Molecular mechanisms underlying staphylococcal induced osteomyelitis.

Tania Claro
Royal College of Surgeons in Ireland

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MOLECULAR MECHANISMS UNDERLYING STAPHYLOCOCCAL INDUCED OSTEOMYELITIS

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

by

Tânia Claro

(January, 2012)

Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin

Under the supervision of Dr. Steven W. Kerrigan
I. DEDICATION

Para os “Ms” da minha vida, Mia e Miguel.
## II. TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. DEDICATION</td>
<td>1</td>
</tr>
<tr>
<td>II. TABLE OF CONTENTS</td>
<td>2</td>
</tr>
<tr>
<td>III. ACKNOWLEDGEMENTS</td>
<td>6</td>
</tr>
<tr>
<td>IV. ABBREVIATIONS</td>
<td>8</td>
</tr>
<tr>
<td>V. ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>12</td>
</tr>
<tr>
<td>General Introduction</td>
<td>12</td>
</tr>
<tr>
<td>1.1. Bone</td>
<td>13</td>
</tr>
<tr>
<td>1.2. Bone cells</td>
<td>16</td>
</tr>
<tr>
<td>1.2.1. Osteoclasts</td>
<td>16</td>
</tr>
<tr>
<td>1.2.2. Osteoblasts</td>
<td>16</td>
</tr>
<tr>
<td>1.2.3. Osteocytes</td>
<td>17</td>
</tr>
<tr>
<td>1.3. Bone cells surface receptors</td>
<td>17</td>
</tr>
<tr>
<td>1.3.1. Cadherins</td>
<td>18</td>
</tr>
<tr>
<td>1.3.2. Immunoglobulin superfamily</td>
<td>19</td>
</tr>
<tr>
<td>1.3.3. Integrins</td>
<td>20</td>
</tr>
<tr>
<td>1.3.4. Other important receptors in bone: RANK/RANKL, OPG, TNFR-1</td>
<td>24</td>
</tr>
<tr>
<td>1.4. Bone remodeling</td>
<td>28</td>
</tr>
<tr>
<td>1.4.1. Osteoclastogenesis</td>
<td>30</td>
</tr>
<tr>
<td>1.4.2. Bone resorption</td>
<td>31</td>
</tr>
<tr>
<td>1.4.3. Bone formation</td>
<td>32</td>
</tr>
<tr>
<td>1.5. Bone disease</td>
<td>32</td>
</tr>
<tr>
<td>1.5.1. Osteoporosis</td>
<td>33</td>
</tr>
<tr>
<td>1.5.2. Septic arthritis</td>
<td>34</td>
</tr>
<tr>
<td>1.6. Osteomyelitis</td>
<td>36</td>
</tr>
<tr>
<td>1.6.1. Osteomyelitis definition</td>
<td>36</td>
</tr>
<tr>
<td>1.6.2. Different types of Osteomyelitis</td>
<td>36</td>
</tr>
<tr>
<td>1.6.3. Acute and Chronic Osteomyelitis</td>
<td>41</td>
</tr>
<tr>
<td>1.6.4. Osteomyelitis prevalence</td>
<td>43</td>
</tr>
<tr>
<td>1.6.5. Osteomyelitis diagnosis</td>
<td>43</td>
</tr>
<tr>
<td>1.6.6. Treatment of osteomyelitis</td>
<td>46</td>
</tr>
<tr>
<td>1.6.7. Osteomyelitis causative microorganisms</td>
<td>50</td>
</tr>
<tr>
<td>1.7. Staphylococci</td>
<td>52</td>
</tr>
<tr>
<td>1.7.1. Staphylococci biology</td>
<td>52</td>
</tr>
<tr>
<td>1.7.2. History and division into S. aureus and S. epidermidis</td>
<td>52</td>
</tr>
<tr>
<td>1.8. S. aureus</td>
<td>53</td>
</tr>
</tbody>
</table>
# Table of Contents

1.8.1. *S. aureus* virulence factors ................................................................. 54
  1.8.1.1. Surface proteins ............................................................................. 54
  1.8.1.2. capsule ......................................................................................... 65
  1.8.1.3. Extracellular toxins ........................................................................ 66
  1.8.1.4. Epidermolytic toxin ....................................................................... 68
  1.8.1.5. Superantigens ............................................................................... 68
  1.8.1.6. Cell Wall ....................................................................................... 69

1.9. *S. epidermidis* .................................................................................. 70
  1.9.1. *S. epidermidis* virulence factors ................................................... 71
    1.9.1.1. Biofilm ......................................................................................... 71
    1.9.1.2. Surface proteins ........................................................................... 74
    1.9.1.3. Extracellular proteins .................................................................... 74

1.10. Bone Infection .................................................................................. 76

Chapter 2..................................................................................................... 81

Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process ........................................... 81

2.1. Introduction ....................................................................................... 82
  2.1.1. Chapter Aims ................................................................................... 85

2.2. Chemicals and general conditions ..................................................... 86

2.3. Methods .............................................................................................. 87
  2.3.1. Bacterial culture conditions ............................................................... 87
  2.3.2. Cell culture conditions ..................................................................... 89
  2.3.3. Functional assays ............................................................................. 92
  2.3.4. Protein biochemistry .......................................................................... 93
  2.3.5. Statistical analysis ............................................................................ 97

2.4. Results ................................................................................................ 98
  2.4.1. *S. aureus* binding to osteoblast ......................................................... 98
  2.4.2. Contribution of the cell surface molecules in the *S. aureus* binding to osteoblast .......................................................... 100
  2.4.3. The role of *S. aureus* Fibronectin protein A and B in the binding to osteoblast .......................................................... 103
  2.4.4. The role of *S. aureus* surface proteins in the binding to osteoblast .......................................................... 105
  2.4.5. Confirmation of the role of *S. aureus* protein A in the binding to osteoblast .......................................................... 107
  2.4.6. Contribution of the *S. aureus* protein A domains in the binding to osteoblast .......................................................... 109
  2.4.7. The role of *Staphylocococcus aureus* Sbi in the binding to osteoblast .......................................................... 111
  2.4.8. The role of *S. aureus* protein A in osteoblast proliferation .......... 113

2.5. Discussion ............................................................................................ 115
<table>
<thead>
<tr>
<th>Chapter 3</th>
<th>Investigation of the osteoblast receptor involved in binding <em>S. aureus</em> protein A and the downstream signalling events that occur as result of this interaction</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.</td>
<td>Introduction</td>
<td>121</td>
</tr>
<tr>
<td>3.1.1.</td>
<td>Chapter Aims</td>
<td>123</td>
</tr>
<tr>
<td>3.2.</td>
<td>Chemicals and general conditions</td>
<td>124</td>
</tr>
<tr>
<td>3.3.</td>
<td>Methods</td>
<td>125</td>
</tr>
<tr>
<td>3.3.1.</td>
<td>Bacterial culture conditions</td>
<td>125</td>
</tr>
<tr>
<td>3.3.2.</td>
<td>Osteoblast culture conditions</td>
<td>125</td>
</tr>
<tr>
<td>3.3.3.</td>
<td>Functional assays</td>
<td>125</td>
</tr>
<tr>
<td>3.3.4.</td>
<td>Protein biochemistry</td>
<td>126</td>
</tr>
<tr>
<td>3.3.5.</td>
<td>Flow cytometry</td>
<td>127</td>
</tr>
<tr>
<td>3.3.6.</td>
<td>Confocal microscopy</td>
<td>128</td>
</tr>
<tr>
<td>3.3.7.</td>
<td>Apoptosis</td>
<td>129</td>
</tr>
<tr>
<td>3.3.8.</td>
<td>ELISA</td>
<td>132</td>
</tr>
<tr>
<td>3.3.9.</td>
<td>siRNA mediated silencing of the TNFR-1 gene</td>
<td>134</td>
</tr>
<tr>
<td>3.4.</td>
<td>Results</td>
<td>136</td>
</tr>
<tr>
<td>3.4.1.</td>
<td>Identification of the <em>S. aureus</em> protein A ligands that are involved in binding osteoblast</td>
<td>136</td>
</tr>
<tr>
<td>3.4.2.</td>
<td>Investigating the expression of the Tumor Necrosis Factor Receptor 1 on osteoblast surface</td>
<td>140</td>
</tr>
<tr>
<td>3.4.3.</td>
<td>Investigating the osteoblast receptor that binds to <em>S. aureus</em> protein A</td>
<td>142</td>
</tr>
<tr>
<td>3.4.4.</td>
<td>Investigating downstream events as a result of <em>S. aureus</em> protein A binding to osteoblast TNFR-1: Apoptosis</td>
<td>145</td>
</tr>
<tr>
<td>3.4.5.</td>
<td>Investigating downstream events as a result of <em>S. aureus</em> protein A binding to osteoblast TNFR1: NFκB activation</td>
<td>150</td>
</tr>
<tr>
<td>3.4.6.</td>
<td>Investigating downstream events as a result of <em>S. aureus</em> protein A binding to osteoblast TNFR-1: Cytokine release</td>
<td>152</td>
</tr>
<tr>
<td>3.5.</td>
<td>Discussion</td>
<td>154</td>
</tr>
</tbody>
</table>

Chapter 4 | Study of the molecular mechanisms involved in *S. epidermidis* binding to osteoblasts: early steps in implant infection | 158 |
| 4.1. | Introduction | 159 |
| 4.1.1. | Chapter Aims | 161 |
| 4.2. | Chemicals and general conditions | 162 |
| 4.3. | Methods | 162 |
| 4.3.1. | Bacterial culture conditions | 162 |
| 4.3.2. | Cell culture conditions | 163 |
| 4.3.3. | Functional assays | 163 |
### Table of Contents

4.3.4. Protein biochemistry .............................................................. 164
4.3.5. Statistical analysis ................................................................. 165

4.4. Results .................................................................................. 166
4.4.1. Investigating the ability of *S. epidermidis* to interact with osteoblast ........................................................... 166
4.4.2. A proteomic approach to identify the differential protein expression between *S. epidermidis* ...................................................... 168
4.4.3. Investigating the role of *S. epidermidis* SdrG in binding osteoblast ................................................................. 169
4.4.4. Investigating the mechanism through which *S. epidermidis* SdrG interacts with osteoblast ........................................................... 171
4.4.5. Investigating the osteoblast receptor that recognises *S. epidermidis* SdrG ................................................................. 173
4.4.6. Investigating the effect of *S. epidermidis* binding on osteoblast proliferation ................................................................. 175
4.4.7. Investigating the downstream events as a result of *S. epidermidis* SdrG binding to osteoblast  : Cell Death ................................................................. 177

4.5. Discussion ............................................................................. 179

Chapter 5 ...................................................................................... 183

General Discussion ..................................................................... 183

5.1. Future work ......................................................................... 191

VI. APPENDIX .................................................................................. 193
i. Oral presentations ................................................................ 194
ii. Poster presentations ......................................................... 195
iii. Publications ........................................................................ 196

VII. REFERENCES ............................................................................. 197
III. ACKNOWLEDGEMENTS

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### IV. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>Alpha Minimal Essential Medium</td>
</tr>
<tr>
<td>α-toxin</td>
<td>Alpha toxin</td>
</tr>
<tr>
<td>Aae</td>
<td>Autolysin adhesin E</td>
</tr>
<tr>
<td>AAP</td>
<td>Accumulation-Associated Protein</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
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<td>Asp</td>
<td>Aspartic acid</td>
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<td>AtlE</td>
<td>Autolysin E</td>
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<tr>
<td>β-toxin</td>
<td>Beta toxin</td>
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<tr>
<td>β2-GPI</td>
<td>β2-glycoprotein I</td>
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<td>Bbp</td>
<td>Bone sialoprotein-binding protein</td>
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<td>BCA</td>
<td>Bicinchoninic Acid</td>
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<td>BHI</td>
<td>Brain Heart Infusion</td>
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<td>Bp</td>
<td>Base-pair</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>ClfA</td>
<td>Clumping factor A</td>
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<tr>
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<td>Clumping factor B</td>
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</tr>
<tr>
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</tr>
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<td>Delta-toxin</td>
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<td>Dithiothreitol</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGFR</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorben Assay</td>
</tr>
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<td>ESR</td>
<td>Erythrocyte Sedimentation</td>
</tr>
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<td>FACS</td>
<td>Fluorescent- Activated Cell Sorting</td>
</tr>
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<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
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<tr>
<td>FAME</td>
<td>Fatty Acid Modifying Enzyme</td>
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<td>Fbe</td>
<td>Fibronectin binding protein from S. epidermidis</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FDG</td>
<td>Fluorine- 18-fluoro-D-deoxyglucose</td>
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<td>Fibronectin-binding protein A</td>
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</tr>
<tr>
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</tr>
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<td>Glycine</td>
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<td>Horseadish Peroxidase</td>
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<td>ICAMs</td>
<td>Intercellular adhesin molecules</td>
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<td>Immunoglobulin</td>
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<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>IkB</td>
<td>Inhibitory Kinase β</td>
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<td>Description</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>LBP</td>
<td>Lipopolysaccharide -Binding Protein</td>
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<td>LDV</td>
<td>Leu-Asp-Val</td>
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<td>LFA-1</td>
<td>Lymphocyte Function- Associated antigen</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LPXTG</td>
<td>Leu-Pro-X-Thr-Gly (Leucine-Proline-Variable amino acid-Threonine-Glycine)</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
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<td>MAP</td>
<td>Microtubule-Associated Protein</td>
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<td>M-CSF</td>
<td>Macrophage- Colony Stimulating Factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial Surface Components Recognizing Adhesive Molecules</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin Susceptible <em>S. aureus</em></td>
</tr>
<tr>
<td>MurNAc</td>
<td>N-acetylmuramic acid</td>
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<td>NCAM</td>
<td>Neural cell binding molecule</td>
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<td>NFkB</td>
<td>Nuclear Factor Kappa B</td>
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<td>OD</td>
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</tr>
<tr>
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<td>Osteoprotegrin</td>
</tr>
<tr>
<td>PAGE</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PET</td>
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</tr>
<tr>
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</tr>
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<td>Polysaccharide Intercellular Adhesin</td>
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<td>PIC</td>
<td>Protease Inhibitor Cocktail</td>
</tr>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
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<td>PPP</td>
<td>Platelet Pour Plasma</td>
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<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PV</td>
<td>Panton and Valentine</td>
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<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<td>RANK</td>
<td>Receptor Activator of Nuclear factor kB</td>
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<td>RANK ligand</td>
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<td>Arg-Gly-Asp-Ser</td>
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<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
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<td>RT</td>
<td>Room Temperature</td>
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<td>Sbi</td>
<td><em>S. aureus</em> binder of IgG</td>
</tr>
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<td>Sdr</td>
<td>Serine-aspartate repeat family of proteins</td>
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<td>Serine-aspartate repeat protein F</td>
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<tr>
<td>SD</td>
<td>Ser-Asp dipeptides repeats</td>
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<td>SDS</td>
<td>Sodium Dodecil</td>
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<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
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<td>Serine</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA or silencing RNA</td>
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<td>SpA</td>
<td><em>S. aureus</em> protein A</td>
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<td>SrtA</td>
<td>Sortase A</td>
</tr>
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</table>
Abbreviations

SSP  S. epidermidis surface proteins
SSSS Staphylococcal Scalded Skin Syndrome
TBS  Tris Buffered Saline
TBST Tris Buffered Saline Tween
TEMED Tetramethylethylendiamine
TLR2 Toll Like Receptor 2
TLR4 Toll Like Receptor 4
TNF  Tumor Necrosis Factor
TNF-α Tumor Necrosis Factor alpha
TNFR-1 Tumor Necrosis Factor Receptor 1
TRADD Tumor necrosis factor Receptor type 1-Associated Death Domain
TRAIL Tumour necrosis factor Receptor Apoptosis Inducing Ligand
TSS Toxic Shock Syndrome
TSST-1 Toxic Shock Syndrome Toxin-1
VCAM Vascular Cell Binding Molecule
vWF Von Willebrand factor
WHO World Health Organization
Abstract

Osteomyelitis is a debilitating infectious disease of the bone. It is mainly caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*, being the second one, the major cause of implant-related osteomyelitis. Osteomyelitis is associated with significant morbidity and mortality and it is mainly characterised by weakened bones due to progressive bone loss and inflammation. However, currently the mechanism through which either bone destruction or inflammation occurs in osteomyelitis patients is poorly understood.

The data presented in this thesis shows that *S. aureus* and *S. epidermidis* bind differently to bone and follow distinct pathways in the initiation and progression of disease. While *S. epidermidis* interacts with osteoblast via SdrG binding to fibrinogen which in turn binds to an integrin receptor on the osteoblast surface, *S. aureus* uses a very specific interaction mediated by *S. aureus* protein A (SpA) and Tumor Necrosis factor receptor 1 (TNFR-1) to bind osteoblast. A study of the interaction between *S. epidermidis* and osteoblast demonstrated that *S. epidermidis* SdrG protein is involved in the inhibition of osteoblast growth but not in osteoblast death following infection. On the other hand, investigation of the effect following SpA binding to TNFR-1 revealed this to be crucial in the activation of the receptor pathway mediating multiple signals ultimately leading to suppression of bone growth together with bone destruction and inflammation. Here, it is shown that upon infection with *S. aureus*, osteoblasts undergo apoptosis, through caspase 3 cleavage, and the activation of inflammatory processes occurs via NFkB activation associated with IkB degradation and the release of IL6. All of these events were prevented when blocking SpA or TNFR-1 or both, confirming the importance of this interaction in the onset and development of disease.

The findings presented in this thesis describe for the first time interactions between Staphylococci and osteoblast and their role in the progression of Staphylococcal induced osteomyelitis. Furthermore, this study may also highly contribute for the development of a novel or improved therapy for osteomyelitis patients using the TNFR-1.
Chapter 1

General Introduction
Chapter 1 - General Introduction

1.1. Bone

The skeleton plays a central role in the life of a human being. It gives every body an individual form, architecture and it also determinates its size. Bone is a highly specialized tissue embedded within soft tissues with two main different functions: support and storage (Adler 2000). This complex tissue is composed of extracellular matrix that mineralizes, conferring strength and rigidity to the skeleton but also some elasticity. In addition, bone is a major source of inorganic ions, actively participating in calcium homeostasis in the body (Ducy 2000). Bone is mainly composed of type I collagen, impregnated with crystals of calcium hydroxyapatite, accounting for approximately 95 % of the organic matrix; the remaining 5 % are proteoglycans (highly glycosylated proteins) and numerous non-collagenous proteins.

Morphologically, there are two distinct types of bone: compact (or cortical) bone which constitutes 85 % of the total bone in the body, and spongy (or trabecular or cancellous) bone constituting the remaining 15 % (Figure 1-1) (Ilvesaro 2001). The medullary cavity in the centre of the bone is composed of bone marrow, responsible for the production of the majority of blood cells. Cortical bone is the dense, rigid outer layer that provides strength and structural integrity while the cancellous bone is a loosely organized porous matrix that makes up much of the enlarged ends of the long bones and ribs (Rogers 2010). Differences in the structural arrangements of these two bone types are related to their primary functions: compact bone provides mechanical and protective functions whereas spongy bone provides metabolic functions (Bilezikian, Raisz et al. 2008).
During the first 20 to 30 years of life, there is more bone being formed than resorbed, resulting in an increase in bone mass (Matkovic et al. 1994, Mundy 1995). Following the growth period the adult skeleton is remodelled every year with equal ratio of localized bone resorption and formation (Parfitt 1994). Later in life, the remodeling activity starts to fail, deregulating the capacity of maintaining the skeletal mass. Therefore skeletal strength is reduced and the risk of fracture increases over time, depending on the magnitude by which resorption and formation are uncoupled.

Bone is composed of three main cell types: osteoclasts, osteoblasts and osteocytes. While osteoclasts are bone resorbing cells, osteoblasts are bone forming cells, and finally osteocytes are mature bone cells that permeate the interior mineralized matrix and account for 90% of all cells in the adult skeleton (Figure 1-2). Osteoclasts are hematopoietic descent and their precursors are located in the monocytic fraction of the bone marrow (Fujikawa, Quinn et al. 1996). On the other side, osteoblasts and osteocytes share the same origin both deriving from pluripotent mesenchymal stem cells (Aubin 1998).
Osteoclast: Multinucleate cell that secretes acids and enzymes to dissolve bone matrix

Osteocyte: Mature bone cell that maintains the bone matrix

Osteoblast: Immature bone cell that secretes organic components of matrix

Figure 1-2 – Different types of bone cells, their origin and location (Martini and Bartholomew 2003)
1.2. Bone cells

1.2.1. Osteoclasts

Derived from hematopoietic stem cells, osteoclasts are defined as specialized macrophages, highly migratory and multinucleated with a life span reasonably short, varying among species from days to weeks (Teitelbaum 2000; Tanaka, Miyazaki et al. 2006). The cross sectional diameter of an osteoclast ranges from twenty to hundred microns, being several times the size of a robust osteoblast (Tanaka, Miyazaki et al. 2006). Osteoclasts are also called bone resorbing cells as their main function is to resorb bone by destroying the extracellular bone matrix through dissolution of hydroxyapatite and degradation of the organic matrix components (Teitelbaum 2007; Pietrzak 2008). The number of osteoclast must be balanced to maintain normal bone remodeling and to prevent excess of bone resorption that would lead to pathological bone loss (Bruzzaniti and Baron 2006).

1.2.2. Osteoblasts

Osteoblasts are fully differentiated cells responsible for the production of bone (Bilezikian, Raisz et al. 2008). Osteoblasts arise from the same pluripotent stem cell with chondroblasts, adipocytes, myoblasts and fibroblasts (Grigoriadis, Heersche et al. 1988; Bennett, Joyner et al. 1991; Yamaguchi and Kahn 1991). These bone forming cells are located at the bone surface and are morphologically mono-nucleated and cuboid in shape. They maturate from osteoprogenitor cells into pre-osteoblasts and some
differentiate into osteocytes (Figure 1-3) (Sommerfeldt and Rubin 2001; Bilezikian, Raisz et al. 2008). Osteoblasts are strongly alkaline-phosphatase positive cells and synthesise bone matrix proteins, hormones receptors, cytokines and growth factors, with the ultimate aim of making a tissue recognizable as bone (Bilezikian, Raisz et al. 2008).

1.2.3. Osteocytes

Considered the most differentiated cells from the osteoblast lineage, osteocytes are highly specialized and fully differentiated osteoblasts incorporated within the extracellular matrix. Only 10 to 20 % of the osteoblasts differentiate into osteocytes, however osteocytes end up being the most abundant cells in bone (Parfitt 1977; Bilezikian, Raisz et al. 2008). Despite sharing the same lineage, osteoblasts and osteocytes are very distinct in morphology, location and function. Osteocytes are smaller star shaped cells with an increased nucleus to cytoplasm ratio, contain less organelles and have decreased alkaline phosphatase activity than osteoblasts (Sommerfeldt and Rubin 2001). They reside in a regular spacing within lacunas in the mineralized bone matrix and newly formed osteoid (un-mineralized bone matrix) (Kogianni and Noble 2007) and are functionally responsible for the mechanical sensing on bone which initiates bone matrix turnover or bone remodeling (Burger and Klein-Nulend 1999; Mikuni-Takagaki 1999; Petersen 2007). This mechanical sensing is initiated via the osteocytes through their long cytoplasmic extensions denominated canaliculi, which allow them to contact neighbouring osteocytes, osteoblasts, internal and external surfaces of bone, and blood vessels traversing the matrix (Doty 1981; Menton, Simmons et al. 1984; Boneveld 1999).

1.3. Bone cells surface receptors

Osteoblasts, osteocytes and osteoclast express a wide range of surface molecules such as cadherins and integrins that mediate binding and signaling
pathways that are involved in maintaining normal functioning of bone. The processes of cell to cell and cell to matrix communication play a central role in the physical and mechanical changes that lead to bone remodeling. The bone cells surface receptors are complex and diverse and this is easily explained by the number of functions that they exercise. Beyond adhesive functions, surface receptors have been increasingly identified to mediate signaling pathways in immune response regulation. Conversely, intracellular events can also lead to receptor affinity and activity changes. For example, in platelet–fibrinogen interaction, the platelet integrin gpllbIIIa only binds the extracellular matrix protein after alteration on integrin conformation, following its activation via ligand binding such as the thrombin receptor.

1.3.1. Cadherins

Cadherins are a large family of binding molecules that play a major role in intercellular binding and signaling (Takeichi 1991; Geiger and Ayalon 1992), tissue morphogenesis and tumor suppression (Takeichi 1993). These calcium dependent proteins share a common primary structure composed of an amino-terminal extracellular domain, a single trans-membrane domain and a conserved carboxyl-terminal cytoplasmic region (Figure 1-4). The extracellular domain can be divided into highly homologous regions whereas the calcium cations articulate with the ligand binding site, the conserved HAV motif (Ac-Cys-His-Ala-Val-Cys-NH₂) located in the first extracellular repeat, rigidifying the extracellular domain into a rod like conformation. The cytoplasmic region binds to catenins which are important in gene transcription and binding regulation. Cells often express a repertoire of different cadherins simultaneously, and they tend to be concentrated at cell-cell junctions on the cell surface (Siegel and Agranoff 1999; Bilezikian, Raisz et al. 2008). The known cadherins of bone cells, their respective ligand and function are summarized below in Table 1-1. On osteoclasts, blocking
antibodies to E-cadherin have been demonstrated to inhibit osteoclast formation and fusion in vitro, as well as resorption by mature osteoclasts (Mbalaviele, Chen et al. 1995; Ilvesaro, Lakkakorpi et al. 1998). While on osteoblasts, intercellular binding is likely to be mediated by cadherins such as N-cadherin, cadherin -4, -6 and -11 (Babich and Foti 1994; Okazaki, Takeshita et al. 1994; Cheng, Lecanda et al. 1998; Mbalaviele, Nishimura et al. 1998; Ferrari, Traianedes et al. 2000), however the knowledge about their role in osteoblast function is limited (Babich and Foti 1994).

### Table 1-1 – Bone cells cadherins, respective ligand and function

<table>
<thead>
<tr>
<th>Cadherin</th>
<th>Cell type</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>N- an other cadherins</td>
<td>Osteoblast</td>
<td>N-cadherin</td>
<td>Osteoblast development</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Osteoclast</td>
<td>E- cadherin</td>
<td>Osteoclast differentiation</td>
</tr>
</tbody>
</table>

**1.3.2. Immunoglobulin superfamily**

The human genome encodes 765 distinct Ig domains, making it the most abundant domain in human proteins. Within the immunoglobulin superfamily most of the members are well known for their involvement in immune functions, however some of these proteins also mediate cell to cell binding (Karp 2008). Structurally the immunoglobulin family of receptors are characterized by an Ig-like domain in their extracellular domain and by one or more Ig fold copies organized into two anti-parallel β sheets held together by hydrophobic bonds (Figure 1-5) (Juliano 2002; Belkin, Gu et al. 2010). Members of this family such as VCAM (vascular cell binding molecule) or NCAM (neural cell binding molecule) play an important role in nerve outgrowth, synapse formation, and other events during the development of the nervous system (Karp 2008). Their ligands include members of the Ig family such as NCAM binding to itself, but also members of
the integrin family such as for the ICAMs (Intercellular binding molecules), which bind β2 integrins and components of the extracellular matrix (e.g. collagen binding for myelin-associated glycoprotein). Signaling pathways activated by Ig family members include MAP (Microtubule-Associated Protein) kinase pathways (Isacke and Horton, 2000; Hubbard and Rothlein, 2000). Ig superfamily of receptors in bone cells and their specific role in bone cell functioning is summarized in Table 1-2 below. Osteoblasts express ICAM-1 and VCAM-1 on their surface and some studies showed that these receptors are involved in osteoblast interactions with T-lymphocytes followed by cytokine release (Tanaka, Morimoto et al. 1995) which may be important in the regulation of skeletal turnover during inflammation.

Table 1-2 - Ig superfamily of receptors in bone cells, respective ligand and function.

<table>
<thead>
<tr>
<th>Ig superfamily</th>
<th>Cell type</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>Osteoblast</td>
<td>LFA-1 (lymphocyte function-associated antigen) on leukocytes</td>
<td>Osteoblast differentiation Production of cytokines</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Osteoblast</td>
<td>α4 Integrins on leukocytes</td>
<td>Osteoblast differentiation Production of cytokines</td>
</tr>
</tbody>
</table>

1.3.3. Integrins

Integrins are heterodimeric transmembrane molecules important in a variety of cellular functions such as growth, development, immune response, and wound repair (Juliano 2002). These receptors are composed of two subunits, alpha and beta, linked non-covalently whereas both subunits are amino-glycosylated glycoproteins with a large extracellular domain, a single hydrophobic transmembrane region, and a short intracellular cytoplasmic tail (Figure 1-6). Several isoforms of the alpha and beta subunits have been identified to date; 18 different mammalian α subunits and 8 β subunits, forming 24 distinct heterodimers dictating their integrin-binding specificity (Zamir and Geiger 2001). α subunits vary in size from 120 to 180 KDa, while β subunits are 90 to 110 KDa, apart from the 210 KDa β4 chain. Most integrins can bind different types of ligands such as extracellular matrix molecules or signal molecules, such as
growth factors. Many of those ligands are recognized by multiple integrins and this is often through recognition of a common sequence. In many extracellular matrix molecules this is an RGD peptide sequence, although other proteins can recognize other sequences, such as collagen, recognizing DGEA (Asp-Gly-Glu-Ala) or fibronectin that recognizes LDV (Leu-Asp-Val) sequences (Plow et al., 2000; Humphries et al., 2006).

Figure 1-6 – Basic structure of an integrin. (http://www.scq.ubc.ca/the-role-of-integrins-in-wound-healing/)

Osteoclast integrins

Binding of osteoclasts to the bone surface is mediated by the interaction between integrins and extracellular matrix proteins within the bone matrix. Phenotypic and biochemical analyses demonstrated that osteoclasts express integrins $\alpha_\nu\beta_3$ and $\alpha_\nu\beta_2$, receptors for vitronectin; and $\alpha_2\beta_1$, a collagen/laminin receptor on their surface (Helfrich, Nesbitt et al. 1996; Horton and Rodan 1996). The $\alpha_\nu\beta_3$ vitronectin receptor mediates RGD peptide-dependent binding to a wide variety of proteins containing the RGD sequence, including bone sialoproteins and several extracellular matrix and plasma proteins. This interaction is involved in inducing a change on integrin conformation which in turn leads to a number of outside-in signaling events including cytoskeletal
remodeling (Teltelbaum 2007). Moreover, studies on osteoclasts $\alpha_2\beta_1$ integrin blockade suggest a role for this integrin in osteoclast fusion (Helfrich, Nesbitt et al. 1996). Although the downstream effects of integrin-mediated signaling in osteoclasts have not been clearly investigated, osteoclasts seem to respond to integrin ligands, in a number of ways indicative of direct signal transduction (Duong, Lakkakorpi et al. 2000). These include the release of intracellular calcium stores, induction of protein tyrosine phosphorylation, and as mentioned before reorganization of the structural and signaling components of the cytoskeleton (Paniccia, Colucci et al. 1993; Shankar, Davison et al. 1993; Zimolo, Wesolowski et al. 1994). Additional downstream effects of osteoclast integrin-mediated signaling are transcytosis, regulation of cell motility and induction of binding-related apoptosis (Ruoslanti and Reed 1994). Osteoclasts use transcytosis to remove degraded matrix from active sites of bone resorption, enabling the osteoclast to maintain the integrity of the enclosed resorption site and facilitate cell migration and penetration into bone (Nesbitt and Horton 1997; Salo, Lehenkari et al. 1997). Reports have suggested that the integrins $\alpha_2\beta_1$ and $\alpha_v\beta_3$ may also be involved in the uptake of the bone matrix at the ruffled border area (Nesbitt, Nesbit et al. 1993; Helfrich, Nesbitt et al. 1996). Some of the known osteoclast integrins, their ligands and potential functions are summarized below in Table 1-3.

<table>
<thead>
<tr>
<th>Osteoclast integrin</th>
<th>Ligand</th>
<th>Known/potential function</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_v\beta_3$</td>
<td>Vitronectin, osteopontin, bone sialoprotein, fibronectin, fibrinogen, denatured collagen</td>
<td>Matrix binding, Signal transduction, Osteoclast polarization, Cessation of resorption*</td>
</tr>
<tr>
<td>$\alpha_2\beta_1$</td>
<td>Native collagens</td>
<td>Matrix binding</td>
</tr>
<tr>
<td>$\alpha_v\beta_1$</td>
<td>Vitronectin</td>
<td>Matrix binding</td>
</tr>
</tbody>
</table>

* Potential function

Table 1-3 – Osteoclast integrins, their ligands and respective function
Osteoblast integrins

A diverse range of integrins have been shown to be expressed by osteoblasts (Table 1-4) particularly of the $\beta_1$ class (Brighton and Albelda 1992; Clover, Dodds et al. 1992; Grzesik and Robey 1994; Ganta, McCarthy et al. 1997). However, there is some contradiction between different studies which may reflect the heterogeneity of osteoblast-like populations that might show different patterns of integrin expression along the different stages of osteoblast differentiation. Functional studies have demonstrated that $\alpha_2\beta_1$ binding to collagen leads to expression or up-regulation of markers of osteoblastic differentiation (Xiao, Wang et al. 1998). Others have shown that ligand binding by the $\alpha_2$ integrin modulates cell motility and contraction of collagen gels in vitro (Riikonen, Westermarck et al. 1995). Moreover, the osteoblast integrin $\alpha_3\beta_1$ binds collagen, fibronectin and function-perturbing antibodies inhibiting the formation of mineralized nodules in rat calvarial osteoblast cultures (Moursi, Globus et al. 1997). Finally, in vitro studies have shown that the selective fibronectin receptor, $\alpha_5\beta_1$, is expressed by osteoblasts, and it seems to be important in both cell survival (Globus, Doty et al. 1998) and mechanical sensing (Salter, Robb et al. 1997) for bone maintenance.

