HHS Public Access

Author manuscript

J Leukoc Biol. Author manuscript; available in PMC 2024 September 27.

Published in final edited form as:

J Leukoc Biol. 2023 September 27; 114(4): 347–357. doi:10.1093/jleuko/qiad089.

Inflammatory macrophages exploited by oral streptococcus increase IL-1B release via NLRP6 inflammasome

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Abstract

Chronic inflammatory periodontal disease develops in part from the infiltration of a large number of classically activated inflammatory macrophages that release inflammatory cytokines important for disease progression, including inflammasome-dependent interleukin (IL)-1β. *Streptococcus gordonii* is a normally commensal oral microorganism; while not causative, recent evidence indicates that commensal oral microbes are required for the full development of periodontal disease. We have recently reported that inflammatory macrophages counterintuitively allow for the increased survival of phagocytosed *S. gordonii* over nonactivated or alternatively activated macrophages. This survival is dependent on increased reactive oxygen species production within the phagosome of the inflammatory macrophages, and resistance by the bacterium and can result in *S. gordonii* damaging the phagolysosomes. Here, we show that activated macrophages infected with live *S. gordonii* release more IL-1β than non-activated macrophages infected with either live or dead *S. gordonii*, and that the survival of oral Streptococci are more dependent on macrophage activation than other Gram positive microbes, both classical pathogens and commensals. We also find that *S. gordonii*–dependent inflammatory macrophage inflammasome activation requires the cytoplasmic NLRP6. Overall, our results suggest *S. gordonii* is capable of evading immune destruction, increasing inflammatory mediators, and increasing inflammatory macrophage response, and that this ability is increased under conditions of inflammation. This work reveals additional mechanisms by which normally commensal oral streptococci-macrophage interactions can change, resulting in increased release of mature IL-1β, potentially contributing to an environment that perpetuates inflammation.

Keywords

commensal; inflammasome; macrophages; streptococcus gordonii

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Author contributions

J.G.K. and S.M conceptualized the study; S.M. and M.P. performed the experimental investigations; S.M, M.P., and J.G.K. analyzed the data; S.M. and J.G.K. wrote the original manuscript draft; all authors contributed to review, editing, and revision of the manuscript.

Conflict of interest statement. The authors declare no conflict of interest.

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

1 Introduction

Macrophages are an important cell type in the oral cavity. They function in the maintenance of mucosal immunity, including tolerance and tissue homeostasis, by physically interacting with, and responding to, oral microbes.[1–4] During the development and progression of periodontal disease from gingivitis to periodontitis, the number of inflammatory or classically activated (M1) macrophages in the oral cavity increases, along with the inflammatory immune response that they promote.[5–8] These inflammatory macrophages contribute to periodontal disease by further promoting inflammation and alveolar bone resorption.[6,7,9–11] The importance of inflammatory macrophages in disease progression has been demonstrated through studies in which depletion of macrophages reduces alveolar bone resorption by modulating the host immune response,[9] and recruitment of unactivated or alternatively activated (M0 or M2) macrophages by CCL2 reduces alveolar bone loss in mouse models of periodontitis.[12]

As part of the inflammatory response, macrophages produce cytokines and chemokines to orchestrate the immune response.[13] One such cytokine, interleukin (IL)-1β, is produced in an inflammasome-dependent manner and acts to promote inflammation, stimulate fever, and recruit and activate other immune cells.[13,14] Increased release of IL-1β along with enhanced inflammasome component expression occurs in gingivitis and chronic periodontitis[7,10] and has emerged as a possible therapeutic target in periodontal disease as well as in other chronic inflammatory diseases.[11,15,16]

Streptococci are part of the normal oral flora; however, they are also able to colonize extraoral sites and contribute to systemic disease.[17–19] Classically, periodontal disease was thought to be driven by specific pathogenic bacteria, mainly those classified as red complex bacteria.[20] However, recent evidence indicates periodontal disease is not caused by a single organism, but rather is due to the development of a dysbiotic, or imbalanced from healthy, community of microorganisms that includes some normally commensal organisms along with a minority of a keystone pathogen, such as *Porphyromonas gingivalis*, capable of driving this dysbiosis.[21–24] *Streptococcus gordonii*, an oft-studied model oral streptococcus, can promote periodontal disease by working in conjunction with *P. gingivalis*, thus acting as an accessory pathogen to enhance the pathogenicity of *P. gingivalis*.[25–27] In addition, *S. gordonii* can penetrate dental tubules, and is found in patients with apical periodontitis, allowing for *P. gingivalis* migration.[28,29] While such intramicrobiome interactions are becoming better understood, there remains a gap in the mechanistic understanding of how dysbiosis of the oral microbiome alters oral microbial-immune cell interactions, including how normally commensal members such as *S. gordonii* may contribute to disease.

We have previously shown that *S. gordonii* is better able to survive within, and damage the phagosomes of, inflammatory-activated macrophages over nonactivated macrophages in a reactive oxygen species–dependent manner.[30] Here, we show that live *S. gordonii* taken up by inflammatory activated macrophages results in increased production of the inflammasome-dependent and periodontal disease–relevant cytokine IL-1β, and that this is mechanistically dependent on the NLRP6 inflammasome.

2 Materials and methods

2.1 Cell culture

RAW264.7 macrophages (ATCC) were grown in RPMI medium (Lonza or Corning) supplemented with 10% fetal bovine serum (FBS) (Corning) and 2 mM L-glutamine (Corning) at 37 °C in 5% CO_2. Prior to bacterial killing assays macrophages were stimulated with 20 ng/mL recombinant mouse interferon γ (IFNγ) (GenScript) for 24 h and with 0.1 μg/mL lipopolysaccharide (LPS) (*Salmonella enterica* serotype Minnesota strain Re595; MilliporeSigma) for 2 h. Prior to cytokine analysis experiments macrophages were stimulated with 20 ng/mL IFNγ only.

Human monocyte-derived macrophages were isolated from blood obtained from healthy donors in accordance with our institutional review board–approved protocol (ID: MODCR00005631) as previously described.[30] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by isosmotic density gradient centrifugation using 1-Step polymorphs (Accurate Chemical). The PBMCs were plated on glass coverslips, allowing only PBMCs to adhere in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin (Corning) at 37 °C and 5% CO_2. The adherent monocytes were differentiated for 5 to 7 d with 50 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GenScript). Differentiated macrophages were activated with 20 ng/mL recombinant human IFNγ (BioLegend) for 48 h.

