Enterococcus faecalis modulation of NF- κ B signaling in macrophages

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NANYANG
TECHNOLOGICAL
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***Enterococcus faecalis* modulation of NF- κ B signaling in
macrophages**

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**Interdisciplinary Graduate School
Singapore Center on Environmental Life Science and
Engineering**

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A thesis submitted to the Nanyang
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Contents

Acknowledgements	2
List of figures	5
Abstract	10
 1 Introduction: <i>Enterococcus faecalis</i> modulation of NF- κ B signalling in macrophages.	11
1.1 <i>Enterococcus faecalis</i>	11
1.2 <i>Enterococcus</i> as an opportunistic pathogen	12
1.3 Antibiotic resistance in <i>Enterococcus</i>	14
1.4 <i>Enterococcus</i> virulence factors	15
1.5 Cellular immune response to <i>E. faecalis</i> infection.....	16
1.6 Intracellular survival and dissemination of <i>Enterococci</i>	17
1.7 Host cell receptors and the NF-KB signalling pathway	19
1.8 Signalling pathways involved in <i>E. faecalis</i> infection	22
1.9 Sortase and substrates	24
1.10 Autolysin.....	25
 2 Chapter 1 – <i>E. faecalis</i> actively suppresses NF- κ B activity by a heat-modifiable, surface associated factor	28
2.1 Establishing and Optimizing NF- κ B reporter assay in RAW macrophages .	28
2.1.1 Growth curves for <i>E. faecalis</i>	28
2.1.2 LPS 100ng activates NF- κ B reporter assay in macrophages	28
2.1.3 <i>E. faecalis</i> OG1RF does not have alkaline phosphatase activity	29
2.2 <i>Enterococcus faecalis</i> disrupts NF- κ B activation in murine macrophages...	31
2.3 <i>E. faecalis</i> V583 disrupts NF- κ B activity in a dose-dependent manner	34
2.4 A heat modifiable, surface associated molecule suppresses NF- κ B activity	36
2.5 <i>E. faecalis</i> actively prevents NF- κ B activation.....	38
2.6 <i>E. faecalis</i> prevents NF- κ B activation in the presence of <i>E. coli</i>	40
2.7 <i>E. faecalis</i> does not activate the TLR2 NF- κ B or the TLR4 NF- κ B pathway	42
2.8 Discussion	45
2.9 Future Directions.....	49

3	Results: Chapter 2 – SortaseA modulates NF- κ B activity	50
3.1	Panel of mutants tested for their NF- κ B activity	50
3.2	D-alanine Lipoteichoic acid and capsular polysaccharide are not required for prevention of NF- κ B activity	55
3.3	Sortase substrates modulating NF- κ B activity	58
3.4	Sortase A has a cellular invasion defect.....	59
3.5	Discussion	62
3.6	Future directions	64
4	Results: Chapter 3 – <i>E. faecalis</i> genomic DNA fails to activate TLR9	66
4.1	Genomic DNA complements NF- κ B activity of SortaseA and Autolysin....	66
4.2	DNA from other bacterial strains differ in their NF- κ B activity.....	69
4.3	<i>E. faecalis</i> genomic DNA fails to activate NF- κ B	71
4.4	Sheared <i>E. faecalis</i> genomic DNA activates NF-KB.....	74
4.5	<i>E. faecalis</i> genomic DNA is not recognized by TLR9.....	76
4.6	Discussion	78
4.7	Future Directions.....	81
5	Materials and methods	82
5.1	Bacterial strains and growth conditions	82
5.2	Cell Culture and Media	85
5.3	NF- κ B reporter assay	85
5.4	LDH assay.....	86
5.5	Gentamicin protection assay	87
5.6	Cytokine assay	87
5.7	Genomic DNA extraction and amplification	88
5.8	Supernatant, heat killed and UV killed and transwell treatment.....	89
5.9	Measurement of eDNA	90
5.10	Sonication and denaturation of genomic DNA	90
5.11	Statistical analysis	90
6	References.....	91

List of Figures

Fig 1.1 <i>E. faecalis</i> OG1RF (TEM image, Adeline Yong, Kline lab)	11
Fig 1.2 Schematic representation of TLR- NF- κ B signalling pathway	19
Fig 2.1 (A) Growth curve of <i>E. faecalis</i> strain OG1RF. The X-axis represents the time in hours and Y axis represents the optical density at 600nm, (B) Growth curve of <i>E. faecalis</i> . The X-axis represents the time in hours and Y axis represents CFU/ml. Murine RAW 267.4 macrophages were stimulated with LPS concentrations from 10ng to 10ug/ml represented on the X axis, and either (C) NF- κ B reporter activity or (D) cytotoxicity, with % cell death represented on Y-axis were measured. (E) NF- κ B SEAP reporter activity induced by <i>E. faecalis</i> OG1RF in the absence of macrophages and macrophage alone controls, Y axis represents NF- κ B reporter activity (C, E & F). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Data are combined data (C, D, & E) from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to media alone (-), where * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.	30
Fig 2.2 Murine RAW 267.4 macrophages were stimulated with (A) <i>E. faecalis</i> OG1RF, (B) <i>E. faecalis</i> and LPS simultaneously, where on the X-axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity (A&B), (C) <i>E. faecalis</i> OG1RF (D) <i>E. faecalis</i> and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents % cell death (C&D), (E) <i>E. faecalis</i> OG1RF, or (F) <i>E. faecalis</i> and LPS simultaneously where on the X- axis, MOI indicates the multiplicity of infection, Y-axis represents IL-6 pg/ml (E&F), (G) NF- κ B SEAP reporter activity of macrophages infected with <i>E. faecalis</i> in the log (O.D 0.5) and stationary phase where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test and compared to LPS, where * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.	33
Fig 2.3 Murine RAW 267.4 macrophages were stimulated with (A) <i>E. faecalis</i> V583, (B) <i>E. faecalis</i> V583 and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity (A &B). (C) <i>E. faecalis</i> V583, (D) <i>E. faecalis</i> V583 and LPS simultaneously, Y axis represents % cell death (C&D), Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.	35
Fig 2.4 Murine RAW 267.4 macrophages were stimulated with (A) Live <i>E. faecalis</i> , supernatant from overnight culture of <i>E. faecalis</i> , UV-killed and Heat-killed <i>E. faecalis</i> , (B) Live <i>E. faecalis</i> , supernatant from overnight culture of <i>E. faecalis</i> , UV-killed and Heat-killed <i>E. faecalis</i> , all at an MOI 100:1 (C) <i>E. faecalis</i> in 24 well transwell plates. Exposure to media alone (-) represents background NF- κ B reporter activity and	37

stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	
Fig 2.5 Murine RAW 267.4 macrophages were stimulated with (A) <i>E. faecalis</i> and LPS simultaneously (9 hrs) or <i>E. faecalis</i> (3 hrs) followed by LPS (6 hrs), (B) <i>E. faecalis</i> and LTA simultaneously (9 hrs) or <i>E. faecalis</i> (3 hrs) followed by LTA (6 hrs), Y axis represents NF-κB reporter activity (A & B). (C) <i>E. faecalis</i> and LTA simultaneously (9 hrs) or <i>E. faecalis</i> (3 hrs) followed by LTA (6 hrs), Y axis represents percentage cell death. X-axis, MOI indicates the multiplicity of infection (A,B &C), Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist LPS or LTA shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS (7A) and LTA (7B), where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	39
Fig 2.6 Murine RAW 267.4 macrophages were stimulated with (A) <i>E.coli</i> UTI89, (B) <i>E. coli</i> K12 MG1655, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity, (A&B).(C) <i>E.coli</i> UTI89, (D) <i>E. coli</i> K12 MG1655, X- axis, MOI indicates the multiplicity of infection and Y axis represents % cell death (C&D), (E) <i>E. faecalis</i> and <i>E. coli</i> K12 MG1655 (MOI 1:1) simultaneously. X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity, (F) <i>E. faecalis</i> and <i>E. coli</i> K12 MG1655 (MOI 1:1) simultaneously. X- axis, MOI indicates the multiplicity of infection and Y axis represents % cell death. Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonists LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	41
Fig 2.7 Murine HEKTLR2 cells were stimulated with (A) <i>E. faecalis</i> or <i>E. faecalis</i> and LTA simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity, Murine HEKTLR4 cells were stimulated with (B) <i>E. faecalis</i> or <i>E. faecalis</i> and LPS simultaneously. (C) <i>E. faecalis</i> or <i>E. faecalis</i> and LTA simultaneously (D) <i>E. faecalis</i> or <i>E. faecalis</i> and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents % cell death (C&D). Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonists LPS or LTA show maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	44
Fig 3.1 Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> OG1RF and the mutants Δatn, Δ983, Δ1767, Δ1953, Δ2064, Δ2736, Δ3023, ΔSrtA, ΔEbpABCsrtC, Δeep, ΔhtrA, ΔGelE, Δmprf1, Δmprf2 at an MOI of 100. Fig (A), (B) and (C) represent data from three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (D) Represents macrophage cell death on exposure to <i>E. faecalis</i> and the mutants	52

mentioned above (combined data from three experiments). Y axis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	
Fig 3.2 Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> OG1RF and the mutants Δ atn, Δ 983, Δ 1767, Δ 1953, Δ 2064, Δ 2736, Δ 3023, Δ SrtA, Δ EbpABCSrtC, Δ deep, Δ htrA, Δ GelE, Δ mprf1, Δ mprf2 at an MOI of 50. Fig (A), (B) and (C) represent data from three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (D) Represents macrophage cell death on exposure to <i>E. faecalis</i> and the mutants mentioned above (combined data from three experiments). Yaxis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	53
Fig 3.3 Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> OG1RF and the mutants Δ atn, Δ 983, Δ 1767, Δ 1953, Δ 2064, Δ 2736, Δ 3023, Δ SrtA, Δ EbpABCSrtC, Δ deep, Δ htrA, Δ GelE, Δ mprf1, Δ mprf2 at an MOI of 20. Fig (A), (B) and (C) represent data from three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (D) Represents macrophage cell death on exposure to <i>E. faecalis</i> and the mutants mentioned above (combined data from three experiments). Y axis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	54
Fig 3.4 Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> V583 WT, and the mutants Δ CpsC, Δ dltA (A) at MOI 100 (B) at MOI 50 (C) at MOI 20, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity, (A,B&C). (D) Represents macrophage cell death on exposure to <i>E. faecalis</i> and the mutants mentioned above (combined data from two experiments). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity (n = 2). Statistical analysis was performed by the one way ANOVA test, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	57
Fig 3.5 Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> OG1RF, Δ SrtA and the mutants OG1RF_10485::Tn, OG1RF_10508::Tn, OG1RF_12504::Tn, OG1RF_12506::Tn, OG1RF10785_Tn, OG1RF_12268::Tn, OG1RF_11974::Tn, OG1RF_12303::Tn, OG1RF_10766::Tn, OG1RF_10088::Tn, and OG1RF_10084::Tn at an MOI of 20. Fig (A) represents data from one of three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (B) Represents macrophage cell death on exposure to <i>E. faecalis</i> and the mutants mentioned above (combined data from three experiments). Y axis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	59
Fig 3.6 (A) Murine RAW 267.4 macrophages were incubated with <i>E. faecalis</i> OG1RF, Δ SrtA, and Δ atn at MOI10 and bacterial counts were	61

enumerated for invasion. Murine RAW 267.4 macrophages were incubated with <i>E. faecalis</i> OG1RF and Δ SrtA, at MOI10 and (B) bacterial counts were enumerated for invasion, attachment and total bacteria. Y axis represents NF- κ B reporter activity, (A, &B). (C) Y-axis represents percentage reduction in invasion. (n = 3). Statistical analysis was performed by unpaired t test, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	
Fig3.7 Model for sortase substrate OG1RF_10508	64
Fig 4.1 (A) Murine RAW 267.4 macrophages were stimulated with <i>E. faecalis</i> OG1RF WT, and the mutants' Δ atn, Δ SrtA at MOI 20 (B) eDNA released from overnight cultures of <i>E. faecalis</i> OG1RF WT, and the mutants' Δ atn, Δ SrtA normalised to an OD 1 represented in μ g/ml on Y axis. Murine RAW 267.4 macrophages were stimulated with (C) <i>E. faecalis</i> OG1RF WT, Δ atn, Δ atn and increasing concentration of genomic DNA simultaneously (MOI 20) (D) <i>E. faecalis</i> OG1RF WT, Δ SrtA, Δ SrtA and increasing concentration of genomic DNA simultaneously (MOI 20) where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity, (A,C&D). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity (n = 2). Statistical analysis was performed by the one way ANOVA test compared to WT, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	68
Fig 4.2 A) Murine RAW 267.4 macrophages were stimulated with DNA (1 μ g) from Group B Streptococcus, <i>E. faecalis</i> , <i>E. coli</i> K12, <i>E. coli</i> UTI89, <i>P. aeruginosa</i> in the presence and absence of LPS. Y axis represents NF- κ B reporter activity. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity (n = 2). Statistical analysis was performed by the one way ANOVA test compared to LPS, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. B) Represents the CG% of the bacterial species used in this experiment.	70
Fig 4.3 Murine RAW264.7 cells were stimulated with (A) Increasing concentrations of genomic DNA <i>E. faecalis</i> OG1RF (B) or genomic DNA <i>E. faecalis</i> OG1RF and LPS simultaneously, Y axis represents NF- κ B reporter activity, (C) Represents macrophage cell death on exposure to genomic DNA of <i>E. faecalis</i> . (D) Represents macrophage cell death on exposure to genomic DNA of <i>E. faecalis</i> and LPS simultaneously. Y axis represents % cell death (C&D). (E) Murine RAW264.7 cells were stimulated with increasing concentrations of genomic DNA of <i>E. faecalis</i> V583 or genomic DNA of <i>E. faecalis</i> V583 and LPS simultaneously. (F) Represents macrophage cell death on exposure to genomic DNA of <i>E. faecalis</i> V583, or genomic DNA of <i>E. faecalis</i> V583 and LPS simultaneously. Y axis represents % cell death. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS show maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	73
Fig 4.4 (A) Murine RAW264.7 cells were stimulated with Genomic DNA <i>E. faecalis</i> OG1RF (1 μ g) sonicated and whole gDNA Y axis represents NF- κ B reporter activity Statistical analysis was performed by the one way ANOVA test compared to ODN, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.	75