<table>
<thead>
<tr>
<th>Osteoblast Integrin</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_2\beta_1$</td>
<td>Native collagens</td>
<td>Matrix binding</td>
</tr>
<tr>
<td>$\alpha_3\beta_1$</td>
<td>Collagen, fibronectin</td>
<td>Osteoblast mineralization</td>
</tr>
<tr>
<td>$\alpha_5\beta_1$</td>
<td>Fibronectin (RGD)</td>
<td>Osteoblast differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mechanical sensing</td>
</tr>
</tbody>
</table>

Osteocyte integrins

The ability of osteocytes to adhere and communicate within bone is crucial for the translation of biomechanical signals into chemical signals during bone mechanical sensing. Although there is a lack of knowledge in osteocytes integrins, some studies reported their ability to bind collagen type I, osteopontin, vitronectin, fibronectin and thrombospondin, however the exact integrin
receptor for each ligand have not been yet identified (Table 1-5) (Aarden, Nijweide et al. 1996). Furthermore, expression of integrins $\alpha_6\beta_3$ and $\beta_3$ by osteocytes has been suggested to be involved in their differentiation from osteoblasts and binding to bone matrix molecules (Bennett, Carter et al. 2001).

<table>
<thead>
<tr>
<th>Table 1-5 - Osteocyte integrins and respective function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocyte Receptor</td>
</tr>
<tr>
<td>$\alpha_6\beta_3\beta_3$</td>
</tr>
</tbody>
</table>

1.3.4. Other important receptors in bone: RANK/RANKL, OPG, TNFR-1

Along with numerous cytokines and colony stimulating factors, the members of the Tumor necrosis factor (TNF) superfamily of ligands and receptors have a pivotal role in both the immune system and bone regulation (Hofbauer, Khosla et al. 2000; Lorenzo 2000).

RANKL/RANK

The receptor activator of NF-kB (RANK) ligand (RANKL) is a member of the TNF superfamily (Yasuda, Shima et al. 1998) produced by osteoblasts in response to a variety of stimuli. This receptor is most commonly found as a membrane receptor, however a soluble form also exists (Simonet, Lacey et al. 1997; Teitelbaum 2000). RANKL plays an important role in osteoclasts development by interacting with its receptor RANK in osteoclast precursors (Nakagawa et al., 1998). RANKL induces differentiation, formation, survival, and fusion of pre-osteoclasts (Hsu, Lacey et al. 1999). In addition, RANKL has also been shown to mediate the actin ring formation during cytoskeleton rearrangement before resorption, and to activate mature osteoclasts to resorb bone (Lacey, Timms et al. 1998). In vivo studies revealed an increase in osteoclast formation and activation following administration of soluble RANKL to mice, leading to osteoporosis and hypercalcemia (Simonet, Lacey et al. 1997). On the other hand,
RANKL knockout mice showed an increased in bone mass and impaired tooth eruption because of a lack of mature osteoclasts (Giuliani, Bataille et al. 2001).

**OPG**

Osteoprotegerin (OPG), also a member of the TNF superfamily, is a soluble receptor produced by osteoblasts and acts as an inhibitor of the RANK/RANKL interaction. Once released by osteoblasts OPG competes with RANK in binding RANKL, leading to the inhibition of osteoclast differentiation and activation (Simonet, Lacey et al. 1997). In addition, OPG has also been shown to neutralize the apoptosis inducing factor TNF-related apoptosis-inducing ligand (TRAIL) (Lacey, Timms et al. 1998; Khosla 2001). Studies in mice have revealed that OPG knockout mice develop severe osteoporosis, whereas the over-expression of OPG in transgenic mouse models and OPG treatment of normal mice leads to osteopetrosis (increased bone density) (Hsu, Lacey et al. 1999; Roodman 2001).

**TNFR-1**

The tumor necrosis factor receptor 1 (TNFR-1) is another member of the TNF superfamily and one of the major receptors for the tumor necrosis factor alpha (TNF-α). In bone TNFR-1 is expressed by both osteoblasts and osteoclasts (Bu, Borysenko et al. 2003). TNF-α signaling via interaction with the TNFR-1 plays an important role in the bone system as a potent osteoresorptive cytokine, stimulating the differentiation and activity of osteoclasts (Abu-Amer, Erdmann et al. 2000; Azuma, Kaji et al. 2000) and inhibiting osteoblast differentiation (Gilbert, He et al. 2000; Abbas, Zhang et al. 2003). TNFR-1 can also activate the transcription factor NF-κB, mediating apoptotic events, and function as a regulator of inflammation.

The signalling pathway following TNFR-1 engagement leads to the recruitment of several cytosolic molecules triggering two different pathways: cell survival via the activation of Nuclear factor Kappa B (NFkB) and cell death through caspase
activation (Figure 1-7). NFκB represents a group of transcriptional factors controlling different biological processes such as immune and inflammatory responses, cellular growth and apoptosis (Ghosh and Karin 2002; Kalthoff 2009). On the other hand, Inhibitory kinase β (Ikβ) belongs to a family of cytoplasmic inhibitory proteins that regulate the translocation of NFκB from the cytoplasm to the nucleus. In resting cells, Ikβ binds to NFκB sequestering it on the cytosol, however when the TNFR-1 signalling pathway is activated, the complex TNFR-1-Tumor necrosis factor Receptor type 1-Associated Death Domain (TRADD) induces the degradation of Ikβ allowing the translocation of NFκB to the nucleus where it will mediate the expression of genes ultimately involved in inflammatory responses, such as cytokines or chemokynes (May and Ghosh 1997; Kalthoff 2009; Panzer, Steinmetz et al. 2009). TNFR-1 activation can also induce the binding of the adaptor Fas-Associated protein with Death Domain (FADD) in the cytosol, which is necessary for the cleavage of pro-caspases leading to the activation of executioner caspases that will sentence cell death (Srivastava 2007).

In 2004, Gomez et al showed that SpA from S. aureus has affinity to bind the TNFR-1 receptor triggering a signalling pathway that leads to inflammation, including cytokine release (Gomez et al., 2004). On the other hand, the release of cytokines such as IL-6 by osteoblasts has been shown to modulate bone resorption and osteoclastogenesis (Udagawa, Takahashi et al. 1995; Kwan Tat, Padrines et al. 2004).

Linking this together it is likely that bacterial bone infection may be involved in the stimulation of the TNFR-1 pathway leading to cytokine release and osteoclastogenesis and at the same time reducing osteoblast-mediated bone matrix production and mineralisation, characteristic of bone inflammation and bone destruction (Wright and Nair 2010).
Figure 1-7 - TNFR-1 signaling pathway. Engagement of cell surface receptor TNFR-1 triggers the recruitment of several cytosolic molecules leading to two different pathways: the apoptotic pathway, through the activation of executioner caspases or the NFκB signaling pathway triggering inflammation processes.
1.4. Bone remodeling

Bone remodeling involves the coordinated effort of osteoblasts, osteocytes and osteoclasts. Together these cells ensure localized removal of old bone followed by replacement with newly formed bone (Figure 1-8). Normal bone remodeling is initiated by local events that lead to an increase in osteoclastic activity. This is followed by pre-osteoblast recruitment and differentiation into mature cells, which lay down new bone and repair the resorption defects caused by the resorbing osteoclasts. Formation follows resorption at the resorption site, and the amount of bone formed is almost equal to the amount of bone removed. Bone remodeling occurs to enable the bones to adapt to mechanical stress and to repair micro-damage maintaining its strength. Alterations in the remodeling process will result in metabolic bone disease (Bilezikian, Raisz et al. 2008). In adults, osteocytes are continuously replacing the surrounding calcium salts. While osteoclasts and osteoblasts activities are balanced: as quickly as osteoblasts form osteon, osteoclasts remove bone by osteolysis. In young adults, almost one-fifth of the adult skeleton is recycled and replaced each year. However not every part of every bone is affected equally: the rate of turnover differs regionally and locally, depending on the external forces or stimuli that bone senses in each area (Martini and Bartholomew 2003). Bone adjusts to different environments, and an inability to do so results in disease. The knowledge of bone remodeling physiology is important so that it can be manipulated to treat or prevent bone disease (Bilezikian, Raisz et al. 2008).
Figure 1-8 – Bone remodeling. Old bone is resorbed by osteoclasts, macrophages can be found at the remodeling site to remove the dead cells and debris. Finally, osteoblasts precursors are recruited and after differentiating into mature osteoblasts secrete bone matrix that mineralizes and generates new bone (adapted from © Biomedical Tissue Research, University of York).
1.4.1. Osteoclastogenesis

Osteoclastogenesis is the process by which osteoclasts fully differentiate and become activated. In the earliest step of osteoclastogenesis, osteoclasts progenitors are recruited from hematopoietic tissues such as bone marrow to bone via circulating blood (Bilezikian, Raisz et al. 2008). The macrophage colony stimulating factor (M-CSF), and RANK are sequentially expressed as a requirement for the development of the mature osteoclast (Arai, Miyamoto et al. 1999). Osteoclasts undergo proliferation in response to stimulation by the M-CSF (Takeshita, Namba et al. 2002) and following recruitment of RANK (Figure 1-9(B)). RANK then binds to its ligand RANKL expressed on the osteoblast surface (also called osteoclast differentiation factor). Finally the RANK-RANKL interaction promotes pre-osteoclasts differentiation and fusion, and formation of mature osteoclast, enabling osteoclasts to resorb bone (Petersen 2007).

At the end of the process of bone resorption, osteoclastogenesis is inhibited by the presence of OPG. As osteoblasts differentiate, the relative production of OPG compared with RANKL increases (Figure 1-9(A)). Therefore, OPG binds and
neutralizes RANKL, inhibiting osteoclastogenesis (Figure 1-9(C)) and leading to osteoclast apoptosis which in turn allows the mature osteoblast to form new bone in the remodeling space following osteoclast resorption, completing the process of bone remodeling (Gori, Hofbauer et al. 2000).

1.4.2. Bone resorption

Once fully differentiated, osteoclasts undergo a series of morphological changes that compartmentalize the cell into a clear sealing zone and a ruffled border area (Figure 1-10). The sealing zone is rich in actin filaments and free of organelles and juxtaposed to the bone surface mediating the process of cell attachment (Holtrop and King 1977). The attachment mechanism is thought to be mediated via osteoclast integrins interaction with bone matrix proteins, such as the interactions between $\alpha_v\beta_3$ and $\alpha_v\beta_1$ with vitronectin or between $\alpha_3\beta_1$ and native collagens (Vaaninen and Horton 1995; Nakamura, Takahashi et al. 1996). The ventral membrane coming from the sealing zone is characterized by a highly folded membrane, the ruffled border area that harbours the secretory activity. From the sealing zone, osteoclasts pump an arsenal of lysosomal vesicles, protons and proteases and acid across the ruffled border into the resorption lacuna causing collagen destruction and mineral bone dissolution and a marked decrease of pH (Silver, Murrills et al. 1988; Horne 1995; Pietrzak 2008). Subsequently a secretory domain can be distinguished on the opposite side of the ruffled border, from where the resorbed material is released (Baron, Neff et al. 1990; Salo, Lehenkari et al. 1997; Ilvesaro 2001; Bilezikian, Raisz et al. 2008).

Figure 1-10 - Model of bone resorption by osteoclasts (adapted from (Bilezikian, Raisz et al. 2008)).
1.4.3. Bone formation

Osteoblasts main function is to form bone. In this well-regulated process, osteoblasts regulate bone matrix maturation and mineralization by secreting membrane-associated alkaline phosphatase, bone matrix molecules such as, type I collagen and other non-collagenous proteins such as osteocalcin, osteopontin and bone sialoprotein (Bilezikian, Raisz et al. 2008). Also called osteogenesis, bone formation is initiated and propagated via the deposition of calcium phosphate crystals and the establishment of cytoplasmic connections between osteocytes and adjacent cells, which, respectively, make the matrix impermeable and ensure metabolic survival (Figure 1-11). During bone matrix formation the un-mineralized matrix defined as osteoid is converted into bone (Martini and Bartholomew 2003; Bilezikian, Raisz et al. 2008). Osteocytes are responsible for the bone matrix maintenance (Buckwalter, Glimcher et al. 1996).

Figure 1-11- Bone formation (adapted from www.highered.mcgraw-hill.com).

1.5. Bone disease

Disruption or impairment in the normal functioning of bone growth and maintenance results in a variety of bone diseases and disorders.
Chapter 1- General Introduction

1.5.1. Osteoporosis

Osteoporosis is a bone disease characterized by low bone mass and consequent increase in bone fragility and susceptibility to fractures (Meunier 1998; Klotzbuecher, Ross et al. 2000; Bilezikian, Raisz et al. 2008). Osteoporosis prevalence and incidence worldwide is difficult to establish as there are still uncertainties with its definition and diagnosis. However a 2007 World Health Organization (WHO) report states that osteoporosis is estimated to affect 200 million women worldwide, approximately one-tenth of women aged 60, one-fifth of women aged 70, two-fifths of women aged 80 and two-thirds of women aged 90 (Kanis 2007). Although the pathogenesis of osteoporosis is not fully understood, the characteristic bone loss is a direct consequence of an increase in the osteoclastic activity, whereby bone resorption raises thus weakening bone and leading to an augmented risk of fractures (Odell et al. 1993, Takano-Yamamoto et al. 1990, Tuukkanen et al. 1991). Therefore, a useful way to estimate osteoporosis prevalence is to use the fracture rates. The WHO estimates that one in three women and one in eight men over age 50 risk having an osteoporotic fracture during their lifetime. These fractures cause disability, loss of independence and loss of quality of life. Osteoporotic fractures are a major contributor to medical care costs around the world (Hardman and Stensel 2009).

Osteoporosis is diagnosed by measurement of the bone mass density by X-ray. With age, the human skeleton loses bone density, therefore age is a risk factor for osteoporosis and prevention is very important mostly in elderly people. In women, estrogen is an important hormone for bone density maintenance, after menopause the levels of estrogen drop and may induce bone loss. Other strong risk factors for osteoporosis are family history of osteoporosis, medical conditions such as thyroid disease, rheumatoid arthritis and blocked calcium absorption (Weinstein and Buckwalter 2005; Qaseem, Snow et al. 2008).

Osteoporosis can be classified according to different situations: appearing late in life, or due to hormonal insufficiency, disease of the bone marrow or reduced osteogenesis. All these forms of osteoporosis share an identical histological
pattern, and only further clinical radiological tests allows the correct classification and further treatment administration (Adler 2000).

Treatment focuses on agents that reduce bone loss such as bisphosphonates which are potent inhibitors of bone turnover, together with calcium and vitamin D as supplements for the maintenance of bone strength. Hormone therapy has also been administrated for osteoporosis patients, however, several adverse effects have been observed. As inhibitors of osteoclast activity denosumab and calcitonin have also been administrated in osteoporotic patients acting by reducing bone resorption and consequently bone fractures (Hardman and Stensel 2009).

1.5.2. Septic arthritis

Septic arthritis is an infection of the joints accompanied by inflammatory destruction. The incidence of this disease ranges from 2 to 10 in 100,000. However, in rheumatoid arthritis patients or patients with prosthetic joints it may be as high as 30 to 70 per 100,000 (Goldenberg, 1998; Nade, 2003; Stott, 2001). It is more common in children than adults, and in males rather than females (Levine and Siegel, 2003). Moreover irreversible loss of joint function develops in 25 to 50 % of the patients (Yu, Bradley et al. 1992; Kaandorp, Van Schaardenburg et al. 1995; Kaandorp, Krijnen et al. 1997).

Septic arthritis is mainly caused by bacteria such as Staphylococcus aureus (Le Dantec, Maury et al. 1996; Ryan, Kavanagh et al. 1997), Streptococcus pneumoniae (Morgan, Fisher et al. 1996) or Streptococcus pyogenous (Schattner and Vosti 1998). The incidence of Haemophilus influenza as a cause is significantly decreasing (De Jonghe and Glaesener 1995). In addition, although relatively rare in Western Europe, the Gram-negative bacterial Neisseria gonorrhoeae is the most common cause of septic arthritis in the United States (Le Dantec, Maury et al. 1996; Ryan, Kavanagh et al. 1997). The infecting microorganisms infiltrate and proliferate within the joints triggering
inflammation through the release of cytokines including IL-1β and IL-6 into the joint fluid (Koch, Lemmermeier et al. 1996) and by the recruitment of leukocytes (Goldenberg 1998; Nade 2003).

Most of the septic arthritis infections develop as result of haematogenous seeding of the vascular synovial membrane (Klein 1988; Morgan, Fisher et al. 1996). Bacteria can also gain entry secondary to penetrating trauma or after trauma to a joint. Moreover, direct introduction of bacteria in association with knee and hip replacement has increasingly been a source of infection (Nelson and Koontz 1966; Barton, Dunkle et al. 1987). Bacterial superantigens such as staphylococcal TSST-1 and the staphylococcal enterotoxins A contribute to the collagen breakdown and can lead to destruction of intra-articular cartilage and subchondral bone loss within days of infection (Goldenberg, 1998; Nade, 2003; Shirtliff and Mader, 2002; Stott, 2001(Bremell and Tarkowski 1995). The infectious process within the joint will result in increased pressure, which impedes blood and nutrient supply to the joint worsening joint damage and destruction (Shirtliff and Mader, 2002).

Treatment of septic arthritis requires appropriate antibiotic therapy together with drainage of the infected joint. Antibiotic therapy should be adjusted following identification of the causative microorganism by synovial fluid or blood culture isolation. Antibiotic administration can be oral or intravenous for duration of 2 days to 4 weeks. Drainage can be done by needle aspiration (Ho 1993), however an arthroscope (flexible tool fitted with a mini-camera and source light) is often used in knee or shoulders infections for better visualization and joint irrigation (Le Dantec, Maury et al. 1996; Morgan, Fisher et al. 1996). Open surgical drainage should be performed when conservative treatment fails. Early active motion exercises within the limits of tolerance will hasten recovery (Mathews, Kingsley et al. 2008; Garcia-Lechuz and Bouza 2009).
1.6. Osteomyelitis

1.6.1. Osteomyelitis definition

The word osteomyelitis derives from the ancient Greek words osteon – bone, myelo - marrow and itis meaning inflammation (Sax and Lew 1999; Baltensperger and Eyrich 2009). The term osteomyelitis was first used by the French surgeon Edouard Chassaignac in 1852, who defined the disease as an inflammatory process accompanied by bone destruction and caused by a pyogenic microorganism (Chassaignac 1852; Mader, Mohan et al. 1997). Infection is established when pathogenic microorganisms reach the bone, forming pus and oedema that compromise or obstruct the local blood supply (Figure 1-12). Following this, the infected bone forms an agglomerate of necrotic bone cells, defined as sequestrum, which is considered a classical sign of osteomyelitis (Topazian and Goldberg 1994). Infection can be limited to a single portion of the bone or it may involve several regions, such as marrow, cortex, periosteum, and the surrounding soft tissue. Infection takes place as a result of a large inoculation of pathogenic microorganisms from the blood stream, following trauma or in the presence of implanted devices with subsequent bone damage (Lew and Waldvogel 2004).

1.6.2. Different types of Osteomyelitis

Osteomyelitis can be classified according to the source of the infection. The infecting microorganisms can reach the bone by haematogenous delivery resulting from bacteraemia (bacterial infection of the blood stream); via local

Figure 1-12 – Diagram of osteomyelitis infection bone caused by S. aureus infection.

(Illustration © Lydia V. Kibiuk, CMI 2010)
invasion from a contiguous focus of infection when the infection is spread from local tissue (for example cellulitis); or by direct inoculation often following injury, surgery or implantation of a foreign body or secondary to vascular insufficiency (diabetic patients) (Berendt and Byren, 2004; Simon and Koenigsknecht 2001; Ciampolini and Harding, 2000; Goldenberg, 1998; Lazzarini et al., 2004; Lew and Waldvogel, 2004).

Haematogenous osteomyelitis
Haematogenous osteomyelitis is caused by direct seeding of bacteria from the bloodstream into the bone. It accounts for 20% of all cases of osteomyelitis from which 85% are diagnosed in children between 1 and 15 years of age. In which cases a minor trauma may cause a small hematoma followed by vessel obstruction ending in bone necrosis (Lew and Waldvogel 2004). In teenagers and in young adults it may be seen in those who play contact sports, and often associated with sickle cell anaemia (genetic blood disorder where the red blood cells assume abnormal and rigid shape). In adults, predisposing factors include advanced age, immunodeficiency, chronic bacteraemia, intravenous drug abuse, long-term indwelling catheters, and also sickle cell anaemia (Gentry 1997). In most cases it is usually originated by secondary infection where the bacteria gains access to the bloodstream subsequently seeding the bone. Infections can also reactivate from an inactive focus of haematogenous osteomyelitis developed during childhood (Brady, Leid et al. 2008).

Depending on the site of infection, two types of haematogenous osteomyelitis can occur: long bone and vertebral osteomyelitis. Haematogenous osteomyelitis of the long bone is mainly diagnosed in children occurring as a single focus in the area of the long bones, while vertebral osteomyelitis is most common in older persons (Greenwood 2007). In haematogenous osteomyelitis of the long bone, the infection begins in the metaphysis (wider portion of the long bone adjacent to the bone edges) where the blood supply is denser. The tibia, femur and humerus are frequently involved; from where the infection may spread involving
a larger part of the bone (Chan 1999). The blood flow in these areas is reduced allowing the bacteria to settle and colonize, further inducing an inflammatory response. However, because bone is a rigid structure, it does not allow swelling which greatly compromises the body immune defences. Therefore, following trauma a small hematoma is produced in the bone accompanied with vascular obstruction and consequent bone necrosis. As previously mentioned, the sequestrum is a group of necrotic bone cells formed after trauma. The scheme in Figure 1-13 is representative of a bone sequestrum formation; where (A) is healthy and (B) newly formed bone surrounded by (C) a contiguous layer of newly formed bone, the involucrum that delimits the (E) sequestrum. Abscess formation also takes place and it eventually ruptures into the surrounding soft tissues reaching the surface forming a sinus (D) (Morrissy and Haynes 1989). Finally sequestrum may detach from the surrounding tissues and migrate settling in another part of the bone.

![Bone sequestrum formation](image)

**Figure 1-13** Bone sequestrum formation. Healthy bone (A); New bone (B); Involucrum (C); Sinus (D); Sequestrum (E) (adapted from Darland's Medical Dictionary for Health Consumers © 2007 by Saunders)

- **Clinical Features**

In haematogenous osteomyelitis symptoms can present as fever, lethargy, tenderness over the infection site and decreased movement (Carek, Dickerson et al. 2001). In children sinus formation is not usually seen, but blood cultures are positive in more than half of patients (Lew and Waldvogel 1997). Symptoms are usually present for 3 weeks or less, but in some cases may persist for 1 to 3 months duration. In adults vertebral osteomyelitis is most commonly seen, and
most patients present with a constant pain that progress slowly. For reasons not fully understood, the cervical spine is often the site of infection in long-standing intravenous drugs abusers (Endress, Guyot et al. 1990).

Osteomyelitis secondary to a contiguous focus of infection

Osteomyelitis secondary to a contiguous focus of infection usually develops after trauma, bone surgery, or joint replacement. It can occur at any age and can involve any bone. However, in children, it occurs mainly as result of trauma or related surgery (Stott 2001), while in adults is more secondary to nosocomial contamination during surgical procedures (Wallace, Cinat et al. 1999). This is due to the fact that techniques for joint replacement have improved in the past number of years, and so the number of artificial joints implanted into patients inserted has increased dramatically. As a result of this the number of infections associated with prosthetic joints has also increased. Although the term contiguous focus implies that the infection stems from an adjacent soft tissue infection, chronic contiguous focus osteomyelitis can also begin as an acute infection, with the microorganisms being directly inoculated into the bone at the time of trauma (Healy and Freedman 2006).

- Clinical Features

In the initial stages osteomyelitis secondary to a contiguous focus of infection, patients usually present with pain, fever, swelling, and skin redness. However, during persistent chronic infection, fever drops, and pain and drainage from a sinus tract are often seen. Acute contiguous osteomyelitis infections after hip joint replacement may occur within the first few days or weeks after surgery, resulting directly from infected skin, subcutaneous tissue, or muscle. Chronic contiguous infections are usually diagnosed 6 to 24 months after surgery. Although most infections are probably introduced during surgery but remain quiescent, within sequestrum, for a long time (Laughlin, Armstrong et al. 1997).
**Osteomyelitis secondary to vascular insufficiency**

Osteomyelitis secondary to vascular insufficiency occurs most frequently in older patients with diabetes or severe vascular impairment (Lew and Waldvogel 2004) accounting for 30% of all osteomyelitis cases (Brown and Neumann 2004). In almost all cases, infection follows a soft tissue infection that spreads to bone; often, in the small bones of the feet in patients in whom soft tissue breaks down over weight-bearing or pressure-bearing areas (Southwick 2003). Contributing factors for osteomyelitis development involve the metabolic consequences of diabetes such as bone and soft-tissue ischemia (blood supply restriction); and a combination of neuropathy, atherosclerotic peripheral vascular disease, and repetitive trauma (Brady, Leid et al. 2008). Limb ischemia, combined with restricted blood circulation, impairs wound healing in foot ulcers and allows for the contiguous spread of infection which consequently may contribute to the development of gangrenous scenarios and anaerobic infections. In addition, peripheral vascular disease may compromise the efficacy of antibiotic therapy by preventing the accumulation of adequate drug levels in the infected tissues.

Osteomyelitis should be considered in all diabetic patients with deep or chronic foot ulcers or infections. Twenty-five percent of diabetic patients with superficial mild to moderate foot infections develop bone infections, from which over 50% will have osteomyelitis (Lipsky 1997). Moreover, debridement of the infected area is necessary, whereas two thirds of cases require bone resection or partial amputation (Ramsey, Newton et al. 1999).
Chapter 1 - General Introduction

Figure 1-14 - Foot X-rays films of an osteomyelitis patient. (A) March 1998 no obvious osteomyelitis; (B) September 1999, 5th toe is absent, destruction of the end of the 5th metatarsal, resorption of the end of the 4th metatarsal, destructive changes in the 4th proximal phalanx, and lack of cortical definition at the base of the proximal phalanx of the 2nd toe and the head of the 2nd metatarsal; (C) May 2000, shows demineralization and loss of bone mass with obvious inflammatory skin disintegration.
(http://www.circulatorboot.com/casehistory/case198.html)

- Clinical Features

Patients with generalized vascular insufficiency may present with an ingrown toenail, a perforating foot ulcer, cellulitis, or a superficial or deep wound infection. Fever and systemic signs of infection are not usually present (Brady, Leid et al. 2006). Diabetic patients present with either no pain (with advanced neuropathy) or excruciating pain (if bone destruction has been acute) (Southwick 2008).

1.6.3. Acute and Chronic Osteomyelitis

Upon bacterial colonization when active bone infection takes place, there are three possible outcomes. The infection may resolve, may become an inactive recurrent infection, or may become a chronic infection with associated progressive bone destruction. Osteomyelitis can be therefore classified according
to the clinical status related to the length of infection as acute, developing over days or weeks of disease onset, or chronic a long-standing infection that can last for months or even years.

**Acute osteomyelitis**

Acute osteomyelitis normally evolves over several days or weeks of disease onset (Jauregui 1995), presenting as a suppurative, or pus-forming infection accompanied by inflammation at the site of microbial colonisation (Dwivedi, Soni et al. 2011). Damage to bone matrix and compression and destruction of vasculature is also observed as the infection spreads to surrounding soft tissues, which can further exacerbate bone necrosis. Sequestrum may form and detach to form a separate infectious focus which, due to the lack of vasculature, is protected from immune cells and antibiotics (Lazzarini, Mader et al. 2004; Lew and Waldvogel 2004). Such areas of dead, infected tissues that are inaccessible to antimicrobials or the immune response can lead to a chronic persistence of the infection (Lazzarini, Mader et al. 2004).

**Chronic osteomyelitis**

Chronic osteomyelitis as opposed to acute is defined as long-standing infection that evolves over months or even years. In patients with acute haematogenous osteomyelitis, about 10% will progress to a chronic condition characterised by the persistence of microorganisms, low-grade inflammation, and the presence of sequestrum (Norden, Nelson et al. 1992; Caputo, Cavanagh et al. 1994; Jauregui 1995). Patients who are more susceptible to chronic osteomyelitis include those with relapses in the same area accompanied with fever (Norden, Nelson et al. 1992), patients with open fractures and those for whom therapy was delayed. The presence of an implant is also associated with chronic osteomyelitis, whereby antibiotic treatment is frequently ineffective, and removal of the implant and debridement is often required (Ciampolini and Harding 2000). Relapsing cases of osteomyelitis episodes have been documented with
reactivation fifty or even eighty years after the initial infection (Gallie 1951; Korovessis, Fortis et al. 1991; Greer 1993; Ciampolini and Harding 2000).

1.6.4. Osteomyelitis prevalence

Despite continued progress toward understanding its pathology and optimal management, osteomyelitis causes substantial morbidity and mortality worldwide (Haas and McAndrew 1996; Dwivedi, Soni et al. 2011). The overall incidence of osteomyelitis ranges between 1 in 5000 and 1 in 10,000 (Weichert, Sharland et al. 2008) and in the United States, neonatal prevalence is approximately of 1 case per 1,000. The prevalence of osteomyelitis after foot puncture can be as high as 16 % increasing between 30 and 40 % in diabetic patients (Zimmerli 2010).

Moreover the incidence of implant related osteomyelitis has increased over the past decade (Bozic and Ries 2005; Kurtz, Lau et al. 2008) with post-surgery infections occurring in approximately 1.2 % of primary surgery and 3 to 5 % following revisions (Kurtz, Lau et al. 2008; Bozic, Kurtz et al. 2009; Bozic, Kurtz et al. 2010; Urquhart, Hanna et al. 2010). As medical care improves also the aging population increases with increased demand for joint replacements. Therefore the total number of implanted related infections is estimated to rise from 17,000 to 266,000 per year by 2030 (Bozic and Ries 2005; Kurtz, Ong et al. 2007; Kurtz, Ong et al. 2007; Kurtz, Lau et al. 2008).

1.6.5. Osteomyelitis diagnosis

Histological examination

Diagnosis of osteomyelitis can be very difficult. The most important step in osteomyelitis diagnosis is to isolate the offending organisms so that the appropriate antimicrobial therapy can be chosen (Carek, Dickerson et al. 2001). The causative agent of osteomyelitis can be identified by histological examination, obtained from blood cultures, synovial fluid or by direct biopsy of
bone (Jacobson and Sieling 1987; Howard, Einhorn et al. 1994). Histological examination can be carried by needle biopsy where a hollow needle is used to draw tissue from the infected area; or through an open biopsy, a small surgical procedure in which an incision is made through the skin in order to expose the infected area from where a sample will be taken (Pollard, McCracken et al. 2004). However, material taken from an open infected area can give misleading results as the isolates may include non-pathogenic microorganisms that are colonising the site.

**Laboratories studies**

Although no laboratory test specifically indicates the diagnosis of osteomyelitis, standard laboratory indicators of inflammation such as the total white blood cell count, Erythrocyte Sedimentation Rate (ESR), and C-reactive protein, are all generally elevated (Kaplan 2005). Although it can be of clinical significance to monitor disease, ESR is considered of little value in the diagnosis of osteomyelitis, because increased results do not identify where the inflammation is in the body nor what is causing it, and also because it can be affected by other conditions besides inflammation (Perry 1996; Saadeh 1998; Lovell, Winter et al. 2006). C-reactive protein is synthesised by the liver in response to infection, and even tough testing for C-reactive protein is also non-specific, this test appears to be more reliable for follow-up of the response to treatment than ESR, as the concentration of C-reactive protein increases within hours of infection and returns to normal within a week after adequate treatment have begun in most cases (Unkila-Kallio, Kallio et al. 1994; (Lovell, Winter et al. 2006).

**Imaging procedures**

Plain film radiographs or X-rays are the simplest and most common diagnostic tool used for diagnosis and follow-up of the bone-skeletal disease (Figure 1-15). X-rays pass radiation through the body onto a film. The radiation is absorbed by bones but not by soft-tissue like muscle and fat. So despite its limitations this
method is used to check for narrowing or widening of joint spaces, bone destruction, and periosteal reaction (formation of new bone in response to infection). X-rays are also used to measure bone mass or the weight of the skeleton (bone densitometry) as the amount of bone in it determines its strength.

