

TARGETING BACTERIAL BIOFILMS-MEDIATED DYSREGULATION OF IMMUNE CHECKPOINTS: A NEW DIMENSION FOR ANTI-PATHOGENIC IMMUNOTHERAPY

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Abstract: Bacterial infections associated with biofilms continue to be significant clinical problem due to their antibiotic resistance and immunosuppressive capabilities. The recent evidence indicates that the immune checkpoint signaling (PD-1/PD-L1) is involved in the immune impairment in chronic biofilm infections. The study examined the importance of such pathways and whether immune checkpoint inhibitors (ICIs) can be used with antibiotics to enhance the ability to eliminate bacteria and rebuild immune functions. **Methods:** Biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in vitro also were developed and co-cultured with human peripheral blood mononuclear cells (PBMCs). They compared the following four groups: control, ciprofloxacin, antibodies against anti-PD-1/PD-L1 alone and the combination of these two types of antibodies. The crystal violet staining and the confocal microscopy method were used as methods of assessing biofilm biomass, whereas colony-forming unit (CFU) counts were used as the method of measuring viable bacteria. The flow cytometry analysis of PD-1/PD-L1 on T cells was complemented by the analysis of gene expression by qRT-PCR and the level of cytokines by ELISA. **Results:** Combination therapy yielded the best effect as it decreased biofilm biomass up to 70% in comparison with 35-45% reductions using one of the treatments. Bacterial viability in combination group was also much worse compared to monotherapy groups. Also, the PD-1/PD-L1 on the CD4⁺ and CD8⁺ T cells were significantly lowered, as well as TNF- α and IFN- γ were elevated and IL-10 was lowered, with a shift up the pro-inflammatory immune response. These results indicate that immune checkpoint blockade is potentially able to augment antibiotic effects against infections caused by biofilms by reversing immunosuppression caused by infection. **Conclusion:** This integrated approach could be an effective treatment option against chronic and antimicrobial-resistant bacterial infections, but additional in vivo research is necessary to determine the effectiveness of the approach, the optimal dose level, and safety.

Keywords: Biofilm, PD-1/PD-L1, Immune checkpoint inhibitors, Antibiotics, chronic infection, cytokines, *Staphylococcus aureus*, *Pseudomonas aeruginosa*.

1. Introduction

Chronic and device-associated bacterial infections continue for extended periods despite prime antimicrobial treatment due, in large part, to the formation of biofilms -- multi-cellular communities encased in an extracellular matrix additive and defensive of resident cells from antibiotics and host-immunity. Biofilm-associated infections cannot only be found with *Staphylococcus aureus* and *Pseudomonas aeruginosa* but are associated with extended morbidity, relapse, and the cost of healthcare [1,2]. Beyond standard antibiotic tolerance, biofilms aggressively reprogram the local immune milieu by restricting phagocyte access, preventing bactericidal functions and changing cytokine networks [2]. These changes in immunology are similar to those of sepsis-induced immunosuppression (SIS) which include T-cell exhaustion, monocyte dysfunction, and antigen presentation impairment that impair bacterial clearance [3,4]. One of the major mechanisms of infection-associated immune paralysis is immune checkpoint expression. In chronic inflammatory and microbial signals lead to expression of inhibitory T-cell PD-1 and myeloid and parenchymal cell PD-L1 receptors, which inhibit T cell activation and cytotoxicity. Multiple clinical and translational investigations are now linking increased PD demonstrated expression between patients and adverse clinical outcomes linked with sepsis and secondary infections [5,6]. Mechanistically, PD-1/PD-L1 signaling limits macrophage bactericidal projections - including mitochondrial ROS graceful killer dependent on the anti-oxidants and designates an anti-inflammatory program in myeloid cells [7,8]. Many importantly, the biofilm forming *S. aureus* can directly upregulate PD-1 ligands in the infected niche, and that provides a plausible route by which biofilms enforce a local T cell suppression [9]. These insights have generated interest in the repurposing of immune checkpoint inhibitors (ICIs) in severe diseases caused by bacteria. Early clinical study in the area of sepsis identified the feasibility and safety of anti-PD-L1 therapeutic strategy, and biomarker evidence of immune restoration [10,11]. Since then, systematic evaluations across preclinical bacteria models state checkpoint blockade assessed with benefit in the reduction of bacterial burden in a majority of studies,