, (B) Represents macrophage cell death on exposure to genomic DNA of <i>E. faecalis</i> OG1RF (1 µg) sonicated and whole gDNA, Y axis represents % cell death.	
<p>Fig 4.5</p> <p>(A) Murine HEK_mTLR9 cells were stimulated with increasing concentrations of genomic DNA <i>E. faecalis</i> OG1RF in the presence and absence of ODN Y axis represents NF-κB reporter activity, (B) Represents HEK cell death on exposure to genomic DNA of <i>E. faecalis</i> and LPS simultaneously. Y axis represents % cell death, (C) Murine HEK_mTLR9 cells were stimulated with increasing concentrations of amplified genomic DNA <i>E. faecalis</i> OG1RF in the presence and absence of ODN Y axis represents NF-κB reporter activity. (D) Represents macrophage cell death on exposure to genomic DNA of <i>E. faecalis</i> and LPS simultaneously. Y axis represents % cell death (C&D). Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist ODN shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to ODN, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.</p>	77

Abstract

Enterococcus faecalis is a gram-positive commensal of the mammalian gastrointestinal tract as well as an opportunistic pathogen that can lead to endocarditis, bacteraemia, wound infections, pelvic and soft tissue infections and urinary tract infections. *E. faecalis* has an intrinsic and acquired resistance to several antibiotics and a high mortality rate in immune-compromised patients. It is imperative to understand how *E. faecalis* bacteria evade host immune response and establishes robust opportunistic infections in order to identify novel therapeutic intervention points. In this thesis, we applied a model system to study NF- κ B activity in response to *E. faecalis* and to identify bacterial factors which modulate NF- κ B activity. Employing this model system, we have shown that *E. faecalis* prevents NF- κ B activity in murine macrophages in a dose-dependent manner and prevents NF- κ B activation in response to potent activators like lipopolysaccharide and lipoteichoic acid. We established that the *E. faecalis* NF- κ B suppressing molecule is bacterial surface-associated, heat modifiable and not secreted. We have shown that bacterial virulence factors sortase A and autolysin are important to prevent NF- κ B suppression in macrophages. Furthermore, we identified a novel function for an uncharacterized sortase substrate in preventing NF- κ B activity. Finally, we have identified a previously undescribed function for *E. faecalis* gDNA as an immunosuppressor molecule in murine immune cells. Taken together this work helps to better understand enterococcal pathogenesis and immune modulatory capability.

1 Introduction: *Enterococcus faecalis* modulation of NF- κ B signalling in macrophages.

1.1 *Enterococcus faecalis*

Fig 1.1 *E. faecalis* OG1RF (TEM image, Adeline Yong, Kline lab)

Enterococcus faecalis and *Enterococcus faecium* are gram-positive, facultative anaerobic cocci which are commensals of the gastrointestinal tract of mammals. Enterococci are also found in the human oral cavity (1). In addition, *E. faecalis* are found in ecological habitats like plants, soil, water and dairy products. The ability of *E. faecalis* to thrive in varied environments is attributed to its capability to survive in a wide range of pH and temperatures and in both hypotonic and hypertonic conditions (2). In addition, Enterococci are also tolerant to desiccation, high heavy metal concentration and ionizing radiation (1). Enterococci are homofermentative, lactic acid producing bacteria and are non-spore forming facultative anaerobes (3, 4). *E. faecalis* are found as either

diplococci or arranged in chains. In humans, Enterococci form a part of the gastrointestinal tract consortia comprising of less than 1% of the adult intestinal microflora. However, their concentration in human faeces is between 10^4 to 10^6 per gram (5). Very little is known regarding the role of enterococcal colonization of the human gastrointestinal tract (6, 7).

Among the *Enterococcus* species sequenced so far, enterococcal genomes range from 2.7 to 3.6 Mb, and have a low CG content of 37.5% in *E. faecalis* (1, 2). *E. faecalis* V583 was the first vancomycin resistant strain isolated in the US and has a genome size of 3.36 Mb, of which the mobile genomic content is over 25% (1, 8). In comparison, *E. faecalis* OG1RF is a laboratory generated rifampicin and fusidic acid resistant strain, derived from the human oral isolate OG1, with a genomic size of 2.74 Mb (9, 10). The OG1RF genome lacks plasmids, has only one prophage that is core to the genome, and has little mobile genomic content. Despite this, OG1RF can cause disease in animal models of infection which suggests that even commensal strains of *E. faecalis* can cause opportunistic infections (1, 9).

1.2 *Enterococcus* as an opportunistic pathogen

In the 1970's, Enterococci started emerging as a drug resistant pathogen in the nosocomial environment (11-13). Sixty-five to 80% of hospital acquired enterococcal infections are caused by *E. faecalis* (14).

In the nosocomial environment, patients rapidly acquire multiple antibiotic resistant enterococcal strains in their gastrointestinal tract from contaminated surfaces, further antibiotics administered to hospitalised patients eliminate susceptible organisms, which also contributes to *E. faecalis* establishing an infection (15). In immune-compromised hosts, Enterococci act as an opportunistic pathogen by translocating across the intestinal mucosal barrier (16). Enterococci are causative agents for several infections such as urinary tract infections (UTI); intra-abdominal, pelvic and soft tissue infections; bacteraemia; and endocarditis. Enterococci cause 15% of the nosocomial UTI in the intensive care unit (ICU) setting. Of particular concern are vancomycin resistant Enterococcus (VRE) isolated from ICU patients (17). Intra-abdominal, pelvic and soft tissue infections with Enterococci are frequently polymicrobial. Infection in these sites serve as a source for enterococcal bacteraemia (1).

The genitourinary tract is often the source of infection for enterococcal bacteraemia and endocarditis. As per a study conducted in the year 2004 in the US, *Enterococcus* species are the third leading cause of hospital acquired bacteraemia and 9.4% of all bacteraemias (18). Enterococci are also occasionally isolated from decubiti and foot ulcers in diabetics (1). Antibiotic therapy in critically ill patients alters the balance of the gut microbiota and helps to establish Enterococci as a predominant commensal in the gastrointestinal tract, facilitating Enterococci to cause opportunistic infections (19). *E. faecalis* can persist on inanimate surfaces for as long as 4 months in the absence of regular

decontamination and can hence serve as a reservoir for transmission of infection in the hospital environment (20).

As per the Centre for Disease Control (CDC) threat report published in 2013, vancomycin resistant *Enterococcus* (VRE) has been classified as threat level “serious”. A study conducted in Denmark showed that the 30-day mortality attributed to *E. faecalis* was 21.4% for vancomycin susceptible strains and above 45% for vancomycin resistant strains (21).

1.3 Antibiotic resistance in *Enterococcus*

Intrinsic resistance is encoded within the core genome of all members of enterococcal species while, acquired resistance is present only in some members of the species (1). Enterococci can be intrinsically resistant to several antibiotics such as β -lactams, aminoglycosides, and trimethoprim-sulfamethoxazole (1, 22-24). Enterococci express low affinity penicillin binding proteins, resulting in weak binding to β -lactam antibiotics which explains the basal low level resistance to penicillins (25, 26). Enterococci exhibit poor uptake of aminoglycosides and are therefore resistant to clinically achievable doses (23).

Enterococci are capable of acquiring resistance via horizontal gene transfer as well as chromosomal mutations (1). Enterococci are capable of horizontal gene transfer via transposons, pheromone sensitive plasmids, and broad host range plasmids (27). Enterococci have been reported to have high level acquired

resistance to β -lactams, aminoglycosides, macrolides, glycopeptides, streptogramins, linezolid and daptomycin (1, 17, 28).

1.4 *Enterococcus* virulence factors

Classical enterococcal virulence factors can be classified into secreted factors and surface-associated adhesins. Cytolysin, gelatinase and serine protease are secreted factors which have been shown to have a role in virulence. Cytolysin is a secreted toxin related to the antibiotic class of bacteriocins and has been shown to contribute to virulence in animal models of infection (14, 29). Gelatinase (GelE, encoded by *gelE*), a matrix metalloprotease, and the serine protease (SprE, encoded by *sprE*) are known to have a role in pathogenesis by regulating the formation of biofilm (30). In addition, GelE contributes to pathogenesis *in vivo* in rabbit endocarditis models (1, 31). Adhesins associated with virulence include aggregation substance (AS), Enterococcal surface protein (Esp), adhesion to collagen of *E. faecalis* (Ace), and endocarditis and biofilm-associated pili (Ebp) (14). AS are surface anchored polypeptides encoded on pheromone responsive plasmids. *E. faecalis* strains expressing AS are resistant to intracellular killing by macrophages and human neutrophils (32). In rabbit endocarditis models, AS has been associated with an increase in virulence (1, 32, 33). Enterococcal surface protein (Esp) has been correlated with the formation of biofilms *in vitro* (34). Furthermore, in murine UTI infection models, Esp has been shown to be an important virulence factor with a role in adhesion to the bladder epithelium (1,

35). Ace is required to adhere to several extracellular matrix proteins and has been implicated in the virulence of *E. faecalis* in murine UTI as well as endocarditis models (36, 37). Ebp are also required for the formation of biofilms and deletion mutants cause significantly attenuated UTI infections *in vivo* (38, 39).

1.5 Cellular immune response to *E. faecalis* infection

A study conducted on the cellular constituents of the immune response to enterococcal catheter associated urinary tract infection (CAUTI) revealed that the percentage of myeloid cells in infected murine bladders is as high as 45% (40). The most abundant myeloid cells recovered from the inflamed bladders were neutrophils and macrophages. Strikingly, in the *E. faecalis*-infected animals, there was a 12-fold increase in the proportion of non-activated macrophages to activated macrophages, compared to the un-infected but catheterized controls. The number of basophils and eosinophils recovered from *E. faecalis*-infected mice were also significantly raised, meanwhile other immune cells such as dendritic cells, mast cells and lymphocytes were similar in infected and uninfected controls (40).

Tissue macrophages are present below the mucosal surfaces and thus are well positioned to act and are the first line of defence against infections. In comparison to neutrophils, macrophages have a longer life span and are dominant sources of cytokines. Furthermore, recognition of bacterial pathogens by the macrophages

is essential for the adaptive immune response, as macrophages are ideally situated mobile cell population capable of early recruitment of other immune cells during an infection. A complete understanding of the macrophage-*Enterococcus* interactions is important to understand the pathogenesis of enterococcal infections (41).

1.6 Intracellular survival and dissemination of *Enterococci*

Enterococci are commensal organisms, and in order to establish an infection, they must successfully circumvent or counteract the host defence mechanisms. Previous studies have shown that Enterococci were able to survive within the host macrophages for prolonged periods (32, 42, 43), and Enterococci may use the macrophages as a mechanism to disseminate to distant organs and establish an infection (44). Recently it has been shown that multiple strains of *E. faecalis* resist killing by macrophages up to 12 hours post infection by interfering with the host cell apoptotic pathway (45). Based on electron microscopy studies, *E. faecalis* is rapidly phagocytosed by macrophages into the phagolysosome, are subsequently observed in the cytoplasm of the host cells after 24 to 48 hours, and macrophages are lysed by 48 to 72 hours post infection (46). Neutrophils are the most abundant and predominant phagocytic cells in mammals. *E. faecalis* has been shown to survive inside activated human neutrophils for up to 2 hours (33). Furthermore, clinical isolates of *E. faecalis* can be internalized by human

embryonic kidney cell line (HEK) and the Girardi heart cell line implicating their role in pathogenesis of UTI and endocarditis (47).