![Figure 1-15 - X-ray images of healthy and infected bone with osteomyelitis. Tibia and fibula of an osteomyelitis patient, before infection (left side) and after infection (right side) (source: Encyclopaedia Britannica Online. Web. 20 Sep. 2011. Credit: Goran C.H. Bauer).](image)

Computed tomography (CT) scan and Magnetic Resonance Imaging (MRI) are the most common ultrasound imaging methods. They are useful for the early diagnosis of osteomyelitis and allow simultaneous evaluation of bone and surrounding soft tissues (Kaiser and Rosenborg 1994; Mah, LeQuesne et al. 1994). Both CT and MRI have excellent resolution power and can reveal bone destruction as well as periosteal reaction, cortical destruction, articular damage, and soft-tissue involvement, even when conventional X-rays are normal.

CT produces cross-sectional views of parts of the body whereas the x-ray beam rotates around the patient who has been injected with a special dye to help the area of the body to reflect the x-ray beam. CT provides excellent definition of cortical bone and a fair assessment of the surrounding soft tissues. It is especially useful in identification of sequestrum and devitalized bone. However CT is prone to image degradation, owing to artefacts caused by the
presence of bone or metal, but is nevertheless useful for guiding needle biopsy (Santiago Restrepo, Gimenez et al. 2003).

An MRI scan uses radio waves and a magnet to produce cross-sectional views of specific parts of the body and provides detailed information about soft tissue, bone marrow and bone marrow tumors. Furthermore, MRI is particularly reliable in distinguishing normal from abnormal areas when surgery is being planned for diabetic patients with osteomyelitis (Sammak, Abd El Bagi et al. 1999). However, the expense of MRI precludes its use on a routine basis.

MRI is more useful than CT for soft-tissue assessment and so, more effective to use for early detection of infection. Although MRI it is not helpful in assessing the response to therapy, given the persistence of oedema despite microbiological cure (Berendt and Byren 2004).

Positron Emission Tomography (PET) is a nuclear imaging technique that produces a three-dimensional image in the body by detecting pairs of gamma rays emitted indirectly by a positron-emitting radio-nucleotide which is introduced into the body on a biologically active molecule. PET has been used as an imaging tool for infections of the bone (Robiller, Stumpe et al. 2000). PET with fluorine- 18-fluoro-D-deoxyglucose (FDG) as a biologically active molecule has also been shown to be superior to normal bone scans in diagnosing chronic osteomyelitis of the central skeleton (Guhlmann, Brecht-Krauss et al. 1998; Zhuang, Duarte et al. 2000). Moreover FDG-PET combined with CT scan appears particularly promising for delineation of lesions and their concomitant inflammatory or infectious activity (Robiller, Stumpe et al. 2000; Schmitz, Kalicke et al. 2000).

1.6.6. Treatment of osteomyelitis
Osteomyelitis treatment depends on the stage of the disease when diagnosed. Following establishment of the causative microorganism, treatment includes antibiotic therapy and in most cases, debridement of the dead bone (Gitelis and Brebach 2002). A balance between medical and surgical therapy is needed for
complete healing and total recovery from the disease state (Dougherty 1988; Snyder, Cohen et al. 2001).

**Antibiotic therapy**

Oral therapy brings obvious economic benefits: decreased hospital stay, reduced pharmacy and supply costs, no need for surgical insertion of a catheter, and thus decreased catheter-related complications. Nonetheless, intravenous antimicrobial therapy targeting the causative organism is also used as standard treatment for chronic osteomyelitis (Berendt and Byren 2004). Furthermore, antibiotic-impregnated acrylic beads have been used for local treatment of bone infections. Although the beads deliver a high concentration of antibiotic to the area of infection, bactericidal levels of antibiotic are present locally for only 2 to 4 weeks. Thus, the beads are to be used in conjunction with systemic antibiotics (Gitelis and Brebach 2002). Both biodegradable and non-biodegradable beads have been used, and both require surgical placement. Non-biodegradable beads must be surgically removed after 2 to 4 weeks while the biodegradable do not require surgical removal and provide local bactericidal concentrations for extended periods (Calhoun and Mader 1997; Kanellakopoulou and Giamarellos-Bourboulis 2000).

Early antibiotic therapy produces the best results, whereby antibiotics must be administered for at least 4 to 6 weeks to achieve an acceptable rate of cure (Mader, Shirliff et al. 1999; Lew and Waldvogel 2004). A summary of the antibiotic therapy for osteomyelitis patients is detailed below in Table 1-6.
Table 1-6 – Antibiotic treatment for osteomyelitis patients according to the respective infecting microorganism

<table>
<thead>
<tr>
<th>Infecting microorganism</th>
<th>Antibiotic of first choice</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> or coagulase negative methicillin sensitive staphylococci</td>
<td>Nafcillin, gatifloxacin or Clindamycin phosphate</td>
<td>(Norden, Bryant <em>et al.</em> 1986; Shirtliff, Calhoun <em>et al.</em> 2002; Kaplan 2005)</td>
</tr>
<tr>
<td><em>S. aureus</em> or coagulase negative methicillin resistant staphylococci</td>
<td>Vancomycin, rifampin</td>
<td>(Bailey, Rybak <em>et al.</em> 1991; Eady and Cove 2003; Cohen and Grossman 2004)</td>
</tr>
<tr>
<td>Streptococci (groups A and B ß-hemolytic organisms or penicillin-sensitive <em>Streptococcus pneumoniae</em>)</td>
<td>Penicillin G</td>
<td>(Bradley, Kaplan <em>et al.</em> 1998; Kaplan 2005)</td>
</tr>
<tr>
<td>Penicillin resistant <em>S. pneumoniae</em></td>
<td>Vancomycin</td>
<td>(Friedland, Paris <em>et al.</em> 1993; Naktin and DeSimone 1999)</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>Ampicillin or vancomycin oxazolidinone</td>
<td>(Dresser and Rybak 1998; Till, Wixson <em>et al.</em> 2002)</td>
</tr>
<tr>
<td><em>Serratia</em> species or <em>Pseudomonas aeruginosa</em></td>
<td>Ceftazidime</td>
<td>(Sheftel and Mader 1986)</td>
</tr>
<tr>
<td>Mixed aerobic and anaerobic organisms</td>
<td>Amoxicillin-clavulanate</td>
<td>(Gadepalli, Dhawan <em>et al.</em> 2006; Brook 2007)</td>
</tr>
</tbody>
</table>

Recently, few studies have investigated the treatment of osteomyelitis. This is most likely due to the heterogeneity of the causing pathogens and clinical situations, and also due to the timeline needed for disease follow up for results confirmation. Therefore, most recommendations for antibiotic treatment of osteomyelitis are based on the specific clinical scenario and medical opinion (Carek, Dickerson *et al.* 2001).

**Osteomyelitis related to implanted devices**

For implant-related staphylococcal osteomyelitis patients who have stable implants and symptoms of short duration, long-term treatment with the ciprofloxacin and rifampin has been successful (Zimmerli, Widmer *et al.* 1998). Moreover, the use of ofloxacin together with rifampin for 6 months has also been showed to be efficient in diabetic patients with mild to moderate foot lesions associated with osteomyelitic bones (Senneville, Yazdanpanah *et al.* 2001).
Acute haematogenous osteomyelitis

In acute haematogenous osteomyelitis, when diagnosed early and promptly administrated, the appropriate antibiotic therapy usually lasting 4 to 6 weeks, will be sufficient to treat infection (Berendt and Byren 2004). Children with acute osteomyelitis should receive two weeks of initial intravenous antibiotic therapy before the oral agent (Tetzlaff, McCracken et al. 1978; Mader, Mohan et al. 1997). Cure rates of 95% have been obtained in children administrated with intravenous antibiotic from 4 to 14 days and supplemented with oral therapy for 14 to 26 days (Unkila-Kallio, Kallio et al. 1994). Surgical debridement is rarely recommended in acute haematogenous osteomyelitis in children, however, when patients do not respond to antibiotic therapy, surgery must be considered together with additional and prolonged antibiotic therapy (Mader, Ortiz et al. 1996; Mader, Mohan et al. 1997). In adults with haematogenous osteomyelitis, abscesses drainage and surgical debridement are often necessary (Gentry 1997).

Chronic osteomyelitis

In chronic osteomyelitis, therapy generally couples antibiotic administration and surgical debridement. Depending on the type of chronic osteomyelitis, debridement of the necrotic area will be performed followed by the appropriate intravenous antibiotic administration during 2 to 6 weeks. Adequate debridement of the necrotic area is imperative for better results with the antibiotic therapy (Mader, Mohan et al. 1997; Carek, Dickerson et al. 2001).

Surgical therapy

As previously mentioned when antibiotic therapy fails, but also when the extent of infection and necrotic area requires, such in cases of chronic osteomyelitis, surgical debridement is essential for successful treatment. However, inadequate surgical debridement, regardless of antibiotic therapy, is the most common
cause of treatment failure as sequestered dead bone might serve as a locus for persistent infection (Patzakis, Wilkins et al. 1994).

During surgical therapy, following removal of the dead area it is necessary to fill the dead space created by debridement and re-establish the blood supply. Therefore, healthy bone grafts are commonly used to fill the wound and muscle used for the revascularization of the affected area. Antibiotic-impregnated beads are also used in order to prevent recurrence (Carek, Dickerson et al. 2001).

1.6.7. Osteomyelitis causative microorganisms
The pathogenic microorganisms isolated from patients with osteomyelitis are associated with the type of infection, age of the patient and with pre-disposing clinical condition (see Table 1-7). However, among the several infective microorganisms implicated in causing osteomyelitis, S. aureus is by far the most common, accounting for approximately 80 % of all cases of osteomyelitis (Cole, Dalziel et al. 1982; Ellington, Reilly et al. 1999; Lew and Waldvogel 2004). S. epidermidis is also an important cause of chronic osteomyelitis mostly related with prosthetic joint devices and intravascular catheters, and is commonly isolated from immuno-compromised patients, intravenous drug abusers, patients under immunosuppressive therapy, and premature newborns (Tacconelli et al., 1997; (Carek, Dickerson et al. 2001).
Table 1-7- Common infecting microorganisms recovered from different types of osteomyelitis and pre-disposing condition (Adapted from (Carek, Dickerson et al. 2001))

<table>
<thead>
<tr>
<th>Type of osteomyelitis</th>
<th>Pre-disposing condition</th>
<th>Infecting microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematogenous osteomyelitis (commonly monomicrobiotic)</td>
<td>Overall</td>
<td>S. aureus 60% to 90% Gram-negative bacteria 25% (Lew and Waldvogel 2004; Brady, Leid et al. 2008)</td>
</tr>
<tr>
<td>Newborn</td>
<td>none</td>
<td>S. aureus, S. epidermidis, Streptococcus agalactiae, Escherichia coli, Group B streptococci and Gram-negative bacilli (Song and Sloboda 2001; Lew and Waldvogel 2004; Brady, Leid et al. 2008)</td>
</tr>
<tr>
<td>Children</td>
<td>none</td>
<td>S. aureus, Streptococcus pyogenes, and Haemophilus influenzae (Song and Sloboda 2001; Lew and Waldvogel 2004; Brady, Leid et al. 2008)</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td></td>
<td>Salmonella (Paget, Gibofsky et al. 2005)</td>
</tr>
<tr>
<td>Urinary tract infection or instrumentation</td>
<td></td>
<td>S. aureus, Gram negative group B, Streptococci (Shirtliff ME 1999; Paget, Gibofsky et al. 2005)</td>
</tr>
<tr>
<td>Skin infection</td>
<td></td>
<td>S. aureus, Streptococci</td>
</tr>
<tr>
<td>Respiratory infection</td>
<td></td>
<td>Streptococci, Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Intravenous drug abusers or vascular catheters</td>
<td></td>
<td>Gram negative bacilli, Staphylococci, Pseudomonas aeruginosa, Serratia species and Candida species (Holzman and Bishko 1971; Sapico 1996; Lew and Waldvogel 1997)</td>
</tr>
<tr>
<td>Sickle cell anemia Patients</td>
<td></td>
<td>Salmonella species (Sadat-Ali 1998) and Streptococcus pneumoniae (Brady, Leid et al. 2008)</td>
</tr>
<tr>
<td>Immunodeficient patients</td>
<td></td>
<td>Candida species and Mycobacteria (Miller and Mejicano 2001; Malani, McNell et al. 2002)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>S. aureus and coagulase-negative staphylococci 75% (Mader, Ortiz et al. 1996).</td>
</tr>
<tr>
<td>Bone fractures</td>
<td></td>
<td>S. aureus, S. epidermidis, Gram negative bacilli (Laughlin, Armstrong et al. 1997)</td>
</tr>
<tr>
<td>Puncture wounds</td>
<td></td>
<td>Pseudomonas aeruginosa, S. aureus, anaerobes</td>
</tr>
<tr>
<td>Prosthetic joints</td>
<td></td>
<td>S. aureus, S. epidermidis</td>
</tr>
<tr>
<td>Skin ulcer</td>
<td></td>
<td>Staphylococci, Streptococci, Gram negative bacilli, Anaerobes</td>
</tr>
<tr>
<td>Sinusitis, Dental abscess</td>
<td></td>
<td>Streptococci, Anaerobes</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>Contiguous focus of infection (poli-microbiotic)</td>
<td></td>
<td>Staphylococci, Streptococci and Enterococcus species, Gram-negative bacilli, and anaerobes (Calhoun, Cantrell et al. 1988; Berendt and Byren 2004; Rao and Lipsky 2007)</td>
</tr>
<tr>
<td>Vascular Insufficiency</td>
<td></td>
<td>Aerobic, mixed Gram-positive and Gram-negative bacilli (Calhoun, Cantrell et al. 1988)</td>
</tr>
<tr>
<td>Diabetic foot</td>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>Chronic or previously treated infections</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.7. Staphylococci

1.7.1. Staphylococci biology

Staphylococci are round single cells with a diameter ranging from 0.5 to 1.5 µm. Structurally, they can be found in pairs, tetrads, short chains, but characteristically dividing in more than one plane forming grape-like irregular clusters (Figure 1-16). Bacteria from this genus are Gram-positive meaning that they are protected by a thick cell wall, composed of layers of peptidoglycan, teichoic acid and lipoteichoic acid (Schleifer and Kandler 1972; Ward 1981). Staphylococci are facultative anaerobes that grow most rapidly under aerobic conditions and in the presence of CO₂ (Tegmark, Morfeldt et al. 1998). These microorganisms are also characterized as non-motile, non-sporulating, catalase-positive and highly salt and lipid-tolerant. In addition, Staphylococci can be distinguished from other organisms by their ability to grow on mannitol agar.

1.7.2. History and division into S. aureus and S. epidermidis

Staphylococci were first seen by Koch in 1878 in human pus. Koch recognized that the presence of Gram-positive cocci clusters it was correlated with abscesses diseases. In 1880, the Scottish surgeon Alexander Ogston also verified that a cluster forming coccus was the cause of certain pyogenic abscesses. Shortly thereafter, in 1882, Ogston named that microorganism as Staphylococcus which became the name of the genus and derives from the Greek words staphyle, bunch of grapes and kokkus meaning berry or grain. The name of the genus correlates directly to its morphology; therefore, on microscopical examination, Staphylococci are presented as spherical grains organized in grape-like irregular clusters (Figure 1-16).
like clusters. Two years later, Rosenbach distinguished two different species inside the genus based on the colonies pigmentation and the correspondent pathogenicity, whereby the golden colonies were the most pathogenic species and the white colonies less pathogenic. These were formally named as *S. aureus* and *Staphylococcus albus*, respectively, from the Latin "aureus" meaning golden and "albus" meaning white. Since then, *S. albus* was renamed to *S. epidermidis*, and today around 36 species of staphylococci are recognised (Götz 2006). The measure of pathogenicity it was based on the bacteria ability to cause blood plasma clots via the expression of protein coagulase. Only a few staphylococcal species are coagulase-positive and in clinical practice, *S. aureus* is the only coagulase-positive species colonising humans. However, it is now known that this division into coagulase-positive and negative can be misleading in some cases, as some natural isolates of *S. aureus* are defective in coagulase and as there is no direct evidence of coagulase as a virulence factor (Foster 1996).

Staphylococci are normally found on skin and mucous membranes of mammals. *S. epidermidis* is found on the skin of all humans and *S. aureus* is found in the nose of more than 30% of the healthy human population (van der Mee-Marquet, Achard et al. 2003).

### 1.8. *S. aureus*

*S. aureus* it was the first member of the Staphylococci genus to be identified, and as mentioned previously, is distinguished from the other staphylococci species on the basis of their golden colonies (Figure 1-17) and positive results of coagulase (Crossley and Archer 1997). *S. aureus* is persistently carried by 30% of the population and transiently found in 70% (von Eiff, Becker et al. 2001; Kluytmans and

![Figure 1-17 - Coloured scanning electron micrograph (SEM) of *S. aureus* bacteria. Credit: Juergen Berger, Science Photo Library.]
Wertheim 2005). S. aureus causes a very broad range of human diseases with many clinical manifestations. Infections associated with this microorganism include endocarditis, folliculitis, food poisonings, meningitis, osteomyelitis, scalded skin syndrome, skin abscesses and suppurative arthritis; and is therefore, considered the most virulent of the staphylococci, and consequently the main pathogen (Novick 1990; Lowy 1998; Crossley 2009).

1.8.1. S. aureus virulence factors
The ability of S. aureus to colonise and survive within the host relies on its ability to express a large number of extracellular and cell-associated factors which contribute to its virulence and success as a pathogen.

1.8.1.1. Surface proteins
S. aureus expresses on its surface a wide range of cell-wall-associated proteins that provide attachment to various structures and surfaces. Some of them have been showed to play a central role in the colonization of the host and implanted biomaterials during invasive disease (Patti, Allen et al. 1994; Waldvogel 2000). Also called adhesins, these proteins share some structural characteristics: including a ligand-binding domain at the N terminal that is exposed on the bacterial surface enabling these proteins to function as adhesins; and at the C-terminal end, a hydrophobic region spanning the membrane with an LPXTG motif (Leu-Pro-X-Thr-Gly) that extends into the cytoplasm and allow it to be retained in the cell wall (Foster and McDevitt 1994). The LPXTG motif is recognized by sortase A (SrtA), a membrane-associated enzyme that cleaves the motif between the threonine and glycine residues. The liberated carboxyl group of threonine then forms an amide bond with the amino group of the peptidoglycan, tethering the C-terminus of the protein to the bacterial peptidoglycan (Schneewind, Fowler et al. 1995; Mazmanian, Liu et al. 1999; Ton-That and Schneewind 1999). S. aureus mutants defective in expressing sortase A, fail to anchor several of the
surface proteins on the bacterial cell wall, these strains have been shown to be less virulent in animal models of *S. aureus* infection (Mazmanian, Liu *et al.* 2000). Some of the *S. aureus* surface proteins have been designated as microbial-surface components recognizing adhesive matrix molecules (MSCRAMM) for their ability to recognize and bind to extracellular proteins, such as collagen, fibrinogen and fibronectin (Ponnuraj, Bowden *et al.* 2003). The best characterised are protein A (SpA) (Forsgren and Forsum 1970; Uhlen, Guss *et al.* 1984; Hartleib, Kohler *et al.* 2000), two fibronectin-binding proteins (Fnbps), fibronectin protein A (FnbpA) and fibronectin protein B (FnbpB) (Signas, Raucci *et al.* 1989; Jonsson, Signas *et al.* 1991; Greene, McDevitt *et al.* 1995), the fibrinogen binding proteins clumping factors A and B (ClfA and ClfB) (McDevitt, Francois *et al.* 1994; Ni Eidhin, Perkins *et al.* 1998) and a collagen binding protein (Cna) (Patti, Jonsson *et al.* 1992). Other MSCRAMMs include three members of the serine aspartate family (SdrC, SdrD and SdrE) (Josefsson, McCrea *et al.* 1998) and the bone sialoprotein-binding protein (Bbp) (an allelic variant of SdrE) (Tung, Guss *et al.* 2000).

- **Protein A**

*S. aureus* protein A was the first *S. aureus* surface-associated protein to be characterised (Forsgren and Sjoquist 1966). Since its discovery, it has become the best studied and most widely used surface protein of Gram-positive bacteria.

SpA is expressed at high levels in 97 % of all *S. aureus* strains, (Forsgren and Forsum 1970; Fomenko 1980; Sanford, Thomas *et al.* 1986) and has been recognized as an important virulence factor of *S. aureus* (Palmqvist, Foster *et al.* 2002; Fournier and Philpott 2005).

SpA is a 42 KDa protein composed of five Immunoglobulin (Ig)-G (IgG) binding domains (E - C) lying at the N-terminus end followed by regions of the wall spanning region (W), membrane spanning region (M) (Figure 1-18) and the LPXTG motif at the C-terminus attaches SpA covalently to peptidoglycan layer following sortase cleavage (Uhlen, Guss *et al.* 1984).
Chapter 1- General Introduction

Figure 1-18 - Structural organization of SpA. S, Represents the signal sequence; E, D, A, B, C are the five IgG binding domains; Wr and Wc are the wall spanning regions; and M represents the membrane spanning region (adapted from (Burman, Leung et al. 2008)).

Structural analysis of a single domain revealed that each one of SpA domains comprise a triple helical bundle (Figure 1-19) (Gouda, Torigoe et al. 1992; Zhou and Karplus 1999; Zhou and Karplus 1999). SpA has been shown to interact with different immunoglobulins, such as IgG 1, 2 and 4, IgA, IgM and IgE (Deisenhofer 1981; Inganas 1981; Langone 1982; Sasso, Silverman et al. 1991).

Figure 1-19 - Zhou and Karplus simulation of the folding of SpA that forms a triple helix bundle structure. (Zhou and Karplus 1999)

In serum, each one of SpA domains has the ability to bind the Fc region of IgG (Figure 1-20) (Uhlen, Guss et al. 1984). This SpA- IgG interaction impedes neutrophils to recognize IgG- Fc receptor therefore disrupting opsonisation and phagocytosis. In vitro experiments with S. aureus mutants lacking SpA have been shown to be more efficiently phagocytised by neutrophils than the WT (Gemmell, Tree et al. 1991). Moreover in vivo studies with a murine model of S. aureus arthritis infection and experimental infection of mice with S. aureus WT and SpA defective mutants suggested that SpA enhances S. aureus virulence
(Patel, Nowlan et al. 1987; Palmqvist, Foster et al. 2002). Furthermore, each individual domain of SpA has also the ability to interact with B lymphocytes via the \(V_H^3\) subclass of the Fab region of IgM molecules (Jansson, Uhlen et al. 1998) exposed on their surface and thus inducing B lymphocytes to proliferate and undergo apoptosis resulting in a significant depletion of the antibody secreting B cell repertoire (Figure 1-21) (Goodyear and Silverman 2004; Viau and Zouali 2005).

In addition, SpA also binds to von Willebrand factor (vWf) which main function is to interact with platelets via Gplb-\(\alpha\) receptor and immobilize them at the site of blood vessel damage stimulating clot formation. Thus when interacting with the vWf (A1 domain), SpA mediates the adherence between \(S.\ aureus\) and platelets or to damaged blood vessels (O'Seaghdha, van Schooten et al. 2006).

Finally, SpA has been shown bind to Tumor Necrosis Factor Receptor-1 (TNFR-1) inducing Tumor Necrosis Factor alpha (TNF-\(\alpha\)) responses in epithelial cells and thus acting directly as an immune effector in the lungs and respiratory system (Gomez, Lee et al. 2004; Gomez, O'Seaghdha et al. 2006). In vivo studies demonstrated that TNFR-1 null mice are not susceptible to \(S.\ aureus\) pneumonia.
and *S. aureus* protein A defective mutants of do not cause infection in wild-type animals, emphasizing the role of SpA-TNFR-1 interaction in the pathogenesis of pneumonia.

- **S. aureus** binder IgG (Sbi)

Sbi is a more recently discovered immunoglobulin binding protein from *S. aureus* and therefore denominated *S. aureus* binder IgG. Sbi has also been shown to be expressed at high levels in *S. aureus* strains 8325-4 and Newman (Zhang, Jacobsson *et al.* 1998). This protein is 45 KDa long and shares high homology with SpA via identical IgG binding properties. However, whereas SpA has the ability to interact with both Fc and Fab regions of IgG, on the other hand, Sbi only interacts with the IgG Fc region (Atkins, Burman *et al.* 2008). Moreover, Sbi lacks the LPXTG cell wall anchoring sequence, but it does have a predicted proline-rich cell wall spanning sequence (Wr) (Figure 1-22) and it has also been suggested to be attached to the *S. aureus* surface through electrostatic interactions (Zhang, Jacobsson *et al.* 1998; Bouma, de Groot *et al.* 1999).

![Figure 1-22 - Structural organization of Sbi. S, represents the signal sequence; I, II, III and IV are the IgG binding domains; Wr, is the predicted cell wall spanning proline-repeat region; and Y, is the C-terminal tyrosine-rich region. Extracellular domains are represented in grayscale (adapted from (Burman, Leung et al. 2008)).](image)

Sbi has been shown to interact with the binding protein β2-glycoprotein I (β2-GPI) also termed apolipoprotein H. β2-GPI is a plasma protein reported to have varied biological functions when bound to negatively charged lipids, such as promoting clearance of liposomes and foreign particles from the bloodstream (Chonn, Semple *et al.* 1992), and also in the inhibition of coagulation by affecting the activity of pro-thrombinase in platelets (Nimpf, Bevers *et al.* 1986). It is therefore
suggested that *S. aureus* benefits from the Sbi-β2-GPI interaction in the attachment to host cells for intracellular growth and by inhibition of clot formation.

Recent studies show that Sbi is secreted from the *S. aureus* cell wall surface and binds to the human serum factor H. This interaction is enhanced in the presence of complement component C3 forming a tripartite complex. The complement component C3 binds to specific molecules of the immune system, being this interaction crucial in the induction of immunological memory and optimal antibody response (Nielsen and Leslie 2002; Rickert 2005). Therefore, formation of the tripartite complex Sbi-factor H-C3 will disrupt the natural complement activation of the immune response activated following infection, thus providing *S. aureus* a mechanism for evasion (Burman, Leung *et al.* 2008; Haupt, Reuter *et al.* 2008).

- **Fibronectin binding proteins A and B (FnbpA and FnbpB)**

Fibronectin is a high molecular weight glycoprotein (approximately 440 KDa) found in the plasma and body fluids such as cerebrospinal fluid and urine and also on many cell surfaces and extracellular matrix (Yamada and Olden 1978). Fibronectin is involved in several processes via direct interaction with different proteins such as collagen, heparin and fibrin. These processes include cell binding, spreading and cell alignment (Yamada and Olden 1978); growth, differentiation and migration processes (Tamura, Gu *et al.* 1998), including embryogenesis (George, Georges-Labouesse *et al.* 1993; Francis, Goh *et al.* 2002), wound healing (Herard, Pierrot *et al.* 1996), blood coagulation, host defence (Magnusson and Mosher 1998) and metastasis (Akiyama, Olden *et al.* 1995).

*S. aureus* binding to fibronectin is mediated by two related fibronectin-binding proteins, the FnbpA and FnbpB, expressed by most strains and encoded by two closely related but independently transcribed genes (Jonsson, Signas *et al.* 1991; Greene, McDevitt *et al.* 1995). The fibronectin proteins, as other MSCRAMMs,
are anchored to the staphylococcal cell wall at the C-terminal LPXTG motif (Figure 1-23) and the ability to interact with fibrinogen, was first attributed to FnbpA repeat region D (Wann, Gurusiddappa et al. 2000).

![LPXTG Diagram](image)

Figure 1-23 - Structural organization of FnbpA. S represents the signal sequence; W, represents the wall-spanning region; and M represents the membrane-spanning region and positively charged residues. LPXTG motif and the A-, B-, C- and D-domains are indicated, whereas the D-domains are the ligand-binding domains (Foster and Hook 1998; Fischetti 2000).

The FnbpA and FnbpB proteins have been shown to be crucial factors for the initiation of foreign body infection. *In vitro* studies with *S. aureus* mutants lacking the expression of both FnbpA and FnbpB showed completely defective binding to polymethylmethacrylate coverslips that had been coated with fibronectin and to coverslips that had been removed from tissue cages implanted subcutaneously in guinea-pigs. These results demonstrated that *S. aureus* Fnbps promote binding to fibronectin in both plasma clots and to *ex vivo* biomaterial that has been in long-term contact with the host (Greene, McDevitt et al. 1995; Vaudaux, Francois et al. 1995). In addition Fnbps also play a role in the adherence and invasion of mammalian cells, including epithelial (Sinha, Francois et al. 1999) and endothelial cells (Peacock, Foster et al. 1999). They recruit and bind fibronectin, by recognition of the host fibronectin receptor integrin α5β1 triggering the uptake of *S. aureus* by the host cells and thereby providing an evasion mechanism of the host immune system facilitating bacterial persistence (Peacock, Foster et al. 1999; Sinha, Francois et al. 1999).

The ability of *S. aureus* Fnbps to bind fibronectin is crucial for *S. aureus* pathogenicity. The use of exogenous administration of recombinant fibronectin-binding domain has been showed to block this interaction therefore reducing the risk of staphylococcal abscess formation, preventing wound infection (Menzies,
Kourteva et al. 2002). Also, an S. aureus isolate from a murine sepsis model whereas the gene encoding for Fnbps was knockdown demonstrated that the Fnbps are responsible for mediating systemic inflammation via IL-6 secretion, severe weight loss and mortality (Palmqvist, Foster et al. 2005). Moreover, studies with S. aureus clinical infective endocarditis isolates showed complete inhibition of platelet activation by the use of monoclonal antibodies specific to Fnbps. The same study demonstrated the formation of a bridge between S. aureus Fnbps and fibronectin which in turn interacts with the platelet receptor Fcy RIIa inducing platelet activation (Fitzgerald, Loughman et al. 2006).

**Collagen binding protein**

Collagen is the major insoluble fibrous protein in the extracellular matrix and in connective tissue, being the most abundant protein in mammals (Kreis and Vale 1999). There are several types of collagen, however, approximately 80 to 90% of the collagen in the body consists of types I, II, and III (Lodish 1999). The various collagens types and structures and the interactions between collagen and non-collagen molecules regulate the architecture of several tissues helping in withstanding and stretching (Heinegard, Hultenby et al. 1989; Lodish 1999). S. aureus collagen binding protein Cna mediates bacterial binding to collagen substrates and collagenous tissues (Switalski, Speziale et al. 1989; Patti, Jonsson et al. 1992). The structural organization of Cna is represented in Figure 1-24.

![Figure 1-24 - Structural organization of Cna.](image)

S, represents the signal sequence; W, represents the wall-spanning region; and M represents the membrane-spanning region and positively charged residues. The positions of the LPXTG motif and A and B- domains are indicated, whereas the A domain is the ligand binding domain (Foster and Hook 1998; Fischetti 2000).
In vitro experiments have demonstrated that the cna gene is both necessary and sufficient for the binding of S. aureus to cartilage (Switalski, Patti et al. 1993). Cna binding was shown to be a virulence factor in a rat experimental model of endocarditis and in a mouse septic arthritis model (Patti, Bremell et al. 1994). However, there was some contradictory data published from two separate studies. In one, nearly all of the strains causing infection were Cna positive (Switalski, Patti et al. 1993) while in the other study, the proportion of Cna positive was no greater in strains causing infection than in the control sample. The last one indicating that collagen binding is probably not a pre-requisite for these types of infection and also that the cna gene is only found in approximately 50% of the S. aureus strains (Ryding, Flock et al. 1997; Smeltzer, Gillaspy et al. 1997). Nevertheless, one year later, vaccination with Cna recombinant A domain, previously identified as Cna ligand-binding domain (Patti, House-Pompeo et al. 1995), was reported to provide protection against S. aureus-mediated septic death (Nilsson, Patti et al. 1998).

- **Fibrinogen binding proteins- clumping factors A and B**

Fibrinogen is the main protein of the blood coagulation system. It is 340 KDa large, composed of two identical subunits that contain three polypeptide chains: α, β and γ (Doolittle 1984; Ruggeri 1993). Fibrinogen is the most abundant ligand for platelet interaction via the αIIbβ3 integrin triggering platelet aggregation and formation of platelet-fibrin thrombi in vivo (Hawiger 1995). Fibrinogen clotting underlies the pathogenesis of thrombo-embolism and thromboses of arteries and veins.

S. aureus is known to form clumps in the presence of blood plasma, this is due to its ability to bind fibrinogen (Hawiger, Timmons et al. 1982). Fibrinogen is present in large amounts at wound sites and is quickly deposited on synthetic surgical material such as catheters (Foster and McDevitt 1994). Therefore, binding to fibrinogen is seen as a major primary virulence factor in S. aureus, especially in infections due to the presence of foreign bodies.
S. aureus ClfA and ClfB have been primarily shown to be responsible for promoting cell clumping in the presence of soluble fibrinogen and to mediate adherence to immobilized fibrinogen and to ex-vivo biomaterial (McDevitt, Francois et al. 1994; Ni Eidhin, Perkins et al. 1998). However, S. aureus Fnbps can also promote cell-clumping in a solution of fibrinogen if expressed at sufficiently high levels (Wann, Gurusiddappa et al. 2000).

