylococcus aureus* has the ability to form biofilms under physiological conditions (4, 13, 19). The increased prevalence of antibiotic resistance, including recent isolates demonstrating intermediate resistance to vancomycin, has induced a surge in efforts to better understand the nature of *S. aureus* virulence both in vitro and in vivo (15, 25). The mechanisms behind biofilm resistance, especially with regard to leukocytes, are not clear and have been explained by the paradigm of a lack of penetration by the leukocytes and a decreased ability of phagocytes to actively kill the bacteria, a process termed "frustrated phagocytosis" (11, 27).

The ability of leukocytes to traffic to sites of inflammation and exert their respective effector functions is vital in maintaining overall health. One of the important parameters of leukocyte trafficking is the ability of adhesion molecules to function optimally under laminar-shear conditions. Indeed, the selectin family of adhesion proteins, which are responsible for the first step in the trafficking cascade, have a requirement of a threshold amount of shear for optimal function (8). Here we have investigated the interactions of freshly isolated human leukocytes with 2-day-old (early, maturing) and 7-day-old (fully mature) *S. aureus* biofilms under static and dynamic conditions that mimic physiological shear in vivo.

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MATERIALS AND METHODS

Strains and plasmids. The *S. aureus* strains used is this study were Seattle 1945 and a clinical isolate from the University of Texas Medical Branch in Galveston that we refer to as MRSA. The Seattle 1945 strain of *S. aureus* was transformed with a green fluorescent protein (GFP)-producing plasmid to produce 1945^{GFPuvr}. The plasmid was constructed by inserting the promoter for the global regulator *sarA* into the upstream region of a promoterless GFP adapted for maximal expression in *S. aureus*, GFP*uvr*, as previously described (9). This *sarA*-GFP*uvr* construct is contained within the pSK236 plasmid, which carries a chloramphenicol resistance cassette and a gram-positive origin of replication (14).

Growth conditions. To observe the morphology of *S. aureus* biofilms under shear conditions like those seen in catheter-related infections, artificial heart valve-related infections, and endocarditis, we used an in vitro flow system that allows for reproducible biofilm growth as well as real-time observations by digital time-lapse and scanning confocal laser microscopy (SCLM) as described previously (28). Briefly, square (1- by 1-mm) glass flow cells were inoculated with $\sim 10^6$ CFU of *S. aureus* strain Seattle 1945 at the logarithmic stage and allowed to attach to the internal surface of the glass for 30 min without shear. Subsequently, flow was applied at a rate of 1 ml/min to the cell and the biofilm was allowed to form at 37°C for the specified periods. This flow rate corresponds to ~ 1 dyn/cm^2.

Isolation of human leukocytes. Peripheral blood was collected from healthy human donors by venipuncture into ACD Vacutainer blood collection tubes as described previously (17). Whole blood was diluted 1:1 with Hanks' balanced salt solution (HBSS; Gibco-BRL, Grand Island, N.Y.), underlaid with Histopaque 1077 (Sigma, St. Louis, Mo.), and centrifuged at $700 \times g$ for 30 min. The buffy coat containing peripheral blood mononuclear cells was collected and washed with HBSS, and if red blood cells were present, they were lysed in a hypotonic solution for 10 s and then rapidly diluted in HBSS and centrifuged. In some cases, monocytes were depleted from the sample by incubation for 30 min at 37°C on plastic in Dulbecco modified Eagle medium (Sigma) containing 0.7% bovine serum albumin (Sigma). Purified lymphocytes were collected, centrifuged, and suspended in either HBSS or phosphate-buffered saline (Sigma). The collection, isolation, and use of human peripheral blood leukocytes for these studies was approved by the appropriate institutional review board at Montana State University, and the use of human subjects for these studies followed the IRB and federal guidelines.