Tibias and femurs were isolated from C57BL/J6 wild-type (WT) (purchased from the Jackson Laboratory) and NLRP6 KO mice (provided by Dr. Gabriel Núñez from University of Michigan Medical School).[31] Progenitor cells were seeded at 1×10^6 cells/mL in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2. Adherent cells were differentiated with 50 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GenScript) for 5 d and activated with 20 ng/mL IFNγ for 24 h.

WT, NLRP6 knockout, and NLRP3 knockout immortalized mouse bone marrow–derived macrophages (iBMDMs) were generously provided by the lab of Dr. Gabriel Núñez from University of Michigan Medical School. Cells were grown in RMPI medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate (Fisher Scientific), and 50 μg/mL gentamycin (Amresco) but maintained without antibiotics after initial passages. Cells were stimulated with 20 ng/mL mouse rIFNγ (GenScript) for 24 h before experiments to differentiate to an M1-like macrophage or left unstimulated.[32,33]

Human monocytic THP-1 cells (ATCC), THP-1 ASC-GFP cells (provided by Dr. Emad Alnemri of Thomas Jefferson University), and caspase-4 knockout THP-1 (Invivogen) were maintained in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES (Fisher Scientific), 1.5 g/L sodium bicarbonate, and 0.05 mM 2-mercaptoethanol. Two days prior to an experiment, cells were differentiated to macrophages with 100 nM PMA (Cayman Chemicals) for 24 h then allowed to rest in media without PMA for an additional 24 h.[34] Cells were stimulated with 20 ng/mL human IFNγ for 24 h to activate toward an M1-like macrophage or left unstimulated.[35] For studies

looking specifically at IL-1β production of inflammasome activation, LPS was not used to stimulate cells, as LPS alone can activate inflammasome pathways.[36,37]

2.2 Bacterial culture

S. gordonii strains DL1, SK12, SK9, and 38 were grown in brain heart infusion (BHI) medium (BD Biosciences) supplemented with 0.5% yeast extract (MP Biomedicals) at 37 °C and 5% CO_2. All experiments used mid-log phase bacteria cultures and multiplicity of infection (MOI) was calculated by counting using Petroff-Hausser Counter or based on OD600. For experiments using heat-killed bacteria, *S. gordonii* was grown to mid-log phase then incubated at 80 °C for 60 min prior to use. Throughout the article, *S. gordonii* refers to *S. gordonii* strain DL1, unless otherwise indicated.

Staphylococcus epidermidis clinical isolate 94309–0594 and *S. aureus* strain UAMS-1 (provided by Dr. Anthony Campagnari of the University at Buffalo). Bacteria were grown in BHI medium at 37 °C. *S. mutans* 25175 (provided by Dr. Stefan Ruhl of the University at Buffalo) was grown in BHI medium at 37 °C. For all bacterial killing assay experiments, mid-log cultures were used.

2.3 Bacteria killing assay

To determine bacterial survival within macrophages, a modified gentamycin resistance assay was used.[30,38,39] Briefly, macrophages were seeded in duplicate on 12-well plates and stimulated overnight with 20 ng/mL IFNγ (human [BioLegend] or mouse [GenScript] as required) and with 0.1 μg/mL LPS (*S. minnesota* Re 595) for 2 h. Mid-log *S. gordonii* was sonicated to break up chains and added to macrophages at an MOI of 10:1. Plates were centrifuged to synchronize contact of bacteria with macrophages (125 *g* for 1 min). Cells were incubated at 37 °C for 30 min, then one set of wells was washed extensively with PBS to remove external bacteria, and macrophages were lysed with sterile H_2O and were serial diluted and plated on BHI or Todd-Hewitt plates to determine the initial number of bacteria taken up by the macrophages. For the other set of wells, 150 μg/mL gentamycin was added and incubated for 30 min at 37 °C, after which the media was replace with fresh RPMI and incubated for an additional 1.5 h. Again, macrophages were lysed with sterile H_2O and serial diluted and plated on bacterial media plates and incubated at 37 °C and 5% CO_2 overnight. After incubation, the number of colony forming units (CFU) taken up and CFU survival 2 h postphagocytosis are determined. The ratio of surviving (2.5 h) bacteria to phagocytosed (0.5 h) bacteria gave us percent survival of bacteria within macrophages.

2.4 Cytokine analysis

Macrophages were seeded on 12 or 24-well plates at 5×10^5 cells/mL. Mid-log *S. gordonii* were added to macrophages at an MOI = 10:1. Plates were centrifuged to synchronize contact of bacteria with macrophages (125 *g* for 1 min) and incubated at 37 °C for 6, 12, or 24 h. For experiments with small molecule inhibitors, 20 μM of each inhibitor was added 30 min before addition of bacteria: Ac-LEVD-CHO (caspase-4 inhibitor; Cayman Chemicals), Ac-YVAD-CHO (caspase-1 inhibitor; Cayman Chemicals), and MCC950 (NLRP3 inhibitor; Cayman Chemicals). After incubation, cell supernatants were collected and spun down to remove cell debris and bacteria. Levels of human TNFα and IL-1β were measured

by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions. Concentrations (pg/mL) are normalized to amount per 1×10^5 cells.