The ClfA and ClfB are structurally related but they recognise different parts of the host ligand and might act synergistically allowing S. aureus to adhere more firmly to thrombi under flow conditions in the bloodstream. ClfA defective isogenic mutants have been shown to act as a virulence factor in a rat model of endocarditis and in a murine model of septic arthritis (Vaudaux, Francois et al. 1995; Josefsson, Hartford et al. 2001). The clfA gene is present in almost all S. aureus strains (Peacock, Moore et al. 2002), and its virulence has been related to its potent anti-phagocytic activity, whereas the A domain of ClfA strongly interferes with the binding of leukocytes to fibrinogen (Figure 1-25) (Vernachio, Bayer et al. 2003). The ClfB is only expressed during the exponential phase of growth under aerobic conditions (Ni Eidhin, Perkins et al. 1998) and despite of its fibrinogen binding function it has also been shown to promote in vitro adherence to immobilized epidermal cytokeratins and therefore playing a major role in the S. aureus nasal colonization (O’Brien, Walsh et al. 2002; Wertheim, Walsh et al. 2008).
Figure 1-25 - Structural organization of *S. aureus* ClfA and ClfB. S, represents the signal sequence; W, represents the wall-spanning region; R, represents the Ser-Asp dipeptide repeats and M represents the membrane-spanning region and positively charged residues. The positions of the LPXTG motif and the A domain are indicated, whereas the A domain is the ligand binding domain (Foster and Hook 1998; Fischetti 2000).

- **Sdr family of proteins**

  The ClfA and ClfB are also members of Sdr family of proteins, these are structurally related *S. aureus* proteins characterized by the presence of the R-domain containing the tandem repeats of serine (Ser) and aspartic acid (Arp) (SD) dipeptides, however the number of SD repeats is variable from protein to protein (Josefsson, McCrea *et al.* 1998). *S. aureus* SdrC, SdrD and SdrE have a comparable structural organization (Figure 1-26), however, they are not closely related, with only 20 to 30% identical amino acid residues suggesting that different Sdr proteins might have different roles in *S. aureus* pathogenicity. Some studies in another *S. aureus* protein from the Sdr family of proteins, the bone sialoprotein-binding protein (Tung, Guss *et al.* 2000), which is an allelic variant of the SdrE protein, have demonstrated that despite their similar structure, SdrE is not involved in binding to bone sialoprotein (McCrea, Hartford *et al.* 2000). The Sdr proteins have two, three, or five additional motifs that are tandemly repeated in SdrC, SdrE, and SdrD, respectively. These motifs bind Ca$^{2+}$ with high affinity and their structure unfolds when calcium ions are removed, still their function remains unclear. In all *S. aureus* strains at least two *sdr* genes are present (Josefsson, McCrea *et al.* 1998) and these always include the *sdrC* gene (Peacock,
Moore et al. 2002). Therefore, the lack of sdr genes must be explained by the absence of sdrD or sdrE. Moreover, some studies described a strong correlation between S. aureus invasiveness and the presence of one of the allelic variants of the sdrE gene (Peacock, Moore et al. 2002). In addition, other studies reported a significantly higher prevalence of the sdrD gene in S. aureus strains isolated from bone infections (Trad, Allignet et al. 2004).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sdr.png}
\caption{Structural organization of S. aureus SdrC, SdrD and SdrE. S, represents the signal sequence; W, represents the wall-spanning region; R, represents the Ser-Asp dipeptide repeats and M represents the membrane-spanning region and positively charged residues. The positions of the LPXTG motif and the A and B domains are indicated (Foster and Hook 1998;)
\end{figure}

1.8.1.2. Capsule

The capsule or capsular polysaccharide is an important component of the S. aureus surface. In 1931, Isabelle Gilbert reported that production of capsular polysaccharide by S. aureus covered approximately 90 % of the S. aureus isolates (Gilbert 1931; Hochkeppel, Braun et al. 1987). S. aureus can produce 11 different capsular serotypes (Sompolinsky, Samra et al. 1985). The serotypes 5 and 8 are the most common, being found in respectively, 16 to 26 % and 55 to 65 % of all S. aureus human isolates (Arbeit, Karakawa et al. 1984; Sompolinsky, Samra et al. 1985; Hochkeppel, Braun et al. 1987; Boutonnier, Nato et al. 1989), and accounting for 75 % of human infections (Lee 1996; Lowy 1998). The capsule forms a layer on the outer surface of the peptidoglycan layer in the bacterial cell wall. Its function is not clear but according to several reports it is thought to
enhance staphylococcal virulence by impairing phagocytosis (Peterson, Wilkinson et al. 1978; Xu, Arbeit et al. 1992). In an endocarditis model it was suggested that the expression of capsular polysaccharide impeded colonization of damaged heart valves by *S. aureus* (Foster 1996). Moreover, in a mouse model of arthritis, mice inoculated with positive capsular polysaccharide strains had a higher incidence of arthritis and a more severe form of the disease than animals inoculated with the non-encapsulated mutant strain (Nilsson, Lee et al. 1997). Furthermore, *in vitro* experiments demonstrated that *S. aureus* capsule modulates adherence to endothelial cells promoting colonisation and persistence on mucosal surfaces *in vivo* (O'Riordan and Lee 2004).

The use of capsular polysaccharide vaccines serotype 5 and 8 in treating *S. aureus* infections has been reported (Fattom, Schneerson et al. 1990; Fattom, Sarwar et al. 1996; Tollersrud, Zernichow et al. 2001; Shinefield, Black et al. 2002) although the differential expression on capsular serotypes narrows their use and effectiveness and the inclusion of other antigens is essential for the development of an effective *S. aureus* vaccine.

### 1.8.1.3. Extracellular toxins

*S. aureus* produces several different types of toxins grouped on the basis of their mechanisms of action that are responsible for symptoms during infections.

Cytotoxins cause pore formation and induce pro-inflammatory events leading to cellular damage, thereby contributing to manifestations of the sepsis syndrome (Bhakdi and Tranum-Jensen 1991; Walev, Reske et al. 1995).

- **Alpha-toxin (α-toxin)** is a 34 kDa toxin composed of 293 amino acids (Bernheimer 1965) and is the best characterized and most potent cytotoxin of *S. aureus*. It is a cytolytic pore-forming toxin secreted by almost all *S. aureus* strains into the extracellular environment as monomer that binds to the membrane of susceptible cells (Bhakdi and Tranum-Jensen 1991). Susceptible cells have a specific receptor for α-toxin which allows the monomeric toxin to oligomerise.
forming a ring-shaped heptameric waterfilled transmembrane pore (Valeva, Weisser et al. 1996; Fischetti 2000). In humans, platelets and monocytes are sensitive to α-toxin (Hildebrand, Pohl et al. 1991). They carry affinity sites which allow toxin to bind, following binding, a complex series of reactions triggers cytokines release leading to production of inflammatory mediators. These events are associated with the symptoms of septic shock that occur during severe infections caused by *S. aureus* (Novick 1990).

- **Beta-toxin (β-toxin)** is a catalytic enzyme secreted by *S. aureus* into the extracellular environment (Cohen and Van Heyningen 1982). β-toxin causes invaginations of selected regions of the host cell membrane and is also leukotoxic. In human monocytes, beta-toxin inhibits migration and stimulates the release of Interleukin (IL) 1β, IL-6 receptor and soluble CD14 (Walev, Weller et al. 1996). Impaired chemotaxis and Fc binding in neutrophils is also caused by beta-toxin. Although beta-toxin has been shown to contribute to *S. aureus* pathogenesis in a murine mastitis model (Foster, O'Reilly et al. 1988; O'Callaghan, Callegan et al. 1997) and in an ocular keratitis model (O'Callaghan, Callegan et al. 1997), its effects are thought to be less significant than those from alpha-toxin.

- **Gamma-toxin (γ-toxin) and Leukocidin** are bicomponent toxins that damage membranes of susceptible cells. These toxins are expressed separately but act together to damage membranes. The Panton and Valentine (PV) leukocidin is released by some *S. aureus* strains and is strongly associated with severe skin infections such as furunculosis and is active against both human and rabbit leukocytes (Prevost, Cribier et al. 1995). On the other hand, γ -toxin is active against erythrocytes, and is thought to attack cells by inducing pore formation (Ozawa, Kaneko et al. 1995).
1.8.1.4. Epidermolytic toxin

*S. aureus* expresses two distinct forms of the epidermolytic or exfoliative toxin. Both have been implicated in Staphylococcal Scalded Skin Syndrome (SSSS) in neonates and children with skin erythema and loss of epidermis. The SSSS is characterized by blister formation and a widespread of epidermal splitting (Bailey, Lockhart *et al.* 1995). It is possible that these toxins target a very specific protein which is involved in maintaining the epidermis integrity, however, their mechanism of action remains controversial (Cribier, Prevost *et al.* 1992).

1.8.1.5. Superantigens

*S. aureus* expresses two different types of superantigen activity toxins: enterotoxins, of which there are approximately nine serotypes and the Toxic Shock Syndrome Toxin-1 (TSST-1) (Ulrich, Bavari *et al.* 1995). The enterotoxins are responsible for staphylococcal food poisoning causing nausea, diarrhea and vomiting (Harris, Grossman *et al.* 1993). The TSST-1 is responsible for 75% of Toxic Shock Syndrome (TSS), whereas patients present with fever, rash and desquamation during convalescence and have involvement of at least three additional organs systems. TSS can be caused by *S. aureus* TSST-1 expressing strains colonising the vaginal or cervical mucosa (menstrual form); or mediated by either TSST-1 or enterotoxins affecting other areas in the body (non-menstrual). These superantigens can bind to Major Histocompatibility Complex (MHC) class II proteins and stimulate T cells non-specifically, inducing massive cytokines release, causing the symptoms of toxic shock syndrome and certain autoimmune diseases (Marrack and Kappler 1990; Schlievert 1993). These toxins are known for their potency and severely debilitating effects (Ler, Lee *et al.* 2006) and are effective at picogram per litre concentration (Fraser and Proft 2008).
1.8.1.6. **Cell Wall**

The staphylococcal cell wall is mainly composed of peptidoglycan and teichoic acid making up, respectively, about 50 to 60% and 30 to 50% of the dry weight (Figure 1-27) (Schleifer and Kandler 1972). Peptidoglycan is therefore, the main structural polymer in the wall and consists of alternating polysaccharide subunits of β-1,4 linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). The peptidoglycan chains are cross-linked by tetrapeptide chains bound to N-acetylmuramic acid and by a pentaglycine bridge specific for *S. aureus* (Schleifer 1973). The cell-wall teichoic acid of *S. aureus* is a water-soluble polymer covalently linked to the peptidoglycan acid. The peptidoglycan biological activities have been associated with inflammatory skin reaction, stimulation of cytokines release by macrophages, inhibition of leukocyte migration and adjuvant and complement activation (Franken, Seidl *et al.* 1984). The major cell wall component of *S. aureus*, has also been reported to cause pro-inflammatory responses and platelet aggregation in the host, leading to disseminated intravascular coagulation in cases of severe septicaemia (Kessler, Nussbaum *et al.* 1991) and to induce tissue factor expression in monocytes activating the coagulation cascade (Mattsson, Herwald *et al.* 2002). *S. aureus* peptidoglycan is also recognized by the toll-like receptor 2 (TLR2) (Yoshimura, Lien *et al.* 1999; Kyburz, Rethage *et al.* 2003). In fewer amounts there is also the lipoteichoic acid (LTA) a glycerol phosphate polymer linked to a glycolipid terminus anchored in the cytoplasmic membrane, that has been reported to activate immune cells via TLR2, lipopolysaccharide (LPS)-binding protein (LBP) and CD14 (Morath, Stadelmaier *et al.* 2002; Schroder, Morath *et al.* 2003).
1.9. **S. epidermidis**

*S. epidermidis* (Figure 1-28) is the most frequently isolated species of coagulase-negative staphylococci. It is distinguished from *S. aureus* for its inability to produce coagulase and also by their characteristic grey to greyish-white colonies as opposed to the yellowish *S. aureus* colonies (Balows 1992).

For a long time, *S. epidermidis* was considered as a non-pathogenic organism, but it is now recognised as the most widespread and persistent microorganism colonizing the skin and mucous membranes of the human body, representing a major part of the human micro-flora accounting for 65 to 90% of all staphylococci isolated from these environments (O'Gara and Humphreys 2001; Vuong and Otto 2002). *S. epidermidis* has become the most important pathogen associated with nosocomial bloodstream infections, cardiovascular infections and foreign body device infections (Kloos and Bannerman 1994; Vuong and Otto 2002). In contrast to *S. aureus*, *S. epidermidis* does not usually cause pyogenic infections due to its distinctly reduced arsenal of toxins compared to *S. aureus*. Infections caused by *S. epidermidis* are often persistent and relapsing, however, *S. epidermidis* requires a predisposed host in order to cause infection, and therefore it is described as an opportunistic pathogen. Compromised patients, such as drug abusers and immuno-compromised patients and also premature newborns are the main risk group, whereas the main port of entry for these infecting microorganisms is the intravascular devices (Tacconelli, Tumbarello *et al.* 1997; Domingo and Fontanet 2001).

![Figure 1-28 - Scanning electron microscopy of *S. epidermidis* arranged in grape-like clusters. Credit: David Scharf, Science Photo Library](image-url)
Chapter 1 - General Introduction

1.9.1. **S. epidermidis virulence factors**

The success of *S. epidermidis* as a pathogen is associated with its ability to adhere and accumulate onto surfaces, under the cover of a self-produced extracellular substance (Vuong and Otto 2002). This process is referred to as biofilm formation and is considered as one of the important pathogenic factors of *S. epidermidis* in indwelling devices infections (Raad, Alrahwan et al. 1998). A summary of some of the *S. epidermidis* virulence factors and respective functions is showed below in Table 1-8.

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Attachment of the bacteria to the foreign material direct and via host matrix proteins</strong></td>
<td></td>
</tr>
<tr>
<td>AtlE</td>
<td>Abundant autolysin and adhesin that affects surface hydrophobicity and binds to fibrinogen, fibronectin and vitronectin</td>
</tr>
<tr>
<td>Aae</td>
<td>Autolysin and adhesin that binds to fibrinogen, fibronectin and vitronectin</td>
</tr>
<tr>
<td>SSP-1 and SSP-2</td>
<td>Present in fimbria-like appendages (unknown ligand)</td>
</tr>
<tr>
<td>Telchoic acids</td>
<td>Enhances binding to fibrinectin</td>
</tr>
<tr>
<td><strong>Attachment of the bacteria to the foreign material via host matrix proteins</strong></td>
<td></td>
</tr>
<tr>
<td>SdrF</td>
<td>Binds to collagen</td>
</tr>
<tr>
<td>SdrG</td>
<td>Binds to fibrinogen</td>
</tr>
<tr>
<td><strong>Biofilm formation</strong></td>
<td></td>
</tr>
<tr>
<td>PIA</td>
<td>Intracellular polysaccharide adhesin</td>
</tr>
<tr>
<td><strong>Extracellular proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Cysteine protease</td>
<td>Degradation of several host matrix proteins and components of the immune system</td>
</tr>
<tr>
<td>Accumulation-associated protein Aap</td>
<td></td>
</tr>
<tr>
<td>Lipases</td>
<td>Skin colonization</td>
</tr>
<tr>
<td>FAME</td>
<td>Detoxification of bactericidal fatty acids, destroys bactericidal properties</td>
</tr>
<tr>
<td><strong>Toxins</strong></td>
<td></td>
</tr>
<tr>
<td>Delta-toxin</td>
<td>Lysis of erythrocytes; Pro-inflammatory cytolysin</td>
</tr>
<tr>
<td><strong>Lantibiotics</strong></td>
<td></td>
</tr>
<tr>
<td>Lantibiotics</td>
<td>Anti-bactericidal activity</td>
</tr>
</tbody>
</table>

1.9.1.1. **Biofilm**

*S. epidermidis* biofilm formation is thought to protect the bacteria from host defence mechanisms and antimicrobial agents (Hoyle and Costerton 1991; Costerton, Stewart *et al.* 1999; Konig, Schwank *et al.* 2001; Stewart and Costerton 2001). Furthermore, *S. epidermidis* growth adapts to the biofilm mode,
with down-regulation of basic cell processes such as nucleic acid, protein and cell wall biosyntheses (Yao, Sturdevant et al. 2005). These changes may directly affect antibiotic activity in targeting *S. epidermidis* biofilms (Konig, Schwank et al. 2001).

In foreign device infections two different steps occur, firstly the attachment of the bacteria to the foreign material followed by biofilm formation (Mack, Nedelmann et al. 1994).

**Bacterial attachment to the foreign material**

The first stage of bacterial adherence to the foreign material is mediated by the physiochemical properties, hydrophobicity and surface charges of the foreign material (Vuong and Otto 2002). Within this first step, *S. epidermidis* can colonize the implanted material either by direct attachment of the bacterial cells to the foreign device, or by binding the host matrix proteins, previously coated onto the foreign material (Gottenbos et al., 2000).

When bacteria binds directly to the implanted material, a variety of factors such as the major autolysin E (AtlE) (Heilmann, Gerke et al. 1996; Heilmann, Hussain et al. 1997), the teichoic acids (Gross, Cramton et al. 2001; Vuong and Otto 2002) and the *S. epidermidis* surface proteins (SSP) 1 and 2 (Veenstra, Cremers et al. 1996; Fischetti 2000) can be involved.

On the other hand, when bacteria attaches to host matrix proteins previously coated on the implanted device, binding occurs via MSCRAMMs, considered significant virulence factors of *S. epidermidis*. The most important include the covalently anchored fibronectin binding protein (Fbe) also denominated serine-aspartate repeat protein G (SdrG) (Davis, Gurusiddappa et al. 2001; Hartford, O’Brien et al. 2001) that has been demonstrated to bind fibrinogen and the serine-aspartate repeat protein F (SdrF) that has been reported to bind collagen (Arrecubieta, Lee et al. 2007). Moreover, the non-covalently bound AtlE and autolysin-adhesin E (Aae) even though with a less-specific interaction, can also bind to fibrinogen, fibronectin and vitronectin (Heilmann, Hussain et al. 1997; Heilmann, Thumm et al. 2003). Finally the
teichoic acids for their ability to enhance binding to immobilized fibronectin (Hussain, Heilmann et al. 2001) have been also reported to play a role in S. epidermidis binding to host extracellular matrix proteins.

**Biofilm formation**

After the initial binding to the foreign material, biofilm formation develops as multilayered S. epidermidis cell clusters linked by a slimy extracellular substance that supports cell to cell binding (Figure 1-29) (O'Toole, Kaplan et al. 2000). This process requires intercellular binding, provided by the Polysaccharide Intercellular Adhesin (PIA) which has been suggested to be a crucial for the biofilm accumulation process (Mack, Siemssen et al. 1992; Mack, Nedelmann et al. 1994). PIA is a homoglycan molecule located on the S. epidermidis surface as fibrous strands and is an important component of the extracellular matrix connecting the bacterial cells in a biofilm. Several studies emphasized the role of PIA as a virulence factor when in animal models a PIA negative mutant revealed reduced ability to cause infection when comparing with the wild-type bacteria (Rupp, Ulphani et al. 1999; Rupp, Ulphani et al. 1999; Rupp, Fey et al. 2001; Francois, Tu Quoc et al. 2003; Fluckiger, Ulrich et al. 2005; Chokr, Leterme et al. 2007).

![Figure 1-29 – S. epidermidis biofilm formation (adapted from Otto 2009).](image-url)
1.9.1.2. Surface proteins

Fbe/SdrG

The serine-aspartate repeat-containing protein G (SdrG) also known as fibrinogen-binding protein (Fbe) is the most intensively studied MSCRAMM and the most predominant fibrinogen-binding protein on S. epidermidis (Otto 2009). Being present in approximately 67 to 91 % of the clinical strains tested (Arciola, Campoccia et al. 2004; Brennan, Loughman et al. 2009) this protein that belongs to the sdr family of proteins and has been described as necessary and sufficient to promote bacterial attachment to both soluble and immobilized forms of fibrinogen in a dose-dependent manner. SdrG binds to the thrombin cleavage site in the fibrinogen using a 'dock, lock and latch' mechanism (Ponnuraj, Bowden et al. 2003) which is thought to stabilize the SdrG–fibrinogen interaction. SdrG antibodies are present in human blood (McCrea, Hartford et al. 2000) and in vivo environment expression of SdrG has been shown to increase (Sellman, Timofeyeva et al. 2008). Moreover, SdrG has also been implicated in promoting central venous catheter-associated infection in vivo (Guo, Zhao et al. 2007). For these reasons SdrG is considered a major factor for S. epidermidis infection.

Several additional S. epidermidis MSCRAMMs have been predicted and have undergone preliminary characterization (Bowden, Chen et al. 2005), although their role in matrix protein binding and virulence remains to be clarified.

1.9.1.3. Extracellular proteins

S. epidermidis has been described to secrete a cysteine protease (Sloot, Thomas et al. 1992) implicated in the degradation of several host matrix proteins and components of the immune system in vitro. Also the extracellular Accumulation-Associated Protein (AAP), has been shown to be contribute for the accumulative growth of S. epidermidis on polymer surfaces (Hussain, Herrmann et al. 1997). Other studies also suggested that two very similar lipases found in S. epidermidis
might be important for skin colonization (Longshaw, Farrell et al. 2000). Moreover the Fatty acid modifying enzyme (FAME) secreted by *S. epidermidis* seems to work by esterifying fatty acids to cholesterol and thereby destroying their bactericidal properties (Chamberlain and Brueggemann 1997).

**Toxins**
In contrast to the vast toxin repertoire of *S. aureus* and many other bacteria, *S. epidermidis* toxin production is limited to the haemolytic peptide delta-toxin (McKevitt, Bjornson et al. 1990) implicated in the lysis of erythrocytes by forming pores in the cytoplasmic membrane (Gemmell and Thelestam 1981). Moreover, *S. epidermidis* delta-toxin (δ-toxin) has been showed to contribute to sub-acute and chronic infections by *S. epidermidis* (Vuong and Otto 2002; Otto, O'Mahoney et al. 2004). Although the biological role of this toxin deserves specific further investigation, δ-toxin has also been reported to take part on inflammatory response modulation for its involvement in cytokine and NFκB production in cells of macrophage lineage (Mehlin, Headley et al. 1999).

**Lantibiotics**
Some *S. epidermidis* strains produce lantibiotics which are extremely stable antibacterial peptides (Fischetti 2000) that seem to play an important role in bacterial interference on skin and mucous membranes by the exclusion of competing microorganisms sensitive to their bactericidal activities (Bierbaum, Gotz et al. 1996).
1.10. Bone Infection

Osteomyelitis involves bone inflammatory destruction and is mainly caused by *S. aureus* and *S. epidermidis*. This infectious disease is painful, debilitating and associated with serious morbidity and often difficult to diagnose (Berendt and Byren 2004). Moreover, due to the rise of antibiotic resistance, treatment is increasingly difficult. In addition, the number of prosthetic joint replacement procedures is also increasing providing new opportunities for the development of new infections (Wright and Nair 2010). Therefore, a lot of studies have been undergoing in order to understand the mechanisms by which Staphylococci interacts and destroys bone.

Microbiological virulence and osteomyelitis

Several studies investigated the correlation between Staphylococci virulence factors and invasive bone disease. A combination of virulence factors such as adhesins and toxins or genes involved in biofilm formation were associated with invasive bone disease, and increased severity of infection (Peacock, Moore *et al.* 2002). In 2004, a study from a range of orthopaedic infections, identified the genes *fnbA* and *fnbB* encoding for the *S. aureus* fibronectin-binding proteins FnbP and FnbB in 98% and 99% of the clinical isolates, respectively. On the other hand, the *cna* gene encoding for the collagen-binding protein was present only in 46% of the isolates (Arciola, Campoccia *et al.* 2004). Another study identified the gene encoding for the Panton-Valentine leukocidin in 59 of 89 *S. aureus* isolates and suggested an association between this virulence factor with enhanced inflammatory response in a case of haematogenous osteomyelitis in children (Bocchini, Hulten *et al.* 2006). However, a major problem associated with these findings is that there was no report of strain typing, so it is unclear how representative they are around the world. Therefore, there is a lack of knowledge related to the association of specific microbiologic genetic features and bone infections.
Host physiology and predisposition

Bacterial colonization is not sufficient to cause infection, and many surgical wounds become contaminated but not all undergo infection. The wound location can be limited to the medullary surface of bone, or exposed in the bone surface at the base of the wound, but it can also be localized in a cortical sequestration that can be removed without compromising bony stability or it can be diffused requiring resection of bone and loss of stability. On the other side, the host predisposition is also important in the development of infection, if it is systemically compromised such as in cases of malnutrition, renal failure, and diabetes, immune disease or even if the treatment is worse than the disease.

Molecular mechanisms underlying bone inflammation and destruction

Osteoblasts are known to modulate osteoclast differentiation and activity through the interaction between RANK expressed by osteoclasts and its ligand RANKL expressed by osteoblasts (Henderson and Nair 2003; Matsuo and Irie 2008). Following RANK-RANKL interaction a signalling pathway is triggered activating a number of transcription factors including the NFκB which ultimately will end in osteoclast differentiation (Wada, Nakashima et al. 2006; Matsuo and Irie 2008). On the other hand if OPG, an endogenous inhibitor of RANKL signalling, is present it will function as a decoy receptor for RANKL blocking the interaction with RANK and therefore inhibiting osteoclastogenesis (Yasuda, Shima et al. 1998; Wada, Nakashima et al. 2006).

Staphylococci induction of cytokine production after infection has also been reported as an evidence of bone inflammation and destruction in osteomyelitis disease. The inflammatory cytokines TNF-α and IL-6 seem to be greatly involved in bone physiology and pathology (Kwan Tat, Padrines et al. 2004) as high levels of these cytokines were identified to be up-regulated locally and systemically in cases of acute osteomyelitis (Klosterhalfen, Peters et al. 1996; Evans, Jellis et al. 1998), in rats experimentally implanted with S. aureus infected needles (Garcia-Alvarez, Navarro-Zorraquino et al. 2009) and in a murine model of osteomyelitis (Yoshii, Magara et al. 2002). The mechanism by which
Staphylococci induces the release of these cytokines is not clear; however the effects of it are highly implicated in osteomyelitis pathology. The release of IL-6 by osteoblasts in response to *S. aureus* infection has been reported (Ishimi, Miyaura *et al.* 1990; Bost, Ramp *et al.* 1999), and further associated with the increase of bone resorption by direct stimulation of osteoclasts differentiation in a mouse model (Ishimi, Miyaura *et al.* 1990). In a murine model following LPS injection, deletion of TNFR-1 and TNFR-2 receptors has been demonstrated to decrease osteoclast number and bone resorption (Chiang, Kyritsis *et al.* 1999).

Several investigations with different cell-types have demonstrated that TNF-α inhibits osteogenesis (Lacey, Simmons *et al.* 2009) and suppresses the production of osteogenic markers such as osteonectin, osteopontin, type I collagen, osteocalcin and alkaline phosphatase, consequently reducing bone matrix deposition and mineralisation (Canalis 1987; Li and Stashenko 1992; Nanes 2003). Moreover, studies with *S. aureus* and *S. epidermidis* surface material revealed that these bacterial components induce bone resorption, and that blocking TNF-α totally abolishes this effect (Nair, Song *et al.* 1995; Meghji, Crean *et al.* 1997; Meghji, Crean *et al.* 1998). Furthermore induction of bone resorption with *S. aureus* surface material did not require co-culture with osteoblasts suggesting a total independence on the RANKL signalling (Lau, Wang *et al.* 2006).

**Staphylococcal invasion of bone cells**

Following infection, *S. aureus* has been shown to be able to invade osteoblasts, therefore leading to bacterial persistence within the host cells. *S. aureus in vitro* uptake has been reported in different cell types such as epithelial and endothelial cells, keratinocytes and also in osteoblasts (Hudson, Ramp *et al.* 1995; Ellington, Reilly *et al.* 1999; Jevon, Guo *et al.* 1999; Reilly, Hudson *et al.* 2000; Kintarak, Whawell *et al.* 2004; Khalil, Williams *et al.* 2007; Garzoni and Kelley 2009). In 2000 Reilly *et al.* captured *in vivo* internalisation of *S. aureus* bacteria by embryonic chick osteoblasts by electron microscopy following infection (Figure 1-30) (Reilly, Hudson *et al.* 2000).
Figure 1-30- Transmission electron microscopy of human osteoblasts infected with *S. aureus*. Scale represented by black bar corresponds to 1 μm. From right to left, arrow points to *S. aureus* interacting with the osteoblast cell surface, arrow indicates an internalized *S. aureus* within the osteoblast membrane (Jevon, Guo et al. 1999).

This bacterial internalization process by the host cells is thought to prolong infection by providing an evasion system to the bacteria from the host immune system and from the action of antibiotics (Henderson and Nair 2003; Wright and Nair 2010). The Fnbps on the *S. aureus* surface are essential for *S. aureus* internalisation as mutants deficient in Fnbps have been shown to have a significant reduction in invasion rates by osteoblasts (Sinha, Francois et al. 1999; Ahmed, Meghji et al. 2001; Garzoni and Kelley 2009). Uptake of *S. aureus* by osteoblasts occurs therefore, by interaction between the Fnbps on the bacterial surface with the integrin α5β1 on the osteoblast surface via fibronectin binding (Dziewanowska, Patti et al. 1999; Sinha, Francois et al. 1999; Fowler, Wann et al. 2000). However, *S. epidermidis* internalisation by osteoblasts seems to be independent of the fibronectin-integrin mechanism (Khalil, Williams et al. 2007). Furthermore, expression of Tumor necrosis factor Receptor Apoptosis Inducing Ligand (TRAIL) has been shown to be up-regulated after *S. aureus* internalization by osteoblasts which may be triggering the activation of a cascade of caspases leading to apoptotic events, previously reported in *S. aureus* infected osteoblasts (Alexander, Rivera et al. 2003; Mahalingam, Szegozdi et al. 2009). TRAIL has also been reported to induce apoptosis in human osteoclasts and to inhibit
osteoclastogenesis (Zauli, Rimondi et al. 2004; Colucci, Brunetti et al. 2007) which might be contributing to bone loss in osteomyelitis patients (Henderson and Nair 2003). Therefore, Staphylococcal invasion of osteoblasts may play a role in the pathogenesis of bone infections by providing a sheltered environment for bacteria, enabling evasion of antimicrobials and host immune mechanisms and causing further bone destruction by inducing apoptosis prolonging the persistence of infection.
Chapter 2

Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process
2.1. Introduction

The integrity of the human skeleton is maintained by the normal growth, modeling and remodeling of the bone. Osteoblasts together with osteoclasts, osteocytes and bone lining cells, are the basic units sustaining these processes (Frost 1986; Bilezikian, Raisz et al. 2008). Also defined as bone forming-cells, osteoblasts produce an organic collagen matrix that undergoes mineralization and forms new bone (Bilezikian, Raisz et al. 2008). An inability to perform their function and generate new bone, results in abnormal bone growth.

In bone diseases such as osteomyelitis, the processes that maintain the integrity of the skeleton are compromised. Osteomyelitis is a debilitating infectious disease mainly characterised by bone destruction and inflammation caused by infecting microorganisms (Lew and Waldvogel 1997). Among these pathogenic microorganisms, *S. aureus* is by far the most commonly found in any type of osteomyelitis, accounting for 80% of all cases (Lew and Waldvogel 2004).

*S. aureus* expresses a range of extracellular and cell-associated factors that contribute to its virulence. Bacterial binding to host tissues is the first step in the establishment of an infection. *S. aureus* expresses on its surface MSCRAMMs that promote bacterial attachment to the host (Waldvogel 2000). The MSCRAMMs are covalently bound to peptidoglycan by the LPXTG motif (Navarre, Ton-That et al. 1998) and can only be released from the cell by enzymatic degradation. This sorting reaction is catalysed by an enzyme called sortase. Sortase recognizes the LPXTG sequence and cleaves the LPXTG motif between the Thr and Gly residues (Navarre and Schneewind 1994; Ton-That and Schneewind 1999).