Time-lapse video microscopy. To observe leukocyte interactions with the biofilm under flow, human peripheral blood leukocytes (5×10^7) were isolated on the morning of the assay as described above and injected into the assay mixture without turning off the shear. The interactions were observed and recorded by videotape for analysis. Grey-scale images were captured by a COHU 4612-5000 charge-coupled device camera and a Scion VG-5 PCI framestore board. After the assay, the flow cell was removed and stained with the BacLight Live/Dead prokaryotic staining kit (Molecular Probes) as specified by the manufacturer and observed by confocal microscopy. Representative micrographs from three separate experiments are shown. Of note, although the stain was a prokaryotic stain, it did stain the leukocytes, most probably because the solvent contained dimethyl sulfoxide. Thus, the leuko-

TABLE 1. Cytokine response to *S. aureus* biofilms

Cytokine	Medium alone	WBCs alone	Biofilm alone	Amt of cytokine produced (pg/ml) (mean ± SEM) WBCs/ biofilm[d]	Flow cell
IL-1β	0.07 ± 0.08	0.02 ± 0.63	1.04 ± 0.31[a]	8.12 ± 1.85[a] (0.01)	ND
			2.11 ± 0.78[b]	10.14 ± 1.39[b] (0.01)	
IL-12	0.12 ± 0.04	0.21 ± 0.18	0.44 ± 0.36[a]	2.48 ± 0.44[a] (0.01/0.02)	ND[a,c]
			0.90 ± 0.87[b]	11.40 ± 1.88[b] (0.01)	2.16 ± 0.23[b] (0.08/0.03)
IFN-γ	0.06 ± 0.08	0.12 ± 0.04	0.14 ± 0.10[a]	0.38 ± 0.07[a] (0.04/0.01)	ND[a]
			0.30 ± 0.13[b]	2.09 ± 0.09[b] (0.01)	0.65 ± 0.18[b] (0.09/0.02)

[a] Values are for MRSA biofilm.
[b] Values are for Seattle 1945 biofilm.
[c] ND; not determined
[d] *P* values are listed in parentheses; the first value is biofilm alone versus WBCs/biofilm, and the second value is WBCs alone versus WBCs/biofilm. If the *P* value was equal for the two comparisons, only one value is listed.

cytes appear both green and red in the pictures presented here, but we do not interpret this to definitively show that the leukocytes are alive or dead.

***S. aureus* 1945^{GFPuvr} flow cell assay.** Strain 1945^{GFPuvr} (described above) was grown as a planktonic culture (i.e., with shaking) to log phase (growth at 37°C in brain heart infusion plus 10 μg of chloramphenicol per ml), washed, and incubated for 1 h at 37°C with chloromethylbenzoylaminotetramethyl-rhodamine (CMTMR)-stained leukocytes (at 10^7/ml of RPMI buffer with 20% autologous serum). The leukocytes were fluorescently labeled using the CMTMR Cell Tracker probe (Molecular Probes, Eugene, Oreg.) as specified by the manufacturers. Incubated suspensions were then observed by using a Leica TCS-NT SCLM instrument, and digital images were acquired. Simultaneous pseudocolor dual-channel SCLM imaging with 488-nm excitation was used to display green fluorescence and red fluorescence, representing 1945^{GFPuvr} and isolated leukocytes, respectively. We also examined the interaction of leukocytes with 7-day-old *S. aureus* biofilms of this GFP-producing strain (1945^{GFPuvr}) grown in square glass flow cells (1 by 1 mm). Briefly, medium flow (0.1 × brain heart infusion broth plus 10 μg of chloramphenicol per ml) was administered in this "once-through" system via silicon tubing with a mixing chamber positioned immediately before the flow cell, allowing for media aeration. The chamber also acted as a bubble trap and maintained the system at 37°C. Approximately 10^6 CFU of *S. aureus* 1945^{GFPuvr} at the logarithmic growth stage was inoculated into the flow cells after medium flow was arrested. Microbes were incubated for 30 min to allow their attachment to the internal surfaces of the flow cell. Flow was then reapplied, and biofilms were grown for 7 days. On day 7, the flow system was reconfigured from a once-through design to a recirculation mode and CMTMR-stained leukocytes (5×10^7) were added to the flow system. The interaction of the leukocytes with the *S. aureus* biofilm was monitored in the flow cell for 1 h. Data are representative of three separate experiments.