2.5 ASC speckle

To quantify inflammasome activation in human macrophages we analyzed ASC speckle formation as described.[36] Briefly, THP-1 ASC-GFP cells were seeded at 5×10^5 cells/mL on 12-well plates with 18-mm cover glass and differentiated to macrophages and stimulated as described previously . Prior to adding bacteria, 20 μM Z-VAD-FMK (AdooQ Bioscience) was added to prevent cell death, then bacteria were added at an MOI = 100:1. After 1 h, any external bacteria were killed by incubation with 150 μg/mL gentamycin for 30 min, after which the media was replaced to fresh RPMI. Bacteria were incubated for a total of 6 or 12 h, then coverslips were fixed in 4% PFA, permeabilized with 0.1% Triton-X100, and counterstained with 0.5 μg/mL DAPI. For a positive control of ASC-associated inflammasome activation, cells were stimulated with 1 μg/mL *E. coli* LPS for 4 h then incubated with 5 μM nigericin (Cayman Chemicals) for 30 min. Cells were imaged using a 20 × objective on a Nikon Eclipse TE2000-u instrument equipped with a Spot RT740 Camera with at least 1,000 cells imaged per coverslip for at least 3 independent experiments. Percent speckle-positive was calculated using thresholding and analyzing particles on FIJI,[40] in which the ratio of cell number (as determined by DAPI) and speckle number (as determined by high intensity small green fluorescent protein [GFP] positive points) were calculated, giving percent cells with activated ASC positive inflammasomes. For imaging cells with bacteria, *S. gordonii* was labeled with 25 μg/mL Alexa Fluor 568 carboxylic acid, succinimidyl ester (Invitrogen) in 0.1 M sodium bicarbonate for 5 min.

2.6 Immunoblot

Macrophages were incubated with bacteria (as with cytokine analysis) at an MOI = 10:1 in 0.3% FBS-supplemented RPMI in 6-well plates. After 6 or 24 h incubation, protein lysates were collected by rinsing cells with PBS, then scraping and collecting using extraction buffer (50 mM Tris HCl (pH 7.4), 0.5 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EDTA, 1% Triton-X100, 1 × Pierce Protease Inhibitor [Thermo Fisher Scientific]). Protein isolates were concentrated by precipitating with ice-cold acetone then resuspending in extraction buffer. Protein concentrations were measured and normalized using Bio-Rad Bradford Assay. Antibodies used for detection of protein in the cell lysate and supernatant were human reactive α-IL-1β and α-caspase-1 (Cell Signaling Technology; #43811, proptosis sampler kit) or mouse reactive α-IL-1β and α-caspase-1 (Cell Signaling Technology; #20836, inflammasome sampler kit) and α-Actin (BD; #612656).

2.7 Caspase-1 fluorometric assay

The 1×10^6 macrophages were incubated with mid-log *S. gordonii* at an MOI = 10:1 for 6 h. Cleaved caspase-1 was detected with YVAD-AFC in cell lysates according to manufacturer instructions (Caspase-1/ICE Fluorometric Assay Kit; BioVision). Fold increase of caspase-1 activity was determined by comparing bacteria infected macrophages with uninfected macrophages.

2.8 Cell death assays

Propidium iodide (Biotium) acquisition was measured by flow cytometry. Lactate dehydrogenase (LDH) cytotoxicity was measured using a CyQUANT LDH Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer's directions. Percent maximum LDH release was compared with maximum LDH release by addition of lysis buffer to macrophages.

3 Results

3.1 Representative oral streptococcus *S. gordonii* consistently survives better within inflammatory macrophages

Inflammatory activated macrophages generally have increased microbicidal activity[41,42]; however, we have shown that *S. gordonii* has a higher survival rate within M1 activated macrophages over M2 macrophages.[30] Further testing found that this increase in survival within activated macrophages was consistent regardless of the macrophage source (Fig. 1A–D); the survival within inflammatory macrophages relative to nonactivated macrophages increased about 2-fold, though the absolute survival rates varied between macrophage source. The intracellular survival rate was analyzed for a variety of traditionally commensal bacteria with pathogenic potential and was found to be unique to the tested viridans streptococci *S. gordonii* and *S. mutans* (Fig. 1E). While *S. epidermidis* and *S. aureus* had survival similar to *S. gordonii* DL1 within inflammatory activated macrophages, this survival did not depend on the macrophage activation state. We also tested survival of various *S. gordonii* strains with varying pathogenicity in an animal model of infective endocarditis. *S. gordonii* SK12 and SK9 were shown to be less pathogenic in the model, whereas *S. gordonii* DL1 and 38 were shown to have more pathogenic potential.[43] Previously, our lab has shown that this pathogenicity is a good indicator of survivability in phagocytes in which *S. gordonii* DL1 survives better than SK12. Along these lines, we see that overall *S. gordonii* DL1 and 38 survive better within macrophages compared with SK12 and SK9 (Fig. 1F). However, the differential survival between activation state of the macrophage was only seen with *S. gordonii* DL1.

3.2 IL-1β release is increased in inflammatory activated macrophages infected with live *S. gordonii*

Inflammatory, or M1, macrophages release a myriad of proinflammatory cytokines to recruit other immune cells and elicit an immune response. We next examined 2 proinflammatory cytokines highly prevalent in periodontal disease, IL-1β and tumor necrosis factor α (TNFα),[7,8,44,45] to determine if there were inducible changes to the proinflammatory response by human macrophages in response incubation with *S. gordonii* (Fig. 2). In THP-1–derived macrophages activated to an inflammatory state with IFNγ, IL-1β, but not TNFα, release was significantly increased over nonactivated macrophages when incubated with live *S. gordonii* (Fig. 2A, B). We also found that macrophages produced significantly less IL-1β when incubated with heat-killed bacteria (Fig. 2C), which are not able to damage the macrophage phagosome.[30] This difference was not seen for TNFα release (Fig. 2D), suggesting that the combination of both inflammatory activation of macrophages and live *S. gordonii* was a prerequisite for maximal IL-1β production and release.

3.3 Live *S. gordonii* stimulated inflammasome activation in inflammatory activated macrophages

Given that IL-1β processing and release requires activation of the inflammasome complex and caspase-1,[46,47] we next tested inflammasome, and ultimately caspase-1, activation in macrophages upon interaction with *S. gordonii*. Activation of the inflammasome complex itself often requires a cytosolic stimulus, and we have previously shown that *S. gordonii* is capable of damaging and potentially escaping the phagosome[30]; therefore, we hypothesized that this allows for activation of the cytoplasmic inflammasome. To examine inflammasome activation, we took advantage of THP-1 macrophages that express ASC, an important scaffold protein in most inflammasomes, conjugated to GFP. When the inflammasome is activated and the large multimeric protein complex is formed, ASC oligomerizes and the diffuse GFP forms a large single speck within the cell (Fig. 3A).[36] Quantification of this oligomerization showed that IFNγ-stimulated human macrophages incubated with live *S. gordonii* had active inflammasomes in significantly more cells than both macrophages alone and macrophages incubated with heat-killed *S. gordonii*, after both 6-h (Fig. 3B) and 12-h (Fig. 3C) incubations. We confirmed that this inflammasome oligomerization does in fact occur concomitantly in macrophages associated with *S. gordonii* bacterium and that the THP-1 macrophages do indeed phagocytose the bacteria (Fig. 3D and Supplementary Fig. 1). In addition to inflammasome speck formation, we found increased activation (cleavage) of IL-1β and caspase-1 in the macrophage supernatant upon infection with live *S. gordonii* (Fig. 4A). Measurement of functional caspase-1 also indicated a significant increase in active caspase-1 when macrophages were infected with live vs heat-killed *S. gordonii* (Fig. 4B). Overall, these results show that live *S. gordonii* activates inflammasomes in proinflammatory human macrophages.