Luminal bacteria adhere to intestinal epithelial cells, adherent bacteria could also be phagocytosed by intraepithelial phagocytes. The bacteria then migrate to the apical end of the epithelial cells or migrate through the phagocytes to the mesenteric lymph nodes and then spread to distant sites via the haematogenous route (48). In a murine model, *E. faecalis* translocation was demonstrated across intact intestinal epithelium after which *E. faecalis* infection was observed in mesenteric lymph nodes, liver, and spleen (44, 49).

1.7 Host cell receptors and the NF- κ B signalling pathway

Fig 1.2 Schematic representation of TLR- NF- κ B signalling pathway

The mammalian immune response can be divided into innate and acquired immunity, although there is clear overlap and interplay between the two. Innate immunity has been described as the first line of defence against pathogens (50). Innate immunity is present in all species, while only vertebrates and cartilaginous fish have evolved adaptive immunity.

Upon encountering a pathogen, innate immunity initiates an immune response and instructs acquired immunity. Acquired immunity has memory function and mounts an efficient antigen specific immune response (50, 51). The adaptive immune system uses somatic mechanisms of gene rearrangement to generate a vast repertoire of antigen receptor specificities while innate immune recognition is mediated by germline encoded nonclonal receptors. These receptors are referred to as pattern recognition receptors (PRRs)(52).

A trinity of innate immune receptors are Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) and Retinoic Acid Inducible Gene-I (RIG-I)-like receptors (RLRs) (53). TLRs recognize pathogen associated molecular patterns (PAMPs) from bacteria, viruses and fungi. Currently, NLRs are only known to recognize bacterial PAMPs, meanwhile RLRs are known to recognize only viruses. The innate immune response is tightly controlled by coordination between the TLRs, NLRs and RLRs (53). TLR, NLR and RLR signalling pathways culminate in the activation of transcription factor nuclear factor-kappa-B (NF- κ B) (54). NF- κ B was first identified by David Baltimore and Ranjan Sen while characterizing proteins which bind to immunoglobulin heavy chain and the κ light chain enhancers (55). NF- κ B is a transcription factor which regulates the expression of several genes which are involved in inflammation, immunity, cell growth and development (56).

Bacterial pathogen associated molecular patterns (PAMPs) such as peptidoglycan, lipoteichoic acid (LTA), lipopolysaccharide (LPS), and bacterial DNA are recognized by pattern recognition receptors (PRRs) which are present

on the host cells and include TLRs and NLRs (57). RLRs recognize viral PAMPs (57). Recognition of bacterial PAMPs leads to the activation of signal transduction cascades which activates the transcription factor, NF- κ B (57). NF- κ B is a key factor which regulates immune and inflammatory signalling pathways and is responsible for expression of chemokines, cytokines and anti-apoptotic factors (58). TLR-mediated induction of the NF- κ B pathway drives the production of inflammatory cytokines like tumor necrosis factor- α (TNF- α) and IL-6 (54).

TLR signalling to NF- κ B proceeds via myeloid differentiation-primary response protein 88 (MyD88)-dependent or MyD88-independent pathways. The MyD88 dependent pathway, can be briefly described as follows and is summarized in **Figure 1.2**; after PAMP recognition and binding by the appropriate TLR, the Toll interleukin 1-receptor (TIR) homology domain of the TLR receptor undergoes a conformational change that promotes interaction with the TIR domain of MyD88, which then associates with IL1-receptor associated kinase 4 (IRAK4). IRAK4 phosphorylates IRAK1, IRAK1 autophosphorylates its N-terminal residues which facilitates tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to bind to this complex. TRAF6 recruitment is followed by dissociation of phosphorylated IRAK1-TRAF6 from the receptor, and both now form a complex at the plasma membrane with a pre-formed complex consisting of transforming growth factor β (TGF- β) activated kinase (TAK1) and TAK1-binding proteins (TAB1 and TAB2), leading to TAK1 and TAB2/TAB3 phosphorylation and translocation with TRAF6 and TAB1 to the

cytoplasm. The rest of the complex comprising of TRAF6, TAK1, TAB1, and TAB2 dissociates and associates with ubiquitin ligases in the cytosol. Meanwhile TAK1 is activated in the cytoplasm, TAK1 then phosphorylates both MAPK and the IKK complex which consists of IKK- α , IKK- β , IKK- γ (inhibitor of NF- κ B kinase complex). NF- κ B dimers in an inactive form are sequestered in the cytoplasm by I κ B proteins. The IKK complex further phosphorylates I κ Bs leading to their polyubiquitylation and proteosomal degradation and then NF- κ B translocates to the nucleus to bind responsive promoters to induce the expression of its target genes (54, 59).

To date, 13 TLRs have been identified in mammals, of which TLR1-9 are conserved in humans and mice, TLR 10 is non-functional in mice, and TLR 11-13 are absent in humans. TLRs recognize a wide range of ligands such as lipids, lipoproteins, proteins, glycans and nucleic acids. TLR2 recognizes lipopeptides, peptidoglycan, lipoteichoic acid (LTA), TLR4 recognizes lipopolysaccharide (LPS), and TLR9 recognizes unmethylated CpG motifs (Cytosine linked to Guanine by a phosphate bond) in bacterial DNA (57, 59).

1.8 Signalling pathways involved in *E. faecalis* infection

Recent studies conducted by Wang et al., investigating the immune response to *E. faecalis* in intestinal epithelial cell lines, has shown that a majority of the *E. faecalis* strains isolated from the healthy infant guts prevented or down-regulated pro-inflammatory responses as measured by IL-8 expression in epithelial cell

lines infected with *E. faecalis* at an MOI (multiplicity of infection) of 100 (60). The authors suggested that this modulation of the immune response by *E. faecalis* could prevent the occurrence of inflammatory diseases such as necrotizing enterocolitis (NEC). Immune gene expression studies in the intestinal epithelial cell line Caco-2 after *E. faecalis* infection showed that cytokine signalling, MAPK signalling, as well as the NF- κ B signalling were significantly reduced (≥ 1.5 fold) , suggesting that *E. faecalis* modulates multiple immune pathways (60). Intestinal epithelial cell lines infected with the same immune modulatory *E. faecalis* isolated from infants showed a down regulation of TLR4, TLR9 and TRAF6 (61).

In contrast, a recent study conducted by Jun et al. to study the macrophage and enterococcal interaction reported that *E. faecalis* strain E99 activates the JNK, ERK and the p38 MAPK along with the NF- κ B pathways when infected at an MOI of 10. They also showed that the MAPK and NF- κ B activation was largely mediated by MD88 (62). These discrepant findings may suggest strain-dependent or MOI-dependent differences in immune modulation by *E. faecalis*.

The only enterococcal factor associated with immune modulation to date is the TIR domain containing protein TcpF that has been identified in some strains of *E. faecalis* and has been shown to interfere with the TLR signalling pathway (63). TcpF directly interacts with MyD88 and blocks NF- κ B induction. Furthermore, a TcpF deletion mutant not only activated NF- κ B, but also had a lower intracellular survival in macrophages and therefore could be attributed to promoting survival within macrophages (63). Also, TcpF was found to be more

predominant in enterococcal UTI isolates as compared to commensal *E. faecalis* strains (63). Notably, TcpF is absent in the lab strain OG1RF that has been used in the studies in this thesis.

1.9 Sortase and substrates

Sortases are required for anchoring surface proteins to the cell wall. Sortase enzymes recognize cell wall sorting signals (CWSS) within the substrate proteins (64). The CWSS consists of a C-terminal LPxTG motif, a transmembrane domain, followed by a positively charged tail. Sortases cleave CWSS-motif containing proteins between the threonine and glycine bond and after transpeptidation of the protein to a lipid II intermediate, the protein is incorporated to the cell wall (64). Aggregation substance (AS), endocarditis and biofilm associated pili (Ebp), enterococcal surface protein and collagen binding (Ace) are CWSS-containing proteins and sortase substrates which have been demonstrated to have a role in pathogenesis of *E. faecalis* (65-67). Sortases have been classified from A to F enzymes based on their sequence and function (64). Sortase A is present in all gram-positive bacteria, also referred to as housekeeping sortases (68). Sortase A has been shown to be essential for adherence and the formation of robust biofilms in *E. faecalis* (69). In a murine CAUTI model, Sortase A was shown to be important in the establishment of infection (70).

1.10 Autolysin

The bacterial cell wall provides structural integrity to the cell and helps to maintain osmotic balance. Peptidoglycan is a major component of the bacterial cell wall. Peptidoglycan is made up of polysaccharides such as *N*-acetylglucosamine and *N*-acetylmuraminidase, linked together by short peptides. During cell division, peptidoglycan cleavage and regeneration is required. Bacterial autolysins, also called murein hydrolases, are required for cell wall cleavage and growth. The major autolysin (Atn) in *E. faecalis* is an *N*-acetylglucosaminidase and is responsible for daughter cell separation during cell division. Disruption of the autolysin gene (*atn*) in *E. faecalis* results in increased chaining, adhesion defects, and defective biofilm formation (69, 71, 72). In a murine CAUTI model, a *atn* mutant strain was able to establish a robust infection and persist in bladder and kidney tissues similar to the wild-type *E. faecalis*. However, it has been shown that an *atn* deletion mutant is defective in the release of eDNA which is an important constituent of *E. faecalis* biofilm matrices (30). Together, these data suggest that eDNA release is not essential to biofilm formation or virulence in murine CAUTI.

Bacterial DNA is typically thought to be an immunostimulatory molecule which is recognized by TLR9. TLR9 recognizes unmethylated CpG-containing motifs of the bacterial DNA (73). TLR9 is located in the endoplasmic reticulum, once CpG DNA is internalized by clathrin-dependent endocytotic pathway and moves into the lysosomal compartment, TLR9 translocates to the lysosomal

compartment and binds to the CpG DNA (74). TLR9 signals to NF- κ B via the MyD88 pathway (54). Different bacterial species have different %GC content. Since, TLR9 recognizes unmethylated CpG motifs, different bacteria vary in their TLR 9 activating capability (75). DNA from *E. faecalis* (37.5% GC) gave rise to less TLR 9-mediated immune activation compared to *E. coli* (50.8% GC), when IL-8 was used as a read out in the HEK-TLR 9 cell line (75).

The aim of this thesis is to study the interaction of *E. faecalis* with macrophages in relation to the immune response and to identify bacterial factors which play a role in modulating immune response in macrophages.

In this thesis, I have shown *E. faecalis* prevents the NF- κ B-driven gene expression in a dose dependent manner. I also show that *E. faecalis* is able to actively prevent the NF- κ B activity in the presence of LPS and LTA which are known activators of NF- κ B. *E. faecalis* infection did not result in macrophage cell death in the MOIs we tested. I found that the *E. faecalis* molecule responsible for the prevention of NF- κ B activity is a heat modifiable surface associated molecule that is not secreted. I show that both Sortase A and Autolysin are important factors which have a role in the prevention of the NF- κ B activity in macrophages and that the Sortase A mutant has a survival defect in macrophages. I was also able to identify two sortase substrates which, when mutated, relieve the NF- κ B suppression. Further I observed that the genomic DNA of *E. faecalis* itself can prevent the NF- κ B response in macrophages and is able to complement the NF- κ B activity of the eDNA-deficient autolysin mutant back to wild-type levels using genomic DNA. Taken together, my PhD thesis work advances the

field of enterococcal pathogenesis by demonstrating immune modulatory capability of *E. faecalis* leading to the prevention or suppression of NF- κ B activity, identifying a novel function for one *E. faecalis* factors sortase A and autolysin, further identifying lack of immune stimulatory effect of *E. faecalis* DNA. Our lab has shown that *E. faecalis* promotes growth of *E. coli* in polymicrobial wound infections. We suggest that in immunocompromised hosts, prevention of NF- κ B activity by *E. faecalis* helps other organisms to establish an infection. Our data could lead to development of host directed immunomodulatory therapy for auto-immune diseases and chronic inflammatory diseases which are associated with NF- κ B activation (Rheumatoid arthritis, ulcerative colitis, COPD, inflammatory bowel disease, asthma) by employing *E. faecalis* DNA to modulate NF- κ B.

2 Chapter 1 – *E. faecalis* actively suppresses NF- κ B activity by a heat-modifiable, surface associated factor

2.1 Establishing and Optimizing NF- κ B reporter assay in RAW macrophages

2.1.1 Growth curves for *E. faecalis*

In order to test the ability of *E. faecalis* to modulate NF- κ B activity in macrophages, we began first by determining the growth rate and colony forming units (CFU)/ml for *E. faecalis* in BHI, so as to determine the bacterial numbers to add to macrophages for accurate multiplicity of infection (MOI) estimations in our *in-vitro* macrophage assay. Briefly, overnight cultures were diluted to 1:20 in BHI and grown at 37⁰C. Optical density (OD 600) values were measured at the time-points indicated (**Fig 2.1.A**), and CFU were measured by serial dilution in PBS and plating on BHI agar plates in triplicate (**Fig 2.1.B**). These data helped us to determine bacterial numbers used in the experiments in this study.