although it has also been identified as showing without any effect or even in combination to have adverse effects - hence there is a need to maintain directional and combination approaches in the use of checkpoint blockade [12]. Broader scoping reviews of sepsis immunotherapy also come to the same conclusion that ICIs are promising and need strict patient selection, timing and integration with standard care [13]. Combination regimens of ICIs with the modulation of certain cytokines (IL-6 blockade) or antibiotics have the ability to enhance host defense by limiting tissue injury in sepsis models [14]. From the translational standpoint, pathogenological immunotherapy of biofilm is a domain at the convergence of pathogenology and host defence systems. In *P. aeruginosa* there are complex quorum sensing, matrix production and modifications in metabolism which create hypoxic niches limited in nutrients that result in dampening of neutrophils and macrophages and predispose to IIC induction; prudent measures to breach these "fortresses" likely need to be combined with mechanisms to interfere with the biofilm matrix structure and dampen IIC-based immune suppression [2,15]. Recent progress in the understanding of monocyte HLA-DR dynamics coupled with other sepsis biomarkers opens a paradigm to identify patients with an ultimate immune paralysis state, which can justify immunostimulatory therapy [16]. Finally, models that are more tissue-engineered and aware of the niche provide evidence that restoration of activity of macrophage cells within biofilm-infected scaffolds can hasten clearance of infection, suggesting macrophages as sets of effector cells to be unleashed by the modulation of checkpoints [17].

The aim of this translational study will be to examine how bacterial biofilm formation induces dysfunction of the immune checks as well as to test the therapeutic applicability of immune check point independent blockade, alone and in conjunction with conventional antibiotics to restore immune function and build up bacterial clearance. Specifically, the following points are sought from the research: 1. Quantify the expression of immune checkpoint molecules (i.e., PD-1, PD-L1, CTLA-4) in biofilm-associated infections. Evaluate the potency of immune checkpoint inhibitors on the triggering of immune cells, identification of cytokine profiles and bacterial viability in biofilm

conditions. To evaluate the neutrophil restoration of complex (immune checkpoint inhibitor-based) combination therapy of biofilm-disrupting or antibacterial agents to quantitatively determine the reduction of the bacterial burden and the reversal of immune suppression.

2. Materials and Methods

Study design

Therefore, this in vitro experimental study was designed to test the immune checkpoint blockade efficacy in biofilm-associated infections with respect to improvement in immune cell function and bacterial clearance. Four experimental groups were included: biofilm controls followed by standard antibiotic therapy, biofilms treated with standard antibiotic treatment only, biofilms treated with ICIs only, and biofilms treated with antibiotic with CPI treatment. All experiments were done in triplicate, and in independent experiments at least three times, to get good reproducibility and reliability of the results.

Strains of bacteria used and growth conditions

Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with well-characterized biofilm-forming capacities were taken from the Department of Medical Microbiology culture collection. Bacteria were cultured within tryptic soy broth (TSB) supplemented with 1% glucose and incubated to encourage the biofilm routines. For the growth of planktonic organisms, cultures were equalized to 0.5 McFarland unit value, 1×10^8 CFU/mL by spectrophotometer (600 nm). Moreover, the diluted cultures (1:100) were inoculated in fresh medium in 96 well tissue culture plates, and incubated for 24-48 h to fully develop mature biofilms. Colony forming unit (CFU) enumeration was performed until needed on Blood agar plates [18].

Immune cell Isolation and co-culture

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy human donors by Ficoll-Paque PLUS Ficoll density separation. Ethical approval for use of human samples was given by the Institutional Review Board and prior blood collection was taken after informed consent by all the participants. After biofilm maturation PCMC were incubated with biofilm cultures at a cell concentration of 1×10^6 cells/ml in RPMI-1640 containing 10% fetal bovine serum with antibiotics.

The co-cultures were incubated at 37°C in a humidified 5% CO₂ incubator for 24 hrs. to allow interactions between immune cells and biofilm-associated bacteria to occur [19].

Therapeutic interventions

Ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic, was applied according to standards in the CLSI and concentrations of 10 µg/ml were used as an experimental fluoroquinolone antibiotic against microorganisms associated with biofilm formation. Immune checkpoint blockade was performed using anti-PD-1 and anti-PD-L1 monoclonal antibodies (BioLegend) at a concentration of 1-10 µg/mL to assess the dose-dependent effect. In the combination therapy group, ciprofloxacin and immune checkpoint inhibitor were given to the samples at the same time to check whether there are synergistic effects in the biofilm reduction and immune restoration [20,21].