3.4 Cell death upon inflammasome activation

Inflammasome-dependent caspase-1 activation leads to cleavage of gasdermin D, which can form pores in the cell membrane. Extended periods of gasdermin D pore formation can result in ion flux, membrane destabilization, and eventual cell death (pyroptosis).[48–50] We measured cell cytotoxicity as an indicator of pyroptosis using propidium iodide influx and LDH release (Fig. 4C, D). There was a significant increase in propidium iodide–positive cells when IFNγ THP-1 cells were incubated with live *S. gordonii* over THP-1 cells alone (Fig. 4C). Additionally, there was a significant increase in LDH release from THP-1 cells incubated with live *S. gordonii* over HK *S. gordonii* or cells alone (Fig. 4D).

3.5 Mechanism of inflammasome activation in macrophages by *S. gordonii*

There are many ways that inflammasomes can be activated, including via sensing ionic or enzymatic changes within the cell cytoplasm as well as through direct interaction with and activation by bacterial components. For example, NLRP3 can be activated by cathepsin B release from the phagolysosome upon damage.[51] Previous work from our lab has shown that *S. gordonii* can cause phagolysosomal damage,[30] so we initially wanted to determine if NLRP3 was required for inflammasome-dependent IL-1β release. Looking at THP-1 macrophages treated with an NLRP3-specific small molecule inhibitor (MCC950) we did

not see significant changes in IL-1β release when the activated macrophages were incubated with *S. gordonii* (Fig. 5A).

Recently, it has been found that the lesser described NLRP6 can be directly activated by Gram-positive bacteria cell wall component lipoteichoic acid (LTA) and that this NLRP6 inflammasome involves activity of both caspase-1 and caspase-4/5 (caspase-11 in mice).[31] Therefore, we next examined if NLRP6 activation is the mechanism through which *S. gordonii* activates macrophage inflammasomes. When caspase-4 was inhibited in human THP-1 cells with a small molecule inhibitor (Ac-LEVD-CHO), we saw a significant decrease in IL-1β release (Fig. 5A). There was also a significant decrease in IL-1β production when caspase-1 was inhibited (Ac-YVAD-CHO), confirming that IL-1β release is dependent on caspase-1 activation. LTA and nigericin were used as controls for NLRP6 and NLRP3 inflammasome activation, respectively, confirming the effectiveness of the small molecule inhibitors (Supplementary Fig. 2A, B).

To verify the inhibitor results, we also tested NLRP3, NLRP6, and capase-11 knockout mouse macrophages and capase-4 knockout THP-1 cells. Consistent with the inhibitor results, NLRP3 knockout iBMDMs did not show a decrease in IL-1β production upon incubation with *S. gordonii*, while NLRP6 iBMDMs did (Fig. 5B). In BMDMs isolated directly from NLRP6 knockout mice, we also saw a significant decrease in IL-1β release as compared with WT BMDMs (Fig. 5C). Transfected LTA was used as a control for NLRP6 inflammasome activation, confirming the knockout. We also tested for activation of caspase-1 and IL-1β cleavage and found reductions of both in NLRP6 knockout BMDMs as compared with WT BMDMs (Fig. 5D).

Further confirmation of this phenotype was done through examining IL-1β release in mouse caspase-11 knockout iBMDMs, in which we saw a significant decrease in IL-1β production by caspase-11 knockout cells incubated with live *S. gordonii* compared with WT iBMDMs (Fig. 5E). We additionally looked at the human caspase-4 knockout THP-1 cells and saw a decrease in caspase-1 and IL-1β cleavage by Western blot in the knockout cells compared with WT THP-1 s (Fig. 5F). Together, these data indicate NLRP6 and caspase-4 (or mouse caspase-11) are important for *S. gordonii*–dependent active IL-1β production and caspase-1 cleavage.

4 Discussion

The multiple pathways of inflammasome activation exist for immune protection; however, many bacteria have mechanisms to either promote or inhibit inflammasome activation and pyroptosis for their benefit.[52] For example, many of the bacteria associated with periodontal disease are inflammophilic, feeding off nutrients provided from the inflammatory immune response and tissue breakdown.[53] The well-studied periodontal pathogen *P. gingivalis* is capable of inhibiting some aspects of macrophage inflammation such as iNOS-mediated killing and Toll-like receptor signaling while promoting destructive inflammation.[54–57] Recent research has also come to appreciate traditionally commensal bacteria as important players in periodontal disease,[23,24,58,59] including oral streptococci and the representative species *S. gordonii*.[26,27,60,61] Here, we show that when macrophages interact with *S.*

gordonii under conditions of existing inflammation, which allows the bacterium to increase its ability to evade immune destruction, the macrophages have increased NLRP6-dependent inflammasome activation and increase the release of the inflammatory cytokine IL-1β.

Previous research has described the involvement and importance of general inflammation, including the inflammasome and IL-1β, in periodontal disease.[8,10,11,62–64] Inflammasomes are activated by recognition of pathogen-associated molecular patterns and damage-associated molecular patterns within the cytosol by cytosolic pattern recognition receptors.[52] A multitude of mechanisms can activate a large variety of cytosolic pattern recognition receptors; the well-studied NLR NLRP3 can be activated by several stimuli, including sterile and pathogen associated molecules, as well as damage to phagosomes.[49–51,65,66] We have previously shown that *S. gordonii* can damage phagolysosomes,[30] so we initially hypothesized that *S. gordonii* may activate an NLRP3 inflammasome through this pathway. In fact, we found that the NLRP3 inflammasome is not the primary inflammasome pathway activated by *S. gordonii* but instead is dependent on NLRP6 and caspase-4 (mouse caspase-11) (Fig. 5). Interestingly, the NLRP3 knockout cells showed a significant increase in IL-1β release compared with WT that was not seen with the small molecule inhibitors. The reasoning for this discrepancy is unclear. The NLRP6 inflammasome has recently been described to sense LTA of Gram-positive bacteria,[31] suggesting that bacterial products released from the damaged phagosome into the cytosol may be the primary activator in this case.