2.1.2 LPS 100ng activates NF- κ B reporter assay in macrophages

Lipopolysaccharide (LPS) is a PAMP from the outer membrane of gram-negative bacteria that activates NF- κ B via the TLR4 receptor pathway. To determine the lowest concentration of LPS which maximally activates the RAW264.7 cells, using NF κ B-induced secreted alkaline phosphatase (SEAP) as a readout, we examined concentrations ranging from 10ng to 10 μ g and found that 100 ng of

LPS significantly activates the NF- κ B reporter activity when compared to untreated RAW264.7 cells (**Fig 2.1.C**). We monitored the lactate dehydrogenase (LDH) released into the cell culture supernatants to evaluate cell death after exposure to the various concentrations of LPS and observed no increase in cell death at any LPS concentration examined, as compared to the positive (high) control of Triton-X lysis (**Fig 2.1.D**). Based on these observations, we chose to use 100 ng of LPS as a positive control for NF- κ B reporter activity for future experiments.

2.1.3 *E. faecalis* OG1RF does not have alkaline phosphatase activity

The NF- κ B reporter activity is detected by a colorimetric assay, where the reagent QUANTI-Blue detects alkaline phosphatase in supernatants of cell-culture. QUANTI-Blue changes in colour from pink to blue or purple, which is measured spectrophotometrically at 640nm. To test whether *E. faecalis* itself has any alkaline phosphatase activity that could confound our assay, we colorimetrically tested the supernatant from *E. faecalis* grown in BHI and noted that *E. faecalis* does not release alkaline phosphatase and thus will not affect the NF- κ B reporter activity assay (**Fig 2.1.E**).

Fig 2.1 (A) Growth curve of *E. faecalis* strain OG1RF. The X-axis represents the time in hours and Y axis represents the optical density at 600nm, (B) Growth curve of *E. faecalis*. The X-axis represents the time in hours and Y axis represents CFU/ml. Murine RAW 267.4 macrophages were stimulated with LPS concentrations from 10ng to 10ug/ml represented on the X axis, and either (C) NF-κB reporter activity or (D) cytotoxicity, with % cell death represented on Y-axis were measured. (E) NF-κB SEAP reporter activity

induced by *E. faecalis* OG1RF in the absence of macrophages and macrophage alone controls, Y axis represents NF- κ B reporter activity (C, E & F). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Data are combined data (C, D, & E) from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to media alone (-), where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.2 *Enterococcus faecalis* disrupts NF- κ B activation in murine macrophages

Recently, there have been conflicting reports regarding the *E. faecalis* inflammatory response, where *E. faecalis* strains isolated from healthy infants were shown to down-regulate NF- κ B in intestinal epithelial cell-lines when infected at an MOI 100, whereas *E. faecalis* E99 activated NF- κ B in macrophages when infected at an MOI 10 (61, 62). These studies suggested a strain dependent or MOI-dependent NF- κ B activity. To first determine MOI-dependent NF- κ B activity in our assay, we tested macrophage NF- κ B activity in response to *E. faecalis* at a range of MOIs. We observed that *E. faecalis* OG1RF failed to activate NF- κ B activity at high multiplicities of infection (MOIs), and full activation only occurred only at low MOIs (**Fig 2.2.A**). These results suggest that NF- κ B activity in macrophages is MOI-dependent.

To determine whether *E. faecalis* actively prevents NF- κ B activation or simply fails to activate at high MOIs, we tested whether it would also prevent activation in the presence of TLR agonists that initiate NF- κ B signalling. When we simultaneously exposed macrophages to LPS and *E. faecalis* for 6 hours and quantified NF- κ B activation, we observed a dose dependent prevention of LPS-mediated NF- κ B activation by *E. faecalis* (**Fig 2.2.B**). We simultaneously

monitored the LDH activity in cell culture supernatants, to assay cell death on exposure to *E. faecalis* or *E. faecalis* and LPS simultaneously, and observed minimal cell death in either conditions at all MOIs tested (**Fig 2.2.C & 2.2.D**).

NF- κ B is a transcription factor which drives production of chemokines and cytokines (58). In order to evaluate the physiological outcome of prevention of NF- κ B activity, we measured the cytokine IL-6 released into the cell-culture supernatants of macrophages upon exposure to *E. faecalis*. We observed that *E. faecalis* OG1RF prevented the release of IL-6 even in the low MOIs we tested (**Fig 2.2.E**). Similarly, simultaneous exposure to *E. faecalis* and LPS prevented the release of IL-6 at all MOI's tested (**Fig 2.2.F**). These results suggest that even at low MOI, *E. faecalis* prevents immune activation.

In order to determine if there is any difference in NF- κ B reporter activity to actively dividing log phase bacteria and stationary phase bacteria in macrophages, we compared the NF- κ B reporter activity of *E. faecalis* in both stationary (OD 1) and log phase (OD 0.5), and found that both have similar SEAP activity (**Fig 2.2.G**). Therefore, in subsequent experiments, we used *E. faecalis* grown to stationary phase.

Fig 2.2 Murine RAW 267.4 macrophages were stimulated with (A) *E. faecalis* OG1RF, (B) *E. faecalis* and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity (A&B), (C) *E. faecalis* OG1RF (D) *E. faecalis* and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents % cell death (C&D), (E) *E. faecalis* OG1RF, or (F) *E. faecalis* and LPS simultaneously where on the X- axis, MOI indicates the multiplicity of infection, Y-axis represents IL-6 pg/ml (E&F), (G) NF-κB SEAP reporter activity of macrophages infected with *E. faecalis* in the log (O.D 0.5) and stationary phase where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test and compared to LPS, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.3 *E. faecalis* V583 disrupts NF- κ B activity in a dose-dependent manner

To determine whether prevention of NF- κ B activity was strain specific, we tested NF- κ B activity in response to *E. faecalis* V583, which is the first sequenced vancomycin resistant strain, and observed that V583 could also prevent NF- κ B activity in a dose-dependent manner. We further tested NF- κ B activity when macrophages were exposed to *E. faecalis* V583 and LPS simultaneously and observed that the strain V583 was also capable of actively preventing NF- κ B activity (**Fig 2.3.A & 2.3.B**). We simultaneously monitored lactate dehydrogenase release into culture supernatants to ensure that lack of NF- κ B activation was not a result of cell death at high MOIs, and observed no increase in LDH release at any of the MOIs used in this study (**Fig 2.3.C & 2.3.D**). Thus we concluded that the prevention of NF- κ B activity is not strain specific.

Fig 2.3 Murine RAW 267.4 macrophages were stimulated with (A) *E. faecalis* V583, (B) *E. faecalis* V583 and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity (A & B). (C) *E. faecalis* V583, (D) *E. faecalis* V583 and LPS simultaneously, Y axis represents % cell death (C&D), Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.4 A heat modifiable, surface associated molecule suppresses NF- κ B activity

To begin to determine which *E. faecalis* factor is responsible for the prevention of NF- κ B activity and if this phenomenon is contact dependent, we tested NF- κ B activity in response to UV-killed *E. faecalis*, heat-killed *E. faecalis*, culture supernatants, and live *E. faecalis* separated from macrophages by semi-permeable cell inserts. We independently confirmed that UV-treatment resulted in death, but the cells remained intact, whereas heat-killing resulted in cell lysis (data not shown). We observed that, UV-killed *E. faecalis* failed to activate NF- κ B at any MOI and heat-killed *E. faecalis* efficiently activated NF- κ B at all MOIs (**Fig 2.4.A & 2.4.B**), suggesting that *E. faecalis* actively prevent NF- κ B activation via a process that requires a heat-modifiable factor and intact cells. Surprisingly, culture supernatants taken from each infecting MOI activated NF- κ B at higher densities and in a dose dependent manner (**Fig 2.4.A & 2.4.B**). Separation of *E. faecalis* from macrophages by semi-permeable filters resulted in activation of NF- κ B at all MOI's tested (**Fig 2.4.C**). Hence, we conclude that the NF- κ B suppressing *E. faecalis* factor is not secreted but is heat-modifiable, surface associated molecule. Moreover, these findings suggest that a factor released from *E. faecalis* is NF- κ B activating, and this activation can be prevented in the presence of live intact cells.

Fig 2.4 Murine RAW 267.4 macrophages were stimulated with (A) Live *E. faecalis*, supernatant from overnight culture of *E. faecalis*, UV-killed and Heat-killed *E. faecalis*, (B) Live *E. faecalis*, supernatant from overnight culture of *E. faecalis*, UV-killed and Heat-killed *E. faecalis*, all at an MOI 100:1 (C) *E. faecalis* in 24 well transwell plates. Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.5 *E. faecalis* actively prevents NF- κ B activation

Since *E. faecalis* prevents NF- κ B activation, we predicted that macrophage exposure to *E. faecalis* would prevent subsequent NF- κ B activation by NF- κ B activators such as LPS and LTA. To test this, we pre-incubated macrophages with *E. faecalis* for 3 hours followed by exposure to either LPS or LTA. We observed that pre-incubation with *E. faecalis* prior to LPS or LTA exposure prevented NF- κ B activation by the TLR agonists (**Fig 2.5.A & 2.5.B**). LTA from multiple strains of *E. faecalis* activates release of chemokines and cytokines from immune cells (76, 77). We demonstrated that simultaneous exposure to *E. faecalis* and LTA prevented NF- κ B activity at high MOIs (**Fig 2.5.B**), similar to what we observed for LPS, and suggesting that *E. faecalis* is also able to circumvent the stimulatory activity of LTA, the gram-positive bacterial PAMP. We also monitored lactate dehydrogenase release into culture supernatants on exposure of macrophages to LTA and *E. faecalis*, and observed minimal cell death in all the MOIs tested (**Fig 2.5.C**). Together, these data show that *E. faecalis* actively prevents inflammatory signalling process that culminates in NF- κ B activation and suggests that pre-exposure to *E. faecalis* blocks or down-regulates PRRs or key signalling molecules, hence preventing subsequent NF- κ B activation by known TLR activators LPS and LTA.

Fig 2.5 Murine RAW 267.4 macrophages were stimulated with (A) *E. faecalis* and LPS simultaneously (9 hrs) or *E. faecalis* (3 hrs) followed by LPS (6 hrs), (B) *E. faecalis* and LTA simultaneously (9 hrs) or *E. faecalis* (3 hrs) followed by LTA (6 hrs), Y axis represents NF-κB reporter activity (A & B). (C) *E. faecalis* and LTA simultaneously (9 hrs) or *E. faecalis* (3 hrs) followed by LTA (6 hrs), Y axis represents percentage cell death. X- axis, MOI indicates the multiplicity of infection (A,B &C), Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist LPS or LTA shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS (7A) and LTA (7B), where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.6 *E. faecalis* prevents NF- κ B activation in the presence of *E. coli*

E. faecalis is frequently isolated with *E. coli* in clinical UTI samples, as well as in wound infections (78, 79). In order to design a physiologically relevant model to test the NF- κ B activity of *E. faecalis* in the presence of *E. coli*, we first tested the NF- κ B activity of macrophages in response to *E. coli* strain UTI89 which was isolated from a patient with acute bladder infection (80). We found that the strain UTI89 by itself suppresses the NF- κ B activity, consistent with previous reports where minimal cytokine response was observed in bladder epithelial cells (81) (**Fig 2.6.A**). Furthermore, as evident from the LDH activity of this infection, *E. coli* UTI89 causes macrophage cell death at all MOIs tested (**Fig 2.6.C**). We therefore tested the ability of another *E. coli* strain, K12 MG1655 (82), which has been maintained as a laboratory strain for its ability to modulate NF- κ B activity and found that it activates NF- κ B at all MOIs tested (**Fig 2.6.B**), but did not result in cytotoxicity at MOI 1:1. We therefore infected macrophages with *E. faecalis* (at a 100:1, 50:1, 20:1, 10:1 and 1:1 MOI) and *E. coli* K12 (MOI 1:1) simultaneously and found that *E. faecalis* prevents the NF- κ B activity that would otherwise be induced by *E. coli* (**Fig 2.6.F**). The percentage cell death as measured by LDH release shows that there is approximately 50% cell death at *E. faecalis* 50 and 100 MOI, at 20 and 10 MOI there is approximately 28% cell death. Therefore, these data show that *E. coli* + *E. faecalis* coinfection at *E. faecalis* MOI 20 elicits low cytotoxicity yet prevents *E. coli*-driven immune activation.