Cytokine assay. To measure soluble cytokine production, a new assay by Pierce Endogen was used as specified by the manufacturer. Importantly, this assay allows the simultaneous observation of up to nine different cytokines by using a cooled charge-coupled-device camera for image capture and data acquisition. Biofilms were grown under static conditions in six-well plates with both strains for 48 h at 37°C. Freshly isolated human peripheral blood leukocytes (10^7) were incubated with the biofilms and control planktonic cultures for 2 h at 37°C, and the supernatant fluid was collected, centrifuged twice, and incubated with the anti-human cytokine monoclonal antibodies (MAbs) coated onto the bottom of the Pierce Endogen 96-well plate. The samples were treated as specified by the manufacturer, and the presence of cytokines was noted by monitoring the luminol reaction with an Alpha Innotech IS-1000. Both visual and numerical data were obtained, and the numerical data are reported as picograms of cytokine per milliliter as determined from standard curves. Aliquots of all samples were sent directly to Pierce for on-site analysis. The values listed in Table 1 are the average from four separate human leukocyte samples run in duplicate on two separate occasions. Control supernatant fluid samples from leukocytes and biofilms incubated separately were run parallel to each test sample. For each cytokine, we conducted two *t* tests for each of the two strains, for a total of four tests. According to the Bonferroni multiple-comparison procedure, an adjusted *P* value less than 0.125 would provide a simultaneous significance level of 0.05 for the tests at each cytokine. Thus, after applying the Bonferroni procedure to our *t* tests, we have reported the data in terms of the *P* value of each cytokine for each bacterial strain. Values of *P* less than 0.05 were considered significant.

RESULTS

Human leukocytes adhere to 7-day-old *S. aureus* biofilms under shear. Since human leukocytes function under conditions of laminar shear, we chose to observe the interactions of leukocytes with biofilms under similar conditions. By real-time

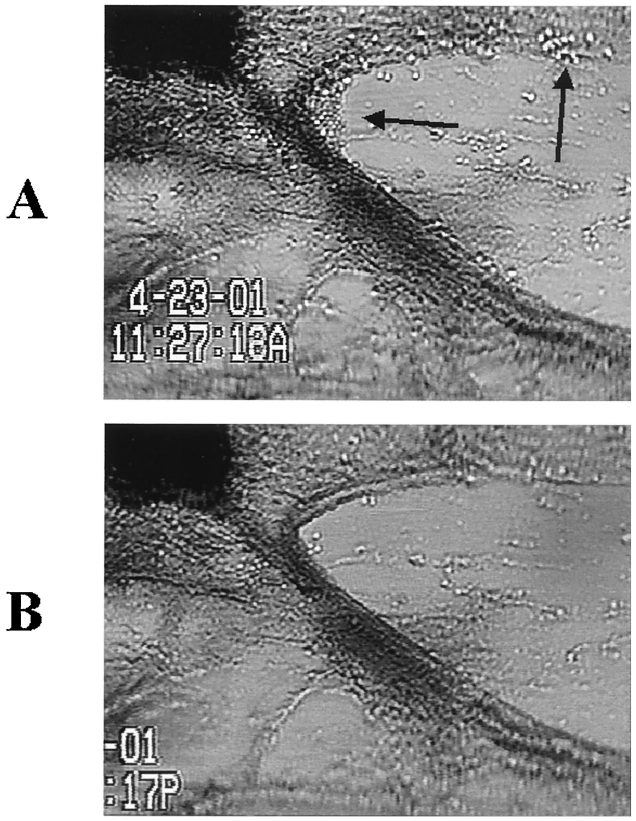

FIG. 1. Digital time-lapse microscopy of human leukocytes interacting with a *S. aureus* biofilm (strain 1945). (A) Micrograph snapshot of leukocytes interacting with a 7-day-old *S. aureus* biofilm grown under flow conditions. Arrows identify biofilm-bound leukocytes. Note the large number of attached leukocytes in the pocket that are not present in panel B. (B) Micrograph snapshot of leukocytes interacting with the same biofilm as in panel A (~1 h following leukocyte addition to the system). Note the reduction in leukocyte numbers attached to the surface of the biofilm later in the assay.