NLRP6 is important for activation of both IL-1β and IL-18 in macrophages and epithelial cells,[31,67] but it can also be important for increased bacterial pathogenicity in infection.[31,68,69] Conflicting information exists on the importance of NLRP6 in the exacerbation of colitis, another major disease associated with microbial dysbiosis, with recent research showing no change in gut microbiome and a gender-based susceptibility to induced colitis between WT and *Nlrp6*$^{-/-}$ mice.[70,71] However, research is coming to appreciate that there may be an important role NLRP6 could play in the oral cavity as NLRP6 is increased, along with other inflammasome components, in periodontal disease.[72] NLRP6-activated caspase-1 can lead to the release of IL-1β and IL-18 in gingival fibroblasts,[72] though NLRP6 may also inhibit inflammatory responses in human periodontal ligament cells associated with apical periodontitis.[73] NLRP6 is known to be activated by LTA from Gram-positive bacterial cell walls,[31] and LTA from *S. mutans*, the major etiological agent of dental caries, can activate NLRP6 in human dental pulp cells.[74] However, many of the bacteria traditionally associated with periodontal disease, including *P. gingivalis*, are Gram-negative inflammophilic bacteria.[75–77] These traditional periodontal pathogens, however, generally make up a minority of the periodontal disease microbiome, with much of the disease-associated dysbiotic microbiome consisting of accessory pathogens, many of which are Gram positive.[78,79] Our results suggest that upon streptococci interaction with inflammatory macrophages, an increase of IL-1β production is produced via NLRP6 that in turn could be beneficial to the periodontal-associated bacteria through feeding into the positive feedback loop of disease development.[80] The results presented here thus suggest that, in addition to the role that *S. gordonii* has in promoting the attachment and growth of periodontal pathogens,[81] it may have an active role in driving chronic inflammation in the oral environment once inflammation is initiated.

Important future studies will need to examine the in vivo requirements of NLRP6 in the development of periodontal disease, as well as the potential changing interactions of oral streptococci with other immune cells known to be important in periodontal disease, including neutrophils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. Núñez, Alnemri, Campagnari, and Ruhl for providing reagents, as noted in the Materials and Methods. The graphical abstract was created with BioRender.com. Microscopy was performed in the Optical Imaging and Analysis Facility (UB Dental Sciences).

Funding

This work was funded in part by National Institutes of Health grants R03DE025062 (J.G.K), R01DE028307 (J.G.K), F31DE029400 (S.M.), and T32DE02526.

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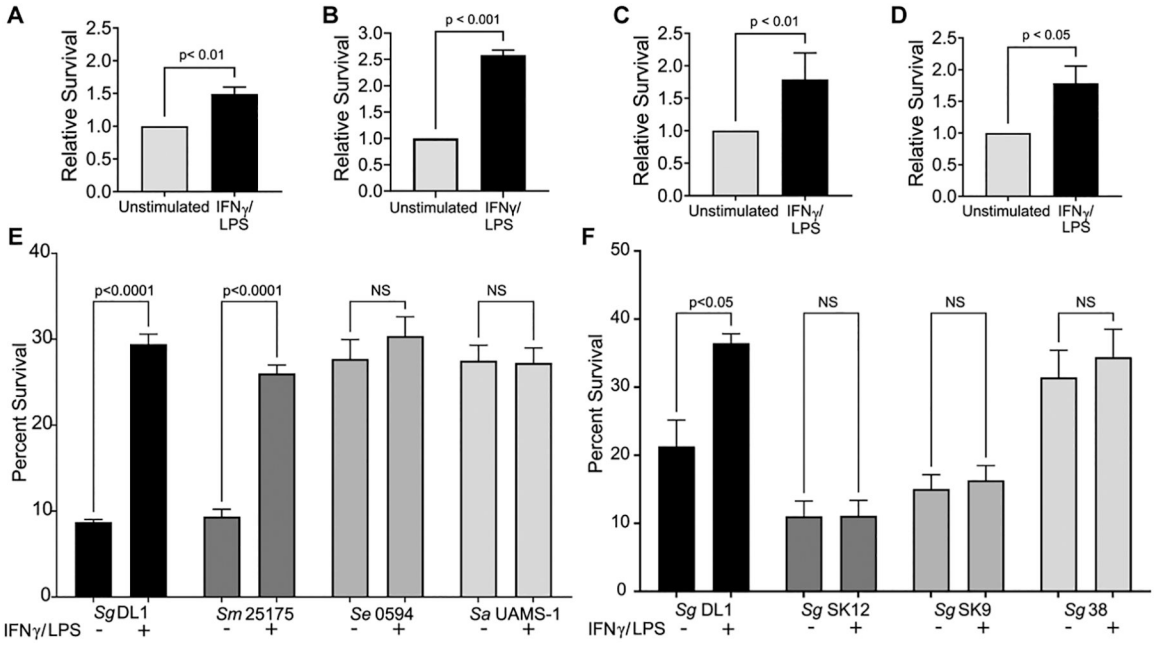

Fig. 1.