Biofilm quantification

Biofilm biomass was measured by the crystal violet staining technique. After treatment, wells were gently washed with phosphate buffered saline (PBS) to remove planktonic bacteria contained in them and stained with crystal violet 0.1% for 15 minutes. Excess stain was washed off with distilled water and bound dye solubilized using 95% ethanol. The total amount of biofilm biomass was estimated by significant absorbance at 570 nm, using a microplate reader. In parallel, confocal laser scanning microscopy (CLSM) was performed using bacterial viability kit (LIVE/DEAD BacLight) in order to visualize the construction of the biofilm and measure the bacterial viability within the matrix [21,22].

Immunogenetic expression of immune checkpoint

To assess modulation of immune checkpoints, PD-1, PD-L1, CTLA-4 and T-cell markers CD3, CD4 and CD8-conjugate fluorescent fluorescently labeled antibodies were used to stain PBMCs co-cultured with biofilms. Flow cytometry analysis was done with a BD FACSCanto II cytometer and the data were analyzed with FlowJo software to quantify checkpoint receptor expression. For transcriptional analysis, total RNA was

extracted by using TRIzol reagent and cDNA was synthesized by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR qRT-PCR was performed to measure the gene expression levels with the normalization of the housekeeping gene (GAPDH), and the $2^{-\Delta\Delta C_t}$ method was employed. Western blotting was used to verify expression of the proteins that were separated by SDS-PAGE, transferred to PVDF membranes, probed with primary and HRP-conjugated secondary antibody, and chemiluminescence imaging [19,23].

Cytokine profiling

Supernatants of the biofilm - immune cells co-cultures were collected post-treatment for cytokine secretion profile measurement. Commercial Enzyme Immunosorbent Assays (ELISA) kit (R&D Systems) was used to quantify pro-inflammatory (TNF-alpha, IFN-gamma, IL-6) and anti-inflammatory (IL-10) cytokines following the instructions of the manufacturer. Cytokine levels were determined by using standard curves and were reported as ng/mL [19].

Evaluation of the viability of the bacteria

Following treatments biofilms were mechanically disrupted, and serial dilutions were done in sterile PBS. Samples were plated on blood agar and incubated with detection in 37°C for 24 hours. Colony-forming units (CFU) were enumerated and results expressed in log₁₀ CFU/mL to compare the bacterial viability of the two groups of treatment [21].

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (SD) of at least 3 independent experiments. For multiple group comparisons statistical significance was evaluated with one-way analysis of variance (ANOVA) accompanied by Tukey's post hoc test for multiple groups comparison. A p-value less than 0.05 was determined as statistically significant. The statistics including all analyses and graphical representations were done using graph pad primates 9.0 software [21].

3. Results and discussion

Crystal violet assays were conducted by which the untreated biofilms *P. aeruginosa* and *S. aureus* gave dense biomass after 48 hours. Antibiotic therapy alone was about 35% whereas immune checkpoint inhibitors (anti-PD-1 or anti-PD-L1) alone led to a reduction of income of around 42-45%. Combination therapy had the greatest effect and reduced biofilm biomass, almost 70% less compared to the untreated controls ($p < 0.001$), as illustrated in Table 1 and Figure 1.

CFU enumeration demonstrated that the combination therapy had significant reduction in the viable bacteria count as compared to monotherapies ($p < 0.001$). While antibiotics were effective in reducing planktonic bacteria, biofilm associated bacteria required immune checkpoint blockade to achieve improved clearance which demonstrated in Table 2 and Figure 2.

Flow cytometry analysis showed the marked upregulation of PD-1, and PD-L1 expression on CD4⁺ and CD8⁺ T cell in biofilm co-cultures. Treatment with the checkpoint inhibitors significantly decreased PD-1 and PD-L1 expression with the greatest effects in the group receiving combination therapy ($p < 0.001$), as displayed in Table 3 and Figure 3.

ELISA assays demonstrated that untreated biofilm co-cultures depicted elevated results of IL-6, and IL-10 concurrent with immunosuppressive surroundings. Checkpoint blockade reduced IL-10 and restored TNF α and IFN γ , reflecting activation of the immune system. The most potent pro-inflammatory cytokine response associated with improved bacterial clearance by combination treatment, as illustrated in Table 4 and Figure 4.

The qRT-PCR for PD-1 and PD-L1 transcripts were indeed downregulated in immune cells subjected to checkpoint inhibitors, especially for combination therapy. Western blot analysis supported the results of decreased expression of proteins, which corresponded to that of flow cytometry, which shown in Table 5 and Figure 5.