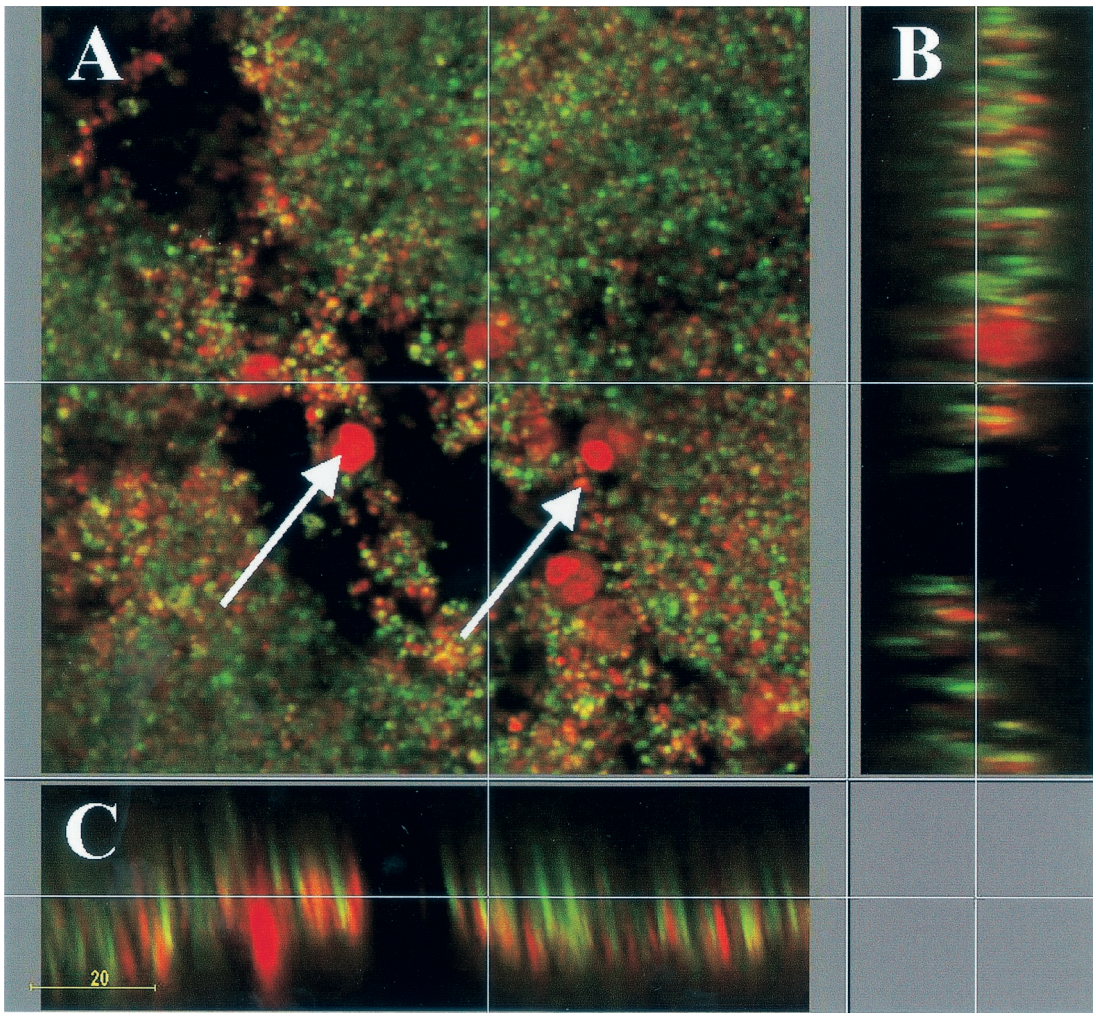

FIG. 2. SCLM of human leukocytes interacting with an *S. aureus* biofilm. (A) Micrograph showing leukocytes (arrows) attached to and within an *S. aureus* biofilm. Note the presence of leukocytes and channels within the biofilm. (B and C). *x* (B) and *Y* (C) cross-sections, showing that the leukocytes have penetrated to the base of the biofilm. Bar, 20 μm.

video microscopic observation in a closed-loop system, we observed that leukocytes attached to 7-day-old *S. aureus* biofilms under conditions mimicking physiological flow (Fig. 1A) (2). As seen with other leukocyte-trafficking studies, additional recruitment was initially observed from immobilized leukocytes at the sites of attachment (on-line supplemental data which include an avi movie of the still frames seen here [http://www.erc.montana.edu/Res-Lib99-SW/movies/default.htm]) (2). Interestingly, our data demonstrated that leukocytes accumulated in the pockets, creases, and channels of the biofilm, as demonstrated in Fig. 1.

Over the duration of the assay (1 h), progressively fewer circulating leukocytes were observed both in the main flow channel and adherent to the pockets and channels within the biofilm (Fig. 1B). After 1 h of leukocyte exposure to the biofilm, the biofilm was stained with BacLight prokaryotic live/dead stain and observed by SCLM (2).

