



IL-26 mediated human cell activation and antimicrobial activity against *Borrelia burgdorferi*

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ABSTRACT

Lyme disease is an inflammatory disease caused by infection with *Borrelia burgdorferi* (Bb). Inflammatory sequelae of Bb infection appear to be refractory to antibiotics. An antimicrobial peptide with the ability to bind the DNA in the tissue could serve as a viable option of treatment for chronic complications of Lyme borreliosis. DNA of Bb can remain in tissues causing a prolonged inflammatory response that lead to chronic joint pain. Here we examined the effect of IL-26, a newly reported antimicrobial protein, against Bb DNA.

An antimicrobial effect of IL-26 on the spirochete was observed. In human macrophages, IL-26 treated cells showed an increase in IRF activation upon Bb stimulation. Moreover, IL-26 treated macrophages showed an increased in phagocytic activity compared to untreated cells. Although no Bb DNA degradation was observed using a TUNEL assay run in an agarose gel, a Comet assay on whole bacteria showed cellular and Bb DNA degradation by IL-26.

Our results showed that IL-26 (monomer and dimer) has not only the potential to control Bb growth in vitro, but it also enhances the anti-borrelial response of human macrophages. Further research aiming to characterize the role of IL-26 in controlling other aspects of the inflammatory response that could provide insight of its potential therapeutic applications are needed.

1. Introduction

Lyme disease (LD) is an infection caused by *Borrelia burgdorferi* (Bb), a spirochete carried by *Ixodes* ticks. Bb infection causes inflammation, which in some patients with LD can lead to arthritis, carditis, and central nervous system disorders (Sanchez, 2015). While erythema migrans and Lyme carditis often present within several weeks, Lyme arthritis is normally found during the later stages of the disease, even after antibiotic treatment. The late onset of musculoskeletal symptoms following Bb infection and their refractoriness after antibiotic treatment is called 'Post-Lyme disease Syndrome' (PLDS) (Aucott, 2015). Since initial studies failed to detect spirochetal DNA in human synovial fluid following treatment with antibiotics, antibiotic-resistant borrelial arthritis was considered an autoimmune disease (Benoist and Mathis, 2001; Steere, 2012). Studies have been able to detect Bb specific DNA in patients with different forms of Lyme borreliosis, including joint fluid from arthritis patients after therapy (Picha et al., 2008; Li et al., 2011; Picha et al., 2013). Presence of Bb DNA has been reported to persist for as long as 11 months in patients with antibiotic-refractory arthritis (Li et al., 2011). In mice models, DNA and antigen deposits have been

shown to persist after antibiotic treatment in cartilage of mice deficient in MyD88 (Bockenstedt et al., 2012),

Since antibiotics do not target bacterial DNA, persistent DNA from the spirochete may be present in the tissue of the host (Embers et al., 2012; Hodzic et al., 2008) after antibiotic therapy (Bockenstedt et al., 2002; Marques et al., 2014). Antimicrobial peptides (AMPs) exhibit diverse modes of action, which include binding to bacterial nucleic acids (Brogden, 2005; Libardo et al., 2015). IL-26 is an antimicrobial protein that can bind and form a complex with microbial DNA. Specifically, it has been reported to interact with DNA from *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Meller et al., 2015).

IL-26 is a cytokine from the IL-10 family, initially described to be produced by helper T cells (Wilson et al., 2007) although recent reports demonstrate it is also produced by natural killer cells, macrophages, and fibroblasts (Tengvall et al., 2016). Besides its antimicrobial action, IL-26 also promotes immune sensing of DNA by forming complexes with bacterial DNA, triggering subsequent activation of TLR-9 (Meller et al., 2015). Th17 cells are essential in the control of bacterial infections, and are also involved in autoimmune inflammation in synovia and CNS tissue. Th17 cells participate in the defense against extracellular bacteria

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in skin and mucosa, which makes them likely to be involved in the response against Bb (Grygorczuk et al., 2016). A synovial fluid Th17 cell response may play a crucial role in the pathogenesis of Lyme arthritis (Codolo et al., 2008), and neuroborreliosis (Henningsson et al., 2011).

Bb can reside in the extracellular matrix of host cartilage (Bockenstedt et al., 2012; Cabello et al., 2007). Antibiotics with good tissue penetration like third generation cephalosporins have good efficacy against infection. However, in cases of antibiotic refractory Lyme arthritis, DNA may be persisting in tissue (Cervantes, 2017). The use of a human AMP able to attach to bacterial DNA, such as IL-26 could become a form of therapy that may have an impact on patients suffering from persistent clinical symptoms after treatment for LD.