Some of these *S. aureus* MSCRAMMs have been well characterised and play an essential role in *S. aureus* pathogenicity. For example the Fnbp A and FnbpB (Signas, Raucci et al. 1989; Jonsson, Signas et al. 1991) have affinity to bind to fibronectin, a glycoprotein that occurs in body fluids and in the extracellular matrix. *S. aureus* can bind to fibronectin on the host via α5β1 integrin whereas
fibronectin forms a bridge between the Fnbps on the bacterial surface and the mammalian cell integrin stimulating the internalization of the bacteria. This process has been shown in *S. aureus* invasion of fibroblasts (Usui, Murai et al. 1992; Fowler, Wann et al. 2000), endothelial (Ogawa, Yurberg et al. 1985; Peacock, Foster et al. 1999) and epithelial cells (Dziewanowska, Patti et al. 1999; Lammers, Nuijten et al. 1999; Sinha, Francois et al. 1999). Additionally this process is also involved in bacterial escape from the blood stream leading to invasion of the internal organs and initiation of invasive diseases. These adhesins can have multiple interactions with the host, the Fnbps have also been shown to bind fibrinogen, together with the ClfA and ClfB, also members of the MSCRAMMs. Fibrinogen is the most abundant ligand for the glycoprotein gpllb/IIIa on the surface of platelets. The interaction between fibrinogen and the MSCRAMMs results in platelet aggregation and consequently thrombi formation likely to be important in endovascular infections such as endocarditis (McDevitt, Francois et al. 1994; Hawiger 1995; Ni Eidhin, Perkins et al. 1998; Wann, Gurusiddappa et al. 2000; Rivera, Vannakambadi et al. 2007). Other important MSCRAMMs are the Sdr family of proteins. These proteins contain a distinct serine-aspartate repeat region and include the SdrC, SdrD, and SdrE. Strains lacking the sdrD and sdrE genes have been demonstrated to have decreased potential to infect bones (Foster and Hook 1998; Sabat, Melles et al. 2006).

Moreover, Bbp also a member of the Sdr family of proteins has been shown to bind the bone sialoprotein and collagen on the host (Ryden, Tung et al. 1997; Arrecubieta, Lee et al. 2007). Finally, SpA is one of the best characterised MSCRAMMs and is an exceptionally complex virulence factor known to have multiple interactions with host molecules, interfering with their functions. SpA is known primarily for its ability to bind the Fc region of IgG with anti-phagocytic properties (Cedergren, Andersson et al. 1993). SpA also binds to the vWF a large glycoprotein involved in mediating platelet binding at sites of endothelial damage promoting blood clot formation (Hartleib, Kohler et al. 2000; O'Seaghdha, van Schooten et al. 2006) and to the V_{H}3 receptor of B-cells resulting in depletion of a significant part of the B cell repertoire (Sasso,
Silverman et al. 1989; Hillson, Karr et al. 1993; Goodyear and Silverman 2004; Viau, Longo et al. 2005). In addition SpA has been shown to mimic TNF-alpha’s role and directly interact with the TNFR-1 in airway epithelial cells stimulating inflammatory processes (Gomez, Lee et al. 2004; Gomez, O’Seaghdha et al. 2006) and regulating TNFR-1’s availability on mucosal and immune cells by direct binding of its IgG binding domain to the epidermal growth factor receptor (EGFR) (Gomez, Seaghdha et al. 2007). Finally recent studies have proposed SpA as a vaccine target for Methicillin Resistant S. aureus (MRSA) (Kim, Cheng et al. 2010).

In order to successfully colonize and infect bone tissue S. aureus must bind to osteoblasts. The main aim of this chapter was to investigate the molecular interactions between S. aureus and osteoblasts. As such the contribution of S. aureus cell surface molecules, such as the capsule, teichoic acids and surface proteins were investigated. Therefore, isogenic mutants of S. aureus lacking the expression of capsule and a wide range of cell surface proteins previously shown to be involved in host recognition were tested. Additionally the role of the cell wall teichoic acid was investigated in this interaction by the blockage of the respective receptor TLR2 on osteoblasts surface. These studies demonstrated that only the lack of SpA on the surface of different S. aureus strains significantly inhibited the binding to osteoblasts. Moreover, further studies examining osteoblasts proliferation after infection with S. aureus revealed that in the presence of SpA, osteoblasts growth was inhibited. In summary it can be concluded that SpA binds to osteoblasts and plays a crucial role in inducing the inhibition of osteoblast proliferation.
2.1.1. Chapter Aims

- Investigate the ability of a range of different *S. aureus* strains to interact with osteoblasts.
- Identify the cell wall component that is responsible for binding to osteoblasts.
- Identify the effects of the deletion of the identified cell wall component on osteoblast function.
2.2. Chemicals and general conditions

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Dublin, Ireland). The other suppliers are listed below in Table 2-1.

All solutions were prepared using pure deionised water. Room temperature (RT) was 20±5 °C.

Table 2-1- List of chemicals and equipment used in this chapter.

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
<th>Address</th>
</tr>
</thead>
<tbody>
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<td>Alpha Minimal Essential Medium</td>
<td>Biosera Ltd.</td>
<td>East Sussex, UK</td>
</tr>
<tr>
<td>Blinchorinic acid assay</td>
<td>Merck Chemical Calbiochem</td>
<td>UK</td>
</tr>
<tr>
<td>Brain Heart Infusion broth</td>
<td>Oxoid</td>
<td>Basingstoke, UK</td>
</tr>
<tr>
<td>Colour plus pre-stained protein ladder (10-20 KDa)</td>
<td>New England Biolabs</td>
<td>UK</td>
</tr>
<tr>
<td>Developing solution</td>
<td>FUJIFILM Ireland Ltd</td>
<td>Ireland</td>
</tr>
<tr>
<td>Fixer solution</td>
<td>FUJIFILM Ireland Ltd</td>
<td>Ireland</td>
</tr>
<tr>
<td>Immobilon-P polyvinylidene difluoride (pore size 0.45μm)</td>
<td>MILLIPORE</td>
<td>Ireland</td>
</tr>
<tr>
<td>Immobilon Western Chemiluminescent HRP Substrate</td>
<td>MILLIPORE</td>
<td>Ireland</td>
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<tr>
<td>MC37-F1 mouse anti-lactate cell line</td>
<td>ATCC</td>
<td>Middlesex, UK</td>
</tr>
<tr>
<td>Clear flat bottomed high binding 96-well plates</td>
<td>Corning® Costar®, VWR International</td>
<td>Blanchardstown, Ireland</td>
</tr>
<tr>
<td>Mini PROTEAN electrophoresis apparatus</td>
<td>Bio-RAD</td>
<td>UK</td>
</tr>
<tr>
<td>Non-fat dried milk</td>
<td>Marvel</td>
<td>UK</td>
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<tr>
<td>Protogel</td>
<td>Bio Sciences</td>
<td>Dun Laoghaire, Ireland</td>
</tr>
<tr>
<td>Trans-Blot Electrophoretic Cell</td>
<td>BioRAD</td>
<td>UK</td>
</tr>
<tr>
<td>Ultraspec III spectrophotometer</td>
<td>Pharmacia Biotech</td>
<td>UK</td>
</tr>
<tr>
<td>Wallac Victor2 micro-plate reader system</td>
<td>Perkin Elmer</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Array film</td>
<td>FUJIFILM Ireland Ltd</td>
<td>Ireland</td>
</tr>
</tbody>
</table>
2.3. Methods

2.3.1. Bacterial culture conditions

Bacterial strains

All the bacterial strains used in this chapter are listed in Table 2-2.

Table 2-2- List of strains used in this chapter.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newman</td>
<td>NCTC 8178 wild type</td>
<td>(Duthie and Lorenz 1952)</td>
</tr>
<tr>
<td></td>
<td>Isolated from a case of secondarily infected tubercular osteomyelitis in man</td>
<td></td>
</tr>
<tr>
<td>8325-4</td>
<td>NCTC 8325 cured of prophages</td>
<td>(Novick 1967)</td>
</tr>
<tr>
<td>SH1000</td>
<td>8325-4 with repaired defect in rbsU</td>
<td>(Horsburgh, Aish et al. 2002)</td>
</tr>
<tr>
<td>S. aureus mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newman Δ cap</td>
<td>cap581:: Tn917; DU5912</td>
<td>(Wann, Dassy et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Defective in Capsular polysaccharide</td>
<td></td>
</tr>
<tr>
<td>Newman Δ clfA</td>
<td>clfA2:: Tn917; DU5876</td>
<td>(McDevitt, Francois et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>Defective in Clumping factor A</td>
<td></td>
</tr>
<tr>
<td>Newman Δ clfB</td>
<td>clfB:: Tc; DU5943</td>
<td>(Ni Eidhin, Perkins et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>Defective in Clumping factor B</td>
<td></td>
</tr>
<tr>
<td>Newman Δ clfA clfB</td>
<td>clfA:: Tn917, clfB:: Tc; DU5944</td>
<td>(Ni Eidhin, Perkins et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>Defective in Clumping factor A and B</td>
<td></td>
</tr>
<tr>
<td>Newman Δ spa</td>
<td>spa:: Ka ; DU5971</td>
<td>(O’Brien, Kerrigan et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Defective in Protein A</td>
<td></td>
</tr>
<tr>
<td>Newman Δ sdrC</td>
<td>sdrC:: pG+Host9; DU5988</td>
<td>(O’Brien, Kerrigan et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Defective in Serine aspartate repeat protein C</td>
<td></td>
</tr>
<tr>
<td>Newman Δ sdrD</td>
<td>sdrD:: pG+Host9; DU5989</td>
<td>(O’Brien, Kerrigan et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Defective in Serine aspartate repeat protein D</td>
<td></td>
</tr>
<tr>
<td>Newman Δ sdrCDE</td>
<td>sdrCDE:: Tc</td>
<td>(O’Brien, Kerrigan et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Defective in Serine aspartate repeat protein C, D and E</td>
<td></td>
</tr>
<tr>
<td>Newman Δ sbi</td>
<td>sbi::Ka’</td>
<td>(Sibbald, Winter et al. 2010)</td>
</tr>
<tr>
<td>Newman Δ spa/sbi</td>
<td>spa:: EtbR’, sbi::Ka’</td>
<td>(Sibbald, Winter et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Defective in Spa and Sbi</td>
<td></td>
</tr>
<tr>
<td>Newman pCU1spa</td>
<td>Cm’ Ap’ spa gene cloned into pCU1. Insertion of pCU1spa capable of replicating in E. coli and S. aureus expressing Protein A.</td>
<td></td>
</tr>
<tr>
<td>8325-4 Δ spa</td>
<td>spa:: Tc</td>
<td>(Hartleib, Kohler et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Defective in Protein A</td>
<td></td>
</tr>
<tr>
<td>SH1000 Δ spa</td>
<td>spa:: Tc; DU5912</td>
<td>(Greene, McDevitt et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>Defective in Protein A</td>
<td></td>
</tr>
<tr>
<td>SH1000 Δ fnbA/B</td>
<td>fnbA::Tet’, fnbB::Erm’</td>
<td>(Provenzano, Provenzano et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Defective in Fnbps</td>
<td></td>
</tr>
<tr>
<td>SH1000 Δ fnbA/B/spa</td>
<td>spa:: Ka’, fnbA::Tet’, fnbB::Erm’</td>
<td>(Provenzano, Provenzano et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Defective in Fnbps and Protein A</td>
<td></td>
</tr>
</tbody>
</table>
* The \textit{S. aureus} strains Newman pCU1spa and SH1000Δspa were a kind gift from Prof. Timothy Foster in Department of Microbiology in Trinity College Dublin, Ireland.

**Growth conditions**

\textit{S. aureus} strains were grown in Brain Heart Infusion (BHI) broth (calf brain infusion 12.5 g/l, beef heart infusion 5 g/l, protease peptone 10 g/l, glucose 2 g/l, sodium chloride 5 g/l, disodium phosphate 2.5 g/l) in sealed tubes overnight at 37 °C. For some experiments, 1 ml of the \textit{S. aureus} SH1000 strains, WT, ΔFnbpA/B and ΔFnbpA/B/SpA overnight cultures was inoculated in 9 ml of BHI and incubated at 37 °C for 4 h of early exponential phase of growth.

**Bacterial stocks**

Original strains were provided as frozen glycerol stocks. The bacterial stocks were grown in 50 ml BHI statically overnight at 37 °C. One ml aliquots were stored in cryovials containing 10 % glycerol and kept at -80 °C for future use. Aliquots were thawed only once and each time one volume was used to inoculate nine volumes of BHI broth. Sub-culturing was minimized by preparation and storage of large numbers of aliquots. Periodically aliquots were tested for cross-contamination by streaking samples on agar plates and inspected by eye for contaminants.

**Bacterial isolation**

Overnight bacterial cultures were harvested by centrifugation at 15,000 g for 5 mln. The supernatants were discarded and the bacterial pellets were washed by re-suspending pellets in 5 ml of Phosphate Buffered Saline (PBS) pH 7.5 and further centrifuged for 5 min at 15,000 g. The washing step was repeated once more and the bacterial supernatants discarded.
Chapter 2- Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**Bacterial cell count**
Following bacterial isolation, the bacterial pellets were re-suspended in 1 ml PBS, the bacterial suspensions were then adjusted to the relevant optical density (OD) measured at 600 nm wavelength using the Ultraspec III spectrophotometer by dilution in PBS. By means of colony-forming units (CFUs) it was determined that the number of *S. aureus* for an OD$_{600}$ nm of 1 corresponds approximately to $1 \times 10^9$ cells. An OD$_{600}$ nm of 1 was used in all binding studies and an OD$_{600}$ nm of 2 was used for osteoblasts proliferation and infection studies.

**Bacterial fixation in 4.8 % formaldehyde**
Following bacterial growth overnight and isolation, the bacterial pellets were re-suspended in 5 ml of 4.8 % formaldehyde followed by 10 min agitation in a shaker platform. The formaldehyde fixed bacterial suspensions were further centrifuged for 10 min at 15,000 g and washed by re-suspension in 5 ml PBS. The bacterial suspensions were re-centrifuged for 5 min at 15,000 g and the washing step repeated once more. Fixed bacterial pellets were then re-suspended in 5 ml PBS and the OD adjusted to an OD$_{600}$ nm of 2 by dilution in PBS. Following re-centrifugation for 10 min at 15,000 g pellets were re-suspended in the same volume of Alpha Minimal Essential Medium (α-MEM) osteoblast media.

**2.3.2. Cell culture conditions**

**Cell type**
The MC3T3-E1 pre-osteoblastic adherent cell line ATCC, Middlesex (UK) was used in this study.
Routine cell culture

The MC3T3-E1 osteoblasts (passage 22-28) were cultured in standard tissue culture flasks in α-MEM supplemented with 10% Foetal Bovine Serum (FBS), 2% Penicillin/Streptomycin and 1% L-Glutamine in a tissue culture incubator at 37 °C in 5% CO₂ atmosphere. The media was replaced every three days. When 100% confluent, cells were routinely passaged to maintain optimum cell number per flask. In order to do this, media was removed from the flask and cells were gently washed by addition of 10 ml PBS. After removing the used PBS, cells were harvested by addition of 3 ml of 0.25% trypsin-EDTA solution for 3 min in tissue culture incubator at 37 °C in 5% CO₂ atmosphere. The flask was gently tapped in order to detach all the cells from the flask surface. Following this cells were re-suspended in 5 ml of warm medium. Finally the total volume of cells suspension was divided into 2 new flasks, 4 ml into each flask with 11 ml of previously added warm medium.

Cell counting

Osteoblasts were washed in PBS and harvested using 3 ml of 0.25% trypsin-EDTA solution. Following this cells were re-suspended in 5 ml of media. The osteoblasts suspension was centrifuged at 9,000 g for 5 min. Finally the pellet was re-suspended in 1 ml of media. Ten µl of the cell suspension was diluted into 10 µl of 0.4% trypan blue solution. Trypan blue solution is a dye that can be applied to living cells without harming them, therefore it is used to distinguish between live and dead cells. Trypan blue is not absorbed by viable cells, however in a dead cell the membrane is damaged, and the dye is able to traverse the membrane staining the cytoplasm with a distinctive blue colour that can be seen under the microscope. Cell counting was performed using a haemocytometer. A haemocytometer is a widely used counting chamber (see Scheme 1) in tissue culture. Prior addition of the cell suspension, a coverslip is placed over the counting surface. Then the haemocytometer is placed under the microscope. The full haemocytometer grid contains nine squares; the counting area is the central square that contains 25 large squares and each of these contain 16 smaller
squares. To calculate the number of cells in the sample, the number of live cells in the counting area was multiplied by $10^4$ and by the trypan blue dilution factor 2 that is the total number of counted cells per ml.

Scheme 1 – Haemocytometer grid where the middle square is the counting area. Source: http://www.hdacultures.org.uk/technical/ccp/cellcounting.jsp (2011).

Cell storage in liquid nitrogen

Cells from a 100 % confluent T175 flask were washed and harvested as previously described (see Routine cell culture). Following this cells suspension was centrifuged at 9,000 g for 5 min. The pellet was re-suspended in freezing media (72 % alpha-MEM, 20 % FBS, 2 % Penicillin/Streptomycin, 1 % L-Glutamine and 8 % Dimethyl Sulfoxide (DMSO)) and transferred to cryovials in 1 ml aliquots. Vials were placed into an isopropanol container and kept at -80 °C overnight to ensure cells integrity by dropping temperature gradually. The next day vials were transferred to liquid nitrogen cell storage chamber at approximately -196 °C.

Cell recovery from storage in liquid nitrogen

Cryovials from liquid nitrogen storage were defrosted at 37 °C in a waterbath ensuring that the vial lid was not in contact with the water thus avoiding contamination. Defrosted cells were transferred to a tube with 5 ml of warm media and gently re-suspended to dilute the toxic DMSO present in the freezing media. The cells suspension was then centrifuged at 9,000 g for 5 min, the supernatant was discarded and the pellet re-suspended in 5 ml of new warm
media. Following this cells were transferred to a new T175 flask containing 11 ml of media. Flask was maintained in tissue culture incubator at 37 °C with 5% CO₂ atmosphere.

2.3.3. Functional assays

Osteoblasts binding assay

Clear flat bottomed high binding 96-well plates (Corning® Costar®, VWR International) were coated with 100 μl of OD_{600nm} = 1 bacteria (1x10⁹ cells/ml) or purified protein (SpA or ClfA= 50 μg/ml). The plate was sealed with a hydrophobic porous sealing film to minimize cross-contamination, spillage or evaporation and incubated at 37 °C for 2 h. Following this the plate was washed with 100 μl of PBS per well to remove any unbound bacteria or protein. The plate was then blocked with 100 μl of 1% bovine serum albumin (BSA) per well and incubated for a further 1h at 37 °C. The BSA was then gently removed and the plate washed once with PBS. MC3T3-E1 cells (1.5x10⁶ cells/ml) were added into each well and allowed to bind for 45 min at 37 °C. Each well was carefully washed with 100 μl of PBS to remove any non-adhered osteoblasts. Adherent osteoblasts were then lysed with 100 μl of lysis buffer containing a substrate for osteoblast acid phosphatase (0.1 M Na acetate pH 5.5, 0.1 % Triton X-100, and 10 mM p-nitrophenol phosphate) and incubated for 20 min at 37 °C. Adherent osteoblasts were quantified by measuring the content of acid phosphatase at an absorbance of 405 nm wavelength using the Wallac Victor2 micro plate reader system.

- Trypsin treatment

In some osteoblasts binding assays S. aureus was pre-incubated with 1 U/ml trypsin-EDTA for 30 min at 37 °C prior to addition to the 96-well plate. Trypsin cleaves the peptides on the C-terminal side of lysine and arginine amino-acid residues, allowing the detachment of the anchored proteins from the bacterial surface.
• **Pre-incubation of osteoblasts with inhibition antibodies**

In some binding assays studies, osteoblasts were pre-incubated with 10 μg/ml anti-TLR2 and anti-TLR4 antibody for 30 min at RT prior to their addition to the 96-well plate.

**Osteoblasts proliferation assay**

One ml of 4.8 % formaldehyde fixed bacteria (see section Bacterial fixation in 4.8 % formaldehyde) or 50 μg/ml of purified SpA was allowed to adhere onto a 6-well plate for 2 h in tissue culture incubator at 37 °C with 5 % CO₂ atmosphere. Unbound bacteria were carefully removed from each well using a transfer pipette and 3 ml of MC3T3-E1 osteoblasts (5x10⁵ cells/well) were added. Uninfected osteoblasts were seeded and cultured in the absence of bacterial suspension as control. The 6-well plate was returned to the tissue culture incubator at 37 °C with 5 % CO₂ atmosphere. Twenty-two and 46 h later, old media was carefully discarded using a transfer pipette and replaced with new warm media. The plate was returned to the incubator and 2 h later, osteoblasts were harvested using 1 ml of 0.25 % trypsin-EDTA, and cells counted using a haemocytometer in a 1:1 dilution with the dye trypan blue as detailed previously (see Cell counting section).

**2.3.4. Protein biochemistry**

**Bacterial cell wall preparation**

Overnight bacterial cultures were harvested by centrifugation at 15,000 g for 10 min at 4 °C. Supernatants were discarded and pellets washed in 1 ml PBS and transferred to 1 ml eppendorf tubes. Samples were micro-centrifuged for 10 min at 13,000 rpm at 4 °C to pellet the cells. Bacterial pellets were then re-suspended in 250 μl Trls EDTA Lysozyme (TEL) buffer [1 M Tris pH 8, 10 mM EDTA and 1 % Lysozyme] plus 10 μl of 10 x Protease Inhibitor Cocktail (PIC) [23 mM 4-(2-
aminoethylbenzenesulfonyl fluoride (AEBSF), 2 mM Aprotinin, 130 μM Bestatin, 100 mM EDTA, 0.3 mM E-64 and 0.3 mM Pepstatin A] followed by 37 °C incubation for 3 h with inversion every 15 min. Samples were then microcentrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants were collected and transferred into chilled eppendorf tubes and stored at -20 °C.

**Cell lysate preparation**

Protein extraction from osteoblasts and 4.8 % formaldehyde fixed bacteria lysates were performed by re-suspending each sample pellet in 100 μl of Radioimmunoprecipitation assay (RIPA) buffer, 10 x PIC and 100 mM of Phenylmethylsulfonyl Fluoride (PMSF). Samples were left on ice for 10 min and subsequently centrifuged at 13,000 rpm and 4 °C for 10 min. The supernatant was transferred into chilled eppendorf tubes and stored at -20 °C.

**Protein quantification**

The protein content of all samples was quantified using a bicinchoninic acid (BCA) assay. The principle of the BCA assay is based on a biuret reaction, the reduction of Cu$^{2+}$ to Cu$^{1+}$ under alkaline conditions. The amount of reduction is proportional to the protein present. Bicinchoninic acid is a chromogenic reagent that chelates with the reduced copper, producing a purple reaction that can be read at 562 nm wavelengths (an interval of 540-590 nm can also be used successfully). The absorbance is directly proportional to the protein concentration (Smith, Krohn et al. 1985; Kessler and Fanestil 1986). Standard curve samples were prepared by diluting 2 mg/ml of BSA solution in RIPA buffer from a 1,000 μg/ml to 0 μg/ml concentration. Twenty-five micro-litres of each standard or protein sample were added in replicate into individual wells of a 96-well plate. Following this 200 μl of BCA working reagent was added into each well. The plate was sealed with hydrophobic porous sealing film and incubated for 30 min at 37 °C. Plate was read at 570 nm wavelength absorbance using the Wallac Victor2 micro plate reader system. The standard curve was obtained by
plotting the absorbencies of the standard samples versus the correspondents BSA concentrations. The equation of the line from the standard curve was then used to calculate the protein concentration of each sample. Final protein concentration was adjusted to 500 µg/ml per sample by dilution in RIPA buffer for Western blot analysis.

**Western blot and SDS-PAGE analysis**

Western blot analysis is used to detect specific proteins of interest in cell lysates. In order to separate the protein mix based on their size a Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used. The separated proteins in the gel are then transferred to a membrane using electrical current and finally probed with specific antibodies that recognize and bind the target proteins.

SDS-PAGE analysis was carried out using a Mini PROTEAN electrophoresis apparatus (Laemmli 1970). Separating and stacking gel were prepared at 10 % and 5 %, respectively, as described below in Table 2-3. The 10 % separating gel was poured into the electrophoresis apparatus and overlaid with ethanol in order to maintain a flat surface. The separating gel was allowed to set at RT, the stacking gel was then added on top of the first one followed by comb insertion to allow the wells formation for sample application.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Separating Gel 10 %</th>
<th>Stacking gel 5 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.8</td>
<td>5.84 ml</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>SDAPS</td>
<td>3.5 ml</td>
<td>------</td>
</tr>
<tr>
<td>Protogel (acrylamide:bisacrylamide)</td>
<td>------</td>
<td>1.88 ml</td>
</tr>
<tr>
<td>MOPS</td>
<td>4.66 ml</td>
<td>1.15 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>82 µl</td>
<td>55 µl</td>
</tr>
<tr>
<td>10% ammonium persulphate (APS)</td>
<td>82 µl</td>
<td>55 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.8 µl</td>
<td>7.5 µl</td>
</tr>
</tbody>
</table>

Table 2-3- SDS-PAGE composition
Chapter 2- Study of the molecular mechanisms involved in S. aureus binding to osteoblasts: early steps in the infection process

Samples were prepared by adding 20 µl of each lysate plus 5 µl of 5 X sample buffer (250 mM Tris-HCl, pH 6.8, 10 % SDS, 0.5 % bromophenol blue, 50 % glycerol plus 50 µg/ml of DTT that were added to the sample buffer immediately prior use) and boiled at 95 °C for 5 min. After being briefly centrifuged at 13,000 rpm for 2 min, samples were loaded into the gel alongside a pre-stained protein ladder (10-230 KDa). The SDS-PAGE running buffer (25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1 % SDS) was added to the electrophoresis tank and samples and marker were resolved through the gel at constant current of 200 V.

The resolved proteins were transferred to PVDF membrane using a Trans-Blot Electrophoretic Cell. The pads and filter paper were soaked in transfer buffer (25 mM Tris-HCl pH 8.0, 0.2 M glycine, 20 % methanol) and the PVDF membrane activated in methanol for 5 sec. The gel was placed onto a layer of filter paper and sponge, the PVDF membrane was placed on the top and overlaid with another piece of filter paper and sponge. The entire assembly was placed into the transfer cassette and inserted in the apparatus chamber filled with transfer buffer. A constant current of 200 mA was used for transfer for 1 h at RT.

The PVDF membrane with the transferred proteins was removed from the transfer apparatus and incubated for 1h in blocking buffer (5 % non-fat dried milk in Tris Buffer ed Saline (TBS) – 0.1 % (v/v) Tween (TBST)) in a roller platform. The primary antibody was diluted in blocking buffer according to the required concentration as documented in Table 4. Membrane was then incubated with the primary antibody overnight at 4 °C in a roller platform. After primary incubation the membrane was washed three times over 10 min each time in TBST. The respective horseradish peroxidase (HRP) conjugated antibody was prepared in blocking buffer according to the respective concentration (see Table 2-4). Membrane incubation on secondary antibody run for 1 h at RT in a roller platform followed by three washes in TBST for 10 min each time. Finally equal volumes of Luminol Reagent and Peroxide Solution of the Immobilon™ Western Chemiluminescent HRP Substrate solution were mixed in a cleaned tube. The PVDF membrane was incubated with the HRP substrate for 5 min at RT. After
draining the exceeding substrate membrane was exposed to X-ray film using a dark room, and developed by immersion in developing solution followed by fixer solution and water.

Table 2-4- Western blot conditions

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Molecular weight (KDa)</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpA</td>
<td>50</td>
<td>Monoclonal chicken IgY anti-SpA produced in mouse</td>
<td>Goat anti-mouse IgG HRP 1:1000</td>
</tr>
<tr>
<td>IgG</td>
<td>50</td>
<td>Monoclonal IgG antibody produced in mouse</td>
<td>Goat anti-mouse IgG HRP 1:1000</td>
</tr>
</tbody>
</table>

2.3.5. Statistical analysis

Statistics were performed using SSC-Stat V2.12. Data shown are the means plus or minus standard error of the mean (SEM). Comparisons between mean values were performed using a 2-tailed paired Student’s t-test.
2.4. Results

2.4.1. *S. aureus* binding to osteoblast

Binding of bacteria to osteoblasts is the first stage of infection in bone disease. Osteoblast binding to immobilized *S. aureus* strains including the Wild-Type (WT) Newman, 8325-4 and SH1000 was assessed. *S. aureus* Newman strain is a commonly used laboratory strain originally isolated from a case of secondarily infected tubercular osteomyelitis patient (Duthie and Lorenz 1952). Previously it has been shown that *S. aureus* Newman does not possess cell wall-anchored fibronectin binding proteins (Grundmeier, Hussain et al. 2004) so it is unlikely to be internalized rapidly by the osteoblasts. The *S. aureus* 8325-4 and SH1000 strains are two modified laboratory strains with a common progenitor and, in contrast to *S. aureus* Newman they have Fnbps anchored on their surface. In this section it is demonstrated that *S. aureus* strain Newman bound with slightly more intensity to osteoblasts than *S. aureus* 8325-4 and SH1000 strains (Figure 2-1).
Chapter 2- Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**Figure 2-1- Distinct *S. aureus* strains bind to osteoblasts.** Osteoblasts were allowed to bind to immobilized *S. aureus* wild-type strains Newman, 8325-4 and SH1000 (1x10^9 cells/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. Bars indicate SEM, n=6.
2.4.2. Contribution of the cell surface molecules in the *S. aureus* binding to osteoblast

After confirming that distinct *S. aureus* strains bind to osteoblasts, the contribution of bacterial surface molecules in this interaction was investigated. *S. aureus* is surrounded in a polysaccharide capsule. Many of the sugars found in the capsule have been previously shown to interact with other cell types (Pohlmann-Dietze, Ulrich *et al.* 2000). For that reason it was explored whether the *S. aureus* capsule could be mediating the interaction with osteoblasts. *S. aureus* Newman expresses capsule whereas *S. aureus* strain SH1000 is naturally defective in capsule. Here it was demonstrated that using a *S. aureus* Newman strain defective in capsule increased the binding to osteoblasts. On the other hand, the capacity of *S. aureus* SH1000 strain to bind to osteoblasts it was comparable to that of *S. aureus* Newman WT. Thus neither disruption of the cap gene in the Newman strain nor the use of the strain naturally defective in capsule inhibited the binding to osteoblasts (Figure 2-2A). These results suggest that *S. aureus* capsule is not mediating bacterial interaction with osteoblasts.

Teichoic acids are constitutively produced glycopolymers anchored to the peptidoglycan layer [Wall teichoic acid (WTA)] or to the cytoplasmic membrane [Lipoteichoic acid (LTA)] in Gram-positive bacteria including *S. aureus*. A variety of roles have been assigned to the teichoic acids in bacterial survival under disadvantageous conditions and in other basic cellular processes (Weidenmaier and Peschel 2008). Some studies demonstrated that the teichoic acids are crucial for *S. aureus* nasal colonization and endovascular infection (Weidenmaier, Kokai-Kun *et al.* 2004; Weidenmaier, Peschel *et al.* 2005; Weidenmaier, Kokai-Kun *et al.* 2008). Previously, it has been shown that teichoic acids in Gram-positive bacteria are recognized by the TLR2 on the host (Fournier and Philpott 2005; Kaji, Kiyoshima-Shibata *et al.* 2010), while Lipopolysaccharide (LPS) in Gram-negative bacteria is recognized by Toll Like receptor 4 (TLR4) (Poltorak, He *et al.* 1998; Akira, Uematsu *et al.* 2006). The contribution of the teichoic acids in *S. aureus* binding to osteoblast was therefore investigated. Anti-TLR2 antibody was used to block the interaction between the teichoic acids on *S. aureus* and its recognition.
molecule, TLR2, on the osteoblasts surface (Figure 2-2B). Anti-TLR4 antibody was also used as negative control. The blockade of this interaction had no effect on the binding intensity of *S. aureus* to osteoblasts, thus teichoic acids do not seem to be involved in *S. aureus* binding to osteoblasts.