Fig 2.6 Murine RAW 267.4 macrophages were stimulated with (A) *E. coli* UTI89, (B) *E. coli* K12 MG1655, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity, (A&B). (C) *E. coli* UTI89, (D) *E. coli* K12 MG1655, X- axis, MOI indicates the multiplicity of infection and Y axis represents % cell death (C&D), (E) *E. faecalis* and *E. coli* K12 MG1655 (MOI 1:1) simultaneously.

X- axis, MOI indicates the multiplicity of infection, Y axis represents NF-κB reporter activity, (F) *E. faecalis* and *E. coli* K12 MG1655 (MOI 1:1) simultaneously. X- axis, MOI indicates the multiplicity of infection and Y axis represents % cell death. Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonists LPS shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.7 *E. faecalis* does not activate the TLR2 NF- κ B or the TLR4 NF- κ B pathway

RAW-Blue cells (Invivogen) express all TLRs except TLR5. Different reports have suggested that TLR family of receptors have the ability to cross-talk through their respective ligands and responses (83, 84). In RAW264.7 cells, higher expression of TLR4 has been noted on treatment with ligands such as CpG-ODN and peptidoglycan (85). Based on this we wanted to test whether the NF- κ B-modulating activity of *E. faecalis* proceeds via the TLR2 and TLR4 receptor-mediated pathway. Thus, we studied NF- κ B reporter activity to *E. faecalis* in HEK293 cell line transfected with SEAP reporter gene and murine TLR2 or murine TLR4 receptor.

We monitored NF- κ B activity of the HEKmTLR2 cells after infection with *E. faecalis* alone, or *E. faecalis* and LTA simultaneously. We found that *E. faecalis* does not activate NF- κ B activity in the HEKmTLR2 cells, either alone or in the presence of LTA, which otherwise activates NF- κ B activity in this cell line (**Fig 2.7.A**). When the HEKmTLR4 cell line was infected with *E. faecalis* alone, no increase in the NF- κ B activity was seen above the basal NF- κ B activity at all the MOIs tested. This was expected because *E. faecalis* is a gram-positive organism which lacks the LPS the ligand for TLR4. However, upon simultaneous infection with *E. faecalis* and LPS, NF- κ B activation only occurred at low MOIs (**Fig 2.7.B**). We measured the percentage cell death of the HEK cell lines on exposure to *E. faecalis* and at high MOIs we found the cell death to be approximately 25% and minimal at the low MOI's tested (**Fig 2.7.C & 2.7.D**). Therefore, from these

data we conclude that *E. faecalis* does not activate NF- κ B via TLR2 mediated pathway.

Fig 2.7 HEK μ TLR2 cells were stimulated with (A) *E. faecalis* or *E. faecalis* and LTA simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity, HEK μ TLR4 cells were stimulated with (B) *E. faecalis* or *E. faecalis* and LPS simultaneously. (C) *E. faecalis* or *E. faecalis* and LTA simultaneously (D) *E. faecalis* or *E. faecalis* and LPS simultaneously, where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents % cell death (C&D). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS or LTA show maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.8 Discussion

E. faecalis is associated with the establishment of opportunistic infections in the nosocomial environment despite being a commensal of the gastrointestinal tract and oral cavity of mammals. In a healthy individual with normal physiological functions such as salivation, gastric acidity, peristalsis and an intact epithelial barrier, *E. faecalis* is a commensal, whereas in an immunocompromised individual with disrupted physiological functions and/or exposure to antibiotics, a favourable environment for transmission and establishment of infection by *E. faecalis* at distant sites can arise (16). Very little is known regarding how *E. faecalis* overcomes host defence mechanisms and establishes robust infections in the urinary tract, endocardium, blood-stream and in chronic wounds. Recent works studying *E. faecalis* inflammatory response, have shown *E. faecalis* strains isolated from healthy infants down-regulate NF- κ B in intestinal epithelial cell-lines when infected at an MOI 100, on the other hand *E. faecalis* E99 activated NF- κ B in macrophages when infected at an MOI 10 (61, 62). In this chapter we have shown that high titer (high MOI) *E. faecalis* infection fails to activate transcription factor NF- κ B which controls the expression of cytokines and chemokines, while low titer (low MOI) *E. faecalis* activates NF- κ B (58). Our findings are thus in agreement with previously published work (61, 62) and clarify that *E. faecalis* immunomodulation of macrophages is dose dependent but strain independent. Since we demonstrated a dose-dependent prevention of NF- κ B activity in murine macrophages, this suggests that high titres of *E. faecalis* possessing larger amounts of surface-associated virulence factors block or down-

regulate receptors and/or down-stream signalling molecules to prevent NF- κ B pathway activation. This may suggest that in order to efficiently establish an infection, a high infection dose of *E. faecalis* is required. The physiological outcome of NF- κ B activity is the release of cytokines and chemokines (58). To validate our observation that *E. faecalis* prevents NF- κ B activity, we measured the release of cytokine IL-6 in culture supernatant on exposure to *E. faecalis*. To our surprise we noted that even at a low MOI of 1 there was minimal release of cytokine IL-6 from macrophages on exposure to *E. faecalis* alone, however at low titres of MOI 1 and 10 in the presence of LPS, IL-6 release was noted. It has been established in Jukat-T cells that release of cytokines is not associated with early transient induction of NF- κ B (86), to the best of our knowledge this phenomenon has not yet been identified in RAW264.7 cells. Since we observed IL-6 activity in RAW264.7 cells in the presence of LPS at low MOI, it is unlikely that in RAW264.7 cells cytokine release is not associated with early induction of NF- κ B. Thus, we suggest that *E. faecalis* prevents the release of cytokines even at low MOI. Unpublished data from our lab has further demonstrated that *E. faecalis* suppresses *E. coli* driven inflammation in the catheterised mouse bladder.

To determine which bacterial factor is involved in prevention of NF- κ B activity to *E. faecalis*, we exposed macrophages to filtered supernatants from overnight growth cultures of *E. faecalis*, UV-killed *E. faecalis* as well as heat-killed *E. faecalis*. We noted that bacterial supernatants and heat-killed bacteria were capable of activating NF- κ B, while UV-killed bacteria and live bacteria failed to activate NF- κ B. Separation of *E. faecalis* from macrophages by the use of cell-

inserts elicited strong NF- κ B activity. We concluded that the *E. faecalis* factor which prevents NF- κ B activity is possibly a surface-associated, heat-modifiable factor which is not secreted.

Several bacterial factors have been identified which interfere with NF- κ B pathway by blocking or mimicking host cell signalling molecules (87). Molecular mechanisms utilized by bacterial pathogens to interfere with NF- κ B are phosphorylation, acetylation and ubiquitination of molecules in NF- κ B pathway (87).

A TIR-like protein (TlpA) which has a structural homology to the mammalian TIR domain impairs NF- κ B activation in *Salmonella. enterica* (88). TIR domain containing proteins (Tcps) in *E. coli* (TcpC) and *Brucella melitensis* (TcpB) similarly interfere with NF- κ B activity (89, 90). Recently it has been demonstrated in *E. faecalis* V583 that a TIR domain containing protein (TcpF) interferes with NF- κ B activity however, *E. faecalis* OG1RF has a truncated *tcpF* gene (63). Thus we suggest that additional *E. faecalis* factors may be working together to prevent NF- κ B activity. Secreted bacterial factors such as non-LEE encoded effectors (NleE) from *E. coli*, Yersinia outer protein J (YopJ) from *Yersinia pestis* interfere and block IKK complex of the NF- κ B pathway (91, 92). While secreted factors from *S. aureus* superantigen-like proteins (SSLs), which bind to TLR2 and interfere cytokine production (93). In our study, we have been able to rule out the role of *E. faecalis* secreted factors in prevention of NF- κ B.

E. faecalis is frequently co-isolated with both gram-positive and gram-negative pathogens in UTI and wound infections (78, 79). To study NF- κ B activity with gram positive and gram negative bacterial PAMPS *in vitro*, we exposed

macrophages to LPS which is a PAMP associated with outer membrane of gram negative bacteria or LTA which is a PAMP associated with the cell wall of gram-positive bacteria and *E. faecalis* simultaneously. We noted that *E. faecalis* prevents NF- κ B activity in the presence of both gram-negative and gram-positive bacterial PAMPs. These results suggest that in multi-organism infections, *E. faecalis* may modulate the NF- κ B activity induced by co-infecting organisms, resulting in a more robust overall infection.

To understand at what stage *E. faecalis* interferes with NF- κ B activation, we tested the hypothesis that it may interfere with TLR2-mediated signalling, which classically recognizes PAMPs on Gram-positive bacteria. In contrast to our observations using RAW264.7 cells, using a HEK cell-line transfected with murine TLR2 SEAP gene did not induce any NF- κ B activity to *E. faecalis* at any MOI in the absence or presence of the TLR2 agonist LTA. This suggests that *E. faecalis* blocks or down-regulates TLR2, or that in contrast to LTA from other *E. faecalis* strains which activate immune response, *E. faecalis* OG1RF LTA is modified in such a way that it is not recognized by TLR2 (76, 77).

Bacteria are known to employ several immune evasion mechanisms and even directly interfere with NF- κ B signalling pathway (87, 94). Capsulated strains of *E. faecalis* V583 are known to evade opsono-phagocytosis. *E. faecalis* V583 capsule was demonstrated to prevent detection of LTA by agglutinating antibodies (95). Recently it has been shown that LTA from a clinical strain of *E. faecalis* P25RC activates NF- κ B signalling pathway (76). Alteration of bacterial peptidoglycan is a well-established mechanism by which bacteria evade detection by host immune cells (96). Gram-positive bacteria such as *Staphylococcus aureus*

and *Streptococcus pneumoniae* are known to modify their peptidoglycan resulting in resistance to lysozyme and contributing to intracellular survival in macrophages (97).

From all of these observations we conclude that *E. faecalis* actively prevents NF- κ B activity in macrophages. Further our data suggests that the bacterial factor responsible is a surface-associated, heat-modifiable factor which is not secreted. We hypothesize that *E. faecalis* factor could bind to PRRs and not bring about conformational change required to trigger NF- κ B signalling pathway or downregulate expression of PRRs. Another possibility is that *E. faecalis* could block or downregulate downstream signalling molecules of NF- κ B pathway.

2.9 Future Directions

Our data provides an interesting insight to *E. faecalis* immune modulating capability. Based on this, future work will entail identifying the bacterial factor or factors which enable *E. faecalis* to prevent NF- κ B activity. We propose to do this by testing a nearly saturated transposon mutant library of *E. faecalis* OG1RF in our NF- κ B assay (98). Future work will also comprise of western blots to detect expression of downstream signalling molecules of NF- κ B pathway in macrophages upon exposure to *E. faecalis* and flow cytometry studies to detect expression of PRRs, to identify which host factor could be either blocked or downregulated.

3 Results: Chapter 2 – SortaseA substrate modulates NF- κ B activity

3.1 Panel of mutants tested for their NF- κ B activity

In the previous chapter we demonstrated that *E. faecalis* failed to activate NF- κ B in macrophages. To identify bacterial factors which could modulate NF- κ B activation in macrophages we tested a panel of deletion mutants in *E. faecalis* OG1RF background. We exposed macrophages to our panel of deletion mutants at MOI 100, 50, and 20 (**Fig 3.1, 3.2 & 3.3**). We observed that Δatn & $\Delta srtA$ significantly activated NF- κ B. We have represented data from individual experiments depicting technical replicates where we show that the general trend remains the same.

The major *E. faecalis* autolysin Atn, is an *N*-acetylglucosaminidase required for the separation of daughter cells after cell division (69). We examined the contribution of Atn to immune modulation and found that the Δatn mutant had strong NF- κ B reporter activity compared to wild-type (**Fig 3.1, 3.2 & 3.3**). Sortase enzymes catalyze the attachment of specific sortase substrate proteins, via recognition of a canonical cell wall sorting signal (CWSS), to the cell wall in gram-positive bacteria. There are approximately 20 predicted Sortase A (SrtA) substrates, based on the presence of a CWSS, encoded in the genome of *E. faecalis* OG1RF (annotated cell wall anchor family protein) (64). We examined the contribution of SrtA to NF- κ B modulation and also found that $\Delta srtA$ mutant had strong NF- κ B reporter activity compared to wild-type (**Fig 3.1, 3.2 & 3.3**). To determine specific sortase substrate protein contributing to immune

modulation, we tested NF- κ B activity to five sortase substrate deletion mutants $\Delta 1953$, $\Delta 2064$, $\Delta 2736$, $\Delta 3023$, and $\Delta ebpABCsrtC$ which is the pilus deficient mutant and observed no difference compared to wild-type (**Fig 3.1, 3.2 & 3.3**). We were further able to rule out the role of $\Delta gelE$, which is a matrix metalloprotease (31), Δeep , which is an intramembrane protease deficient in biofilm formation (99), $\Delta ahrC$, which is a transcription regulator and deficient in biofilm formation (75), $\Delta htrA$, which is high temperature requirement serine protease (100), $\Delta mprf1$, and $\Delta mprf2$ which are membrane protein responsible for aminoacylating anionic phospholipids (101) in NF- κ B reporter activity (**Fig 3.1, 3.2 & 3.3**).