Oral viridans streptococci have a unique ability to survive within M1-activated macrophages over unstimulated macrophages. (**A–D**) Relative survival of *S. gordonii* DL1 within macrophages from different sources comparing unstimulated and IFNγ/LPS-activated macrophages: (**A**) RAW264.7 mouse macrophages, (**B**) WT iBMDMs, (**C**) THP-1 human monocytic cell line–differentiated macrophages, (**D**) human primary peripheral blood monocyte–derived macrophages. Shown are mean ± SEM of 3 or more independent experiments. *P* values calculated by Student's *t* test. (**E**) Survival of Gram-positive bacteria within RAW264.7 macrophages. Shown are mean ± SEM of 3 independent experiments. (**F**) Survival of various *S. gordonii* strains within RAW264.7 macrophages. *S. gordonii* SK12 and SK9 are strains known to not cause endocarditis in animal models,[43] and SK12 has reduced reactive oxygen resistance[30] compared with *S. gordonii* DL1. *S. gordonii* DL1 and 38 are known to be more pathogenic in animal models of endocarditis.[43] *P* values calculated by ordinary 1-way analysis of variance followed by Sidak's multiple comparisons test. *Sa* = *Staphylococcus aureus*; *Se* = *Staphylococcus epidermidis*; *Sg* = *Streptococcus gordonii*; *Sm* = *Streptococcus mutans*.

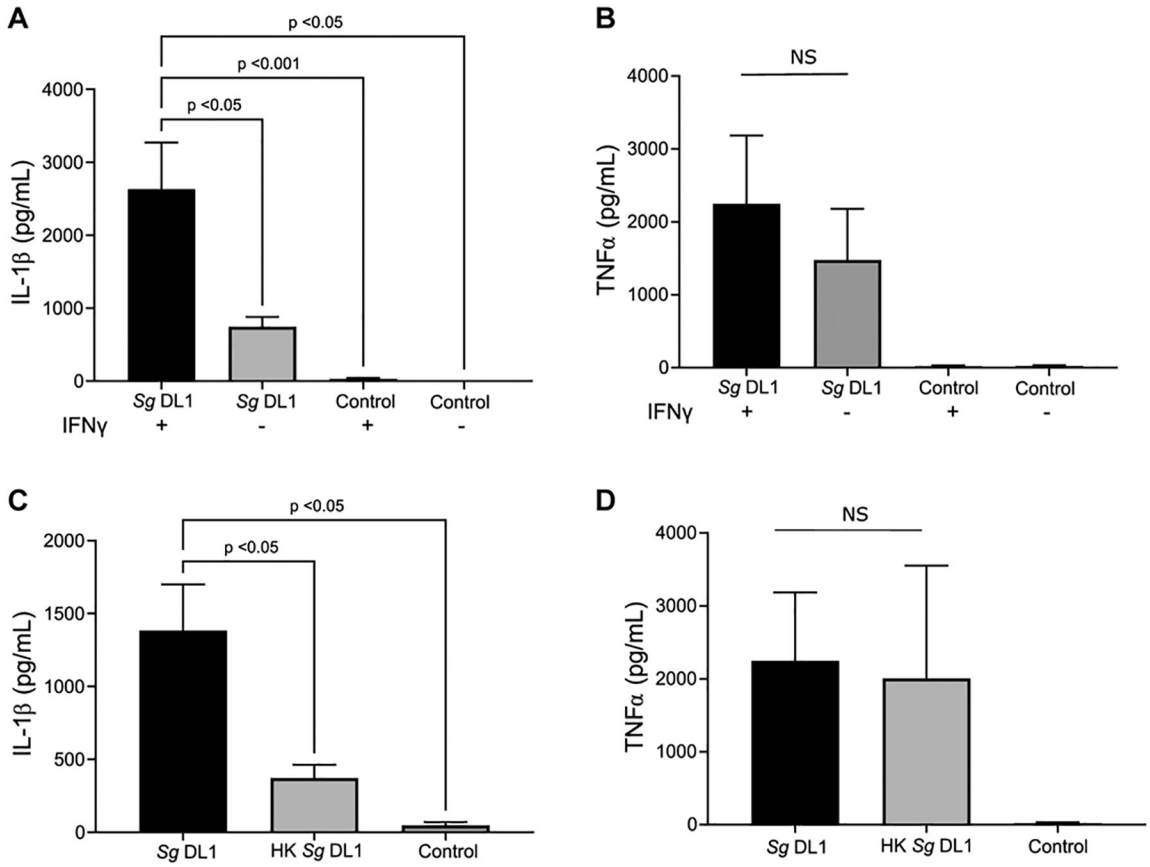

Fig. 2.

Live *S. gordonii* stimulates significantly increased IL-1β release from IFNγ-activated macrophages. Inflammatory cytokine release measured by enzyme-linked immunosorbent assay (ELISA) from PMA differentiated the THP-1 human monocytic cell line. Cytokines measured were IL-1β (**A, C**) and TNFα (**B, D**). (**A, B**) THP-1 cells were polarized with IFNγ or left unstimulated prior to incubation with *S. gordonii* DL1 for 6 h (**B**) or 24 h (**A**). (**C, D**) THP-1 cells were polarized with IFNγ and incubated with live or heat-killed (HK) *S. gordonii* DL1 for 6 h (**D**) or 24 h (**C**). In each case, control indicated the THP-1 cells alone. Shown are mean ± SEM of 4 or more independent experiments. *P* values were calculated by ordinary 1-way analysis of variance followed by Tukey's multiple comparisons test where appropriate. NS indicates that the analysis of variance was not significant with $\alpha = 0.05$. Concentrations (pg/mL) are per 1×10^5 cells/mL. *Sg* = *Streptococcus gordonii*.