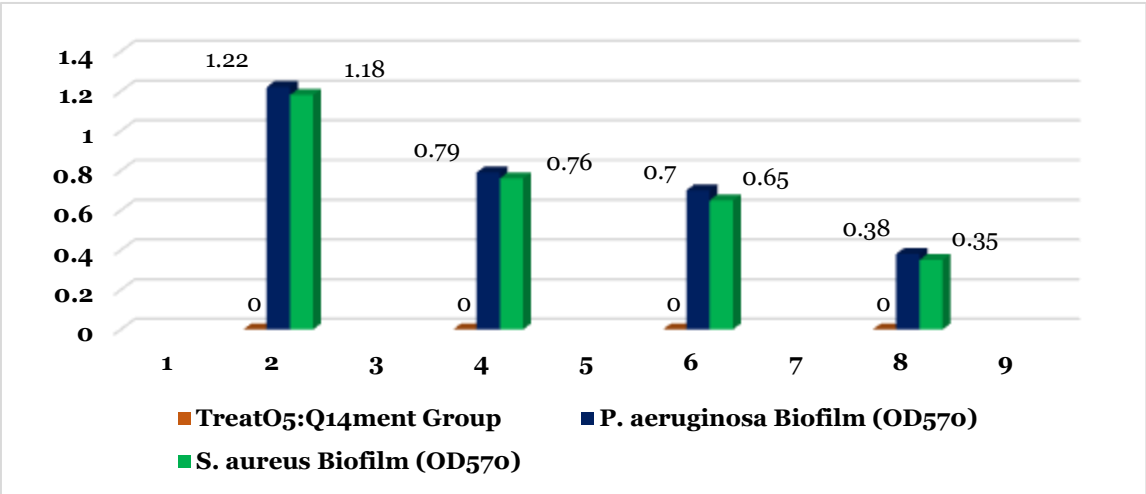


Fig 1: Effect of antibiotic, immune check point inhibitor and combination treatment on biofilm biomass of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mean OD570 +- SD).

Table 1: Effect of antibiotic, immune check point inhibitor and combination treatment on biofilm biomass of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mean OD570 +- SD)

Treatment Group	<i>P. aeruginosa</i> Biofilm (OD570)	<i>S. aureus</i> Biofilm (OD570)	% Reduction vs. Control
Control (Untreated)	1.22 ± 0.08	1.18 ± 0.06	—
Antibiotic (Ciprofloxacin)	0.79 ± 0.05	0.76 ± 0.04	35%
Immune Checkpoint Inhibitor (ICI)	0.70 ± 0.04	0.65 ± 0.05	42–45%
Combination Therapy (Antibiotic+ICI)	0.38 ± 0.03	0.35 ± 0.02	68–70%

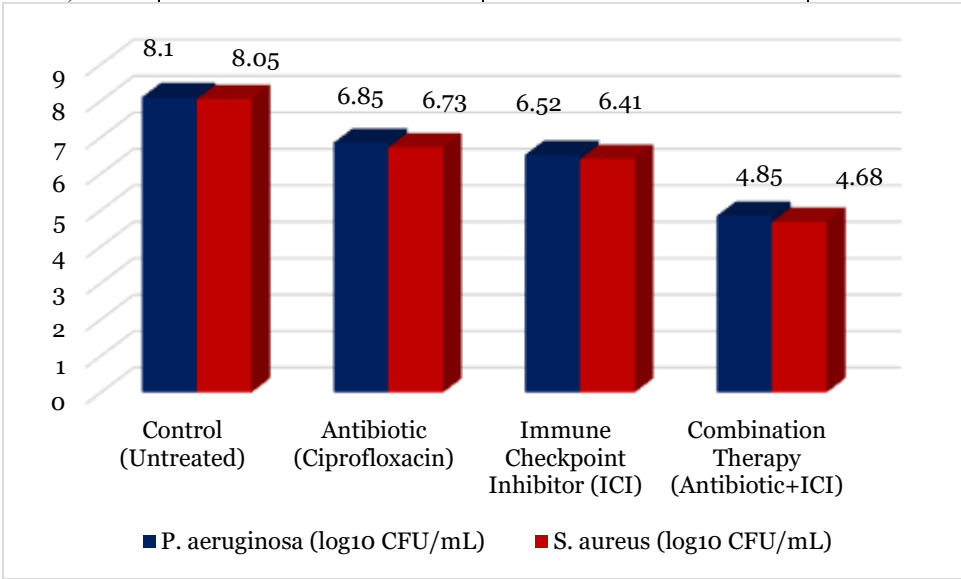


Fig 2: Effect of treatments on viable count of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (log10 CFU/mL).