Human leukocytes penetrate 7-day-old *S. aureus* biofilms under laminar-shear conditions. To observe any changes in the biofilm after incubation with human leukocytes for 1 h, SCLM was employed. Surprisingly, leukocytes were found attached to and embedded within the *S. aureus* biofilm, demonstrating that the leukocytes had been able to penetrate the biofilm under physiological flow (Fig. 2 and 3 and on-line supplemental data). Analysis of single leukocytes embedded within the biofilm consistently demonstrated that a zone of clearance existed around the leukocytes, creating what we term a "leukocyte halo" (Fig. 3). Additionally, both live and dead bacteria surrounded the embedded leukocytes, suggesting that although the leukocytes penetrated the biofilm, they had not killed the bacteria in close proximity over the duration of our flow assays (1 h). Importantly, dead leukocytes that were treated with paraformaldehyde, and thus did not have the active ability to extravasate, did not fully penetrate the 7-day-old biofilms (data not shown).

Human leukocytes actively phagocytose planktonic *S. aureus* but not *S. aureus* within the biofilm. To further explore the significance of both leukocyte penetration and the halo observed around the leukocytes, we constructed a GFP-expressing *S. aureus* (Seattle 1945 strain) in an attempt to observe

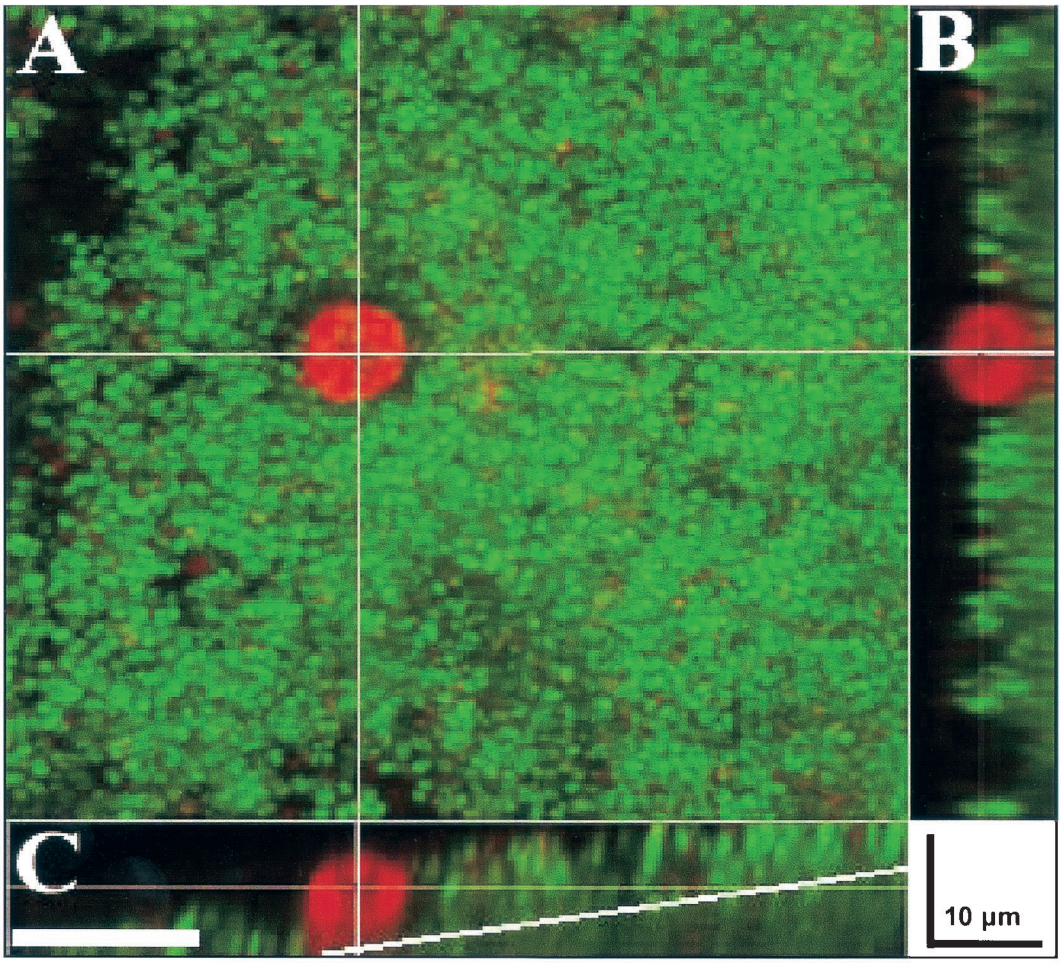

FIG. 3. High-magnification SCLM of a single representative leukocyte bound within an *S. aureus* biofilm. (A) Micrograph showing the "halo" of space surrounding the leukocyte. (B and C) Representative cross-sections (see Fig. 2 Legend). Bar, 10 μm.