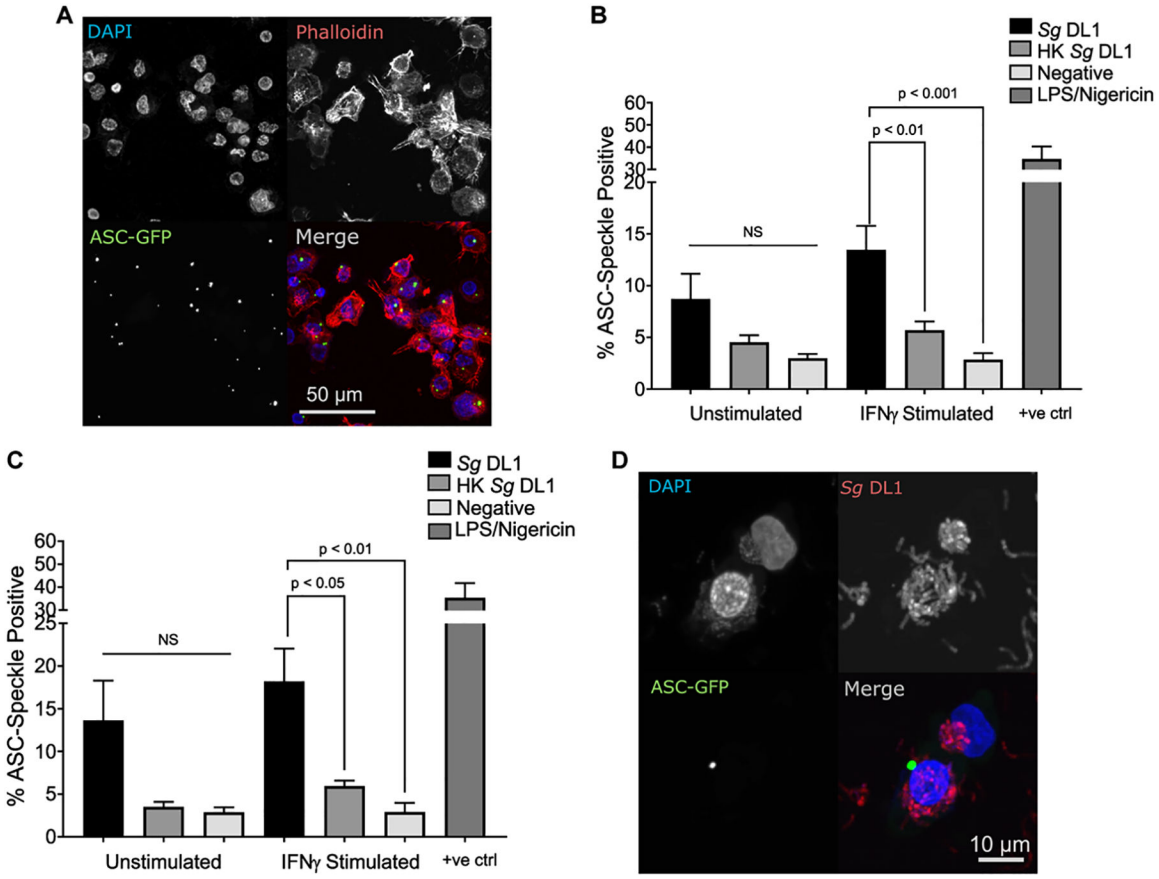

Fig. 3.

Live *S. gordonii* activates inflammasomes in IFNγ-stimulated macrophages. (**A, B**) Percent ASC speckle–positive cells after incubation (A: 6 h; B: 12 h) with live or heat-killed (HK) *S. gordonii* in PMA-differentiated THP-1 Asc-GFP cells polarized with IFNγ. Positive control: 4 h 1 µg/mL LPS + 30 min 20 µM nigericin. (**C**) Representative image of ASC speckle–positive cells. (**D**) Representative image of ASC speckle–positive cell containing *S. gordonii*. Shown are mean ± SEM of at least 4 independent experiments in which at least 1,000 cells per condition were analyzed. *P* values were calculated by ordinary 1-way analysis of variance followed by Tukey's multiple comparisons test. *Sg* = *Streptococcus gordonii*.

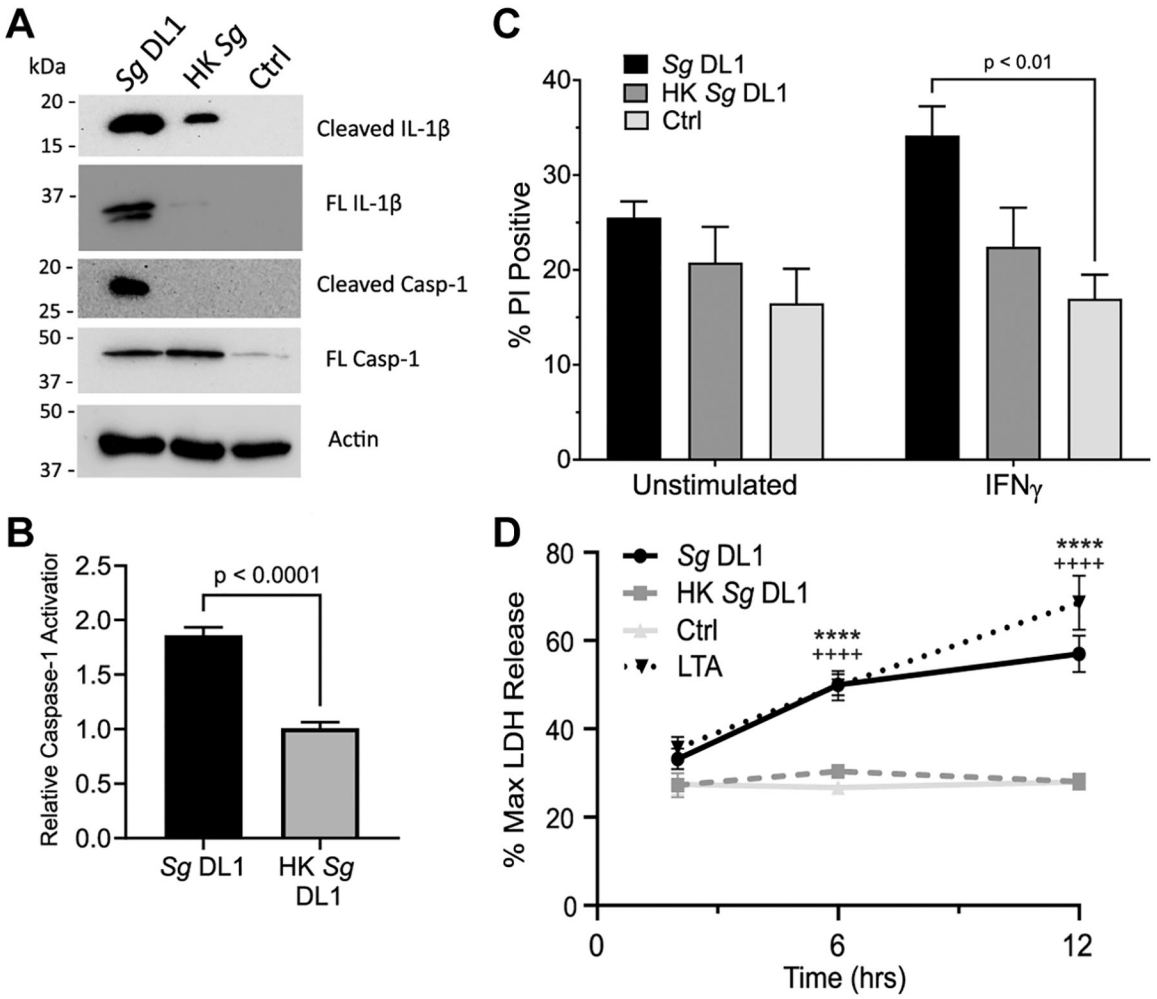

Fig. 4.