We here aimed to assess the antimicrobial effect of IL-26 on live Bb and Bb DNA, as well as its effect in human macrophage response to these stimuli.

2. Material and methods

2.1. Bacterial cell culturing

Bb strain B31, clone 5A2 was obtained from Bei Resources. Bacteria were cultured in 0.5X Revised Barbour-Stoenner-Kelly (BSK) Medium (Sigma-Aldrich). Spirochetes were temperature shifted (Tokarz et al., 2004), aiming to replicate the temperature changes the bacterium experience as it is introduced in the mammalian host by the tick.

2.2. Bacterial viability tests

The resazurin bacterial viability microdilution test, based on a redox reaction where live bacterial cells reduce the dye resazurin (blue) to resofurin (pink), (Elshikh et al., 2016) was used for determining the minimum inhibitory concentration (MIC). The test was performed in a 96-well plate, where 100 µL of Bb culture were dispensed in each well followed by serial dilutions of IL-26 monomer and dimer. Tetracycline was used as a positive control. Plates were incubated at 37 °C for 24 hrs, at the completion of which 10 µL of Resazurin solution (Biotium) was added to each well using a multichannel pipette. Further incubation for 0 to 4 hrs at 37 °C was necessary to allow the color change to take place.

For assessment of bacterial viability after IL-26 treatment, cells were washed with PBS after the incubation period, and stained with the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen, USA) following the instructions from the manufacturer (Yokobori et al., 2019).

2.3. Cell assays

THP1-Dual cells (Invitrogen) containing reporter constructs to simultaneously detect the activation of NF-κB and IRF-mediated pathways were utilized. Prior to stimulation, cells were transformed into macrophages by the addition of 5 ng/mL of PMA (Phorbol 12-myristate 13-acetate) (Sigma) for 72 hrs (Park et al., 2007). Cells were then treated with 1 µM of IL-26 monomer or IL-26 dimer for 24 hrs. Cells were then inoculated with live Bb and incubated for 24 hrs at 37 °C. For evaluation of the effect of IL-26 on Bb and Bb DNA as stimuli, 250 ng of Bb DNA as well as live Bb, were treated with 10 µM of IL-26 monomer or IL-26 dimer (R&D Systems) for 1 hr, prior to be used as stimuli. Readouts of the reporter systems were conducted following manufacturer's instructions. Response under each condition was compared to unstimulated wells and expressed as a response ratio.

2.4. Microscopy

An immuno-fluorescence assay was utilized to assess macrophage phagocytosis. Briefly, after Bb infection, cells were fixed using 2% PFA (fixation media) for 10 min at room temperature, then washed with PBS, blocked with 10% blocking one (Nacalai) buffer, and permeabilized with 0.2% saponin in PBS. Cells were then incubated with primary antibodies

against Bb (Invitrogen) and LAMP-1 (DHSB), and fluorescently-tagged secondary antibodies (Alexa 488, Green, to visualize Bb, and Texas Red, for LAMP-1) (ThermoFisher). Imaging acquisition was done using an Olympus fluorescence microscope, and images processed with ImageJ.

2.5. Bb dna isolation

Bb cultures were centrifuged at 8000 rpm for 15 min, and the pellet was treated with 20 µL of proteinase K and 20 µL of lysozyme prior to using a DNA extraction kit (Qiagen). DNA sample concentration was assessed using a Nanodrop.

2.6. TUNEL assay

To assess development of double or single strand nicks in Bb DNA by IL-26 a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was utilized (Lee and Lee, 2014). The assay is based on the incorporation of a fluorescent tagged dUTP nucleotide. 500 ng of Bb DNA was incubated with 10 µM of IL-26 monomer and dimer for 2 hrs. Untreated Bb DNA was used as a negative control. A positive control tube was created by degrading Bb DNA with 0.005 U of HindIII. Following incubation, TUNEL reagent (*In Situ* Cell Death Detection Kit, Roche) was added according to manufactures instructions and incubated for 10 min. Equal volumes of each reagent were loaded into a 0.8% agarose gel containing a DNA intercalating green fluorescent dye SYTO 13. After gel electrophoresis, gel was imaged using a Molecular ImagerPharosFX System (BioRad) and Quantity One software (BioRad).