Fig 3.1 Murine RAW 267.4 macrophages were stimulated with *E. faecalis* OG1RF and the mutants Δatn , $\Delta 983$, $\Delta 1767$, $\Delta 1953$, $\Delta 2064$, $\Delta 2736$, $\Delta 3023$, $\Delta srtA$, $\Delta ebpABCsrtC$, Δeep , $\Delta htrA$, $\Delta GelE$, $\Delta mprf1$, $\Delta mprf2$ at an MOI of 100. Fig (A), (B) and (C) represent data from three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (D) Represents macrophage cell death on exposure to *E. faecalis* and the mutants mentioned above (combined data from three experiments). Y axis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig 3.2 Murine RAW 267.4 macrophages were stimulated with *E. faecalis* OG1RF and the mutants Δatn , $\Delta 983$, $\Delta 1767$, $\Delta 1953$, $\Delta 2064$, $\Delta 2736$, $\Delta 3023$, $\Delta srtA$, $\Delta ebpABCsrtC$, $\Delta deep$, $\Delta htrA$, $\Delta gelE$, $\Delta mprf1$, $\Delta mprf2$ at an MOI of 50. Fig (A), (B) and (C) represent data from three individual experiments where technical replicates are shown. Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity. Fig (D) Represents macrophage cell death on exposure to *E. faecalis* and the mutants mentioned above (combined data from three experiments). Yaxis represents % cell death. Statistical analysis was performed by the one way ANOVA test compared to wild-type, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2 D-alanine Lipoteichoic acid and capsular polysaccharide are not required for prevention of NF- κ B activity

E. faecalis V583 is the first sequenced vancomycin resistant strain isolated in the US (1, 8). In the previous chapter we demonstrated that *E. faecalis* V583 failed to activate NF- κ B in macrophages. We tested NF- κ B activity of $\Delta dltA$ and $\Delta cpsC$ in macrophages to identify if *dltA* and *cpsC* are required for prevention of NF- κ B activity.

The *dltA* operon in *E. faecalis* V583 incorporates d-alanine into cell wall associated teichoic acid and lipoteichoic acid (102). Inactivation of the *dltA* gene results in strong negative net charge on the bacterial surface and *dltA* mutant is sensitive to cationic anti-microbial peptides (103). Interestingly, *dltA* mutant was deficient in biofilm production when compared to the wild-type *E. faecalis* V583. On the other hand, biofilm production by the *dltA* mutant was similar to the wild type in the *E. faecalis* strain OG1RF (104). We observed no significant difference in NF- κ B reporter activity of *dltA* mutant in the V583 strain background at all MOIs tested suggesting that *dltA* does not play a role in prevention of NF- κ B activity (**Fig 3.4**).

E. faecalis V583 produces two capsular polysaccharide serotypes (C & D) which have been demonstrated to have a role in evasion of host-immune response, suggesting that capsule prevents detection of LTA and other surface associated PAMPs (95). We tested the NF- κ B reporter activity of capsular mutant V583 $\Delta cpsC$ and observed no difference in NF- κ B reporter activity compared to the

wild-type suggesting that *CpsC* does not play a role in prevention of NF- κ B activity (**Fig 3.4**).

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3.3 Sortase substrates modulating NF- κ B activity

We tested NF- κ B modulation of transposon mutants of 12 predicted sortase substrates at an MOI 20:1 and identified OG1RF_10508::Tn which significantly activated NF- κ B. We monitored the percentage cell death on exposure to the 12 predicted sortase substrates and noted minimal cell death (**Fig 3.5**).

3.4 Sortase A has a cellular invasion defect

We performed a gentamicin protection assay to test if $\Delta srtA$ and Δatn in OG1RF strain background have an invasion defect in macrophages which could explain significant NF- κ B activity induced by these mutants. We found that $\Delta srtA$ has an

invasion defect when compared to wild-type *E. faecalis* OG1RF (**Fig 3.6.A**). In order to further determine that our observation in (**Fig 3.6.A**), was not due to an attachment defect of $\Delta srtA$, we repeated gentamicin protection assay to look for invasion, attachment and total CFU and compared it to wild-type. We observed that $\Delta srtA$ did not have any attachment defect (**Fig 3.6.B**). In **Fig 3.6.C** we have represented this data as percentage reduction in invasion. Taken together our data suggests that invasion may be important for the SrtA pathway of immune modulation.

Fig 3.6 (A) Murine RAW 267.4 macrophages were incubated with *E. faecalis* OG1RF, $\Delta srtA$, and Δatn at MOI10 and bacterial counts were enumerated for invasion. Murine RAW 267.4 macrophages were incubated with *E. faecalis* OG1RF and $\Delta srtA$, at MOI10 and (B) bacterial counts were enumerated for invasion, attachment and total bacteria. Y axis represents NF- κ B reporter activity, (A, &B). (C) Y-axis represents percentage reduction in invasion. (n = 3). Statistical analysis was performed by unpaired t test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5 Discussion

In this chapter we identified two bacterial factors, Sortase A and Autolysin, which modulate NF- κ B activity in macrophages. Bacterial autolysins are required for cleavage of cell-wall peptidoglycan and are important for cell wall remodelling during cell division (71). Autolysins from *S. aureus* hydrolyse peptidoglycan and peptidoglycan fragments are not optimally recognised by PRRs, thus in the absence of autolysins decrease in bacterial virulence was noted (105). Similarly, we propose that one of the mechanisms by which Δatn activates NF- κ B could be recognition of unhydrolysed peptidoglycan by TLRs. The role of Autolysin in immune modulation will be further examined in Chapter 3.

In gram-positive bacteria, proteins are attached to the cell wall by sortase enzymes which catalyze attachment of specific substrate proteins, via recognition of a canonical cell wall sorting signal (CWSS). The CWSS consists of a C-terminal LPXTG motif, a transmembrane domain, and a positively charged tail (**Fig3.7**). There are approximately 20 predicted Sortase A (SrtA) substrates, based on the presence of a CWSS, encoded in the genome of *E. faecalis* OG1RF (annotated cell wall anchor family protein) (64, 66). We demonstrated here that an *E. faecalis* $\Delta srtA$ strain has an intracellular survival defect. Recent findings demonstrated that *E. faecalis* can limit vesicle acidification and formation of autophagosome in macrophages (106). We therefore suggest that $\Delta srtA$ is not able limit vesicle acidification and prevent *E. faecalis* delivery to the autophagosome acidification as well as wild type, giving rise to attenuated survival of this mutant strain within macrophages.

In this chapter we identified a previously uncharacterized sortase substrate OG1RF_10508::Tn (**Fig3.7**) capable of significantly inducing NF- κ B activity, phenocopying a Δ *srtA* strain OG1RF_10508::Tn has a putative von-Willebrand factor type A domain (VWA). VWA domains have been identified in eukaryotes, Eubacteria and Archaea (107). A Von-Willebrand factor type A domain has also been identified in *Streptococcus spp.* For example, in GBS, a VWA domain has been identified in the PilA pilus tip adhesin and has been shown to be required for adherence to epithelial cells also, but is not required for biofilm formation (108). VWA domains can also mediate adhesion via metal-ion dependent adhesion sites (108). As per a search for OG1RF_10508 in NCBI (NCBI Reference Sequence: WP_014524960.1), OG1RF_10508 contains a MIDAS motif (109). The pilus tip adhesin in *E. faecalis*, EbpA, also contains a VWA-associated MIDAS motif that is essential for binding fibrinogen and mediating attachment to fibrinogen coated catheters during CAUTI (110). Based on this, we suggest that OG1RF_10508::Tn could have an adhesion defect, limiting uptake into macrophages, which could explain the significant NF- κ B induction we observed by this strain. Alternatively, we suggest that OG1RF_10508::Tn could bring about the expected single membrane vesicle acidification and formation of autophagosome and thereby the processed PAMPs would activate NF- κ B pathway.

3.6 Future directions

To test the hypothesis that *E. faecalis* Δ *srtA* or its substrate *E. faecalis* OG1RF_10508::*Tn* acidifies macrophage phagosomes normally compared to wild-type, we suggest measuring the intra-phagosomal pH in macrophages upon infection to all three strains. The MIDAS motif in *E. faecalis*, EbpA, is required for the function of EbpA pilus *in vivo* (111), since *E. faecalis* OG1RF_10508::*Tn* has a MIDAS domain which is typically required for cell adhesion (107), we suggest mutating the MIDAS motif in *E. faecalis* OG1RF_10508::*Tn* and performing a gentamicin protection assay to test for deficiency in adhesion. Quantitative western blots analysis of downstream signalling molecules in the NF- κ B pathway, coupled with flow cytometry studies of PRR expression in macrophages infected with *E. faecalis* or the mutants Δ *srtA* and Δ *atn* may help identify the bacterial factor/molecule interfering with NF- κ B activity. This will aid in the identification of the mechanism by which *E. faecalis* successfully

prevents NF- κ B activation. Taken together this work will help elucidate a function for the previously uncharacterized sortase substrate and explain how Sortase A strongly activates NF- κ B in macrophages.

4 Results: Chapter 3 – *E. faecalis* genomic DNA fails to activate TLR9

4.1 Genomic DNA complements NF- κ B activity of SortaseA and Autolysin

In the previous chapter, we showed that both sortaseA and autolysin mutants are capable of activating NF- κ B at high MOIs, as compared to WT *E. faecalis* which does not activate at the same MOI (**Fig 3.1, 3.2, 3.3**). We repeated the experiment with only Δ srtA and Δ atn and compared them to WT *E. faecalis* at an MOI 20, as expected we found that both mutants activate NF- κ B (**Fig 4.1.A**).

The *atn* mutant is deficient in the production of eDNA, has a primary attachment defect to polystyrene surfaces, and is deficient in the production of biofilm (69, 71, 112). In order to validate that the *atn* mutant is also deficient in eDNA production in our hands, we tested eDNA released in overnight culture supernatants of WT, Δ srtA and Δ atn, and confirmed that *atn* is indeed deficient in the production of eDNA (**Fig 4.1.B**).

As the Δ atn mutant is deficient in the production of eDNA, we hypothesized that the released eDNA could be an NF- κ B suppressing factor and tested if genomic DNA (gDNA) from *E. faecalis* was able to complement NF- κ B activating activity of Δ atn, to restore NF- κ B suppression. We infected macrophages with Δ atn and increasing concentrations of genomic DNA simultaneously and found that the addition of gDNA was able to complement the NF- κ B activity of *E. faecalis* Δ atn (**Fig 4.1.C**). We tried complementing the *atn* mutant using the plasmid pGCP123::*atn* but were unable to obtain strains which expressed the

protein. This has been reported previously for *atn* complementation, and it has been suggested that due to potential autolytic and cytotoxic effects of Atn, complementation could not be performed (69, 113).

Even though *E. faecalis* Δ *srtA* is not deficient in production of eDNA, we wondered if gDNA would be capable of complementing the NF- κ B activating activity of Δ *srtA*. Surprisingly, we found gDNA could also complement NF- κ B activity of Δ *srtA*, restoring it to WT NF κ B suppression (**Fig 4.1.D**). These data suggest that *E. faecalis* gDNA is sufficient to suppress NF- κ B activity.

Fig 4.1 (A) Murine RAW 267.4 macrophages were stimulated with *E. faecalis* OG1RF WT, and the mutants' Δ atn, Δ srtA at MOI 20 (B) eDNA released from overnight cultures of *E. faecalis* OG1RF WT, and the mutants' Δ atn, Δ srtA normalised to an OD 1 represented in μ g/ml on Y axis. Murine RAW 267.4 macrophages were stimulated with (C) *E. faecalis* OG1RF WT, Δ atn, Δ atn and increasing concentration of genomic DNA simultaneously (MOI 20) (D) *E. faecalis* OG1RF WT, Δ srtA, Δ srtA and increasing concentration of genomic DNA simultaneously (MOI 20) where on the X- axis, MOI indicates the multiplicity of infection, Y axis represents NF- κ B reporter activity, (A,C&D). Exposure to media alone (-) represents background NF- κ B reporter activity and stimulation with agonists LPS shows maximal reporter activity (n = 2). Statistical analysis was performed by the one way ANOVA test compared to WT, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2 DNA from other bacterial strains differ in their NF- κ B activity

Bacterial DNA from different species have been demonstrated to differ in their potential to activate NF- κ B via TLR9 in murine HEK-TLR9 cells and RAW264.7 cells, as measured by IL-8 production, and this difference in activation correlates with CG dinucleotide content in the different bacterial species (75). As we are using NF- κ B activity as a readout in our experiments, and not IL-8, we measured the response of RAW264.7 cells to DNA from various bacterial species (**Fig 4.2**). Our results were in agreement with the above published data, similarly demonstrating that DNA from different bacterial species differ in their NF- κ B stimulating activity. Thus we observed a correlation between GC content of bacterial species and their NF- κ B stimulating activity, wherein gram-positive bacteria with low GC content (GBS and *E. faecalis*) not only had significantly lower NF- κ B stimulating activity, when compared to maximal NF- κ B reporter activity with LPS, but also suppressed LPS-mediated induction of NF- κ B.