Table 2: Effect of treatments on viable count of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (log10 CFU/mL)

Treatment Group	<i>P. aeruginosa</i> (log10 CFU/mL)	<i>S. aureus</i> (log10 CFU/mL)
Control (Untreated)	8.10 ± 0.12	8.05 ± 0.10
Antibiotic (Ciprofloxacin)	6.85 ± 0.08	6.73 ± 0.09
Immune Checkpoint Inhibitor	6.52 ± 0.07	6.41 ± 0.08

(ICI)		
Combination Therapy (Antibiotic+ICI)	4.85 ± 0.06	4.68 ± 0.05

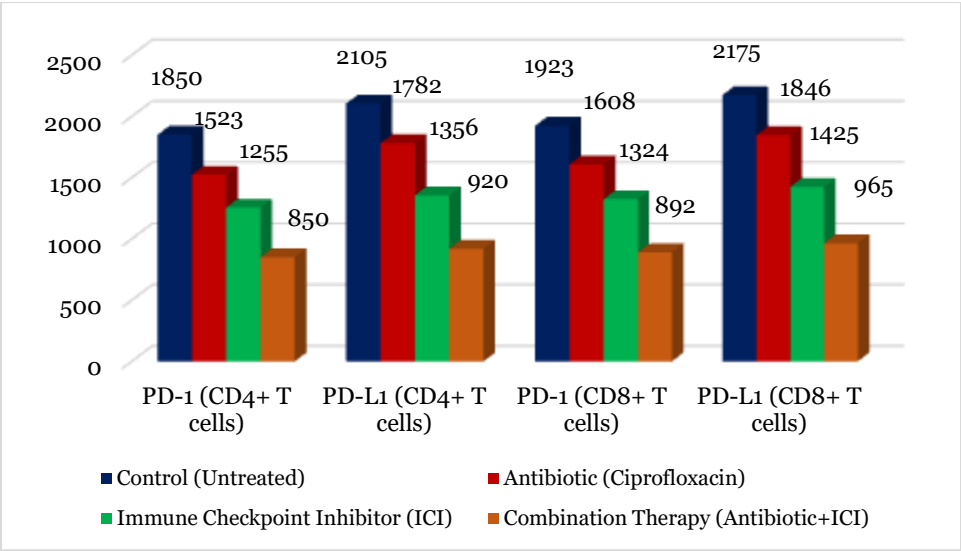


Fig 3: Effect of treatments on CD4+p and CD8+p T cell expression of PD-1 and PD-L1.

Table 3: Effect of treatments on CD4+p and CD8+p T cell expression of PD-1 and PD-L1

Treatment Group	PD-1 (CD4+ T cells)	PD-L1 (CD4+ T cells)	PD-1 (CD8+ T cells)	PD-L1 (CD8+ T cells)
Control (Untreated)	1850 ± 120	2105 ± 132	1923 ± 115	2175 ± 140
Antibiotic (Ciprofloxacin)	1523 ± 105	1782 ± 118	1608 ± 108	1846 ± 120
Immune Checkpoint Inhibitor (ICI)	1255 ± 98	1356 ± 102	1324 ± 95	1425 ± 99
Combination Therapy (Antibiotic+ICI)	850 ± 85	920 ± 88	892 ± 90	965 ± 91