2.7. Bacterial comet assay

A single gel electrophoresis, Comet assay was utilized to assess the effect of IL-26 on whole live Bb cells (Solanki and Haydel, 2012). The assay was performed on a glass bottom dish with a bottom layer of Type A gelatin (5% weight/vol.), and a middle layer of 0.5% agarose with a well in the center. Live Bb was incubated with 10 µM of IL-26 monomer and IL-26 dimer for 1 hr. After incubation 1.8 µM Syto13 and 0.1 µL of RNase A were added to liquid 0.8% agarose. Before the agarose solidified, the mixture was added to the well created in the middle layer of the glass-bottom dish, and allowed to solidify. A final layer of 2 mL gelatin was added to the top of the dish. The plate was then placed in a lysis buffer solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% sodium lauroyl sarcosine, and 1% Triton X-100) for 1 hr at room temperature. Following the lysis buffer the plates were electrophoresed for 50 min at 12 V in a buffer composed of 300 mM sodium acetate and 100 mM Tris pH 9. Dishes were then imaged using an inverted green fluorescent microscope.

2.8. Statistical analysis

General statistical analysis was conducted using GraphPad Prism 8.4 (GraphPad Software, San Diego, CA, USA). Comparison of responses under the different IL-26 treatments were compared to untreated cells, utilizing the Student's *t*-test or a non-parametric test depending if the data followed a normal distribution or not. For each experiment, the standard deviation and the mean were calculated. A *p* value < 0.05 was considered significant.

3. Theory

Since Bb DNA has the ability to persist in host tissue and cause chronic symptoms of disease, an AMP with the ability to bind Bb DNA in human tissue could serve as a viable option of treatment for chronic complications of Lyme borreliosis. We aim to evaluate three aspects: Aim 1: To assess the bacteriostatic and bactericidal effect of IL-26 on Bb.

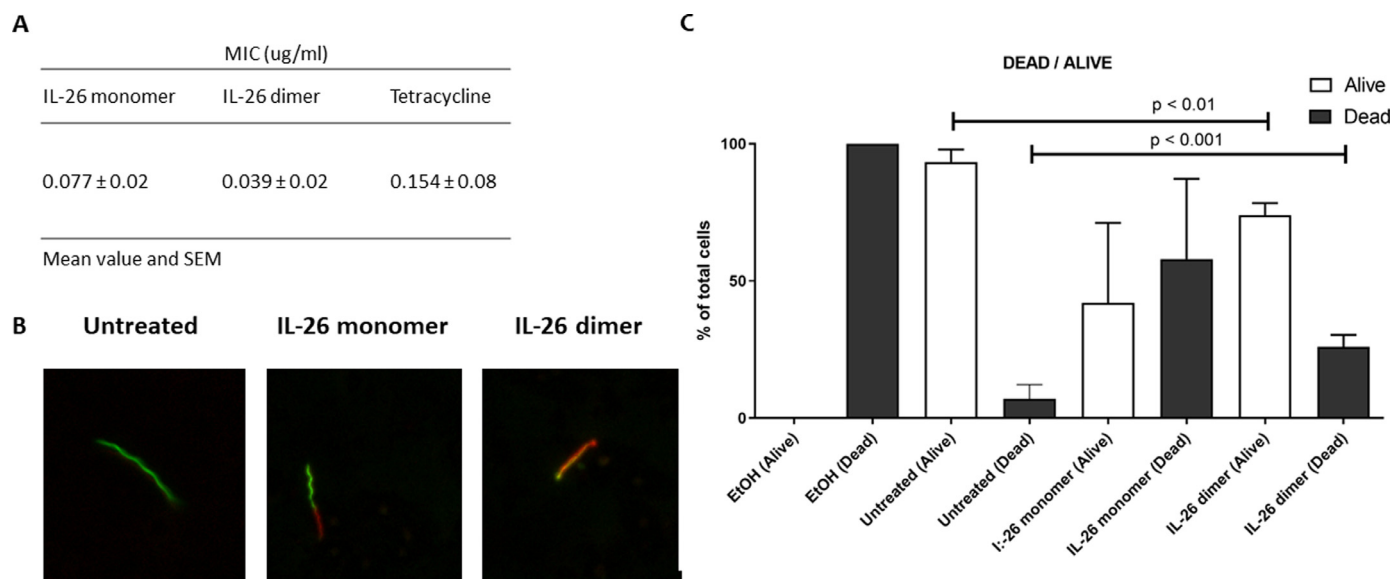


Fig. 1. Antimicrobial effect of IL-26 on Bb. **A:** MIC values for IL-26 M, IL-26D, and Tetracycline. **B:** LIVE/DEAD bacterial viability imaging. **C:** LIVE/DEAD percentages of cells after treatment with 70% ethanol (EtOH), untreated, and treated with IL-26 monomer and dimer for 6 hrs. Graph shows results of three independent experiments. Statistical analysis performed: Paired t-test.