Surface protein interactions are the most common way of bacteria to interact with the host. *S. aureus* expresses a wide variety of proteins localized on the bacterial surface that recognize and bind ligand(s) found within the host (Patti, Allen *et al.* 1994). Trypsin is a proteolytic enzyme that cleaves proteins at the C-terminal of the amino-acids lysine and arginine (Rawlings and Barrett 1994). In order to remove *S. aureus* surface proteins and evaluate their contribution in binding osteoblast, a trypsin-EDTA treatment was performed. The removal of the *S. aureus* surface proteins significantly reduced the bacterial binding to osteoblasts (Figure 2-2C, *P*<0.05), suggesting a major role for these proteins in mediating *S. aureus* binding to osteoblasts.
Figure 2-2 - *S. aureus* surface proteins mediate binding to osteoblasts. Osteoblasts were allowed to bind to immobilized (A) *S. aureus* Newman, Newman Δ cap (Newman capsule defective mutant) and SH1000 (naturally defective in capsule) (1x10^9 cells/ml) for 45 min at 37 °C (B) Osteoblasts were pre-incubated with 10 µg/ml anti-TLR2 and anti-TLR4 blocking antibodies prior to binding to immobilized *S. aureus* Newman (C) Un-treated and 30 min trypsin-EDTA (1 U/ml) treated *S. aureus* Newman for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=3.
2.4.3. The role of \textit{S. aureus} Fibronectin protein A and B in the binding to osteoblast

The \textit{S. aureus} fibronectin binding proteins A and B have been previously shown to be essential in the process of \textit{S. aureus} uptake by osteoblasts and other mammalian cells through the bridging interaction with the host fibronectin receptor integrin $\alpha_5\beta_1$ (Jevon, Guo \textit{et al.} 1999; Sinha, Francois \textit{et al.} 1999; Ahmed, Meghji \textit{et al.} 2001). The role of these proteins was investigated in \textit{S. aureus}-osteoblasts interaction. However, in \textit{S. aureus} Newman strains the Fnbps are completely secreted and, previous studies demonstrated that \textit{S. aureus} Newman does not support binding functions through these proteins (Grundmeier, Hussain \textit{et al.} 2004). Thus, in order to test the role of the Fnbps A and B in osteoblast binding, the \textit{S. aureus} strain SH1000, which optimally expresses these proteins at early exponential phase of growth, was used (Saravia-Otten, Muller \textit{et al.} 1997). \textit{S. aureus} SH1000 strain isogenic mutants defective in FnbpA and FnbpB and in both FnbpA/B and SpA were tested for their role in osteoblast binding. Despite a slight reduction in binding intensity when compared with the WT strain, both \textit{S. aureus} SH1000 FnbpA/B and FnbpA/B and SpA mutants were still able to highly support binding to osteoblasts (Figure 2-3). These results suggest that the FnbpA/B proteins despite being essential for \textit{S. aureus} invasion of osteoblasts are not crucially required to support osteoblast binding in the first contact with the host.
Chapter 2 - Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**Figure 2-3** - *S. aureus* FnbpA/B are not crucial in mediating *S. aureus* – osteoblasts interaction. Osteoblasts were allowed to bind to immobilized *S. aureus* SH1000 WT, ΔSpA, ΔFnbpA/B and ΔFnbpA/B/SpA (1 x 10⁹ cells/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=3.
2.4.4. The role of *S. aureus* surface proteins in the binding to osteoblast

Having established that the *S. aureus* cell surface proteins are partially involved in mediating bacterial binding to osteoblasts, the individual role of specific *S. aureus* surface proteins was investigated. Belonging to the MSCRAMMs family, the Clfa and Clfb, the SpA, and the Sdrc, D and E (Patti, Allen *et al.* 1994; Waldvogel 2000) were previously reported to be involved in host recognition, and therefore, these were tested for their ability to mediate binding to osteoblasts. Several *S. aureus* Newman isogenic mutants were generated by genetic manipulation resulting in strains defective in the expression of Clfa (McDevitt, Francois *et al.* 1994) and B (Ni Eidhin, Perkins *et al.* 1998), SpA and Sdrc, D and E (O'Brien, Kerrigan *et al.* 2002). The results obtained demonstrated that SpA defective mutant bound significantly less than the WT strain. In contrast the other mutant strains tested had similar levels of osteoblast binding when compared to the wild-type strain (Figure 2-4, P<0.05).
Chapter 2- Study of the molecular mechanisms involved in \textit{S. aureus} binding to osteoblasts: early steps in the infection process

Figure 2-4 - SpA mediates \textit{S. aureus} binding to osteoblasts. Osteoblasts were allowed to bind to immobilized \textit{S. aureus} Newman wild type and isogenic mutants (1 x 10^9 cells/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=6.
2.4.5. Confirmation of the role of *S. aureus* protein A in the binding to osteoblast

In order to confirm the involvement of SpA in the binding to osteoblasts, this interaction was further investigated using different *S. aureus* strains. For that reason, the strains *S. aureus* SH1000 and 8325-4 WT along with their SpA defective mutants were used and tested for their ability to bind to osteoblasts. As result, the *S. aureus* SH1000 and 8325-4 strains defective in SpA revealed a significant reduction, of approximately 50%, in the osteoblast binding intensity when comparing with the respective WT (Figure 2-5A, P<0.05). These data support the previous results where *S. aureus* Newman strain binding to osteoblast was significantly reduced when using the respective SpA defective strain (Figure 2-5A, P<0.05). Moreover, complementation of the *S. aureus* protein A mutant with a multicopy plasmid carrying the *spa* gene (strain pCU1spa), showed a total recovery on the capacity to bind osteoblast, with levels similar to those obtained for the *S. aureus* Newman WT strain (Figure 2-5B). To ensure that SpA was being expressed on the surface of the complemented strain *S. aureus* Newman pCU1SpA, Western immunoblotting detection was performed using the cell wall preparations of both *S. aureus* Newman WT and complemented strain pCU1SpA. Immunoblotting analysis revealed a band of 42 KDa, the correspondent molecular weight of SpA, in both lanes for *S. aureus* Newman WT and *S. aureus* Newman pCU1SpA (Figure 2-5C). In addition, the ability of purified SpA and recombinant ClfA to bind osteoblasts was also tested. The purified SpA bound positively to osteoblasts (Figure 2-5D). On the other hand, and complementary to the above findings the recombinant ClfA did not support binding to osteoblasts (Figure 2-5D, P<0.05 when comparing with purified SpA).
Chapter 2 - Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**Figure 2-5** -- SpA binding to osteoblasts is not a strain dependent interaction. (A) Osteoblasts were allowed to bind to immobilized *S. aureus* Newman, 8325-4 and SH1000 wild type, SpA defective mutants and (B) Newman SpA complemented strain (Newman pCU1SpA) (1 x 10^9 cells/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=6. (C) *S. aureus* Newman and pCU1SpA cell wall preparations were separated on a 10 % SDS-PAGE gel and electroblotted onto PVDF membrane. Membranes were probed with anti-SpA antibody. Protein bands were detected using species specific horseradish peroxidase-conjugated secondary antibody and chemiluminescence. (D) Osteoblasts were allowed to bind to immobilized purified SpA or recombinant ClfA (50 µg/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=6.
2.4.6. Contribution of the *S. aureus* protein A domains in the binding to osteoblast

SpA is a major protein from *S. aureus* and it is present in over 90% of *S. aureus* strains (Forsgren 1970; Forsgren and Forsum 1970; Lachica, Genigeorgis et al. 1979; Fomenko 1980; Easmon and Adlam 1983; Sanford, Thomas et al. 1986). This 42 KDa long protein has been shown to comprise 7% of the cell wall (Forsgren 1969). Structurally SpA is composed of five highly homologous extracellular IgG-binding domains in tandem E, D, A, B, and C linked to the *S. aureus* cell wall surface by an LPXTG motif (Figure 2-6A) (Langone 1982; Uhlen, Guss et al. 1984). To further characterise the interaction of SpA with osteoblasts the ability of SpA E-C and D domains to bind osteoblasts along with the full length purified SpA was investigated. The osteoblast binding intensity of SpA D domain by itself was comparable to the levels obtained for the full length SpA. SpA D domain seemed to comprise more than 50% of the full length SpA binding intensity to osteoblasts (Figure 2-6B, P=NS). Alternatively, SpA E-C domains did not support as much osteoblast binding as the full length SpA (Figure 2-6B, P<0.05). The data obtained suggests that SpA D domain may be a crucial domain in SpA contributing to the interaction with osteoblasts.
Chapter 2 - Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**A**

**IgG binding domains**

S E D A B C W M C

**LPXTG**

**B**

Osteoblasts were allowed to adhere to immobilized purified SpA, SpA E-C domains and SpA D domain (50 ng/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. 

*P<0.05, bars indicate SEM, n=6.

Figure 2-6 -SpA D-domain highly contributes to the SpA – osteoblasts interaction. (A) Structural organization of SpA; S, Represents the signal sequence; E, D, A, B, C are the five IgG binding domains; W is the wall spanning region; and M represents the membrane spanning region. (B) Osteoblasts were allowed to adhere to immobilized purified SpA, SpA E-C domains and SpA D domain (50 µg/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. 

*P<0.05, bars indicate SEM, n=6.
2.4.7. The role of *Staphylococcus aureus* Sbi in the binding to osteoblast

*S. aureus* expresses another IgG binding protein that shares high homology with SpA, the *S. aureus* binder of IgG (Sbi). Given their sequence similarity it was investigated whether Sbi plays a role in binding osteoblasts. Using the ClustalW2, a program for alignment of protein sequences, the amino-acid sequences of SpA and Sbi were compared. The results revealed approximately 50% of homology between SpA and Sbi (Figure 2-7A). A *S. aureus* Newman isogenic mutant defective in Sbi expression was used to evaluate the role of Sbi in osteoblast binding. This study shows that unlike for SpA mutant strains, there were no significant differences in the binding between the *S. aureus* Newman WT and the *S. aureus* Newman strain defective in Sbi (Figure 2-7B, P=NS). These data suggest that despite the high homology between SpA and Sbi, Sbi does not play a role in *S. aureus* - osteoblasts interactions.
Chapter 2- Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

**A**

<table>
<thead>
<tr>
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<th>Sbi</th>
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<tr>
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<td>901234567890</td>
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</table>

Figure 2-7 - *S. aureus* Sbi is not involved in *S. aureus* binding to osteoblasts. (A) Alignment of SpA and Sbi from *S. aureus* with ClustalW2 (web automatic program for alignment of protein sequences) (B) Osteoblasts were allowed to bind to immobilized *S. aureus* Newman, SpA and Sbi defective mutants (1 x 10^9 cells/ml) for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro-plate reader. *P<0.05, bars indicate SEM, n=3.
2.4.8. The role of *S. aureus* protein A in osteoblast proliferation

After confirming a crucial role for *S. aureus* protein A in mediating bacterial binding to osteoblasts the outcome of this interaction was further investigated. To explore this, the strains *S. aureus* Newman WT, SpA defective strain, pCulSpA complemented strain and the purified SpA were pre-incubated with osteoblasts and their effect on growth and proliferation over a 48 h period were determined. Addition of live *S. aureus* to tissue culture media will starve osteoblasts of essential nutrients necessary to grow as the bacteria will compete for those nutrients for their own growth. To address this problem, *S. aureus* strains were fixed in a mild formaldehyde solution (4.8 %), to maintain bacterial cell integrity yet stunt bacterial growth. To ensure that formaldehyde fixation of the bacteria had no adverse effect on the expression of SpA by *S. aureus*, detection of SpA in both live and formaldehyde fixed bacterial cell lysates was determined by western blot (Figure 2-8A). Western blot analysis demonstrated consistent levels of SpA, at the correct molecular size 42 KDa, for both live and formaldehyde fixed bacterial cells lysate preparations. Formaldehyde fixed *S. aureus* Newman was added to osteoblasts and proliferation determined after 24 h and 48 h (Figure 2-8B). A proliferation control with resting osteoblasts, in the absence of *S. aureus*, demonstrated a regular proliferation profile as expected. However, osteoblast proliferation was inhibited by the addition of *S. aureus* Newman wild-type (P<0.01). Interestingly, the presence of *S. aureus* Newman defective in SpA did not prevent osteoblast from proliferating (P=NS to uninfected osteoblasts). Complementation of the *S. aureus* protein A defective mutant with pCU1spa restored the inhibitory effect on proliferation to levels comparable for Newman WT (P<0.01). Consistent with this, addition of purified protein A to osteoblasts also prevented proliferation (P<0.01). These results suggest that *S. aureus* protein A binds to osteoblasts triggering a signalling pathway which results in the inhibition of osteoblast proliferation.
Chapter 2- Study of the molecular mechanisms involved in S. aureus binding to osteoblasts: early steps in the infection process

Figure 2-8- S. aureus protein A inhibits proliferation. (A) Live or formaldehyde fixed S. aureus Newman were lysed for 10 min on ice in RIPA buffer containing protease inhibitors and probed with anti-SpA antibody. Protein bands were detected using species specific horseradish peroxidase-conjugated secondary antibody and chemiluminescence (western blot representative of 3 independent experiments). (B) Osteoblasts (5 x 10^5 cells/ml) were pre-incubated with either control buffer or formaldehyde fixed S. aureus Newman wild-type, Newman SpA defective mutant or Newman pCU1SpA (1 x 10^9 cells/ml) or purified protein A (50 μg/ml) over 48 h. After 0, 24 h and 48 h osteoblast were removed by trypsinization and proliferation was determined by counting cells on a haemocytometer. *P<0.01, error bars indicate SEM, n=5.
2.5. Discussion

To date many attempts have been made to understand bone destruction following bacterial infection, most notably on *S. aureus* Infections. Some studies have investigated the effects that *S. aureus* has on osteoblasts, and have demonstrated that the Fnbps on *S. aureus* surface interact with osteoblasts via α5β1 integrin (Sinha, Francois et al. 1999; Ahmed, Meghji et al. 2001). This interaction leads to bacterial invasion of osteoblasts and triggers a series of events that ultimately cause bone destruction. This is not a unique concept, *S. aureus* has evolved with many mechanisms to interact with the host cells, indeed *S. aureus* has many proteins that interact with human blood platelets in cardiovascular disease (Kerrigan, Clarke et al. 2008).

In this chapter, additional specific interactions between *S. aureus* and osteoblasts were exploited. The *S. aureus* cell wall components such as the capsule, teichoic acids and surface proteins were tested for their involvement in promoting bacterial binding to osteoblasts.

The capsular polysaccharides expressed on the *S. aureus* cell surface are a major virulence factor known to promote evasion or interference with the host immune system (Nilsson, Lee et al. 1997; Luong and Lee 2002). Furthermore, the teichoic acids have been demonstrated to play a crucial role in *S. aureus* nasal colonization and endovascular infection (Weidenmaier, Kokai-Kun et al. 2004; Weidenmaier, Peschel et al. 2005; Weidenmaier, Kokai-Kun et al. 2008). In addition, *S. aureus* owes much of its pathogenicity to an array of cell wall-anchored surface proteins, the microbial surface components recognizing matrix molecules (MSCRAMMs). Many MSCRAMMs expressed on *S. aureus* have now been identified and functional studies have demonstrated their ability to bind specific components of various host cells either directly (direct binding between the MSCRAMM and host cell receptor) or indirectly (MSCRAMM binds a plasma protein such as fibrinogen, fibronectin, collagen, bone sialoprotein or von Willebrand factor) (Patti, Allen et al. 1994; Waldvogel 2000).
A series of *S. aureus* mutants defective in specific components of the cell wall of *S. aureus* were used to investigate their interaction with osteoblasts. Deletion of capsule from *S. aureus* Newman or using the capsule negative strain *S. aureus* SH1000 failed to have any effect on binding, suggesting that the polymeric carbohydrates that are found in the capsule do not mediate attachment to osteoblasts (Figure 2-2A). Deletion of the teichoic acids on the *S. aureus* cell wall is very difficult and often leads to defective growth of the bacteria. Therefore, in order to investigate if this interaction plays a role in the bacterial binding to osteoblasts, antibodies (fAb fragments) against osteoblast toll like receptors (which recognise teichoic acids) were used. Blocking TLR2 did not interfere with the *S. aureus* binding to osteoblasts (Figure 2-2B), suggesting that the teichoic acid is not mediating the interaction between *S. aureus* and osteoblasts. Finally, removal of the surface proteins from *S. aureus* using a trypsin-EDTA treatment before binding to osteoblasts, revealed a significant reduction in the binding intensity (Figure 2-2C), and proved the importance of these in *S. aureus* interaction with osteoblasts.

As already mentioned the *S. aureus* Fnbps A and B have been previously shown to be essential in *S. aureus* invasion of osteoblasts (Jevon, Guo *et al.* 1999; Sinha, Francois *et al.* 1999; Ahmed, Meghji *et al.* 2001). However, in *S. aureus* Newman strain the Fnbps are not expressed at stationary phase and therefore do not account for binding to osteoblasts (Grundmeier, Hussain *et al.* 2004). Therefore, the *S. aureus* strain SH1000 expressing Fnbps A and B at exponential phase of growth was used to evaluate the specific role of Fnbps A and B in osteoblast binding (Saravia-Otten, Muller *et al.* 1997). *S. aureus* SH1000 isogenic mutants defective in FnbpA/B revealed a slight decrease in osteoblast binding ability. This is most likely due to the Fnbps capacity to interact with the osteoblasts integrin \(\alpha_\beta_3\), yet this decrease was not significant when compared with the WT strain (Figure 2-3, P=NS). Despite being essential for the internalization of *S. aureus* by osteoblasts, it seems that the FnbpA/B proteins are not important in the initial binding interaction between *S. aureus* and osteoblasts.
Chapter 2- Study of the molecular mechanisms involved in *S. aureus* binding to osteoblasts: early steps in the infection process

*S. aureus* expresses a number of surface proteins that play a critical role in host cell interactions. For example, *S. aureus* ClfA and ClfB can bind specifically to fibrinogen, fibronectin, and IgG which play a critical role in *S. aureus* adhesion to damaged heart valves in both human and rat model of endocarditis (McDevitt, Francois *et al.* 1994; Vaudaux, Francois *et al.* 1995; O'Brien, Kerrigan *et al.* 2002).

*S. aureus* protein A is a major protein expressed on *S. aureus* and has been shown to play an important role in the success of *S. aureus* as a human pathogen in many host cell interactions (O'Seaghdha, van Schooten *et al.* 2006; Foster 2009). Finally, although a relatively newly identified family of proteins the role of the Sdr family of proteins have yet to be fully elucidated. Early studies have demonstrated that the SdrC can bind bone sialoprotein and collagen (Ryden, Tung *et al.* 1997; Arrecubieta, Lee *et al.* 2007). Using a series of *S. aureus* Newman isogenic mutants defective in the expression of several *S. aureus* surface proteins (ClfA, ClfB, SpA and SdrC, D and E) it was demonstrated that only the lack of SpA significantly reduced the osteoblast binding intensity (Figure 2-4).

Furthermore different *S. aureus* strains lacking the expression of SpA also reduced the binding to osteoblasts (Figure 2-5A). In order to confirm the involvement of SpA, a complemented strain *S. aureus* Newman (pCU1SpA) expressing SpA was used, which completely recovered the binding to osteoblasts intensity (Figure 2-5B). Finally, it was also conclusively demonstrated that immobilized purified SpA from *S. aureus* supported osteoblast binding while the control purified recombinant ClfA did not, complementing the previous results (Figure 2-4). Collectively these results suggest that *S. aureus* protein A is critical in mediating and interaction with osteoblasts.

Since its discovery in 1961 the structural make-up of SpA has been the focus of a lot of attention. It comprises five IgG binding domains, and is anchored to the cell wall by a hydrophobic C-terminal region (Figure 2-6) (Patel, Kornblum *et al.* 1992). To further try and identify a particular domain or region on Spa that binds to osteoblasts purified fragments of SpA encoding the EDABC domains were used. Immobilized SpA D domain supported around 70% of the full length SpA
binding intensity to osteoblasts (Figure 2-6B, P=NS), while the SpA E-C domains only sustained approximately 20% (Figure 2-6B, P<0.05). These data suggests that the D domain of SpA may be the specific binding site for binding osteoblast however, further studies are required to support this and identify a more specific amino acid location.

Recent studies have identified another immunoglobulin binding protein from *S. aureus* called Sbi (Zhang, Jacobsson et al. 1998). This surface protein similar to SpA interacts with the Fc part of IgG, however unlike SpA, which requires the presence of both Fc and (VH3) Fab for precipitating human IgG, Sbi only requires the Fc portion (Atkins, Burman et al. 2008). Furthermore, Sbi binds the serum glycoprotein β2-GPI implicated in blood coagulation by affecting the activity of pro-thrombinase (Zhang, Jacobsson et al. 1999). Sequence homology of SpA and Sbi identified their similarities (Figure 2-7A). However, despite the high homology, a Sbi defective strain maintained the capacity to bind osteoblasts, as seen for the WT strain. These results suggest that Sbi is not important for the establishment of the interaction between *S. aureus* and osteoblasts (Figure 2-7B).

After establishing the importance of SpA in binding osteoblasts, its role in developing infection was further investigated. A common feature of infected bone samples from patients is the widespread loss of cells. Therefore studies were carried out to investigate if SpA binding to osteoblasts had downstream effects that resulted in inhibition of proliferation. To do this, osteoblasts were pre-incubated in the presence and absence of *S. aureus* strains and proliferation was asssed over a 48hr period. Osteoblasts failed to proliferate in the presence of *S. aureus* expressing SpA over a 48 h time course (Figure 2-8). Infection of osteoblasts with a an isogenic mutant of *S. aureus* lacking expression of SpA failed to have any effect on proliferation, suggesting that when *S. aureus* protein A binds to osteoblasts it triggers a signal that results in inhibition of proliferation.
To date the mechanism by which *S. aureus* causes weakening of the bones in osteomyelitis is not fully understood. Previous results demonstrate that osteoblasts internalise the *S. aureus* via an indirect interaction between the fibronectin binding proteins that bind fibronectin and form a bridge to osteoblast as a result *S. aureus* can evade immune responses and antibiotics. Here it is described for the first time that *S. aureus* also binds to osteoblasts through a mechanism that involves protein A. This interaction results in the generation of a critical signal that leads to inhibition of osteoblast proliferation. This finding provides new information on the underlying mechanisms likely to be involved in the significant bone loss seen in osteomyelitis patients. Results presented in this study provides evidence for the first time that protein A is likely to play a critical role in the success of *S. aureus* as a human pathogen in osteomyelitis and is a potential novel drug target for the treatment of this debilitating disease.
Chapter 3

Investigation of the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction
3.1. Introduction

The previous chapter demonstrated the importance of *S. aureus* protein A in binding to osteoblasts and inhibiting their proliferation over time. This is a major step into the first stage of infection however more experiments are needed to fully understand the mechanisms underlying the virulence of SpA in osteoblast infection and consequent pathway towards disease.

In this chapter it is hypothesised that SpA from *S. aureus* may play a role in bone destruction and inflammation following infection.

Osteomyelitis is mainly characterized by progressive inflammation and bone destruction. Although there are few studies investigating immune responses in osteomyelitis, production of pro-inflammatory mediators such as cytokines in response to *S. aureus* infection have been previously reported (Lowy 1998; Bannerman, Paape *et al.* 2004; Fournier and Philpott 2005). Interleukin-6 (IL-6) is a member of a family of pro-inflammatory cytokines that play a crucial role in the immune response (Wong and Clark 1988; Heymann and Rousselle 2000). IL-6 can be released by several cell types such as endothelial cells (Jirik, Podor *et al.* 1989; Sironi, Breviario *et al.* 1989), fibroblasts (Helfgott, May *et al.* 1987) and osteoblasts (Ishimi, Miyaura *et al.* 1990) in response to bacterial infection inducing leukocytes recruitment and fever (Gabay 2006; Lipsky 2006). In bone, IL-6 expression has been shown to be regulated by the transcriptional factor NFkB (Libermann and Baltimore 1990; Tak and Firestein 2001). NFkB represents a group of transcriptional factors controlling different biological processes such as immune and inflammatory responses (Ghosh and Karin 2002; Kalthoff 2009). In turn, IkB belongs to a family of cytoplasmic inhibitory proteins that regulate the translocation of NFkB from the cytoplasm to the nucleus. In resting cells, IkB binds to NFkB and sequesters it in the cytosol while in stimulated cells IkB is degraded allowing NFkB translocation to the nucleus where it will affect the expression of genes, such as IL-6 that mediates inflammatory responses (May and Ghosh 1997; Kalthoff 2009; Panzer, Steinmetz *et al.* 2009). Previous studies
using a murine model of staphylococcal osteomyelitis demonstrated the expression of IL-6 by osteoblasts (Marriott, Gray et al. 2004). Moreover, other studies demonstrated that IL-6 is involved in mediating osteoclastogenesis and bone resorption (Kotake, Sato et al. 1996) in human marrow cultures (Kurihara, Bertolini et al. 1990) and in the increase of the interactions between osteoblasts and osteoclasts (Kwan Tat, Padrines et al. 2004).

It is important to note that SpA is present on the surface of over 90% of S. aureus strains (Forsgren 1970; Forsgren and Forsum 1970; Lachica, Genigeorgis et al. 1979; Fomenko 1980; Easmon and Adlam 1983; Sanford, Thomas et al. 1986). SpA is a well-established and exceptionally complex virulence factor known to have multiple interactions with host molecules, interfering with their functions. Particularly, SpA is best known to bind the Fc region of IgG with anti-phagocytic properties (Cedergren, Andersson et al. 1993), the vWf, a large glycoprotein involved in mediating platelet binding at sites of endothelial damage promoting blood clot formation (Hartleib, Kohler et al. 2000; O’Seaghdha, van Schooten et al. 2006) and the Vh3 receptor of B-cells (Sasso, Silverman et al. 1989; Hillson, Karr et al. 1993). However, SpA has also been shown to mimic the role of TNF-α via direct interaction with the TNFR-1 in airway epithelial cells stimulating inflammation processes (Gomez, Lee et al. 2004; Gomez, O’Seaghdha et al. 2006). SpA can also regulate the availability of TNFR-1 on mucosal and immune cells by direct binding of its IgG binding domain to the epidermal growth factor receptor (EGFR) (Gomez, Seaghdha et al. 2007). On the other hand, among the known SpA ligands, both TNFR-1 and EGFR have been implicated in the bone metabolism regulation. The TNFR-1 has been shown to be involved in bone destruction by regulation of osteoclast formation following microbial stimulation (Hussain Mian, Saito et al. 2008; Ochi, Hara et al. 2010). In addition the EGFR has been demonstrated to regulate bone homeostasis by suppressing osteoblast differentiation and mineralization (Zhu, Shimizu et al. 2011)
3.1.1. Chapter Aims

- Identify the ligand for *S. aureus* protein A that is responsible for mediating the interaction with osteoblasts
- Investigate the downstream signalling events that occur as a result of *S. aureus* binding to osteoblasts that may play a role in bone destruction
- Investigate the downstream signalling events that occur as a result of *S. aureus* binding to osteoblasts that may play a role in inflammation
Chapter 3- Investigation the osteoblast receptor involved in binding S. aureus protein A and the downstream signalling events that occur as result of this interaction

### 3.2. Chemicals and general conditions

The chemicals and conditions used in this study are described in detail in section 2.2, additional chemicals and equipment for this specific chapter are stated below in Table 3-1.

Table 3-1- List of chemicals and equipment used in this chapter.

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<tr>
<th>Product</th>
<th>Supplier</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actinin mouse IgG</td>
<td>Santa Cruz Biotechnology</td>
<td>Heidelberg, Germany</td>
</tr>
<tr>
<td>Anti-Gly-Asp-Sep</td>
<td>Merck</td>
<td>UK</td>
</tr>
<tr>
<td>ApoAlert Caspase Colorimetric Assay Kits</td>
<td>Clontech Laboratories</td>
<td>CA, US</td>
</tr>
<tr>
<td>FACSCalibur flow cytometer</td>
<td>Becton Dickson</td>
<td>UK</td>
</tr>
<tr>
<td>Goat anti-mouse IgG HRP</td>
<td>PIERCE, Fisher Scientific</td>
<td>Ireland</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor 488</td>
<td>Invitrogen</td>
<td>Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG HRP</td>
<td>Invitrogen</td>
<td>Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Goat anti-sheep IgG Alexa Fluor 555</td>
<td>GE Healthcare</td>
<td>UK</td>
</tr>
<tr>
<td>Mouse IL-6 ELISA Ready-SET-Go</td>
<td>eBIOSCIENCE LTD</td>
<td>UK</td>
</tr>
<tr>
<td>Poly-L-lysine slides</td>
<td>BDH laboratory supplies</td>
<td>UK</td>
</tr>
<tr>
<td>Platelet Poor Plasma (from blood of a healthy donor)</td>
<td>RCSI</td>
<td>Dublin, Ireland</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-TNF-α, antibody</td>
<td>Santa Cruz Biotechnology</td>
<td>Heidelberg, Germany</td>
</tr>
<tr>
<td>Rabbit polyclonal α-actinin</td>
<td>Santa Cruz Biotechnology</td>
<td>Heidelberg, Germany</td>
</tr>
<tr>
<td>Silence siRNA for TNFR-1</td>
<td>Applied Biosystems</td>
<td>Ireland</td>
</tr>
<tr>
<td>TACS Annexin V kit. Apoptosis detection by flow cytometry</td>
<td>R&amp;D Systems Ltd</td>
<td>UK</td>
</tr>
<tr>
<td>VCA 1001 Cell line nucleofector kit A protocol</td>
<td>Lonza Cologne AG</td>
<td>Germany</td>
</tr>
</tbody>
</table>
3.3. Methods

3.3.1. Bacterial culture conditions

The bacterial culture conditions used in this chapter are described in detail in section 2.3.1.

Bacterial strains

The bacterial strains used in this chapter are listed below Table 3-2.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus Newman</td>
<td>NCTC 8178 wild-type Isolated from a case of secondarily infected tubercular osteomyelitis in man</td>
<td>(Duthie and Lorenz 1952)</td>
</tr>
<tr>
<td>S. aureus mutants Newman Δspa</td>
<td>spa:: Ka'; DU5971 Defective in Protein A Cm r Ap r spa gene cloned into pCU1. Insertion of pCU1spa capable of replicating in E. coli and S. aureus expressing Protein A. Expresses spa with an L17A substitution in each IgG binding domain.</td>
<td>(O'Brien, Kerrigan et al. 2002)</td>
</tr>
</tbody>
</table>

* The S. aureus strain Newman pCU1spaL17A was a kind gift from Prof. Timothy Foster in Department of Microbiology in Trinity College Dublin, Ireland.

3.3.2. Osteoblast culture conditions

The osteoblasts culture conditions of the MC3T3-E1 cell line used in this chapter are described in detail in section 2.3.2.

3.3.3. Functional assays

Osteoblast binding assay

The osteoblast binding protocol is described in detail in section 2.3.3. In some experiments 100 µl of Platelet Poor Plasma (PPP) was added to the immobilised
bacteria for 30 min at 37 °C, prior to adding the osteoblasts. In other experiments osteoblasts were pre-incubated with 50 μg/ml of α-TNF-1 antibody for 30 min at RT prior to their addition to the immobilised bacteria.

**Osteoblasts infection assay**
MC3T3-E1 osteoblasts (5x10^5 cells/ml) were added into a 6-well plate. The plate was incubated for 1 h at 37 °C with 5 % CO₂ atmosphere in tissue culture incubator. Following this one ml of formaldehyde fixed bacterial suspension (section 2.3.1.) was added to each well of the previously incubated 6-well plate. The plate was returned to the tissue culture incubator for different time-points depending on the experiment (0, 15, 30, 45, 60 and 90 min or 24 h and 48 h). Osteoblasts in the absence of bacteria were used as a control. The 6-well plate was then centrifuged for 10 min at 5,000 g at RT. The supernatant of each well was transferred to a new plate and stored at -20 °C for further analysis.

**3.3.4. Protein biochemistry**
The cell lysate preparation, the protein quantification and Western blot analysis were carried out as detailed in section 2.3.4.

The specific Western blot conditions used in this chapter are listed below in Table 3-3.
3.3.5. Flow cytometry

Flow cytometry is used to measure physical and chemical properties of single cells or particles as they pass in a fluid stream by a beam of laser light. As cells capture the light source they scatter light and the fluorochromes are excited to a higher level of energy. This energy is released as a photon of light with specific spectral properties unique and characteristic to different fluorochromes. Scattered and emitted light from cells is then converted to electrical pulses by optical detectors. To measure cells fluorescence a logarithmic amplification is used, spreading out the scale for weak signals and compressing the scale for strong or specific fluorescence signals. After amplification the different signals or pulses are processed by an analog to digital converter (ADC) which plots the events on a graphical scale, histogram (Melamed, Lindmo et al. 1990; Givan 1992; Shapiro 1995).

Seventy five μl of osteoblasts (1x10^6 cells/ml) were incubated with 25 μl of primary α-TNFR-1 rabbit polyclonal antibody or 25 μl of α-actinin rabbit IgG (isotype control) for 1 h at RT followed by 30 min incubation with 2 μl of

---

Table 3-3: Western blot conditions

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Molecular weight (KDa)</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpA</td>
<td>42</td>
<td>Monoclonal chicken IgY anti-SpA produced in mouse</td>
<td>Goat anti-mouse IgG HRP</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monoclonal IgG antibody produced in mouse</td>
<td>Goat anti-mouse IgG HRP</td>
<td>1:1000</td>
</tr>
<tr>
<td>IgG</td>
<td>42</td>
<td>Polyclonal anti-TNFR-1 produced in rabbit</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:1000</td>
</tr>
<tr>
<td>TNFR-1</td>
<td>55</td>
<td>Anti-IκB produced in mouse</td>
<td>Goat anti-mouse IgG HRP</td>
<td>1:1000</td>
</tr>
<tr>
<td>IkB</td>
<td>35</td>
<td>Polyclonal α-actinin produced in rabbit</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:20000</td>
</tr>
<tr>
<td>α-actinin</td>
<td>105</td>
<td>Polyclonal β-actinin produced in rabbit</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-actinin</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---
secondary Goat anti-rabbit IgG R-PE at 4 °C in the dark. Four hundred μl of PBS were then added to both cells suspension to stop antibody binding. A cell suspension with 100 μl of osteoblasts and 400 μl of PBS was used as buffer control. Five thousands viable cells from each sample were counted without gate using a fluorescent-activated cell sorter FACSCalibur™ and analysed using the Cell Quest software Becton Dickinson.