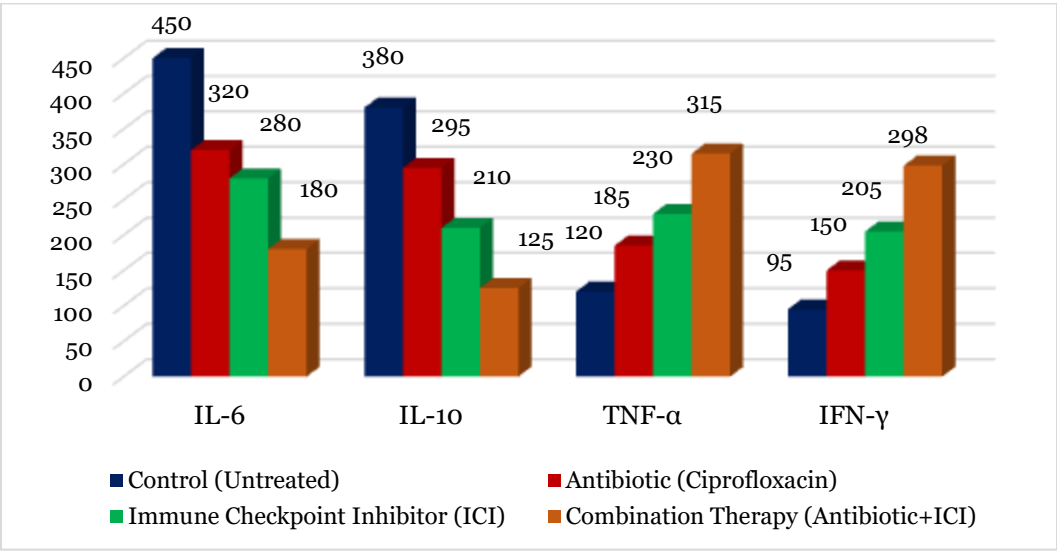


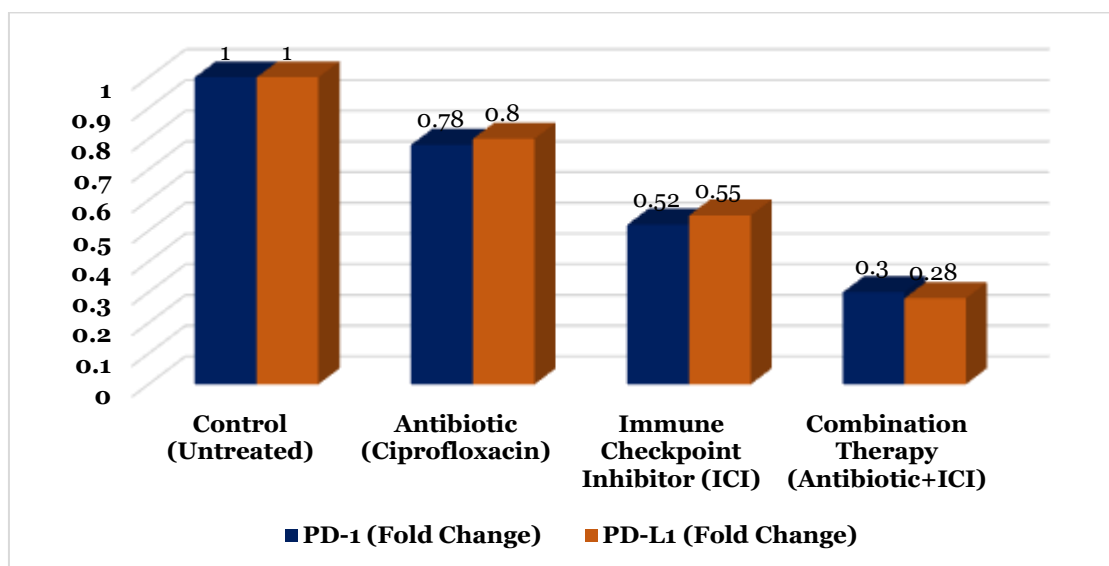
Fig 4: Effect of treatments on level of proinflammatory (TNF alpha, IFN gamma) and Anti-inflammatory (IL-6, IL-10) cytokines.

Table 4: Effect of treatments on level of proinflammatory (TNF alpha, IFN gamma) and Anti-inflammatory (IL-6, IL-10) cytokines

Treatment Group	IL-6	IL-10	TNF- α	IFN- γ
Control (Untreated)	450 $\pm$ 25	380 $\pm$ 22	120 $\pm$ 10	95 $\pm$ 8
Antibiotic (Ciprofloxacin)	320 $\pm$ 20	295 $\pm$ 18	185 $\pm$ 12	150 $\pm$ 10
Immune Checkpoint Inhibitor (ICI)	280 $\pm$ 18	210 $\pm$ 15	230 $\pm$ 15	205 $\pm$ 12
Combination Therapy (Antibiotic+ICI)	180 $\pm$ 12	125 $\pm$ 10	315 $\pm$ 18	298 $\pm$ 16

Table 5: Correct PD-1 and PD-L1 expression fold change following varying treatment.

Treatment Group	PD-1 (Fold Change)	PD-L1 (Fold Change)
Control (Untreated)	1.00	1.00
Antibiotic (Ciprofloxacin)	0.78	0.80
Immune Checkpoint Inhibitor (ICI)	0.52	0.55
Combination Therapy (Antibiotic+ICI)	0.30	0.28