Aim 2: To determine the role of IL-26 in degradation of the genome of Bb.

Aim 3: To determine the effect of IL-26 as macrophage activator (as these are the cells in charge of removing extracellular bacterial DNA in tissues).

4. Results

4.1. Antimicrobial effect of IL-26 against bb

To test the effect of IL-26 in the growth of temperature-shifted Bb, we utilized a colorimetric viability assay to determine a mean MIC value of 0.077 µg/mL for IL-26 monomer and 0.039 µg/mL for the IL-26 dimer (Fig. 1A). Inhibition of Bb growth was observed as early as 2 hrs and persisted for up to 48 hrs (data not shown). Tetracycline showed a higher MIC value (0.15 µg/ml) compared to both forms of IL-26. From these results a bacteriostatic effect and concentration could be deduced.

To evaluate induction of bacterial cell death after IL-26 treatment, we utilized a fluorescent viability stain (LIVE/DEAD). Treatment of IL-26 induced spirochetal membrane damage, evidence by incorporation of propidium iodine (Fig. 1B). A major effect on the viability of Bb was observed after treatment with IL-26 dimer (Fig. 1C).

4.2. IL-26 enhances phagocytosis of bb by human macrophages

We then evaluated the effect of IL-26 on Bb phagocytosis by macrophages. To observe this, PMA-differentiated dTHP1 cells were incubated with monomeric IL-26 and dimeric IL-26 for 24 hrs before a 4-hour-Bb stimulation, and then performed an immunofluorescent assay. Although the IFA revealed that IL-26 monomer-treated macrophages phagocytosed 1.5 fold more Bb compared to untreated macrophages, this was not statistical significant (Fig. 2A). When assessing the number of spirochetes internalized per cell, we observed an increase in IL-26 monomer-treated macrophages (Fig. 2C).

4.3. IL-26 enhances macrophage inflammatory response to bb

Another line of macrophage activation explored was the inflammatory response of human macrophages to live temperature-shifted Bb. Although it was observed that IL-26 monomer-treated macrophages

showed a greater NF-κB pathway activation compared to untreated macrophages by a factor of 2 fold, this was not statistical significant (Fig. 3A). In contrast, IL-26 dimer-treated macrophages showed a similar NF-κB response compared to the untreated macrophages.

When evaluating Interferon Regulatory Factor (IRF)-mediated activation in this cells, we observed that both monomeric IL-26 and dimer IL-26 treated macrophages had a greater IRF-mediated response compared to untreated macrophages (Fig. 3B). Moreover, IL-26 dimer-treated macrophages had a greater response ratio than IL-26 monomer-treated macrophages. This set of experiments showed that treatment of macrophages with the monomeric form of IL-26 may lead to a stronger NF-κB response upon Bb stimulation, while IL-26 dimer treated macrophages produces greater IRF response against Bb.

4.4. IL-26 treated bb dna does not enhance inflammatory response in human macrophages

Since it was reported that addition of IL-26 to DNA from Gram negative bacterium *Pseudomonas aeruginosa* promotes sensing of DNA by dendritic cells (Meller et al., 2015), we aimed to assess the effect of IL-26 on the stimulation of macrophages when it was used in combination with Bb DNA. We observed that IL-26-treated Bb DNA did not increase the NF-κB activation by this stimulus (i.e. Bb DNA alone). Overall IL-26/Bb DNA complexes elicited less NF-κB activation in human macrophages compared to stimulation with live whole Bb organisms (Fig. 4A). No difference was observed regarding IRF activation under these conditions (Fig. 4B).

4.5. IL-26 does not produce double strand nicks in genomic bb dna in

We utilized TUNEL assay on extracted of naked genomic Bb DNA, to observe the occurrence of double strand breaks by IL-26 (Rohwer and Azam, 2000). No positive TUNEL reaction was observed on Bb DNA incubated with either IL-26 monomer or dimer (Fig. 5). While TUNEL is mainly utilized to detect primarily double-strand DNA breaks (Clark et al., 2004), it was still recognizing the sticky ends produced by the digestion of Bb DNA with *HindIII* restriction enzyme (Lee and Lee, 2014). It was possible that the IL-26 was intercalating with the DNA, slowing down its migration in the gel. To assess if this was the case, IL-26 treated Bb DNA was also treated with *HindIII* to observe any