3.3.6. Confocal microscopy

Confocal microscopy is an optical imaging technique that enables deep visualization within living and fixed cells and tissues. It is advantageous over the conventional microscopes for its ability to use spatial filtering that eliminates the out-of-focus light in specimens that are thicker than the focal plane. Moreover, it enables the collection of sharply defined optical sections from which three-dimensional structures can be obtained (Masters 2006; Pawley 2006).

Slide preparation

For confocal microscopy imaging pre-treated poly-L-lysine slides (poly-prep slides) were used. Slides were rinsed with ethanol, dried for 2 min and washed with dH2O and allowed to dry. Following this, slides were blocked for 2 h at 37 °C with 1 % BSA prepared in TBS. All TBS was sterile filtered using 0.22 μm filters.

Osteoblast binding on slides

Slides were washed with TBS and subsequently coated with MC3T3-E1 osteoblasts (5x10^5 cells/ml) in TBS and incubated in a humidity chamber for 1 h at 37 °C, a control without osteoblasts was also incubated with TBS only. Slides were then fixed using 3.8 % formaldehyde for 10 min at RT. After a new wash with TBS, slides were permeabilized for 5 min with acetone at 4 °C and subsequently stained using α-actin mouse IgG and α-TNFR-1 rabbit antibody for 1 h at 37 °C. Slides were washed twice with TBS. Secondary staining followed
using a Goat anti-mouse IgG Alexa Fluor 488 and a Goat anti-rabbit IgG R PE antibodies for 45 min at 37 °C. Slides were washed with TBS to remove excess stain and covered with a glass cover slip and mounting media. Slides were kept at 4 °C until visualization using an oil-immersion 63 X objective lens and a Zeiss LSM 510 confocal microscope.

3.3.7. Apoptosis

Caspase 3

Caspase 3 is a downstream effector caspase important in death receptor apoptotic mechanisms. Caspase 3 activity was detected using the ApoAlert Caspase Colorimetric Assay Kit. The colorimetric assay uses the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after its cleavage by caspases from the labelled caspase-specific substrates (Scheme 3-1). Comparing the reading of an apoptotic sample with an un-induced control allows determination of the protease activity. Units of protease activity are quantified accurately and reproducibly using a standard curve established with free pNA.

\[ \text{Induction of apoptosis in cells} \rightarrow \text{Protease activation} \rightarrow \text{Caspase-3} \]

\[ \text{DEVD-pNA} \]

\[ \text{DEVD} \rightarrow \text{pNA} \]

\[ \text{colorimetric detection} \]

**Scheme 3-1** - The colorimetric assay uses spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA (ApoAlert Caspase Colorimetric Assay Kits brochure, Clontech Laboratories)
Twenty-four hour *S. aureus* infected and un-infected osteoblasts were adjusted to $2 \times 10^6$ cells/ml and cell suspension was centrifuged at 9,000 g for 5 min. Pellets were re-suspended in 50 μl of chilled cell lysis buffer transferred to eppendorf tubes and incubated on ice for 10 min. Cell suspensions were then centrifuged at 15,000 g in a micro-centrifuge for 10 min at 4 °C and supernatants transferred to new eppendorf tubes. Cell lysates were incubated with 50 μl of 2X reaction buffer, 100mM of DTT and 5 μl of 1 mM caspase 3 substrate DEVD-pNA in a 37 °C waterbath for 1 h. Two parallel control reactions were set up with Caspase 3 inhibitor and without conjugated substrate. The pNA calibration curve was generated by diluting 100 mM of pNA solution in cell lysis buffer making 0, 0.5, 1, 2, and 4 mM stock solutions, and further diluted to obtain the final pNA concentrations of 0, 2.5, 5, 10 and 20 nmol. The absorbance of standard and samples was read at 405 nm wavelength using the Wallac Victor2 micro plate reader system. The caspase 3 activity of each sample was then extrapolated from the equation of the line recovered from the standard curve (Scheme 3-2).

![pNA Calibration curve](image)

**Scheme 3-2**- Example of a pNA standard curve. The equation of the line it is used to calculate the units of caspase 3 activity.
Annexin V detection by Flow Cytometry

One of the applications of flow cytometry is the analysis of cell apoptosis. Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Annexin V allows identification of cell surface changes that occur early during the apoptotic process. The AnnexinV-FITC conjugate facilitates rapid fluorometric quantification of apoptotic cells. Early in the apoptosis process, phosphatidylserine (PS) becomes exposed on the cell surface by flipping from the inner to outer leaflet of the cytoplasmic membrane (Scheme 3-3). PS is therefore detected and bound by the Annexin-FITC conjugate. Propidium iodide (PI) is also used to identify cells that have lost membrane integrity. As cells disintegrate, greater access to the inner cell membrane allows for additional Annexin V binding. Thus, double labelling is used to help differentiate between early and late apoptotic/necrotic events (Scheme 3-4).

Scheme 3-3- The flipping of PS from the inner leaflet to the outer leaflet of the cell membrane represents a hallmark (early and widespread) in detecting dying cells (Annexin V brochure, Trevigen®).
Chapter 3- Investigation the osteoblast receptor involved in binding S. aureus protein A and the downstream signalling events that occur as result of this interaction

Scheme 3-4  - (A) Example of a dot plot of untreated and (B) Dexamethasone treated thymocytes showing viable, early apoptotic (Annexin V FITC positive) and late apoptotic or necrotic cells, respectively (Annexin V brochure, Trevigen®).

Apoptosis detection was carried out using the TACS® Annexin V kit- Apoptosis detection by flow cytometry. Un-infected and S. aureus infected osteoblasts over 24 h were collected by centrifugation at 9,000 g for 5 min at RT. Cells were washed once in 500 μl of PBS at 4 °C followed by centrifugation at 9,000 g for 5 min. Cells were then re-suspended in 100 μl of Annexin V Incubation Reagent and left in the dark at RT for 15 min. Finally 400 μl of 1X Binding buffer was added to the cell suspension, and each sample was processed within 1 h by FACSCalibur™ instrument with the Cell Quest software (Becton Dickinson).

3.3.8. ELISA

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique widely used to determine the presence and the amount of a particular protein in a sample.
Chapter 3 - Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

**Scheme 3-5** ELISA assay illustration. A capture antibody is immobilised to a surface. The specific protein present in the sample is applied over the same surface allowing their interaction. After washing, the protein of interest will be recognized by the detection antibody which in turn will be linked to an HRP linked antibody that will be detectable by the addition of a chemical substrate that causes a colorimetric change (Illustration adapted from biosystemdevelopment.com).

The amount of IL-6 released from non-infected and *S. aureus* infected osteoblasts over 24 h and 48 h was quantified using the Mouse IL-6 ELISA Ready-SET-Go (eBIOSCIENCE LTD, UK). A 96-well micro plate was coated with 100 µl per well of IL-6 capture antibody and sealed with hydrophobic porous sealing film. The plate was then incubated overnight at 4 °C. Following this, wells were washed by addition of 250 µl of Wash Buffer. The plate was inverted and blotted on absorbent paper to remove any residual buffer. Each well was blocked with 200 µl of 1 X assay diluent and the plate was incubated at RT for 1 h. Standard curve samples were prepared by diluting mouse recombinant IL-6 in 1 X assay diluent from a 5,000 pg/ml to 0 pg/ml concentrations. The plate was washed again by adding 250 µl of Wash Buffer per well. Finally, 100 µl of standard or sample was added to the 96-well plate. The plate was sealed using hydroporous sealing film and incubated at RT for 2 h. Following washing the plate, 100 µl of detection antibody was added to each well. The plate was incubated for 30 min at RT. After three washes with 250 µl of Wash Buffer per well, 100 µl of Substrate solution was added to each well, the plate was incubated for 15 min at RT. Finally
50 µl of stop solution was added to each well. Absorbance was read at 450 nm wavelength using the Wallac Victor2 micro plate reader system (Perkin Elmer, Cambridge, UK).

3.3.9. siRNA mediated silencing of the TNFR-1 gene

Small interfering RNA (siRNA) or silencing RNA is a short (21-23bp) double stranded RNA with several functions in cells. siRNA transfection involves silencing genes in order to suppress the expression of a particular gene. The success of these experiments depends on the method of delivery of siRNA (transfection or electroporation) and on the transfection agents used, also on the cell type that might be hard to transfect.

The silencer siRNA for TNFR-1 from Applied Biosystems was used to knockdown the expression of the TNFR-1 gene on MC3T3-E1 osteoblasts (Scheme 3-6). To do so the VCA 1001 Cell line nucleofector kit R protocol (Lonza Cologne AG, Germany) was used.

Osteoblasts were harvested by trypsinization and the cell number adjusted to 1x10^6 cells per reaction. After centrifugation at 9,000 g for 5 min, supernatant was removed and each pellet was re-suspended in 100 µl of RT Nucleofector® solution R and 150 nM of silencer siRNA for TNFR-1. The cell- siRNA suspension
was then transferred into the certified cuvette. After selecting the Nucleofector® Program A-024 on the Nucleofector system apparatus the cuvette was inserted and the program runs for 5 s. Immediately after the program finished, 500 µl of 37 °C pre-equilibrated osteoblast media was added to the cuvette and gently transferred into a 6-well plate (final volume of 1.5 ml per well). Plate was incubated at 37 °C with 5 % CO₂ atmosphere until analysis (after 24 h and 48 h).

**Efficiency test**

The efficiency of gene knockdown was tested by Western blot, as detailed in section 2.3.4. using the antibody dilutions described below, in Table 3-4.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Molecular weight (kDa)</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFR-1</td>
<td>55</td>
<td>Polyclonal anti-TNFR-1 produced in rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-actinin</td>
<td>105</td>
<td>Polyclonal α-actinin produced in rabbit</td>
<td>1:5000</td>
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</table>

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Secondary antibody</th>
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<th>concentration</th>
<th>antibody</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
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<td>Polyclonal anti-TNFR-1 produced in rabbit</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:1000</td>
<td>1:10000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyclonal α-actinin produced in rabbit</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:5000</td>
<td>1:20000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4. Results

3.4.1. Identification of the *S. aureus* protein A ligands that are involved in binding osteoblast

*S. aureus* protein A is well known for its multiple interactions with host ligands. Some studies have already demonstrated that SpA residues exposed on the helices I and II bind to the Fc region of IgG and that the binding site for the vWF regions also recognizes a region on helices I and II of SpA, overlapping the IgG Fc binding site (O'Seaghdha, van Schooten et al. 2006). Other studies demonstrated that the TNFR-1 also binds to this face but there are some differences in the residues of SpA that are involved. In particular, leucine 17 is crucial for binding to IgG but not for TNFR-1 binding (Gomez, O'Seaghdha et al. 2006).

Human plasma is rich in several proteins such as albumin, fibrinogen, immunoglobulins and von Willebrand factor (vWf). Platelet poor plasma (PPP), which is rich in plasma proteins, was pre-incubated with *S. aureus* Newman prior to binding to osteoblasts. There was no significant difference between *S. aureus* Newman binding to osteoblast in the absence of PPP compared to PPP pre-incubation (Figure 3-1A, P=NS). These results show that even after bacterial binding to plasma proteins, *S. aureus* Newman strain binding to osteoblasts was not affected.

Previously, it was demonstrated that deletion of a single amino-acid at the Leucine 17 of each IgG binding domain of SpA in *S. aureus* Newman pCu1SpA L17A resulted in the loss of the ability of SpA to bind IgG (unpublished Magnhus O'Seaghdha 2008, Trinity College Dublin). The *S. aureus* Newman pCu1SpA L17A strain was used to investigate whether the IgG binding site was important for binding to osteoblasts. *S. aureus* NewmanpCu1SpA L17A binding to osteoblasts was similar to the binding observed for *S. aureus* Newman WT strain (Figure 3-1B, P=NS). Consistent with the previous results it was demonstrated that the IgG binding site of SpA is not the binding site that mediates *S. aureus* – osteoblast
interaction. More specifically, when SpA binds IgG or vWf it does not affect its interaction with osteoblasts, suggesting a distinct binding site.

In order to confirm loss of IgG antibody binding in *S. aureus* pCu1SpAL17A strain two different antibodies were used, an IgG binding antibody and the anti-SpA IgY antibody. Western blot analysis confirmed the expression of SpA represented by a band at the expected size of 42 KDa for all *S. aureus* Newman strains WT, pCu1SpA and pCu1SpAL17A cell wall preparations when using the IgY binding antibody (Figure 3-1C). However, as expected, the *S. aureus* Newman pCu1SpaL17A strain did not bind the IgG antibody as the SpA binding site was mutated.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

**Figure 3-1 (A)** - SpA ligands vWF and IgG do not mediate the binding to osteoblasts. Osteoblasts were allowed to adhere to immobilised *S. aureus* Newman WT control and pre-incubated with PPP for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader. Error bars indicate SEM, n=4.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

Figure 3-1 (B + C)-- SpA ligands vWf and IgG do not mediate the binding to osteoblasts. (B) Osteoblasts were allowed to adhere to immobilised *S. aureus* Newman WT, pCU1SpA and pCU1SpA L17A for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader. Error bars indicate SEM, n=4 (C) *S. aureus* Newman WT, pCU1SpA and pCU1SpAL17A were lysed, separated on a 10 % SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with either a non-specific IgG or anti-SpA IgY antibody. Protein bands were detected using species specific horseradish peroxidise-conjugated secondary antibody and chemiluminescence.
3.4.2. Investigating the expression of the Tumor Necrosis Factor Receptor 1 on osteoblast surface

Consistent with the literature, a 55 KDa band corresponding to the TNFR-1 molecular size was observed in a Western blot of osteoblast lysates (Figure 3-2A) (Bu, Borysenko et al. 2003). The expression of TNFR-1 on osteoblasts was also evaluated using FACs analysis. Pre-labelling osteoblasts with an isotype control or anti-TNFR-1-FITC antibody revealed a positive shift of the curve representing the TNFR-1 positive osteoblasts when compared with the isotype control curve (Figure 3-2B). In addition confocal analysis was used to visualize the expression of TNFR-1 on the osteoblast surface using α-TNFR-1-PE staining. Actin is abundantly found in most cell types including osteoblasts; therefore labelling osteoblasts with an α-actin-FITC was used as control for this experiment. Osteoblast α-TNFR-1-PE and α-actin-FITC staining are represented respectively in green and red in Figure 3-2C. The results obtained confirmed consistent TNFR-1 expression on the osteoblast surface.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction.

**Figure 3-2** — TNFR-1 is expressed on osteoblasts surface. (A) Osteoblasts lysates were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with rabbit polyclonal anti-TNFR-1. Protein bands were detected using rabbit horseradish peroxidase-conjugated secondary antibody and chemiluminescence. (B) Osteoblasts were pre-incubated with primary rabbit polyclonal anti-TNFR-1 antibody for 1 h at RT followed by 15 min pre-incubation with secondary FITC. Samples were analysed on a flow cytometer. (C) Osteoblasts were added to poly-L-lysine slides previously coated with BSA for 1 h at 37 °C. Slides were fixed using 3.8% formaldehyde for 10 min at RT and permeabilized with 4 °C acetone for 5 min. Osteoblasts were then stained using α-actin-FITC and α-TNFR-1-PE. Visualization was done using an oil-immersion 63 X objective lens in a Zeiss LSM 510 confocal microscope.
3.4.3. Investigating the osteoblast receptor that binds to *S. aureus* protein A

In the previous section it has been demonstrated that *S. aureus* does not use a plasma protein (IgG or VwF) in order to bridge the bacteria to osteoblast and mediate attachment. Therefore binding of *S. aureus* protein A to the TNFR1 was investigated. To do so, osteoblasts were pre-incubated with an anti-TNFR-1 polyclonal antibody. However, this antibody is of IgG origin and not suitable to use with the *S. aureus* Newman WT strain because it binds to SpA. Therefore, a *S. aureus* Newman pCu1L17A strain with affinity to bind osteoblast while lacking the ability to bind IgG, was used. In the presence of the anti-TNFR-1 antibody *S. aureus* binding to osteoblasts was significantly reduced (Figure 3-3A, P<0.001). Moreover osteoblasts binding to purified SpA was also significantly reduced (P<0.001).

In order to investigate that TNFR-1 on osteoblasts surface is essential for binding to SpA on *S. aureus*, siRNA was carried out to knock down the expression of this receptor on osteoblasts surface. Following this, Western blot analysis confirmed successful silencing of the expression of TNFR-1 on osteoblasts (Figure 3-3B). The blot shows a 55 KDa band (TNFR-1 molecular size) for the siRNA negative osteoblasts and no band for the siRNA positive osteoblasts. Alpha-actinin was used as protein loading control. Consistent with the antibody inhibition results above, it was demonstrated that silencing of TNFR-1 expression on the osteoblast surface significantly reduced binding to *S. aureus* (Figure 3-3C, P<0.05). Interestingly binding to purified SpA was also significantly reduced for the TNFR-1 silenced osteoblasts (P<0.05).
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

Figure 3-3 (A)- TNFR-1 is the receptor for SpA on osteoblasts surface. Isotype control and anti-TNFR-1 pre-incubated osteoblasts were allowed to adhere to immobilised *S. aureus* Newman, ΔSpA and pCU1SpAL17A and purified SpA for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a microplate reader. *P<0.05, **<0.001 error bars indicate SEM, n=3.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

**Figure 3-3 (B + C)**- TNFR-1 is the receptor for SpA on osteoblasts surface. (B) siRNA negative and siRNA positive osteoblasts lysates were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with rabbit polyclonal anti-TNFR-1 and α-actinin as equal loading control. Protein bands were detected using rabbit horseradish peroxidise-conjugated secondary antibody and chemiluminescence. (C) siRNA negative osteoblasts and siRNA positive osteoblasts were allowed to adhere to *S. aureus* Newman wild-type and ΔSpA for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a microplate reader. *P<0.05, **<0.001 error bars indicate SEM, n=3.
3.4.4. Investigating downstream events as a result of *S. aureus* protein

A binding to osteoblast TNFR-1: Apoptosis

Given the observation that the ligand for SpA on osteoblast surface is the death receptor TNFR-1, the signalling pathway following infection with *S. aureus* strains was then investigated. *S. aureus* has previously been shown to induce apoptosis in osteoblasts (Tucker, Reilly et al. 2000; Alexander, Rivera et al. 2003). Previously it was demonstrated that SpA is involved in mediating inhibition of osteoblast proliferation. Therefore, the role of SpA binding to osteoblasts in inducing apoptosis and consequent inhibition of osteoblast proliferation was investigated. Osteoblast apoptosis was first determined by measuring the amount of caspase 3 activity following incubation of cultured osteoblasts with and without formaldehyde treated *S. aureus* after a 24 h period. Caspase 3 is a downstream effector caspase important in the apoptotic pathway. In the absence of *S. aureus*, caspase 3 activity was minimal. However, after incubation with *S. aureus* Newman wild-type, caspase 3 activity was significantly increased, indicating that the osteoblasts were undergoing apoptosis (Figure 3-4A, P<0.05). Interestingly, no increase in caspase 3 activity was seen following addition of the *S. aureus* protein A defective mutant (P=NS compared to the resting osteoblasts). The *S. aureus* Newman pCU1SpA complemented strain significantly induced caspase 3 activity as was *S. aureus* Newman WT (P<0.01). Moreover, addition of purified SpA to osteoblasts also considerably increased caspase 3 activity (P<0.05).

Detection of osteoblast apoptotic events was also measured by quantifying the amount of Annexin V - FITC binding following incubation of cultured osteoblasts with and without formaldehyde treated *S. aureus* over a 24 h period. Annexin V binds to the phosphatidylserine (PS) that becomes exposed on the cell surface when it flips from the inner to the outer leaflet of the cytoplasmic membrane of the cell, a main characteristic of early apoptosis. In the absence of *S. aureus* approximately 40% of the resting osteoblasts appeared to bind Annexin V antibody (Figure 3-4B). However, the addition of *S. aureus* Newman to the osteoblasts led to a significant increase in Annexin V binding (Figure 3-4B+C, 145
P<0.01). In contrast, addition of \textit{S. aureus} Newman SpA defective mutant failed to induce apoptosis above the levels obtained for the resting osteoblasts (P=NS). Moreover, addition of \textit{S. aureus} Newman pCU1SpA to osteoblasts significantly increased the levels of Annexin-FITC binding implicating a significant increase on the levels of apoptotic osteoblasts when compared with the resting osteoblasts levels (P<0.05). The amount of dead osteoblasts before and after addition of formaldehyde fixed \textit{S. aureus} was also quantified by measuring the Propidium Iodide (PI) binding intensity over 24 h (Figure 3-4D). PI is used to identify cells that have lost membrane integrity by binding defragmented DNA. The levels of dead osteoblasts for the resting control remained lower than 20 %, while after incubating osteoblasts in the presence of \textit{S. aureus} Newman wild-type increased to more than 40 % (Figure 3-4D, P<0.01). Addition of \textit{S. aureus} Newman SpA defective mutant did not change the levels of dead osteoblasts when compared to un-infected osteoblasts (P=NS). However, the addition of \textit{S. aureus} Newman wild-type and complemented strain Newman pCU1SpA showed a significant increase in the numbers of PI positive osteoblasts (Figure 3-4D, P<0.01 and P<0.05, respectively).
Chapter 3- Investigation the osteoblast receptor involved in binding S. aureus protein A and the downstream signalling events that occur as result of this interaction

Figures 3-4 (A) - SpA expressing strains induce osteoblast apoptosis. Osteoblasts (5 x 10^5 cells/ml) were pre-incubated with either control buffer or formaldehyde fixed S. aureus Newman (1 x 10^9 cells/ml) for 24 h. Osteoblasts were lysed and incubated with caspase 3 substrate (DEVD-pNA) for 1 h at 37 °C. Caspase 3 cleavage was measured at 405 nm in a microplate reader.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction.

**B**

Uninfected WT ASpA___________pCUlSpA osteoblasts

**C**

Annexin V-FITC positive osteoblasts

Figures 3-4 (B+C) - SpA expressing strains induce osteoblast apoptosis. Annexin V-FITC binding by FACS analysis: pellets from un-infected or *S. aureus* Newman infected osteoblasts were re-suspended in 100 μl of Annexin V-FITC labelled antibody. Suspensions were incubated in the dark for 15 min at RT and analysed by flow cytometry and plotted (B) in a graphic bar; and (C) in a histogram. *P<0.01, **P<0.05, error bars indicate SEM, n=3.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

Figures 3-4 (D)- SpA expressing strains induce osteoblast apoptosis. PI staining by FACs analysis: pellets from un-infected or *S. aureus* Newman infected osteoblasts were re-suspended in 100 μl of PI staining. Suspensions were incubated in the dark for 15 min at RT and analysed by flow cytometry and plotted in a graphic bar. *P<0.01, **P<0.05, error bars indicate SEM, n=3.
3.4.5. Investigating downstream events as a result of *S. aureus* protein A binding to osteoblast TNFR1: NFκB activation

As well as the TNFR-1 being recognised as a death receptor it also plays an important role in the control of gene transcription through NFκB. NFκB is a dimeric protein which translocates from the cytoplasm to the nucleus and is controlled by the inhibitory protein IkB (Carmody and Chen 2007; Kalthoff 2009). When the TNFR-1 pathway is stimulated IkB is degraded and NFκB is released to the nucleus where it activates transcriptional factors that mediate the expression of inflammatory cytokines and chemokines (Panzer, Steinmetz et al. 2009).

As a measure of NFκB activation, degradation of IkB was investigated by immunoblotting. Normal osteoblasts were infected over a 90 min period with *S. aureus* Newman WT, ΔSpA and pCu1SpA strains. Anti-TNFR-1 pre-incubated osteoblasts and TNFR-1 silenced osteoblasts were infected with *S. aureus* Newman WT over the same period. HEK293t cells were used as a positive control. IkB degradation was observed over a 90 min period following infection of osteoblasts with *S. aureus* Newman WT (Figure 3-5A) and pCu1SpA (Figure 3-5C). However, following infection with *S. aureus* Newman SpA defective strain, IkB was not degraded over the 90 min period (Figure 3-5B). Consistent with this pre-incubating osteoblasts with an anti-TNFR-1 antibody (Figure 3-5D), in order to prevent the interaction with *S. aureus* protein A, prevented the degradation of IkB. Furthermore using osteoblasts lacking the expression of TNFR-1 also failed to induce IkB degradation in the presence of *S. aureus* Newman (Figure 3-5E). These results demonstrate that in the absence of SpA on the *S. aureus* surface or in TNFR-1 blocked or defective osteoblasts, the TNFR-1 pathway is not stimulated. Consequently IkB retains NFκB in the cytosol compromising the transcription of genes involved in inflammatory response.
Chapter 3- Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

<table>
<thead>
<tr>
<th>Stimulation time</th>
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<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
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<td><img src="image" alt="Ikβ (36 KDa)" /></td>
<td><img src="image" alt="Ikβ (36 KDa)" /></td>
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<td><img src="image" alt="Ikβ (36 KDa)" /></td>
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<tr>
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<td><img src="image" alt="Equal loading α actinin (105 KDa)" /></td>
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<td><img src="image" alt="Equal loading α actinin (105 KDa)" /></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3-5** - SpA binding to TNFR-1 induces NFκB activation. Ikβ expression over 90 min period in normal osteoblasts following infection with *S. aureus* Newman WT, ΔSpA and pCulSpA and in α-TNFR-1 blocked or defective osteoblasts, following infection with *S. aureus* Newman WT. Uninfected and infected osteoblasts lysates were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with monoclonal Ikβ antibody. Protein bands were detected using mouse horseradish peroxidise-conjugated secondary antibody and chemiluminescence. Immunoblots are representative of three independent experiments.
3.4.6. Investigating downstream events as a result of \textit{S. aureus} protein A binding to osteoblast TNFR-1: Cytokine release

IL-6 is a major inflammatory cytokine released at the site of infection in several host cells including osteoblasts. Its expression is controlled by NFkB. IL-6 release in normal and TNFR-1 silenced osteoblast supernatants before and after infection with \textit{S. aureus} Newman over 24 h and 48 h was investigated. The levels of IL-6 release 24 h after infection with \textit{S. aureus} Newman WT and pCu1SpA were significantly higher, than the levels of IL-6 released in un-infected osteoblast controls (Figure 3-6, P<0.05). IL-6 release was further increased 48 h after infection when osteoblasts were infected with both \textit{S. aureus} Newman WT and pCu1SpA (P<0.001). Infecting osteoblasts with \textit{S. aureus} Newman strain defective in SpA expression did not induce release of this cytokine over 24 h or 48 h (P=NS, when comparing with the un-infected osteoblasts control). IL-6 secretion by TNFR-1 silenced osteoblasts remained low before and after infection with \textit{S. aureus} Newman WT, over 24 h and 48 h (P=NS, when compared with the un-infected osteoblasts control). These results suggest that \textit{S. aureus} protein A can induce NFkB activation and regulate the release of IL-6 from \textit{S. aureus} infected osteoblasts by direct interaction with the TNFR-1.
Chapter 3 - Investigation the osteoblast receptor involved in binding *S. aureus* protein A and the downstream signalling events that occur as result of this interaction

![Graph showing IL-6 concentration](image)

**Figure 3-6** - SpA binding to TNFR-1 induces IL-6 release on osteoblasts. The levels of IL-6 released were quantified in supernatants of un-infected and *S. aureus* infected normal and TNFR-1 defective osteoblasts, over 24 h and 48h infection times. *P<0.05, **P<0.001, error bars indicate SEM, n=3.
3.5. Discussion

In the previous chapter it was demonstrated that *Staphylococcus aureus* protein A mediates bacterial binding to osteoblasts inducing inhibition of osteoblast proliferation after infection. SpA is one of the most prevalent cell wall surface proteins on *S. aureus* and it has been shown to play an important role in the success of *S. aureus* as a human pathogen (O'Seaghdha, van Schooten *et al.* 2006; Foster 2009). By binding the Fc portion of immunoglobulin, SpA assists *S. aureus* in evasion of phagocytosis by neutrophils (Foster 2005). SpA also binds to von Willebrand factor which may play a role in supporting platelet binding in the early stages of thrombosis (O'Seaghdha, van Schooten *et al.* 2006). More recently, Gomez and colleagues demonstrated that SpA binds to TNFR-1 resulting in pro-inflammatory signaling (Gomez, O'Seaghdha *et al.* 2006) in lung epithelial cells in staphylococcal pneumonia. In this chapter, it was investigated whether any of these sites were important for *S. aureus* protein A binding to osteoblasts.

In this study, consistent with previous reports, expression of TNFR-1 on osteoblasts surface was confirmed, making this a possible candidate for binding to *S. aureus* protein A. The TNFR-1 on osteoblasts plays a particularly important role in bone disease as engagement of TNFα with its receptor has been implicated in a wide spectrum of bone diseases including osteoporosis and rheumatoid arthritis (Chen and Goeddel 2002; Ochi, Hara *et al.* 2010). To investigate the role of TNFR-1 in binding SpA osteoblasts were pre-incubated with a blocking polyclonal antibody against TNFR-1. Blockade of the receptor resulted in significantly reducing *S. aureus* binding to osteoblasts and totally abolished the binding of purified SpA and SpA D-domain to osteoblast. These results suggest that *S. aureus* protein A binds to the TNFR-1 on osteoblast most likely through its D domain. In support of this, siRNA was also used, to knock down the TNFR-1 on osteoblasts and investigate the interaction with *S. aureus*. Consistent with above osteoblasts lacking expression of TNFR1 significantly reduced the binding to *S. aureus*. Interestingly *S. aureus* lacking expression of
SpA demonstrated reduced binding to the TNFR-1 silenced osteoblasts suggesting other interactions exist.

The downstream signalling functions of TNFR1 on various cell types is the focus of many research groups throughout the world. Probably the best characterised function of TNFR1 is its ability to control apoptosis and inflammatory processes (Locksley, Killeen et al. 2001; Chen and Goeddel 2002). Following engagement of TNFR-1 by its ligand two independent downstream pathways can be activated: the activation of the apoptotic cascade that will end in cell death, and the activation of the NFkB signaling which mediates the expression and secretion of inflammatory cytokines (Ghosh and Karin 2002; Kalthoff 2009).

Several reports have demonstrated that *S. aureus* can induce apoptosis in osteoblasts (Tucker, Reilly et al. 2000; Alexander, Rivera et al. 2003); however the mechanism by which this occurs has not yet been elucidated. Caspase 3 activity and membrane blebbing are key mechanisms that lead to cell apoptosis. Active caspase 3 and annexin V binding (as a measure of membrane blebbing) were detected in un-infected osteoblasts, most likely due to normal apoptosis and cell turnover in the *in vitro* osteoblast tissue culture system. Consistent with previous observations (Alexander, Rivera et al. 2003) when osteoblasts were exposed to *S. aureus*, caspase 3 activity and annexin V binding were significantly increased. However, the SpA mutant yielded similar results to the un-infected osteoblasts whereas the *S. aureus* Newman pCu1SpA complemented strain or addition of purified protein A, increased caspase 3 and annexin V in osteoblasts to levels similar to *S. aureus* Newman wild-type. It is relevant to note that even though previous results demonstrated that *S. aureus* α-toxin induced apoptosis in Jurkat cells via the Bcl-2-controlled mitochondrial death pathway which involves caspase 3 (Menzies and Kourteva 2000) however this mechanism is not playing a role in these experiments as all the *S. aureus* cells were previously formaldehyde-fixed and cannot produce α-toxin. Therefore these findings suggest that *S. aureus* protein A binds to osteoblasts, through an interaction with
the death receptor TNFR-1, inducing caspase 3 activation and membrane blebbing with an end point of apoptosis.

Another path downstream of TNFR-1 results in the degradation of NF-κB, which in turn mediate gene transcription. The nuclear translocation of NF-κB is regulated by cytoplasmic inhibitory protein IκB. Following degradation of IκB, NF-κB is released and activated (Carmody and Chen 2007; Kriete and Mayo 2009). Following *S. aureus* protein A binding to osteoblast TNFR-1, IκB is degraded causing activation of NF-κB. Deletion of either SpA from *S. aureus* or using siRNA silenced TNFR-1 on osteoblasts, the degradation of IκB is inhibited. These results were consistent with previous findings demonstrating that *S. aureus* binding to osteoblasts results in activation of NF-κB (Ning, Zhang et al. 2010), however the present study identifies the mechanism through which NF-κB activation occurs.