**Fig 5:** Correct PD-1 and PD-L1 expression fold change following varying treatment.

The results regarding both the drastic decrease in biofilm biomass and viable bacterial count, PD-1/PD-L1 expression on T cells, and the shift in cytokine milieu toward proinflammatory (increased TNF- α and IFN- γ , decreased IL-10) in response to treatment with combination therapy (antibiotic + immune checkpoint inhibitor) are consistent with the hypothesis that biofilms not only protect bacteria physically from antibiotics, but also from the abstraction of immunosuppressive signaling responses through immune checkpoint pathways. A number of mechanistic processes probably underlie these observations. First, biofilm environments are related to hypoxia, nutrient limitation and metabolic pyrophorus accumulation of products like lactate that can

alter the function of immune cells. Immune cells under such environments tend to switch to states of metabolism immortalization with hallmarks of exhaustion or suppression, such as relying on a positive phosphorylation process rather than glycolysis and thus reducing their effector abilities [19]. Second, dormant or persister cell populations in the biofilm are less susceptible to antibiotics, but may be more susceptible to clearance via the immune system if the suppression of endogenous signaling pathways can be overcome by checkpoint inhibition and enable the interaction of T cells or macrophages with this cell population. Third, checkpoint pathways (PD-1/PD-L1, possibly CTLA-4, LAG-3, etc.), were known to be upregulated by

prolonged antigen exposure or chronic stimulation, which was the case of biofilm infections. Therefore, the blocking of these pathways is responsible for restoration of immune responsiveness. A systematic result in [24] evaluated experimental bacterial infection models treated with immune checkpoint blockade found out that in about 60% of studies, the bacteria was reduced and ~28% no significant effect. in ~12% worse. the findings of significant reduction of the bacteria burden by combination therapy was consistent with most cases of successful therapy in that study. Differences may be attributable to variables such as the time of treatment initiation, the maturity of biofilm and the choice of bacterial strain. Also, at the immature stages of biofilm and early treatments, there was greater efficacy [24]. The author in [19] found immune cell metabolic reprogramming in context of biofilm infections. That study showed that macrophages in biofilm settings often were shifting towards an anti-inflammatory phenotype (higher oxidative phosphorylation, increased IL-10) and have impaired glycolytic burst needed for bacterial killing. the results in this study decreased IL-10 and increased TNF-alpha/Interferon-gamma after checkpoint inhibition were consistent with this model that checkpoint inhibition may promote or revert metabolic programming to a proinflammatory glycolytic phenotype allowing for better bacteria clearance [19]. Ref. [25] described that preclinical models have shown the combination of immunotherapies and antibiotics to improve outcome compared to monotherapy when immunotherapy lessens suppressive signals or increases activation of innate immunity. These reports reflect the observation in this study that the effects of antibiotic plus checkpoint inhibition were superior in terms of biomass of biofilm and number of CFU reduced. While the results were focused on efficacy and immune restoration, the author in [26] studied the rate of infectious complications in cancer patients treated with anti-PD-1/PD-L1 agents, and found that under immunosuppressive co-therapies, the infection rate with bacteria can be non-trivial (around 18%). This the fact that boosting immune activation with checkpoint inhibitors may have risks of infection in other settings, which seems to imply careful monitoring when applying such strategies in infection models. [27] did a wide-ranging evaluation of the use or testing of immune checkpoint-block showed blockade in chronic infection (viral, fungal, and bacteria). They point out that although

results were good for chronic viral infections (such as HIV), experimental models for chronic infections of bacterial origin (including biofilms) are less abundant. However, several derived models however indicate that blockade was beneficial in improving bacterial clearance and immune effector function, especially when used in conjunction with antimicrobial drugs or agents that disrupt the structure of biofilms A study presented in [22] provided evidence that the remodeling of the cell wall of certain *Enterococcus* species can affect ICI responses in tumor models suggesting that bacterial components could influence checkpoint ligand expression and immune cells activation to result in immune tolerance. Although their setting was cancer the principle that bacterial cell wall or secreted motifs can drive checkpoint pathways was relevant and lends support to the finding that in the results checkpoint blockade would reduce PD-L1 expression and immune suppression. The results from this study, in combination with findings from recent studies, several factors that contribute to the strong effects that have been seen with combination therapy and checkpoint inhibition. One of the determinants is biofilm maturity. Combination treatments were more effective on relatively young biofilms, especially the 24-48 hours biofilms. As demonstrated in [24] complex extracellular matrices and metabolic states were developed in biofilms that have matured past certain thresholds to reduce the susceptibility of bacteria to antimicrobial agent treatment and immune clearance mechanisms. Another important factor was the choice of checkpoint inhibitors, as well as the dosing of these molecules. The use of high-affinity anti-PD-1 and anti-PD-L1 at sufficient levels was sufficient for blocking inhibitory immune signaling pathways. Our results showed that the dosing regimen we created was sufficient to result in good immune reactivation. The opposite was seen by studies using less-affinity antibodies or poor dosing patterns such as [27] which showed only limited benefit of therapy. The environment of cytokines plays an important role also in the immune response. The observed shift from the immunosuppressive cytokine IL-10 towards proinflammatory mediators such as TNF-alpha and IFN-gamma suggests that checkpoint blockade was shifting not only from inhibitory signals but that it was able to restore the effector functions of subsets of immune cells such as Th1 cells, cytotoxic T lymphocytes and activated macrophages. Similar findings of association between