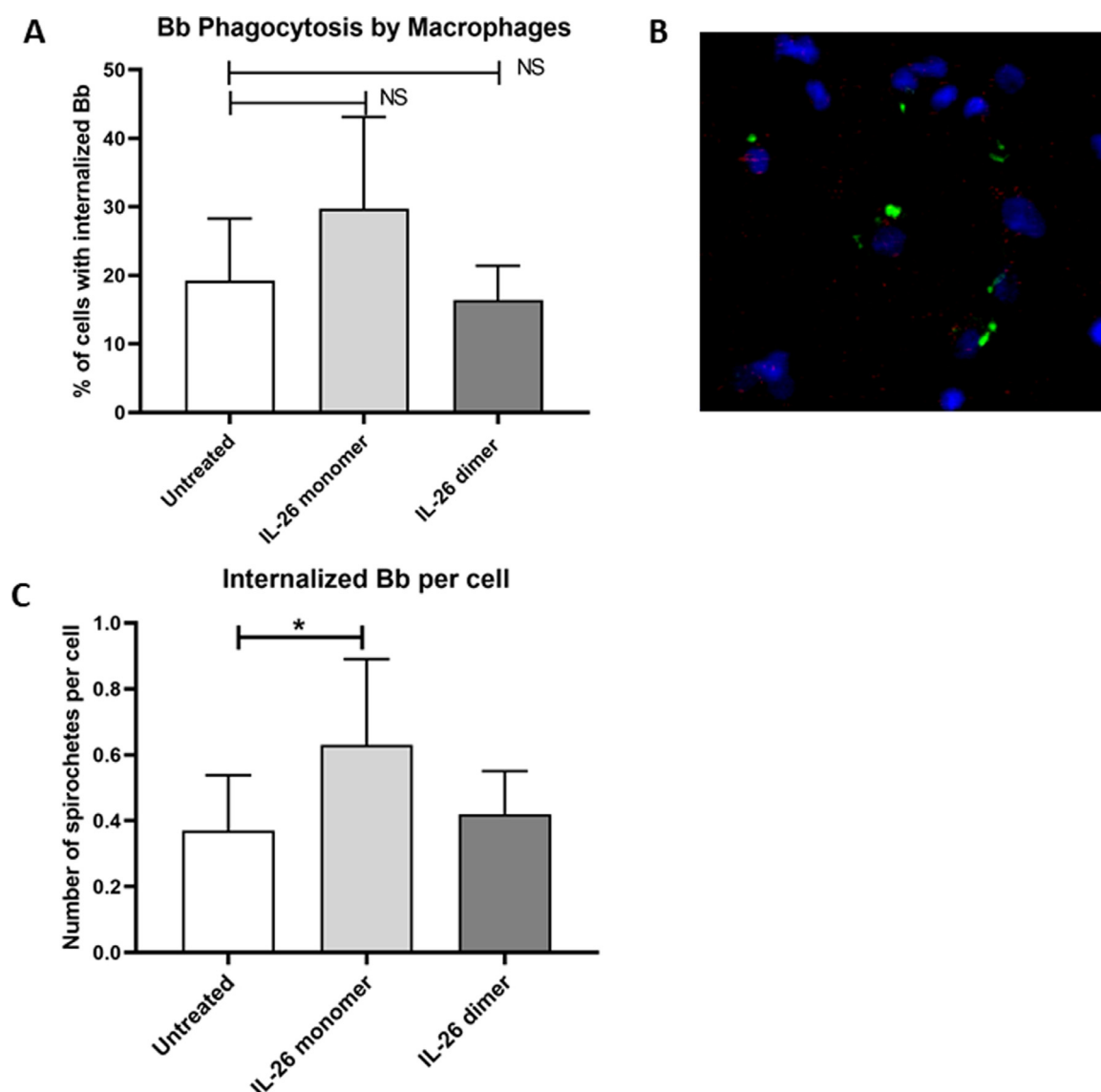


Fig. 2. IL-26 increases internalization of Bb by human macrophages. Immunofluorescence assay assessment of human macrophage phagocytic response to Bb. **A** Number of *Borrelia burgdorferi* cells phagocytosed by human macrophage, untreated (white bars), and treated with IL-26 monomer (light gray) and IL-26 dimer (dark gray). **B** Fluorescence image showing human macrophage nuclei (blue), *Borrelia burgdorferi* (green), and lysosome (red). **C** Number of internalized Bb per cell (bar colors as describe in **B**). Graph shows results of three independent experiments. Statistical analysis performed: Paired *t*-test.

changes of a potential lagged migration. When Bb DNA was co-digested with *HindIII* plus IL-26 (monomer or dimer), an increase in the TUNEL signal was observed, with no apparent change in the size of the digested DNA (Fig. 5B).