ingested bacteria. For this assay, the human leukocytes were stained orange/red with CMTMR (Molecular Probes) and allowed to interact with a GFP-expressing 7-day-old *S. aureus* biofilm under laminar-shear conditions. Although the leukocytes were able to penetrate the biofilm, as observed in the previous assays, we found no internalization of individual biofilm bacteria within the penetrating leukocytes (Fig. 4A). However, these leukocytes were able to engulf planktonic GFP *S. aureus* demonstrating that the phagocytic mechanisms of the leukocytes were intact (Fig. 4B). Thus, although there was ample penetration, the leukocytes were still unable to actively engulf bacteria under these experimental conditions.

Human leukocytes bind to, but do not penetrate, a 2-day-old *S. aureus* biofilm grown under static conditions. We also compared leukocyte interactions of the fully mature, 7-day-old *S. aureus* biofilm to those of a maturing 2-day-old biofilm grown under static conditions. Interestingly, most leukocytes did not fully penetrate the 2-day-old *S. aureus* biofilm, although they were able to readily attach to the biofilm (Fig. 5 and on-line supplemental data). The significance of this observation remains unclear. However, as the micrograph shows, there is a large amount of extracellular polymeric substrate (EPS) associated with the statically grown biofilm that is not present in the 7 day-old shear grown biofilm. Therefore, the large amounts of EPS may hinder the ability of the leukocytes to effectively penetrate the microcommunity. Depending on the type of infection, i.e., in a static setting (prosthetic implant infections) or in a shear setting (endocarditis), this may affect the ability of leukocytes to attack a bacterial biofilm. Previous studies in our laboratory have demonstrated that biofilms grown under shear conditions have much smaller amounts of EPS associated with them (data not shown). Studies are currently under way to address this question.

Human leukocytes produce a Th1-type response to both the 7-day-old shear and 2-day-old static *S. aureus* biofilms. Another important aspect of a successful inflammatory response to invading pathogens is the production of inflammatory and regulatory cytokines. For example, leukocytes mount a Th1-type response to planktonic *S. aureus* bacteria, although Th2-type responses also occur (1, 22). Therefore, we investigated the cytokine response of human leukocytes to *S. aureus* biofilms under static and shear conditions.

In response to incubation with 2-day-old biofilms grown under static conditions, leukocytes produced increased quantities of interleukin-1β (IL-1β), IL-12 and gamma interferon (IFN-γ) as determined by the Pierce Endogen Searchlight hu-