Live *S. gordonii* activates caspase-1 in macrophages. (**A**) Representative immunoblot showing caspase-1 and IL-1β cleavage. THP-1 cells were incubated with live or heat-killed (HK) *S. gordonii* DL1 for 24 h. Actin was used as a loading control. (**B**) Fold change in caspase-1 activation relative to THP-1 cells without bacteria added measured by YVAD-AFC fluorescence. (**C**) Propidium iodide (PI) acquisition by THP-1 cells after 24 h incubation. THP-1 cells were polarized with IFNγ or left unstimulated prior to incubation with live or HK *S. gordonii*. (**D**) LDH cytotoxicity after 2, 6, and 12 h incubation with live or HK *S. gordonii* with IFNγ-stimulated THP-1 cells. Transfected LTA was used as a positive control for pyroptosis. Percent release was determined compared with maximum LDH release by cells incubated with lysis buffer. Shown are mean ± SEM of 3 independent experiments. *P* value was calculated by unpaired *t* test (**B**) or ordinary 2-way analysis of variance followed by Dunnett's multiple comparisons in which each group was compared with control (+ indicates control vs LTA, * indicates control vs *S. gordonii* DL1) (**C**). Ctrl = control; *Sg* = *Streptococcus gordonii*.

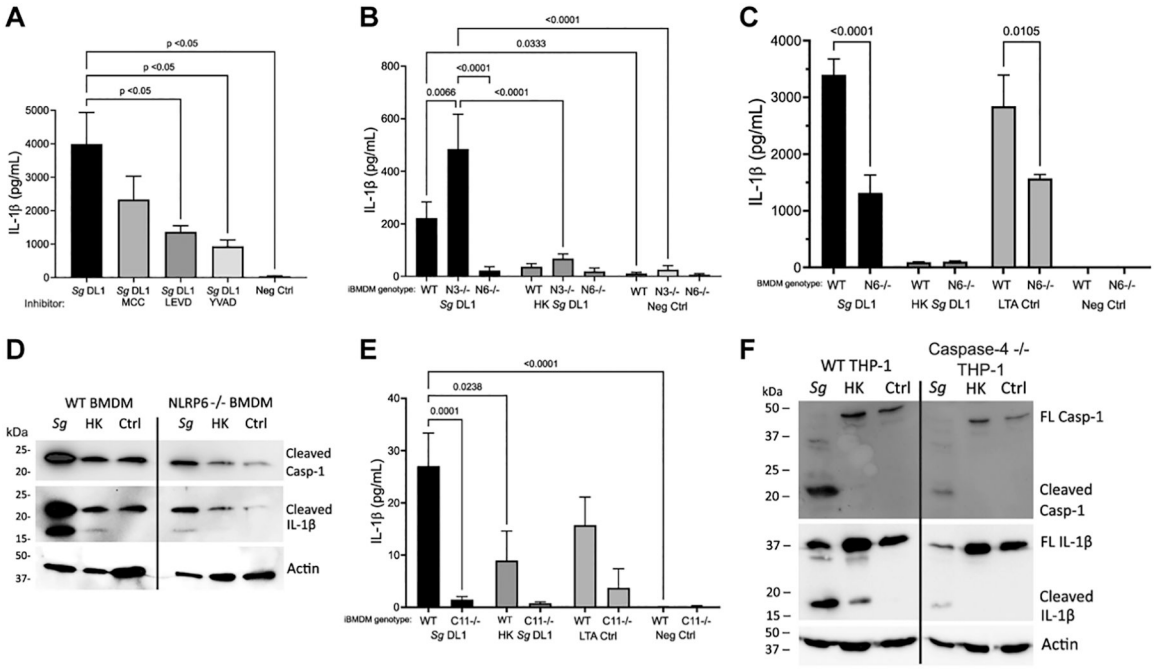

Fig. 5.

Determining inflammasome pathway activation by *S. gordonii* in macrophages. (**A**) IL-β release from PMA-differentiated, IFNγ-activated THP-1 macrophages with indicated inflammasome inhibitor added during incubation with *S. gordonii* for 6 h. MCC950 (MCC) indicates NLRP3 inhibitor, Ac-LEVD-CHO (LEVD) indicate scaspase-4/5 inhibitor, and Ac-YVAD-CHO (YVAD) indicates caspase-1 inhibitor. (**B**) IL-1β release as determined by enzyme-linked immunosorbent assay (ELISA) from IFNγ-stimulated immortalized BMDMs derived from WT, NLRP3 knockout, or NLRP6 knockout mice incubated with like or heat-killed (HK) *S. gordonii* for 24 h. (**C**) IL-1β release as determined by ELISA from BMDM derived from WT or NLRP6 knockout mice incubated with live or HK *S. gordonii* for 24 h. Transfected LTA (28 μg) was used as a positive control for NLRP6 activation.[31] (**D**) Representative immunoblot showing caspase-1 and IL-1β cleavage. WT or NLRP6 knockout primary BMDMs were incubated with live or HK *S. gordonii* for 24 h. Actin was used as a loading control. (**E**) IL-1β release from caspase-11 knockout or WT iBMDMs incubated with live or HK *S. gordonii* for 24 h. (**F**) Representative immunoblot showing caspase-1 and IL-1β cleavage. WT or caspase-4 knockout THP-1 cells were incubated with live or HK *S. gordonii* for 6 h. Again, actin was used as a loading control. Shown are mean± SEM of 3 independent experiments. *P* values were calculated by 1-way analysis of variance followed by Dunnett's multiple comparisons (**A, C, E**) or 2-way analysis of variance followed by Sidak's multiple comparisons test (**B**). Ctrl = control; *Sg* = *Streptococcus gordonii*.