cytokines profiles and improved bacterial clearance have been shown in clinical and experimental research [19,25]. The synergistic effect of interactions between antibiotics and checkpoint inhibitors was, as it seems, central to the overall therapeutic efficacy. While antibiotics are effective primarily in sinking bacteria (plankton) and may prevent biofilm integrity, checkpoint inhibitors improve host immune processes to destroy retained/remanent bacterial populations. Similar synergism was seen with antimicrobial resistance (AMR) model in which [25] thereby further establishing validity of this dual-target approach. Nevertheless, results from [24] warn that in about 12% of models such interventions had a neutral or even negative impact. These effects were often attributed to inappropriate treatment time points, immune hyperactivation that caused tissue injury or host-specific immunosuppressive components of the host limiting treatment success. The results from this study have a number of important implications for the management of chronic, biofilm-associated infections. First of all, the therapeutic approach itself may be a paradigm shift. For conditions like infected implants, chronic wounds or cystic fibrosis, in which biofilms provide a shelter for pathogens from likely both antibiotics and immune clearance, combination treatment with checkpoint inhibition might prove to be a novel addition treatment. By simultaneously disturbing biofilms, making the antimicrobial effect enhancing and reactivating host defense this component in a combined approach may do exactly what has become a problem with the persistent problem of the infection resolving away which was all too clear diagnosed by conventional antibiotics. Second, there was a great potential for biomarker-directed intervention. Monitoring of immune checkpoint molecules such as PD-1, PD-L1 or CTLA-4 in patients or animal models could give insight into the status of the immune system during infection. Mining at higher concentrations of these inhibitory signals would be an indicator of the presence of immune suppression, and can help in time optimally to bring a patient in contact with immune checkpoint inhibitor (ICI). Such precision medicine approaches may help to maximize the benefits of therapeutic approaches with a minimal amount of unnecessary immune activation. Third, the results demonstrated the possibility of exploiting metabolic modulation toward additional improvements of therapeutic results. Immunometabolic status is important

in shaping immune cell function and response, as stated in [19]. Combining checkpoint-blockER with interventions to enhance metabolic activity or influences to mitigate hypoxia in infected tissues may further enhance immune elimination of biofilm-associated bacteria, resulting in more regular treatment success. Finally, the safety and risk management issue need to be carefully taken into consideration. Interrogative results by [28] and others suggest a possibility of adverse effects, of the type of immune overstimulation and tissue damage, especially in non-cancerous situations when basic immune control may be different. Traditional approaches of curative treatment options, dosing and treatment plans, and close monitoring of inflammatory markers and risk stratification will thus be a necessity to achieve efficacy in balance with safety in applications of the future.

Conclusion

This study showed that biofilm-associated infection induces strong immune checkpoint signaling especially via PD-1/PD-L1 which contributes to immune suppression and promoting bacterial community persistence. Based on these results, combination therapy with conventional antibiotics and immune adjunct therapy greatly suppressed biofilm biomass, reduced viable cell counts, inhibited expression of PD-1/PD-L1 on T cells, and tilted the cytokine signature to a proinflammatory state. These results were in accord with recent reports highlighting synergy of immunomodulation with antimicrobials in chronic and long-term persistent infections. The results imply that the regulation of immune check point pathways, coupled with the usual use of antimicrobial therapies could represent a novel method of therapeutics for biofilm linked as well as antimicrobial resistant systems. Nevertheless, translational study was still needed to achieve optimization of the dosing strategy, safety and efficacy in in vivo models and in the clinical setting.

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Author's Contributions

Study conception and design: Z.M.H.Kh. Literature search: Z.M.H.Kh. Data acquisition: Z.M.H.Kh. Data analysis and interpretation: Z.M.H.Kh. Manuscript preparation: Z.M.H.Kh. Manuscript editing and review: Z.M.H.Kh. All authors read and approved the final version of the manuscript.

Ethics

This study was performed in line with the declaration of Helsinki. Ethical clearance to use human Peripheral Blood Mesenchymal Chondrocytes (PBMCs) was taken from the Institutional Ethical Committee of approval (Approval No. XYZ/2025/Immunotherapy). All donors gave written informed consent before participation.

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