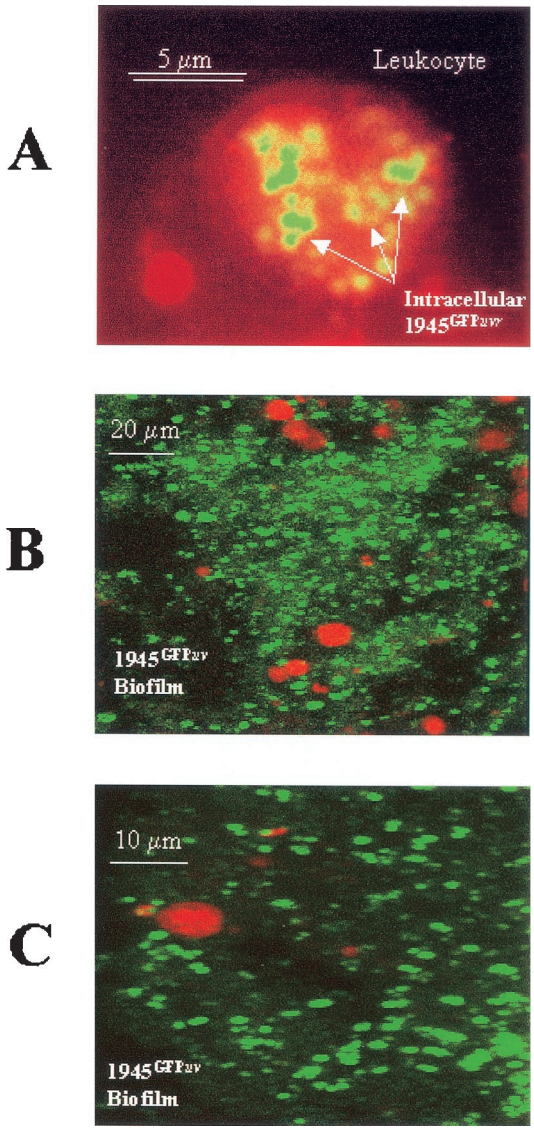

FIG. 4. Epifluorescence microscopy and SCLM of human leukocytes and GFP-expressing *S. aureus.* (A) Epifluorescence micrograph showing GFP-expressing *S. aureus* (planktonic) inside a human leukocyte. Note the numerous planktonic bacteria that have been engulfed (arrows). (B) SCLM of human leukocytes embedded within a GFP-expressing *S. aureus* (7-day-old) biofilm. (C) Higher magnification of a single human leukocyte that has penetrated the 7-day-old *S. aureus* biofilm. Note the absence of GFP-expressing *S. aureus* cells inside the representative leukocyte. Separate scale bars are listed for each micrograph.

man Th1/Th2 cytokine array (Table 1). This new assay allows simultaneous characterization of up to nine different human cytokines in one 96-well plate. Although the assay measures nine cytokines, we have reported only the positive results that were specific to leukocyte incubation with the bacterial biofilms. There were other cytokine responses, such as IL-8, that were observed in the assay but were similar to the responses seen when the leukocytes were incubated alone. Therefore, we determined these results to be nonspecific and have not reported them here. Similar results were also obtained from the supernatant fluid collected from the flow cell assay with the 7-day-old biofilm, although the cytokine response was observed to be less robust. This could have been due to the increased volume of the flow cell diluting the overall cytokine signals as well as the fact that the flow cell assay was run for only 1 h instead of 2 h as in the static assay. Additionally, we incubated planktonic *S. aureus* with human leukocytes and also observed an upregulation of both IL-12 and IFN-γ as previously reported (reference 1 and data not shown).

The fact that the biofilm alone and flow cell samples did not have *P* values smaller than 0.05 is misleading, since anti-cytokine MAb pretreatment experiments were performed with the samples and did not reduce the amount of signal. This was especially apparent with the IL-13 and tumor necrosis factor alpha samples, where large luminescence values were obtained which were not quenched by specific MAb preincubation of the samples. Thus, the inherent background in the biofilm alone samples was probably a nonspecific interaction caused by unknown interactions with the respective anti-cytokine MAbs. Importantly, significant differences were observed between the white blood cells alone and the flow cell samples, suggesting that even under shear, human leukocytes still produced Th1-type cytokines.

DISCUSSION

The data presented here offer new insights into human leukocyte interactions with medically relevant bacterial biofilms under conditions that mimic physiological shear. In contrast to the current literature, which suggests that a physical barrier around the respective biofilm microcommunities serves as a potential resistance mechanism, we demonstrate that human leukocytes do penetrate *S. aureus* biofilms (6, 7, 16, 26). Therefore, other properties of biofilms provide the basis for the inability of the host immune system to eliminate these infections. The previous assays that were employed to study leukocyte-biofilm interactions did not take into account the role of shear on both leukocyte function and biofilm architecture. Thus, one explanation for the results presented here is that under conditions mimicking those found in vivo, human leukocytes are able to more effectively penetrate the biofilm, possibly by using the nutrient/flow channels that exist in a mature biofilm. Additionally, we have previously demonstrated that biofilms are more like an extremely porous hydrogel than a solid, rigid structure (29). Our data further support this observation. Moreover, the fact that the leukocytes were able to penetrate the biofilm but unable to engulf the bacteria present in the biofilm suggests that other mechanisms that inhibit normal leukocyte function are probably present. Further investigation is needed to discern what is occurring in the "halo" that was consistently observed in our studies.