4.6. IL-26 effect on Bb dna in cellulo

We then studied the effect of IL-26 on Bb DNA degradation *in cellulo*, when the whole organism is present in a matrix of collagen or gelatin. We utilized a Comet DNA degradation assay, as this electrophoresis-based method is useful for detecting various types of damage to dsDNA on the individual cellular level (Solanky and Haydel, 2012). The Comet assay not only showed disappearance of the genomic content within the spirochete, translation of degrading effect on the spirochetal membrane by IL26 (Fig. 6), but also presence of chromosomal blebs and a fluorescent shadow (i.e. comet tail extending from the whole Bb organism) after treatment with IL-26.

5. Discussion

The literature has shown the bactericidal effect of IL-26 on various microorganisms (Meller et al., 2015; Dang et al., 2019). Our results showed an antimicrobial effect of IL-26 (monomer and dimer) on live Bb. Not only MIC values were below that one for Tetracycline (Hunfeld and Brade, 2006), but these values were maintained over time.

Our next step was to observe whether there existed an increase in phagocytosis of the pathogen, a phenomenon that has been observed with other AMPs (A.M. van der Does et al., 2010; A.M. van der Does et al., 2010). We found that IL-26 monomer-treated cells had a greater phagocytic activity compared to untreated and dimer IL-26 treated cells. IL-26 induction of intracellular bacteria killing has also been observed in monocyte-derived macrophages inoculated with mycobacteria (Dang et al., 2019). A role of IL-26 in the killing of *Staphylococcus aureus* has also been observed *in vitro* (Woetmann et al., 2018), and by PBMCs from patients with hidradenitis suppurativa (Scala et al., 2019).

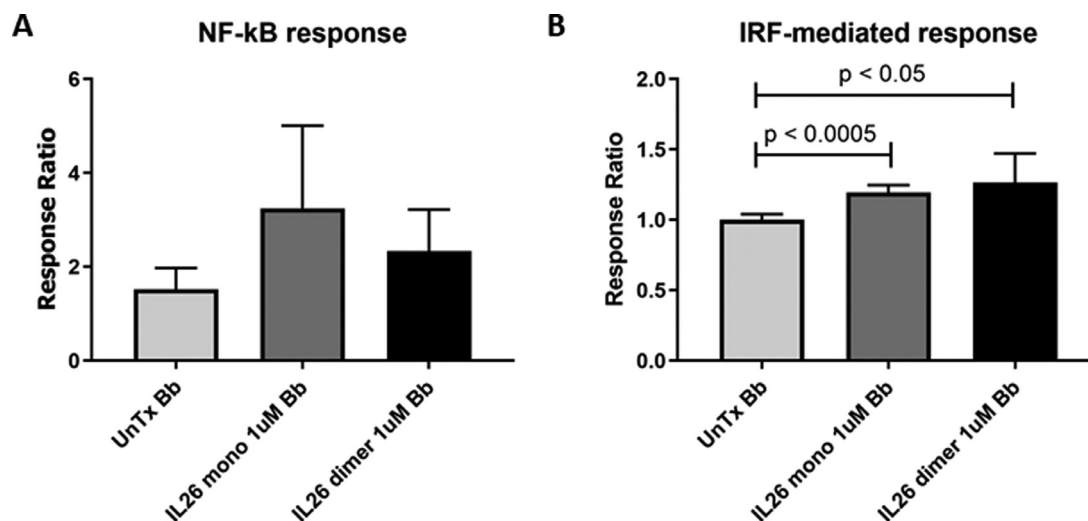


Fig. 3. IL-26 induces inflammatory responses in human macrophages upon Bb infection. **A.** NF-kB Response Ratio of IL-26 stimulated and unstimulated macrophage in the presence of Bb-DNA. **B.** IRF-mediated Response Ratio of IL-26 stimulated and unstimulated macrophage in the presence of Bb-DNA. Graph shows results of at least three independent experiments. Statistical analysis performed: unpaired t-test.