4.3 *E. faecalis* genomic DNA fails to activate NF- κ B

Since we were able to complement the NF- κ B activity to the levels of the parental strain upon addition of *E. faecalis* genomic DNA for both *atn* and *srtA* mutants (**Fig 4.1.C & 4.1.D**), we hypothesized that the enterococcal genomic DNA itself could be preventing NF- κ B activity. In order to test this, we added increasing concentrations (200ng, 500ng, 1 μ g, 5 μ g and 10 μ g) of *E. faecalis* OG1RF genomic DNA to the RAW macrophages. We observed that *E. faecalis* OG1RF gDNA did not activate the NF- κ B response at any of the concentrations we tested (**Fig 4.3.A**). Further, to investigate if *E. faecalis* gDNA was capable of circumventing LPS-mediated NF- κ B activity, we added purified LPS and genomic DNA simultaneously to macrophages and, we observed prevention of NF- κ B activity at high concentrations of *E. faecalis* gDNA (10 μ g) (**Fig 4.3.B**). We also measured the percentage cell death of macrophages on exposure to increasing concentrations of DNA and observed minimal cell death in all the concentrations tested, indicating that cytotoxicity could not explain the absence of NF- κ B activation (**Fig 4.3.C & 4.3.D**). Taken together, this suggests that *E. faecalis* gDNA fails to activate NF- κ B in macrophages and high concentrations of *E. faecalis* gDNA prevent LPS-mediated NF- κ B activity.

E. faecalis V583 has a mobile genomic content of 25% which is absent in *E. faecalis* OG1RF and also has a genomic size of 3.36 Mb compared to OG1RF which is 2.74 Mb (1, 9). In order to test if the difference in the mobile genomic content between the strains V583 and OG1RF had any effect on NF- κ B activity,

we tested NF- κ B activity to gDNA from *E. faecalis* V583 both in the presence and absence of LPS (**Fig 4.3.E**). Similar to *E. faecalis* OG1RF, all the concentrations of gDNA from *E. faecalis* V583 failed to activate NF- κ B in macrophages. Meanwhile, in the presence of LPS, higher concentrations of gDNA (1, 5 and 10 μ g) prevented LPS-mediated NF- κ B activation (**Fig 4.3.E**). These observations are in line with published data, where it was shown that gDNA from *E. faecalis* consistently caused lower release of cytokine IL-8 from RAW 264.7 cells when compared to other bacterial strains (75). We monitored the percentage cell death on exposure to gDNA from *E. faecalis* V583 both in the presence and absence of LPS and noted minimal cell death (**Fig 4.3.F**).

Fig 4.3 Murine RAW264.7 cells were stimulated with (A) Increasing concentrations of genomic DNA *E. faecalis* OG1RF (B) or genomic DNA *E. faecalis* OG1RF and LPS simultaneously, Y axis represents NF-κB reporter activity, (C) Represents macrophage cell death on exposure to genomic DNA of *E. faecalis*. (D) Represents macrophage cell death on exposure to genomic DNA of *E. faecalis* and LPS simultaneously. Y axis represents % cell death (C&D). (E) Murine RAW264.7 cells were stimulated with increasing concentrations of genomic DNA of *E. faecalis* V583 or genomic DNA of *E. faecalis* V583 and LPS simultaneously. (F) Represents macrophage cell death on exposure to genomic DNA of *E. faecalis* V583, or genomic DNA of *E. faecalis* V583 and LPS simultaneously. Y axis represents % cell death. Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonists LPS show maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to LPS, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.4 Sheared *E. faecalis* genomic DNA activates NF- κ B

Eukaryotic DNA is highly methylated when compared to bacterial DNA. Further CpG dinucleotides in eukaryotic DNA are suppressed due to passive deamination of 5-methylcytosine to thymidine, which is not seen in bacterial DNA, and vertebrate DNA is methylated at 5' cytosine, while CpG dinucleotides in bacterial DNA are unmethylated (114-116). TLR9 recognizes bacterial unmethylated CpG DNA and leads to NF- κ B activation (73). The TLR9-CpG DNA interaction has been shown to be sequence specific, wherein murine immune cells respond better to CpG motifs containing the sequence GACGTT, while human immune cells respond well to CpG motifs containing GTCGTT (117). Moreover it's been shown that double stranded CpG-oligodinucleotides (ODN) are weak stimulators compared to single stranded (ss) CpG-ODN (118).

To study the NF- κ B reporter activity of fragmented single stranded (ss) *E. faecalis* DNA, we sonicated and denatured *E. faecalis* genomic DNA and added fragmented DNA to macrophages. We observed that fragmented ss *E. faecalis* DNA activated NF- κ B in macrophages while whole genomic DNA did not (**Fig 4.4.A**). We also measured the percentage cell death of macrophages on exposure to sonicated and whole DNA and observed minimal cell death in all the concentrations tested (**Fig 4.4.B**) This suggests the possibility that it might be the double stranded nature and the folding of gDNA which might be preventing recognition of CpG motifs by TLR9.

4.5 *E. faecalis* genomic DNA is not recognized by TLR9

RAW macrophages have several pattern recognition receptors (PRRs), including TLR9 which recognizes bacterial DNA. In order to address the question whether *E. faecalis* gDNA is recognized by TLR9, we tested the NF- κ B activity in response to *E. faecalis* gDNA in the HEKmTLR9 reporter cell line, using synthetic oligonucleotides (ODNs) as a positive control activator. We observed that *E. faecalis* genomic DNA alone failed to activate TLR9 at any concentration, and was able to prevent the NF- κ B activity in the presence of ODN at high DNA concentration (10 μ g) (**Fig 4.5.A**). We also monitored the percentage cell death on exposure to *E. faecalis* gDNA from both in the presence and absence of ODN and noted minimal cell death (**Fig 4.5.B**).

To test if any molecule bound to *E. faecalis* gDNA was responsible for prevention of NF- κ B activity, we amplified *E. faecalis* gDNA, with the assumption that *in vitro* amplified DNA would not have been exposed or bound by bacterial cellular components. We measured the NF- κ B activity to *in vitro* amplified DNA and we observed that amplified DNA was incapable of activating NF- κ B in HEKmTLR9 cells and there was minimal cell death to all the concentrations of amplified DNA we tested (**Fig 4.5.C & 4.5.D**). These data suggest that it is *E. faecalis* gDNA itself which is responsible for failure of NF- κ B activation.

Fig 4.5 (A) HEKmTLR9 cells were stimulated with increasing concentrations of genomic DNA *E. faecalis* OG1RF in the presence and absence of ODN Y axis represents NF-κB reporter activity, (B) Represents HEK cell death on exposure to genomic DNA of *E. faecalis* and LPS simultaneously. Y axis represents % cell death, (C) HEKmTLR9 cells were stimulated with increasing concentrations of amplified genomic DNA *E. faecalis* OG1RF in the presence and absence of ODN Y axis represents NF-κB reporter activity. (D) Represents macrophage cell death on exposure to genomic DNA of *E. faecalis* and LPS simultaneously. Y axis represents % cell death (C&D). Exposure to media alone (-) represents background NF-κB reporter activity and stimulation with agonist ODN shows maximal reporter activity. Data are combined data from 3 independent experiments (n). Statistical analysis was performed by the one way ANOVA test compared to ODN, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.6 Discussion

The mammalian immune system recognizes and responds to unmethylated CpG motifs in microbial DNA (119). RAW 264.7 macrophages have been shown to ingest plasmid DNA, and are activated by bacterial DNA (75, 120). In our study we noted that the *atn* mutant, which is deficient in the production of eDNA, was capable of inducing NF- κ B reporter activity in comparison to the wild type parental strain. Upon complementing *atn* mutant with increasing concentrations of gDNA, we observed NF- κ B reporter activity similar to wild type levels, suggesting that gDNA modulated NF- κ B activity. Based on this observation, we hypothesized and then showed that *E. faecalis* gDNA itself failed to activate NF- κ B in macrophages, even at high concentrations, and even prevented LPS-mediated activation. Our observation is in line with published data, showing that *E. faecalis* gDNA brought about minimal immune response as measured by IL-8 released in to cell culture supernatants when compared to other bacterial strains (75). DNA from different bacterial species have been shown to differ in the immune response they elicit, and this difference has been correlated to the GC content of the bacterial species, wherein bacterial species with high GC content have stronger immunostimulatory activity and those with low GC content have lower immunostimulatory activity (75). We tested a range of bacterial species with low and high CG content in our assay and our data was in agreement with published data and we observed *E. faecalis* and GBS which have low CG content have minimal NF- κ B activity while *E. coli* and *P. aeruginosa* have strongly

activate NF- κ B. Taken together, our data suggests that low CG content in *E. faecalis* gDNA could be the reason for minimal NF- κ B activity.

In the murine HEK μ TLR9 cell line, we observed that *E. faecalis* gDNA failed to activate NF- κ B at all concentrations tested, suggesting that TLR9 which recognizes bacterial CpG motifs fails to recognize or respond to *E. faecalis* gDNA. We originally hypothesized that this could be due to a molecule or molecules bound to gDNA which prevent recognition by TLR9. To test this we amplified *E. faecalis* gDNA and added amplified DNA to HEK μ TLR9 cells and observed minimal NF- κ B activity. Further, to test if *E. faecalis* amplified gDNA was capable of preventing NF- κ B activity we added amplified gDNA and ODN simultaneously. We observed minimal ODN-stimulated NF- κ B activity in the presence of any *E. faecalis* gDNA concentrations tested. This suggests that *E. faecalis* gDNA actively prevents NF- κ B activity and it's unlikely that this is due to any attached molecule to DNA since the amplified DNA was prepared in the absence of other cellular factors.

TLR9 is the receptor responsible for recognition of bacterial CpG motifs (73). Receptor mediated endocytosis of CpG DNA is a pre-requisite for recognition by TLR9 (121). TLR9-CpG-DNA binding occurs directly and sequence-specifically at an acidic pH (5 to 6.5), which is found in endosomes and lysosomes (117). Moreover ds DNA has been shown to be a poor immunostimulator with weak binding capacity and faster dissociation from TLR9 compared to ss DNA (117, 118, 122). Murine immune cells respond well to CpG motifs containing the sequence GACGTT; changing this core sequence to GTCGTT led to enhanced

TLR9 binding and upon changing the sequence to GACGTC led to decreased binding to TLR9 (117). We suggest that, in addition to having an intrinsically low GC content, the core CpG motif of *E. faecalis* might not have the optimum sequence to bind and activate TLR9.

Additionally, we observed significant NF- κ B activity on addition of *E. faecalis* fragmented gDNA suggesting that the organization and folding of unfragmented *E. faecalis* gDNA may contributed to the minimal NF- κ B stimulation that we observed.

Acidification and maturation of endosomes is required for CpG DNA and TLR9 interaction (121). Recently it has been shown that *E. faecalis* containing vacuoles resist acidification in macrophages (106). We predict that in the presence of *E. faecalis* acidification in vacuoles is prevented which would result in failure of formation of ss DNA could explain poor stimulation of TLR9 by ds DNA.

In conclusion we suggest that failure of *E. faecalis* gDNA to activate NF- κ B via TLR9 could be attributed to multiple factors such as low GC content, lack of optimal TLR9-binding sequence in the CpG motif, and organization and folding of gDNA that masks activating motifs.

4.7 Future Directions

To test the hypothesis that as *E. faecalis* does not acidify vacuoles, which would give rise to TLR9 binding with lower affinity to *E. faecalis* gDNA, we propose measuring TLR9-gDNA real-time binding by altering the pH of the solution and by performing surface plasmon resonance (SPR) analysis (106, 117). If our hypothesis is correct, we would predict that gDNA binds with lower affinity to TLR9 at low pH and higher affinity at high pH levels.