Biofilm-related infections continue to increase in both prevalence and importance in clinical medicine. As more studies are undertaken, it is clear that a wide range of persistent infections may be related to the ability of infectious organisms to form biofilms. If progress is going to be made in treatment and patient health, it is vital to obtain a better understanding of what occurs between the immune system's cells and the bacteria present in the biofilm. Our data suggest that more complex mechanisms are involved in biofilm resistance to attack from the immune system and that the current view of

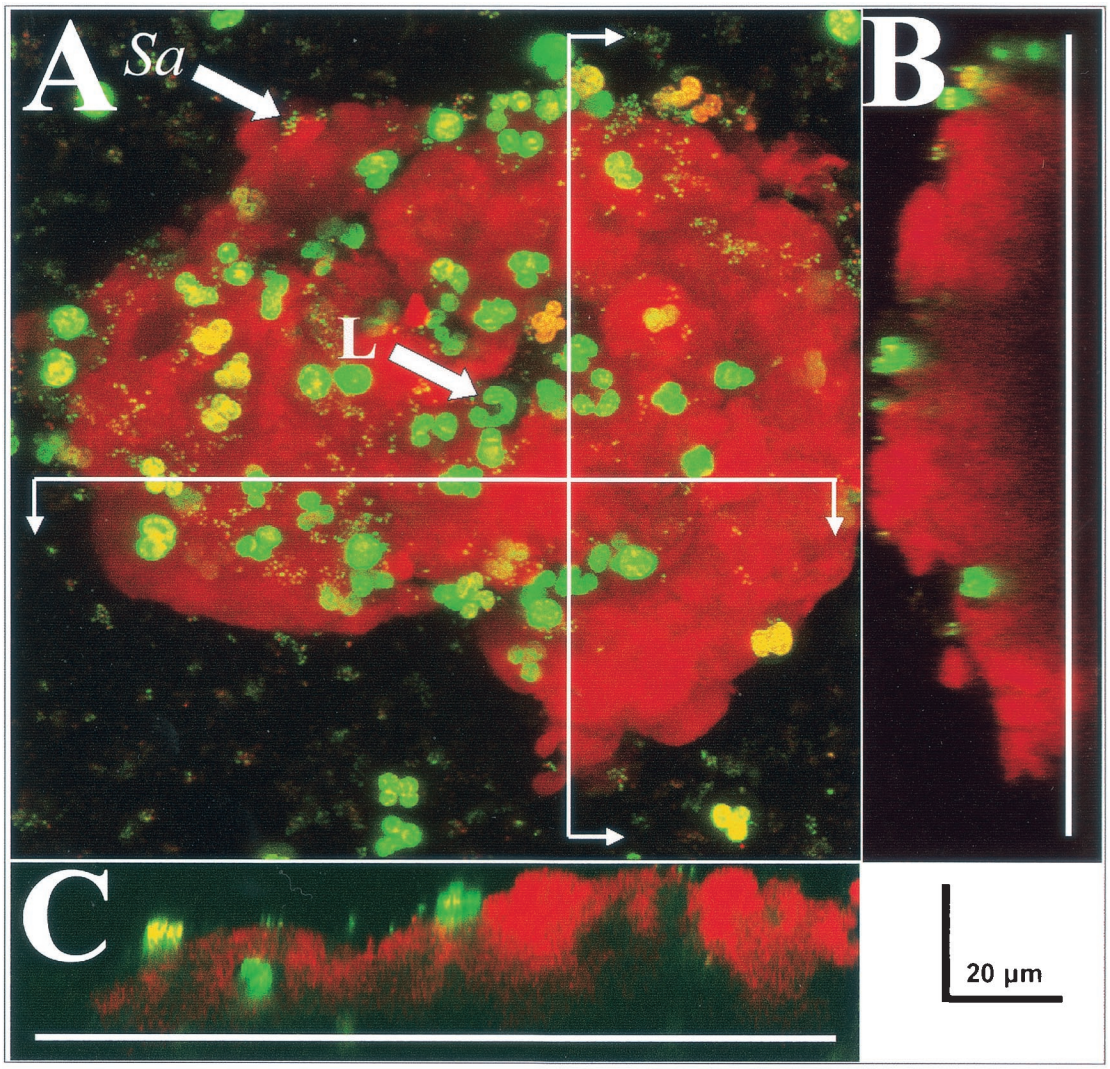

FIG. 5. (A) SLCM showing green-fluorescing leukocytes (L) attached to and within a statically grown, 2-day-old *S. aureus* biofilm (*Sa*). Note the presence of multilobed nucleated leukocytes within the biofilm. (B and C) *x* (B) and *y* (C) cross-sections showing that the leukocytes have not fully penetrated the biofilm but have simply sunk into the physical space that is present in the biofilm's three-dimensional structure. Bar, 30 μm.

leukocyte-biofilm interactions needs to be revisited and more complex analyses need to be performed.

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