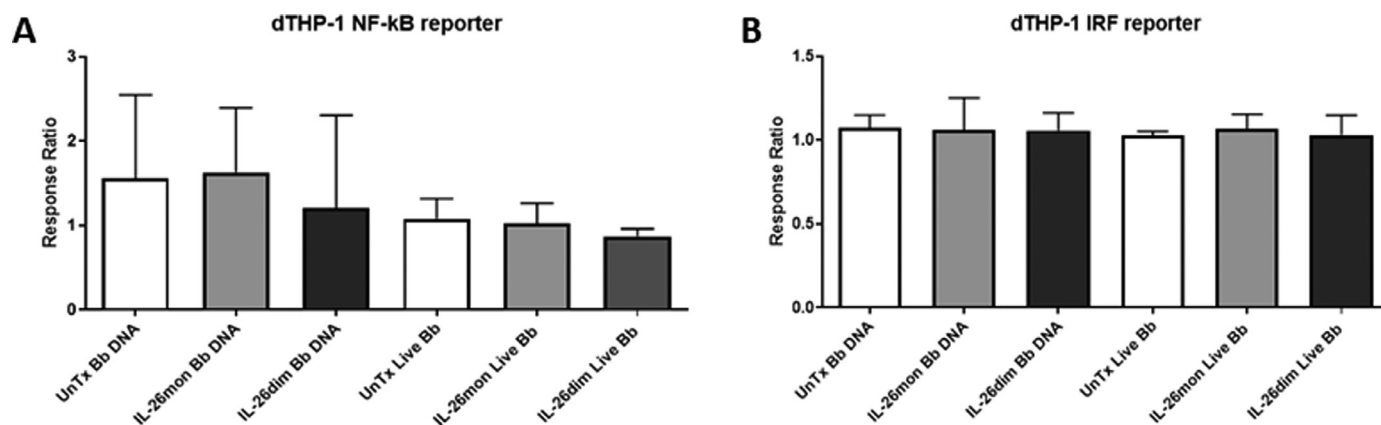


Fig. 4. IL-26 treated Bb DNA does not enhance inflammatory response in human macrophages. **A.** NF-kB and **B** IRF activation in human macrophages stimulated with Bb DNA or whole Bb organisms pre-treated with IL-26 before used for stimulation. Graph shows results of three independent experiments.

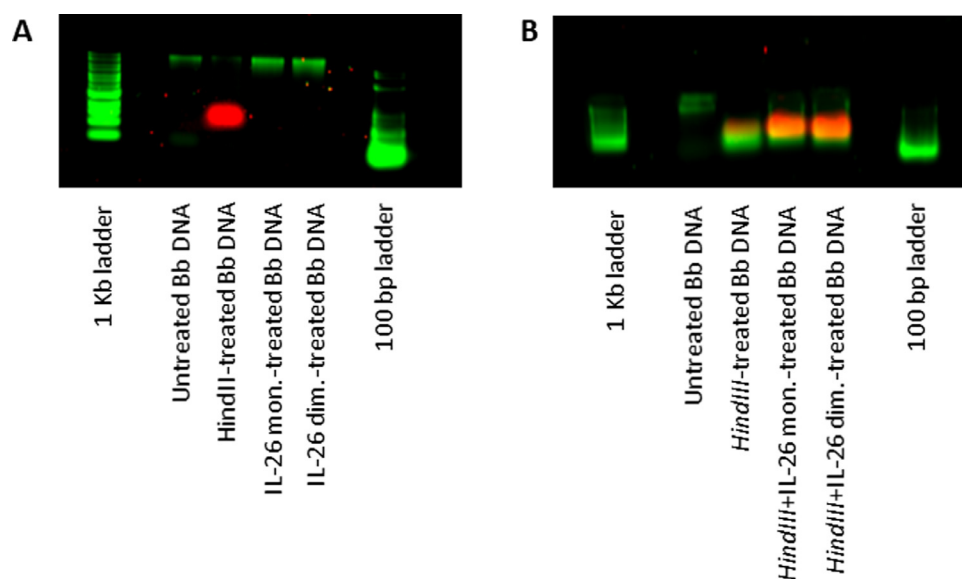


Fig. 5. TUNEL Assay conducted on Bb genomic DNA. **A.** From left to right: 1Kb ladder, Untreated Bb DNA, *HindIII* treated, IL-26 monomer-treated, IL-26 dimer-treated, and 100 bp ladder. **B.** From left to right: 1Kb ladder, Untreated Bb DNA, *HindIII*-treated, *HindIII* and IL-26 monomer-treated Bb DNA, *HindIII* and IL-26 dimer-treated Bb DNA, 100 bp ladder. DNA was labeled with SYTO 13 (Green); TUNEL positive reaction in Red.