E. faecalis have been shown to form biofilms on catheters in murine UTI models (70). Viable *E. faecalis* secrete eDNA in well-defined structures in early biofilms (123). Biofilm matrix comprises of protein, glycoproteins, glycolipids and eDNA (124). We predict that eDNA isolated from *E. faecalis* biofilms will contribute to NF- κ B suppression because bacteria biofilms are typically resistant to host defences (125). To test this, eDNA would be isolated from *E. faecalis* biofilms and NF- κ B activity in macrophages can be compared to similar amount of gDNA. We predict that the distribution of eDNA from wild-type, Δ atn, Δ srtA and Δ OG1RF_10508 would be different because of differing NF- κ B activity of these strains. To test this, ultrastructural analysis of eDNA in biofilms of the above strains can be done by labelling with anti-dsDNA and studying them by scanning electron microscopy (123). To elucidate if there are differences between *E. faecalis* eDNA and gDNA we suggest a study of sequence comparison between *E. faecalis* eDNA and gDNA. Since it has been established that ds CpG ODN are poor stimulators compared to ss CpG ODN (118). To establish whether eDNA is ds or ss in nature, we suggest disruption of *E. faecalis* biofilms by ds nucleases

and ss nucleases. We predict that *E. faecalis* gDNA does not have the optimum sequence in its CpG motifs and thus, is not being recognized by TLR9. We suggest identifying the sequence specific CpG motifs in *E. faecalis* gDNA by bioinformatics tools. Understanding lack of immune stimulation by *E. faecalis* DNA is important not only to better understand *E. faecalis* pathogenesis, but also because of the potential therapeutic uses of suppressive *E. faecalis* DNA in the treatment of inflammatory or auto-immune diseases.

5 Materials and methods

5.1 Bacterial strains and growth conditions

Enterococcus faecalis strains OG1RF, OG1RF Δ *srtA*, and OG1RF Δ *atn* were grown in Brain Heart Infusion Broth (BHI) or agar at 37°C. Antibiotics were added at the following concentrations for *E. faecalis* OG1RF: fusidic acid, 25 mg/L and rifampin, 25 mg/L. *Escherichia coli* strain K12 MG1655 was grown in Luria-Bertani (LB) broth or agar at 37°C. For all experiments single colonies were grown statically overnight for 18 h. Overnight cultures of *E. faecalis* were centrifuged at 6000 x g for 5 minutes and re-suspended in PBS normalized to OD 1.

Strains	Characteristics	Source/Reference
<i>E. faecalis</i> OG1RF	Rifampicin and fusidic acid resistant derivative of human oral isolate OG1.	(10)
<i>E. faecalis</i> V583	First sequenced vancomycin resistant strain	(8)

<i>E. faecalis</i> OG1RF Δ <i>atn</i>	Deficient in the production of eDNA, has an attachment defect and is deficient in the production of biofilm	(69, 71)
<i>E. faecalis</i> OG1RF Δ <i>srtA</i>	Deficient in surface display of all cell-wall anchored proteins.	(69)
<i>E. faecalis</i> OG1RF Δ 983	ahrC, ArgR family transcription factor	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ 1767	Competence protein F	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ 1953	Sortase substrate	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ 2064	Sortase substrate	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ 2736	Sortase substrate	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ 3023	Sortase substrate	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ <i>GelE</i>	Gelatinase E	Gift from Hultgren lab
<i>E. faecalis</i> OG1RF Δ <i>EEP</i>	Intramembrane protease, deficient in biofilm formation	(99)
<i>E. faecalis</i> OG1RF Δ <i>htrA</i>	High temperature requirement serine protease	Adeline Yong, Kline lab
<i>E. faecalis</i> OG1RF Δ <i>EbpABC</i> <i>SrtC</i>	Sortase substrate	Gift from P. Guiton, Hultgren lab
<i>E. faecalis</i> OG1RF Δ <i>mprf1</i>	Membrane protein responsible for	(101)

	aminoacylating anionic phospholipids (paralog 1)	
<i>E. faecalis</i> OG1RF Δ <i>mprf2</i>	Membrane protein responsible for aminoacylating anionic phospholipids (paralog 2)	(101)
<i>E. faecalis</i> V583 Δ <i>dltA</i>	Deficient in biofilm production	(102)
<i>E. faecalis</i> V583 Δ <i>CpsC</i>	Capsular mutant serotypeC	(95)
<i>E. coli</i> MG1655	Laboratory strain	(82)
<i>E. coli</i> UTI89	Clinical isolate from bladder infection	(80)
<i>E. faecalis</i> OG1RF_10485::Tn	Sortase substrate transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_10508::Tn	Sortase substrate transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_12504::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_12506::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_10785::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_12268::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_11974::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_12303::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_10766::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_10088::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_10084::Tn	Sortase substrate Transposon mutant.	(98)
<i>E. faecalis</i> OG1RF_11531::Tn	Sortase substrate Transposon mutant.	(98)

5.2 Cell Culture and Media

RAW-Blue cells derived from RAW 264.7 cells, with a chromosomally integrated gene encoding a secreted embryonic alkaline phosphatase (SEAP) reporter, inducible by NF- κ B (Invivogen) were cultivated in Dulbecco modified eagle medium (DMEM+ high glucose (1X) with l-glutamine, without sodium pyruvate; Gibco) with 10% fetal bovine serum (PAA), 50 U/ml penicillin, 50U/ml streptomycin, 100 μ g/ml Normocin, and 200 mg/ml Zeocin at 37⁰C in 5% CO₂. HEK-Blue-mTLR2, in which the SEAP reporter gene is under the control of the IL-12 p40 minimal promoter, HEK-Blue mTLR4 and HEK-Blue mTLR9 cells, where the SEAP reporter gene is controlled by an IFN- β minimal promoter (Invivogen) were derived from HEK293 cells. These HEK cells were transfected with murine TLR2, TLR4 and TLR9 gene respectively and cultivated in media same as above along with. 100 μ g/ml Normocin, 50 U/ml penicillin, 50 U/ml streptomycin and, 1X HEK-Blue selection antibiotic (Invivogen).

5.3 NF- κ B reporter assay

RAW-Blue cells were seeded in a 96 well plate @ 100,000 cells/well in 200 μ l of antibiotic free media (DMEM+10% FBS) and incubated overnight, the cells were washed once with PBS and 160 μ l of fresh media was added. Wells were stimulated with lipopolysaccharide (LPS) ultrapure from *E. coli* O111:B4 (Invivogen) (100ng/ml) as positive control, exposure to media alone served as

negative control. RAW-Blue cells were infected in three different conditions with *E. faecalis* in PBS (at a 100:1, 50:1, 20:1, 10:1 and 1:1 multiplicity of infection) for 6 hours or *E. faecalis* (at a 100:1, 50:1, 20:1, 10:1 and 1:1 multiplicity of infection) and LPS (100ng/ml) simultaneously for 6 hours or *E. faecalis* (at a 100:1, 50:1, 20:1, 10:1 and 1:1 multiplicity of infection) for 3 hours followed by LPS (100ng/ml) for 6 hours triplicate for each condition. Post infection, 20 µl of supernatant was collected and added to 180 µl of QUANTI-Blue reagent (Invivogen) and incubated at 37°C overnight. SEAP levels were determined at 640 nm using TECAN M200 microplate reader.

5.4 LDH assay

On completion of the infection phase of the RAW-Blue cells, the 96 well plates were centrifuged at 400 x g for 10 mins and 100 µl of the supernatant was collected from each well to perform the LDH assay. LDH assay was performed using the Clontech LDH cytotoxicity determination kit. 100 µl of the cell culture supernatant was mixed with 100 µl of the reaction mixture and incubated for 5 minutes, 50 µl of 1 N HCl was added as the stop solution. For low controls, the wells were mock infected with PBS. For high controls, 0.2% Triton X was added to the wells. Each condition was carried out in triplicate. % cytotoxicity was calculated as per the formula below:

$$\% \text{ Cytotoxicity} = \frac{\text{Triplicate absorbance} - \text{Low control}}{\text{High control} - \text{Low control}} \times 100$$

5.5 Gentamicin protection assay

RAW 264.7 cells in suspension (3 ml), were infected at an MOI of 10:1 for 1 hr at 37°C in 5% CO₂ in DMEM + 10% FBS and divided into three tubes 1 ml each. To the first tube, 500 µg penicillin + 500 µg gentamicin was added to kill the extra-cellular surface attached bacteria, after incubating for 1 hour, followed by centrifugation at 2000 x g for 5 mins. The cell pellet was resuspended in fresh media and RAW-Blue cells were lysed with 0.1% Triton-X100 and serial dilutions were made in BHI agar for CFU enumeration of bacterial invasion. The second tube, was further incubated for 1 hour and after centrifugation the cell pellet was washed twice with PBS. RAW cells were lysed and serial dilution for CFU enumeration of attached bacteria was performed. To the third tube, after incubation for one hour, RAW cells were lysed and serial dilution for CFU enumeration of total bacteria was done.

5.6 Cytokine assay

We measured the cytokine IL-6 released into the cell culture supernatants by enzyme linked immunosorbent assay (ELISA) (BD bioscience) of the RAW-Blue cells after infection with *E. faecalis* or *E. faecalis* and LPS simultaneously. Each condition was tested in triplicate as per the manufacturer's protocol. Uninfected cells represent negative control and LPS stimulated cells were used as positive control.

5.7 Genomic DNA extraction and amplification

Genomic DNA extraction was performed using the Wizard genomic DNA purification kit (Promega) as per the manufacturer's protocol. Overnight bacterial cultures were centrifuged at 16,000 x g for 2 mins in microcentrifuge tubes and the cell pellets were resuspended in EDTA 50mM followed by lysis with 10 mg/ml of lysozyme. Bacterial samples were then incubated at 37⁰C for 60 mins followed by centrifugation at 16,000 x g for 2 mins. The supernatants were discarded and the cell pellets were lysed with 600 µl of nuclei lysis solution at 80⁰C for 5 mins and then cooled to room temperature. 3 µl of RNase solution was added followed incubation for 60 mins at 37⁰ C. After cooling to room temperature, 200µl of protein precipitation solution was added and vortexed for 20 seconds. The microcentrifuge tubes were then placed on ice for 5 mins and then centrifuged at 16,000 x g for 2 mins, the supernatant containing the DNA was transferred to a fresh microcentrifuge tube containing 600 µl of isopropanol and gently inverted till threads of DNA became visible. Isopropanol was discarded after centrifugation and the DNA pellet was resuspended in ethanol. Similarly, ethanol was discarded after centrifugation and the DNA pellet was air dried, 100µl of DNA rehydration solution was added to the tubes and stored at 4⁰ C overnight. DNA content and purity was measured using spectrophotometer. DNA samples within the optical density ratio of A260/A230 of 1.7 to 2.0 were used. Genomic DNA isolated using the above protocol was amplified using the REPLI-G midi kit (Qiagen) as per the manufacturer's protocol. Briefly, 2.5µl of genomic DNA was placed in a microcentrifuge tube to which 2.5µl of was added

and mixed by vortexing for 15 secs followed by an incubation at room temperature for 3 mins. 5µl of buffer N1 was added to the tube and vortexed for 15 secs. REPLI-G midi DNA polymerase was thawed on ice and mastermix was prepared as follows: 10µl nuclease free water + 29µl REPLI-G midi reaction buffer + 1µl REPLI-G midi DNA polymerase. Mastermix was added to 10 µl of reaction mixture and incubated at 30 °C for 16 hours. Post incubation, REPLI-G midi DNA polymerase was inactivated by heating at 65 °C for 3 mins and stored at – 20°C.

5.8 Supernatant, heat killed and UV killed and transwell treatment

Bacteria were heat-killed by exposure to 65°C for 30 mins prior to inoculation of macrophages. Culture supernatants were collected from overnight cultures after centrifugation at 6000 x g and filtered through a 0.2 µm syringe filter. UV-killed bacterial suspensions were prepared by exposure to UV light (254nm) for 30 mins and then was co-cultured with the macrophages. The efficacy of heat and UV killing, and the sterility of culture supernatants, was verified by the absence of CFU after 24 h of growth on BHI agar.

For cell inserts test, RAW-Blue cells were seeded in Corning costar 24 well transwell plates @ 100,000 cells per well and incubated overnight at 37°C in 5% CO₂, cells were washed with PBS and fresh media was added, inserts were introduced and wells were infected in duplicate (at a 100:1, 50:1, 20:1, 10:1 and 1:1 multiplicity of infection). 6 hours post infection 20 µl of supernatant was collected and NF-κB assay was performed as previously described.

5.9 Measurement of eDNA

Stationary phase cultures of *E. faecalis* strains OG1RF, OG1RF Δ *srtA*, and OG1RF Δ *atn* were normalized to an OD of 1, centrifuged at 6000x g for 5 minutes and supernatants were collected after syringe filtration through a 0.2 μ m syringe filter, eDNA was measured using the Qubit dsDNA BR assay kit by QuBit 2.0 fluorometer (ThermoFisher scientific).

5.10 Sonication and denaturation of genomic DNA

Genomic DNA was sonicated at an amplitude setting of 20% for 6 minutes, with a sonication pulse rate of 15 secs on and 15 secs off. This was further denatured at 95°C for 5 minutes. Sonicated and genomic DNA were run on a 1% agarose gel. The gel was placed in ethidium bromide solution for 15 minutes and then visualized using Gel Doc XR+ (Biorad).

5.11 Statistical analysis

All data was analysed using GraphPad Prism 6 software. Statistical analysis was performed by one way ANOVA test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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