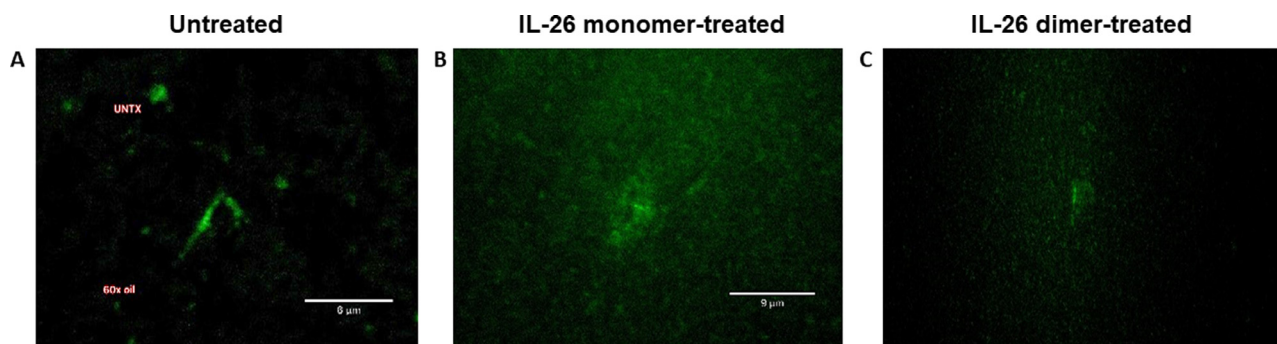


Fig. 6. Comet assay. A. Untreated live Bb. B IL-26 monomer-treated Bb, and C IL-26 dimer-treated Bb. Note the presence of void (B) and a fluorescent tail extending from the right side of the contour of the spirochete. DNA stained with SYTO 13 (Green). Scale = 6 μ m. Images are representative of at least three experiments.

Our findings on macrophage activation (i.e. inflammatory transcription factor activation and phagocytosis) are in line with previous reports of the effect of IL-26 in controlling other bacterial infection in vivo (Meller et al., 2015) and ex vivo (Dang et al., 2019). IL-26 is a member of the IL-10 family, with ability to modulate pro-inflammatory cytokine production (Larochette et al., 2019). IL-26 exhibits priming effects on various immune cells to increase anti-viral and anti-bacterial responses (Stephen-Victor et al., 2016). Despite previous reports of NF- κ B signaling activation on epithelial cells and fibroblasts (Braum et al., 2012; Truong et al., 2017), we did not observe a similar statistical significant phenomena in human macrophages. Our results show, on the other hand, that IL-26-treated macrophages had an increase in IRF activation upon stimulation with live Bb. Both, the monomeric and dimeric forms of IL-26 appeared to have an enhancing effect on this activation. It is uncertain if differences in size or receptor avidity or activation capacity, may account for this results. IRF signaling is triggered after recognition of nucleic acid derived from pathogens by endosomal TLRs (Kawasaki and Kawai, 2014), leading to induction of Type I IFNs and Interferon dependent cytokines which drive an M1 macrophage polarization which are essential in the clearance of Bb (Cervantes et al., 2014). IL-26 is produced by various immune cells (Tengvall et al., 2016). Besides its antimicrobial action (Meller et al., 2015), IL-26 also promotes immune sensing of DNA by forming complexes with bacterial DNA, triggering subsequent activation of TLR-9 (Meller et al., 2015). Unexpectedly IL-26 treatment of Bb DNA prior to macrophage stimulation did not increase the inflammatory response of these cells. Although this could translate in vivo as an inability of IL-26 to increase removal of Bb-DNA in tissues by macrophages, it is still premature to conclude such, as the extracellular matrix presents other cell activators (e.g. matrix metalloproteinases) that need to be explored in this context (Behera et al., 2005; Hyde, 2017).

We expected that IL-26 will effectively bind and degrade Bb-DNA, inside the spirochete or extracellularly. TUNEL assays failed to indicate Bb DNA had been cleaved by IL-26, however, assessment of the effect of IL-26 on whole Bb, via the Comet assay, showed that degradation of the whole organism and its DNA did occur to some extent. Based on prior reports of IL-26 forming complexes with DNA from Gram positive and Gram negative DNA (Meller et al., 2015; Lee and Cua, 2015), it is possible that IL-26 may be intercalating with Bb DNA, without inducing major breakdown.

In conclusion, we have demonstrated the usefulness of IL-26 as an antimicrobial peptide against Bb, and its effect enhancing the inflammatory response of human macrophages against the Lyme disease spirochete. IL-26 monomer-mediated macrophage activation, also promoted a more effective phagocytosis of Bb. Our findings may prompt a new line of research not only to evaluate potential usage of IL-26 in patients with chronic symptoms after Bb infection and Lyme disease therapy.

Declaration of Competing Interest

None.

Author contributions

Conceptualization (JC). Data curation (JC, JH, DP, JB). Formal analysis (JC). Investigation methodology (JC, JH, JB, PK, AS). Project administration (JC, JB). Supervision (JC, JB). Imaging (JH, DP, YR). Writing (JC, PK, JH, AS).

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