Given the observation that SpA interaction with TNFR-1 mediates the signalling leading to NFκB activation, and relying on the fact that activation of the NF-κB transcription factor regulates a number of genes involved in a wide variety of biological processes such as cytokines mediating inflammatory processes (Liou 2002) the next step it was to further investigate whether this activation could be regulating the release of cytokines by osteoblasts in this system. Cytokines are signalling proteins involved in cell-to-cell communication, in disease, these networks can become unbalanced. Measuring amplification or down-regulation of cytokine signaling cascades in response to pathological insults might be beneficial to evaluate disease progression (House and Descotes 2007). Several studies have already published that following *S. aureus* infection, osteoblasts release cytokines and chemokines involved in the activation of inflammatory responses in osteomyelitis disease (Bost, Ramp et al. 1999; Bost, Bento et al. 2001; Marriott, Gray et al. 2004; Wright and Friedland 2004). IL-6 is a pleiotropic cytokine that acts as a regulator of immune response and inflammation during infection or trauma (Nishimoto and Kishimoto 2004). It can be secreted by macrophages, monocytes and T-cells mediating fever and sickness behaviour in response to specific pathogenic molecules (Banks, Kastin et al. 1994; Cartmell,
Poole et al. 2000; Harden, du Plessis et al. 2006). In bone associated diseases, IL-6 inhibitors have been used to treat postmenopausal osteoporosis and rheumatoid arthritis (Edwards and Williams 2010; Jazayeri, Carroll et al. 2010). Moreover studies in osteomyelitis indicate that osteoblasts significantly augment IL-6 secretion following *S. aureus* infection inducing bone resorption (Marriott, Gray et al. 2004; Marriott, Gray et al. 2005; Ning, Zhang et al. 2011). These results confirm that following *S. aureus* infection IL-6 release is up-regulated. However, also in the absence of SpA or using siRNA silenced TNFR-1 on osteoblasts this up-regulation is inhibited. These results suggest that this specific interaction between SpA and TNFR-1 is regulating IL-6 secretion and consequently mediating the activation of an anti-inflammatory response after osteoblast infection with *S. aureus*. Up-regulation of IL-6 release by osteoblasts has been already reported to induce bone resorption via osteoclast activation (Kwan Tat, Padrines et al. 2004). Other work in our laboratory has demonstrated that upon cytokine release from osteoblasts it leads to the migration and subsequent activation of the bone resorbing cells the osteoclasts. Collectively these results may help explain the significant bone destruction and inflammation found at the site of infection in osteomyelitis patients.

In conclusion, this study describes a novel mechanism for *S. aureus* binding to osteoblasts, demonstrating that *S. aureus* virulence factor SpA binds directly to osteoblasts through TNFR-1. This interaction results in the generation of multiple signals leading to inhibition of osteoblast proliferation, osteoblast apoptosis, and activation of the NF-κB pathway in osteoblasts after *S. aureus* infection. Furthermore activation of NF-κB by SpA–TNFR-1 interaction also mediates the activation of inflammation via secretion of IL-6.
Chapter 4

Study of the molecular mechanisms involved in S. epidermidis binding to osteoblasts: early steps in implant infection
4.1. Introduction

The previous chapters focused on the molecular interactions underlying *S. aureus* attachment to osteoblasts and induced bone destruction and inflammation. Major findings were achieved unravelling part of the mechanism by which *S. aureus* infections cause inflammation and bone fractures in osteomyelitis. The second major cause of osteomyelitis and first cause of implant related- infection, *S. epidermidis* is an imperative microorganism to investigate. In order to further study the mechanisms underlying osteomyelitis and bone infections, the next step was to investigate *Staphylococcus epidermis* interaction with osteoblasts.

*S. epidermidis* is recognized as the most widespread and persistent microorganism colonizing the skin and mucous membranes of all humans (O'Gara and Humphreys 2001; Vuong and Otto 2002; van der Mee-Marquet, Achard *et al.* 2003). It rarely causes infection in healthy people, however, over the past two decades it has emerged as an important opportunistic pathogen associated with intravascular catheters and prosthetic implantation (Kristinsson 1989; Bailey, Constance *et al.* 1990; Karchmer 1991; Menzies, MacCulloch *et al.* 1991; Goldmann and Pier 1993; Vuong and Otto 2002) mostly in immuno-compromised, immuno-suppressed, long-term hospitalised and critically ill patients (Domingo and Fontanet 2001; Ziebuhr 2001). *S. epidermidis* has been reported to account for 50 to 80 % of the recovered isolates from implanted related infections (Archer 1990) being therefore considered a leading cause of these infections (Rupp and Archer 1994). Although many of these infections are minor, colonisation and further biofilm formation of *S. epidermidis* into foreign medical devices can develop into major cases of bone infections such as osteomyelitis (Seibold and Betz 1991; Barasch, Mosier *et al.* 1993; De Wit, Mulla *et al.* 1993).

The mechanism by which *S. epidermidis* contributes to the colonization of medical devices promoting bone destruction it is not fully understood. Some studies suggest that colonization of foreign material by *S. epidermidis* relies on
their ability to form biofilms (Mack 1999; Mack, Bartscht et al. 2000). Biofilm formation starts following secretion of an extracellular substance composed of different polysaccharides important in promoting bacterial attachment to foreign devices. This step is crucial to initiate infection and also serves to protect the colonizing microorganism from host defence mechanisms and the action of antimicrobial agents (Hoyle and Costerton 1991; Costerton, Stewart et al. 1999; Stewart and Costerton 2001). Other studies suggest that S. epidermidis surface associated material is an important virulence factor conferring this pathogen osteolytic activity that plays a major role in bone destruction during disease (Meghji, Crean et al. 1997).

S. epidermidis ability to bind to fibrinogen in implant devices has been reported and attributed to the expression of the surface adhesin SdrG (Nilsson, Frykberg et al. 1998; Hartford, O’Brien et al. 2001). SdrG is a well known S. epidermidis MSCRAMM highly homologous to the clumping factor A and B from S. aureus which have also been shown to bind fibrinogen in foreign body infections caused by S. aureus (McDevitt, Francois et al. 1994; Ni Eidhin, Perkins et al. 1998). Moreover during S. aureus-osteoblast interaction, the fibronectin binding proteins also bind to fibrinogen which in turn interacts with the α5β3 integrin triggering bacterial internalization by the bone cells (Hudson, Ramp et al. 1995; Ellington, Reilly et al. 1999; Jevon, Guo et al. 1999). Furthermore, osteoblast integrin α2β1 interaction with type I collagen is involved in the regulation of many cellular activities including the activation of collagenase in bone destruction (Riikonen, Westermarck et al. 1995). These findings support the fact that bacterial interaction with integrin receptors on osteoblasts mediates signaling pathways on staphylococcal bone infections. Nonetheless, despite all the studies in the past, S. epidermidis infections associated with medical devices continues to present at an alarming high rate (Darouiche, Mansouri et al. 2007). Therefore, additional investigations are needed in order to develop novel therapies preventing S. epidermidis adherence and consequent biofilm formation, therefore avoiding infection and further bone destruction, the hallmark in osteomyelitis bone disease.
4.1.1. Chapter Aims

- Investigate the ability of *S. epidermidis* to interact with osteoblasts
- Identify the *S. epidermidis* cell wall surface protein that is involved in binding to osteoblasts: Proteomic approach
- Investigate the role of the identified protein from the proteomic analysis in mediating osteoblast attachment
- Investigate the downstream events in osteoblasts as a result of *S. epidermidis* attachment
4.2. Chemicals and general conditions

The chemicals and conditions used in this study are described in detail in Chapter 2, section 2.2, additional chemicals and equipment for this specific chapter are stated below in Table 4-1.

Table 4-1 - List of chemicals and equipment used in this chapter.

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<th>Product</th>
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<td>Arginine-Glycine-Aspartic-Serine acid peptide</td>
<td>Merck</td>
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<tr>
<td>M17 broth</td>
<td>Oxoid</td>
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4.3. Methods

4.3.1. Bacterial culture conditions

The bacterial culture conditions used in this chapter are described in detail in section 2.3.1.

Bacterial strains

The bacterial strains used in this chapter are listed below Table 4-2.

Table 4-2 - Bacterial strains and isolates used in this chapter

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<td>Newman</td>
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<td>(Duthia and Lorent 1952)</td>
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<td>Isolated from a case of secondarily infected tubercular osteomyelitis in man</td>
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<td>S. epidermidis</td>
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<tr>
<td>HB</td>
<td>Wild type</td>
<td>(Nilsson, Frykberg et al. 1998)</td>
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<tr>
<td></td>
<td>Isolated from a human patient with osteomyelitis</td>
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</tr>
<tr>
<td></td>
<td>fbe:erm^7</td>
<td>(Hartford, O'Brien et al. 2001)</td>
</tr>
<tr>
<td>HB Δ sdrG</td>
<td>Defective in SdrG (fbe)</td>
<td>* (Mack, Siemssen et al. 1992)</td>
</tr>
<tr>
<td>9142</td>
<td>Wild type</td>
<td>** (Conlon, Humphreys et al. 2002)</td>
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<tr>
<td>9142 Δ sdrG</td>
<td>Isolated from blood samples</td>
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</tr>
<tr>
<td></td>
<td>Defective in SdrG</td>
<td></td>
</tr>
<tr>
<td>CS41498</td>
<td>Biofilm positive, cerebrospinal fluid isolate</td>
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</tr>
<tr>
<td>Lactococcus lactis</td>
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</tr>
<tr>
<td>MG1363</td>
<td>Wild type</td>
<td>(Wells, Wilson et al. 1993)</td>
</tr>
<tr>
<td></td>
<td>Insertion of pKS80 (sdrG+) vector</td>
<td></td>
</tr>
<tr>
<td>MG1363 sdrG+</td>
<td>Highly expresses sdrG protein from S. epidermidis</td>
<td>(Hartford, O'Brien et al. 2001)</td>
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* The *S. epidermidis* 9142, 9142ΔsdrG were a kind gift from Prof. Timothy Foster in Department of Microbiology in Trinity College Dublin, Ireland.

** The *S. epidermidis* CSF41498 was a kind gift from Dr. James O’Gara, Conway Institute of Biomolecular and Biomedical Research, UCD Ireland.

**Growth conditions**

*S. epidermidis* strains were grown in Brain Heart Infusion (BHI) broth in sealed tubes overnight at 37 °C. The *Lactococcus lactis* strains were grown in M17 broth (tryptone 5 g/l, soya peptone 5 g/l, Lab-Lemco powder 5 g/l, Yeast extract 2.5 g/l, ascorbic acid 0.5 g/l, magnesium sulphate 0.25 g/l, di-sodium-glycerophosphate 19 g/l) supplemented with 0.5 % glucose in sealed tubes overnight at 30 °C. Erythromycin (5 µg/ml) was added to media when growing *L. lactis* SdrG+ strain.

### 4.3.2. Cell culture conditions

The osteoblasts culture conditions of the MC3T3-E1 cell line used in this chapter are described in detail in section 2.3.2.

### 4.3.3. Functional assays

**Osteoblasts binding assay**

The osteoblasts binding protocol is described in detail in section 2.3.3. In this chapter, for some studies, osteoblasts were pre-incubated with 1 mM of Arginine- Glycine- Aspartic acid- Serine (RGDS) for 30 min at 37 °C prior addition to the 96-well plate. RGDS is a peptide mimetic of the integrin recognition site Arginine- Glycine- Aspartic acid (RGD).

**Osteoblasts proliferation assay**

The osteoblasts binding protocol is described in detail in section 2.3.3.
Osteoblast live - dead assay

Osteoblasts were harvested as described in section 2.3.2. and cell count adjusted to 1.5 x10^5 cells per 300 μl per well, with subsequent addition onto a 96-well tissue culture plate. Plate was transferred to the tissue culture incubator at 37 °C, 5 % CO2 for 24 h. *S. epidermidis* cultures were harvested, PBS washed and fixed in 4.8 % formaldehyde as described in section 2.3.1.. Bacterial concentration was then adjusted to an OD_{600 nm} of 1 in osteoblast supplemented media. After 24 h, osteoblasts were washed in warm PBS and bacteria were added in the same volume to wells with the previously incubated osteoblasts. Bacteria and osteoblasts were co-cultured for further 24 h. The live assessment of osteoblast following infection with *S. epidermidis* was performed using the Live Staining Kit from Sigma-Aldrich, whereas calcein-AM (green – live) was used at a 1:1 ratio in a fluorescence solution. Each well of the 96-well plate was washed by addition of 300 μl of PBS per well. Following this the fluorescence solution was added for 40 min, and absorbencies read at 490 nm for Calcein-AM using a micro-plate reader.

4.3.4. Protein biochemistry

Bacterial cell digestion - Mutanolysin

*S. epidermidis* strains were grown and harvested as described in section 4.3.1. *S. epidermidis* pellets were re-suspended in 1 ml spheroblasting buffer (26 % (w/v) raffinose, 20 mM Tris pH 8.0 containing 10 mM MgCl2) plus 500 U of mutanolysin and 75 μl of PIC. The suspensions were then incubated for 30 min at 37 °C with occasional shaking. Samples were then centrifuged at 3000 x g for 10 min at 4 °C to pellet cells. The supernatants containing the solubilised cell wall associated proteins were transferred to new eppendorf tubes. Finally the appropriate volume of supernatant plus 5 X SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10 % SDS, 0.5 % bromophenol blue, 50 % glycerol) were boiled at 95 °C for 5 min. The cell wall proteins present in the samples were separated in a SDS-PAGE, as previously described in section 2.3.4.
Protein quantification

The protein content quantification of each *S. epidermidis* strain cell wall preparation was quantified according to the protocol described in section 2.3.4.

Mass spectrometry analysis

Mass spectrometry can be defined as an analytical laboratory technique used to separate the components of a sample by their mass and therefore identify the molecular and structural composition of that sample.

In this study, nano-electrospray liquid chromatography mass spectrometry (Nano-LC-MS/MS) analysis was kindly carried out by Kieran Wynne in the Conway Institute in UCD. Peptides identified were searched using the XTandem algorithm against all databases. The probability-based evaluation program, Protein Prophet was used to filter identifications.

4.3.5. Statistical analysis

Statistics were performed using SSC-Stat V2.12. Data shown are the means plus or minus standard error of the mean (SEM). Comparisons between mean values were performed using a 2-tailed paired Student’s t-test.
4.4. Results

4.4.1. Investigating the ability of *S. epidermidis* to interact with osteoblast

*S. epidermidis* biofilm forming strains HB, 9142 and CSF41498 were originally recovered from a human patient with osteomyelitis, blood cultures and cerebrospinal fluid, respectively. The level of staphylococci binding to osteoblasts was assessed for the *S. aureus* strain Newman WT used as control and also for *S. epidermidis* strains HB, 9142 and CSF41498. As result all *S. epidermidis* strains bound to osteoblasts with slightly less intensity when compared to the *S. aureus* Newman strain binding to osteoblasts. Moreover among the *S. epidermidis* strains used, the CSF41498 strain bound significantly less to osteoblasts when compared with the others *S. epidermidis* strains HB and 9142 (Figure 4-1, P<0.05).
Figure 4-1 – *S. epidermidis* strains bind to osteoblasts. Osteoblasts were allowed to bind to immobilized *S. aureus* Newman and *S. epidermidis* HB, 9142 and CSF41498 for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader. *P<0.05, bars indicate SEM, n=4.*
4.4.2. A proteomic approach to identify the differential protein expression between \textit{S. epidermidis}

Given the significant difference in the binding intensities to osteoblasts between the \textit{S. epidermidis} HB and 9142 and CSF41498, the protein expression of these strains was studied. To address this, cell wall preparations of \textit{S. epidermidis} 9142 and CSF41498 were run in a SDS-PAGE for protein separation. Each sample was then analysed by mass spectrometry using Nano- LC-MS/MS carried out by Kieran Wynne in the Conway Institute in UCD. Proteins identified to be present in \textit{S. epidermidis} 9142 but absent in CSF41498 are presented below in Table 4-3. The Serine-aspartate repeat-containing protein G was chosen for further studies as it is one of the best studied \textit{S. epidermidis} surface proteins also because it has been previously shown to be involved in \textit{S. epidermidis} medical devices infections (Foster and McDevitt 1994; Davis, Gurusiddappa \textit{et al.} 2001; Hartford, O’Brien \textit{et al.} 2001; Sellman, Timofeyeva \textit{et al.} 2008).

Table 4-3- Relevant selection of \textit{S. epidermidis} 9142 surface proteins absent in CSF41498.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Acc. No</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTPAL_STAEQ</td>
<td>Q5HPB7</td>
<td>Probable CtpA-like serine protease</td>
</tr>
<tr>
<td>Y1947_STAES</td>
<td>Q8CN89</td>
<td>Uncharacterized lipoprotein</td>
</tr>
<tr>
<td>SDRG_STAEQ</td>
<td>Q9KL13</td>
<td>Serine-aspartate repeat-containing protein G</td>
</tr>
<tr>
<td>GCSH_STAEQ</td>
<td>Q5HQR3</td>
<td>Glycine cleavage system H protein</td>
</tr>
<tr>
<td>TRX8_STAEQ</td>
<td>Q5HQW4</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>ALF1_STAES</td>
<td>Q8CMY5</td>
<td>Fructose-bisphosphate aldolase class 1</td>
</tr>
<tr>
<td>CLPX_STAEQ</td>
<td>Q5HMN9</td>
<td>ATP-dependent Clp protease ATP-binding subunit ClpX</td>
</tr>
<tr>
<td>GDPA_STAEQ</td>
<td>Q5HP71</td>
<td>Glycerol-3-phosphate dehydrogenase [NAD(P)+]</td>
</tr>
<tr>
<td>HEMH_STAEQ</td>
<td>Q5HNA5</td>
<td>Ferrochelatase</td>
</tr>
<tr>
<td>Y1140_STAEQ</td>
<td>Q5HNX4</td>
<td>UPF0365 protein SERP1140</td>
</tr>
<tr>
<td>Y1381_STAEQ</td>
<td>Q5HN91</td>
<td>UPF0342 protein SERP1381</td>
</tr>
<tr>
<td>EBPS_STAEQ</td>
<td>Q5HP65</td>
<td>Probable elastin-binding protein ebpS</td>
</tr>
<tr>
<td>SSAA_STAEQ</td>
<td>Q9KJT6</td>
<td>Staphylococcal secretory antigen ssaA</td>
</tr>
<tr>
<td>HTRAL_STAEQ</td>
<td>Q5HQE2</td>
<td>Serine protease htrA-like</td>
</tr>
<tr>
<td>ENQ_STAEQ</td>
<td>Q5HQV0</td>
<td>Enolase</td>
</tr>
</tbody>
</table>

168
4.4.3. Investigating the role of *S. epidermidis* SdrG in binding osteoblast

One of the major differences in protein expression among the *S. epidermidis* strains in this study was the presence of the SdrG surface protein in strains HB and 9142 cell wall preparations, but not in CFS41498 strain. Therefore, taking into account that the CFS41498 *S. epidermidis* strain showed significant less binding to osteoblasts, the role of this specific protein in *S. epidermidis* interaction with osteoblasts was exploited. To do so, *S. epidermidis* strains HB and 9142, and respective SdrG defective mutants binding to osteoblast was measured. In the absence of SdrG on the bacterial surface the ability of both HB and 9142 *S. epidermidis* strains to bind osteoblasts was significantly reduced (Figure 4-2A, P<0.05). To further confirm the involvement of SdrG in osteoblast-*S. epidermidis* interaction, a *Lactococcus lactis* strain expressing SdrG was used to test its ability to bind osteoblasts. *L. lactis* does not express SdrG naturally, for that reason and also because *L. lactis* is a non pathogenic microorganism it is an ideal surrogate to use. As expected *L. lactis* WT strain showed reduced binding to osteoblasts, while *L. lactis* expressing SdrG revealed a significant increase in osteoblast binding when compared with the WT strain (Figure 4-2B, P<0.05). These results suggest that *S. epidermidis* binding to osteoblasts is mediated by the surface protein SdrG.
Chapter 4 - Study of the molecular mechanisms involved in *S. epidermidis* binding to osteoblasts: early steps in implant infection

**A**

Figure 4-2 - SdrG from *S. epidermidis* mediates binding to osteoblasts. Osteoblasts were allowed to bind to immobilized (A) *S. epidermidis* HB and A9142, WT and SdrG defective strains (B) *Lactococcus lactis* WT and SdrG expressing strains for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader. *P*<0.05, bars indicate SEM, n=4.

![Graph A](image1.png)

**B**

![Graph B](image2.png)
4.4.4. Investigating the mechanism through which *S. epidermidis* SdrG interacts with osteoblast

SdrG is a 119 kDa protein from *S. epidermidis* surface structurally related to the staphylococcal surface proteins, ClfA and ClfB of *S. aureus*. These proteins share the ability to bind to fibrinogen (McDevitt, Francois *et al.* 1994; Pei, Palma *et al.* 1999). Fibrinogen is a soluble plasma protein also present in most of the supplemented media for cell culture use. SdrG expression has been shown to be up-regulated in sera from patients with *S. epidermidis* infections (Galliani, Viot *et al.* 1994). Moreover, *S. epidermidis* interaction with catheters spontaneously coated with fibrinogen has been reported in patients (Desai, Hossainy *et al.* 1992). After showing that SdrG is also involved in *S. epidermidis* binding to osteoblasts, the next step investigated whether SdrG was binding to fibrinogen or if SdrG was directly interacting with any receptor(s) on the osteoblast surface. Therefore *S. epidermidis* binding to osteoblasts in the absence and presence of FBS in the osteoblast culture media was studied. The significant decrease previously obtained for the osteoblasts binding to *S. epidermidis* SdrG defective mutant was comparable to that obtained for the osteoblast binding to *S. epidermidis* WT strain in the absence of FBS (fibrinogen) in the osteoblast culture media (Figure 4-3, P= NS). These results suggest that *S. epidermidis* SdrG is binding to the fibrinogen present in the osteoblast culture media and not directly interacting with the osteoblast surface.
Chapter 4 - Study of the molecular mechanisms involved in *S. epidermidis* binding to osteoblasts: early steps in implant infection

Figure 4-3 – *S. epidermidis* SdrG is binding to the fibrinogen present in the osteoblasts nutrient media. Osteoblasts cultured in normal and media without FBS were allowed to bind to immobilized *S. epidermidis* WT; and osteoblasts cultured in normal media were allowed to bind SdrG defective mutant, for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader.
4.4.5. Investigating the osteoblast receptor that recognises *S. epidermidis* SdrG

Given the finding that SdrG binds to fibrinogen which bridges *S. epidermidis* to osteoblasts, the origin of the receptor for this interaction on the osteoblast surface was investigated.

Integrins are a large family of proteins that serve as major cell surface receptors in cell binding events (Hynes 1992). Most integrins express a three amino acids sequence, described as arginine- glycine- aspartic acid (RGD) which is recognized as an essential binding site that is present in a variety of adhesive proteins including fibrinogen (Shankar, Davison *et al.* 1993; Ruoslahti 1996). In order to identify if the receptor on the osteoblast surface is an integrin, osteoblasts were pre-incubated with an RGD peptide prior to addition to the previously immobilized *S. epidermidis* strains onto a 96-well plate. *S. epidermidis* SdrG expressing and defective strains binding to osteoblast, with and without RGD pre-incubation, showed a significant reduction in the osteoblast binding intensity for the *S. epidermidis* WT strains HB and 9172 (Figure 4-4). However, when using the *S. epidermidis* SdrG defective strains the osteoblast binding intensity remained the same, suggesting that pre-incubating osteoblast with RGD peptide prevents the interaction between osteoblasts and SdrG, and that in the absence of SdrG, the osteoblast binding to *S. epidermidis* is not affected. Therefore, these results implicate that the receptor on osteoblast surface that mediates the interaction between fibrinogen which in turn is binding to SdrG on the *S. epidermidis* surface, is an RGD recognized protein and most likely an integrin.
Chapter 4- Study of the molecular mechanisms involved in *S. epidermidis* binding to osteoblasts: early steps in implant infection

Figure 4-4 - Osteoblasts integrins mediate binding to *S. epidermidis*. Control osteoblasts and osteoblasts pre-incubated with 1 mM RGD were allowed to bind to immobilized *S. epidermidis* HB and A9142, WT and SdrG defective strains in the for 45 min at 37 °C. Binding was determined by measuring the intracellular enzyme alkaline phosphatase content at 405 nm in a micro plate reader. *P<0.05, bars indicate SEM, n=3.
4.4.6. Investigating the effect of *S. epidermidis* binding on osteoblast proliferation

Having established the importance of the *S. epidermidis* SdrG in binding osteoblasts, the effects of this specific protein on osteoblast proliferation was investigated. Osteoblasts were infected with SdrG expressing and SdrG defective strains over 48 h. Formaldehyde fixed *S. epidermidis* 9142 WT strain and respective SdrG defective mutant and *L. lactis* WT strain and respective SdrG expressing strain, were added to cultured osteoblasts and proliferation determined by cells count using a haemocytometer after 24 h and 48 h (Figure 4-5). Un-infected osteoblasts were used as control and as expected a regular proliferation profile was observed. In contrast, addition of SdrG expressing strains *S. epidermidis* 9142 WT and *L. lactis* SdrG+ appeared to limit osteoblast growth over time (Figure 4-5, P<0.01). Interestingly, osteoblast growth was recovered in the presence of either *S. epidermidis* 9142 SdrG defective mutant or *L. lactis* naturally defective in SdrG (P=NS, when compared with the un-infected osteoblasts). These results highly suggest that SdrG is most likely playing a role in the inhibition of osteoblast proliferation after *S. epidermidis* infection.
Figure 4-5 - *S. epidermidis* SdrG Inhibits Proliferation. Osteoblasts (5 x 10^6 cells/ml) were pre-incubated with either control buffer or formaldehyde fixed *S. epidermidis* wild-type and SdrG defective mutant or *Lactococcus lactis* wild-type and SdrG expressing mutant (1 x 10^6 cells/ml) over 48 h. After 0, 24 h and 48 h osteoblasts were removed by trypsinization and proliferation was determined by counting cells on a haemocytometer. *P<0.01, bars indicate SEM, n=5.*
4.4.7. Investigating the downstream events as a result of *S. epidermidis* SdrG binding to osteoblast: Cell Death

In order to further investigate the virulence of SdrG, the viability of osteoblasts following infection with several *S. epidermidis* WT strains and respective SdrG defective mutants was evaluated. The number of live osteoblasts was counted after infection using un-infected osteoblasts as positive control for live cells. As a result, when compared with an un-infected osteoblast control, the number of live osteoblasts following infection with all *S. epidermidis* strains was slightly reduced (Figure 4-6, P<0.001). However, surprisingly no difference was observed among the *S. epidermidis* SdrG defective or SdrG expressing strains (P=NS), suggesting that despite its importance in mediating binding to osteoblasts and inhibiting osteoblast proliferation SdrG is not involved in osteoblast death after infection.
Figure 4-6 - SdrG is not involved in osteoblast death after infection. Osteoblasts (1.5 x10^5 cells/ml) were co-cultured with either control buffer or formaldehyde fixed S. epidermidis wild-type and SdrG defective mutant for 24 h. Un-infected osteoblasts were used as positive control for live osteoblasts. After 24 h infection the number of live cells was determined by detection of Calcein-AM positive cells at an absorbance of 490 nm read in a microplate reader. *P<0.001, bars indicate SEM, n=3.
4.5. Discussion

*S. epidermidis* induced bone infections are mostly associated with the medical device implants during surgery which accounts for almost 50% of the infections associated with catheters, artificial joints and heart valves (Khalil, Williams *et al.* 2007). During surgery insertion of a medical device such as an orthopaedic implant, the wound site is exposed to the external environment, where bacteria from the skin of the patient, medical personnel, or simply airborne bacteria can reach the open site (Donlan and Costerton 2002). Once in contact with the internal environment of the patient, bacteria colonize the cells or the implanted device serving as a septic focus leading to major diseases such as osteomyelitis (Cramton, Gerke *et al.* 2001).

In this chapter the interactions between *S. epidermidis* strains and osteoblasts were investigated, in order to identify specific interactions critical for the development of infection.

In order to address this, the ability of several strains of *S. epidermidis* to bind to osteoblasts was firstly investigated. All of the *S. epidermidis* used in this study bound to osteoblasts with less intensity than *S. aureus* Newman, used as positive control for binding osteoblasts. This result is not surprising, as different bacterial strains have a differential protein expression profile which maybe related to their ability to bind host cells. Characteristic of this, *S. epidermidis* CSF41498 bound significantly less than *S. epidermidis* HB and 9142 suggesting differences in protein expression. Therefore the differential protein expression among the *S. epidermidis* strains under study was investigated, with particular interest in identifying the difference between *S. epidermidis* 9142 and CSF41498. The difference in protein expression may account for the ability to bind osteoblasts. For that reason, the cell wall proteins from *S. epidermidis* 9142 and CSF41498 were removed using mutanolysin and the samples were run through a mass spectrometer for peptide identification. Although several proteins were identified as being present in *S. epidermidis* 9142 and absent in CSF41498, SdrG...
was of particular interest. The *S. epidermidis* SdrG was selected as a good candidate for further studies, as it is one of the most characterised and well studied proteins of *S. epidermidis*. The role of SdrG in *S. epidermidis* binding to osteoblasts was therefore assessed using strains HB and 9142 WT and respective SdrG defective mutants (Figure 4-2). The presence of SdrG revealed to be crucial in supporting osteoblast binding intensity to *S. epidermidis* whereas in the absence of this protein on the bacterial surface the levels of osteoblast binding intensity were significantly lower. In order to confirm the role of *S. epidermidis* SdrG, a surrogate host, *Lactococcus lactis*, was used to express the protein. It is worth noting that *L. lactis* does not express SdrG, therefore serves as a good surrogate host. This model has been previously successfully used to express a number of other proteins from various bacteria (O’Brien, 2002, Kerrigan 2007). Consistent with the previous observation, over expression of SdrG on the surface of *L. lactis* was found to facilitate the binding to osteoblasts.

As previously mentioned SdrG is one of the best studied adhesins of *S. epidermidis*, and various reports demonstrated SdrG to be essential for *S. epidermidis* binding to fibrinogen, promoting central intravenous catheters associated infections (Nilsson, Frykberg et al. 1998; Hartford, O’Brien et al. 2001; Guo, Zhao et al. 2007). Fibrinogen is a dominant glycoprotein found in plasma, extracellular matrix and is often found at sites of infection. Therefore experiments were carried out to investigate if fibrinogen is important in binding to *S. epidermidis* SdrG which in turn binds to an osteoblast receptor. Addition of fibrinogen to media or media rich in fibrinogen (FBS supplemented) resulted in strong binding between *S. epidermidis* and osteoblasts. Removal of fibrinogen from the media significantly reduced the binding of *S. epidermidis* to the osteoblast.

Thus, it seems that SdrG on *S. epidermidis* surface is binding to osteoblasts through a fibrinogen bridging interaction.
Integrins are main receptors involved in cell binding on osteoblasts and are recognized by the presence of an RGD sequence in their ligands (Ruoslahti 1996). The present study showed that pre-incubating osteoblasts with an RGD peptide significantly reduced *S. epidermidis* binding to osteoblasts (Figure 4-4). These results suggest that the receptor on osteoblast surface is an integrin which binds fibrinogen and uses this interaction to bridge with SdrG on *S. epidermidis*. This is not a unique interaction as previous reports demonstrated that *S. aureus* binds to fibronectin via Fnbps bridging to the integrin α5β1 which triggers the internalization of the bacteria into osteoblasts (Reilly, Hudson *et al.* 2000; Ahmed, Meghji *et al.* 2001). In these studies, mutants lacking the two Fnbps on the *S. aureus* surface showed a significant reduction in the invasion of host cells. However other study demonstrated that the mechanism of internalization differs between *S. aureus* and *S. epidermidis* whereas *S. epidermidis* is suggested to bind to a different region of fibronectin from that bound by *S. aureus* (Khalil, Williams *et al.* 2007). The same study also reports that unlike *S. aureus*, *S. epidermidis* invasion mechanism is independent of the integrin α5β1, therefore the interaction behind *S. epidermidis* internalization by osteoblasts remains to identify.

As previously demonstrated in Chapter 2, *S. aureus* binds to osteoblasts inhibiting proliferation. Hence, *S. epidermidis* binding to osteoblast was also investigated for its role in osteoblast proliferation. Osteoblast proliferation was significantly inhibited over 48 h when using *S. epidermidis* expressing SdrG. In contrast to this *S. epidermidis* lacking the expression of SdrG failed to have an effect on proliferation over a 48hr period (Figure 4-5). The data obtained suggests that when SdrG binds to the osteoblast integrin it too generates a signal that prevents proliferation. The idea that integrins play a role in regulating proliferation is not new (Cruet-Hennequart, Maubant *et al.* 2003). Alpha 5 (α5) integrins were previously shown to regulate cell proliferation in ovarian cancer cells. Following α5 integrin-blockade with a specific antibody, the ovarian cancer cells growth was inhibited suggesting a connection between the modulation of
growth factor signalling and the $\alpha_5$ integrins. Integrin-linked kinase (ILK) was also shown to be an important regulator of cell survival and proliferation, as in the same study the authors showed that following blockade of the $\alpha_5$ integrins there was a decrease in ILK activity which consequently regulates positively the cell cycle inhibitor explaining the inhibition of cell proliferation. Our results suggest a similar mechanism to regulation, as SdrG from \textit{S. epidermidis} seems to be interacting with an osteoblast integrin which might be modulating the cell cycle thus inducing the inhibition of osteoblast proliferation.

Interestingly, when evaluating osteoblast viability following \textit{S. epidermidis} infection, no significant difference in the number of live cells recovered with the WT strain and SdrG defective strain was seen (Figure 4-6). Therefore it seems that the signal from the interaction between SdrG and osteoblast integrin triggering inhibition of proliferation it is not associated with cell death. This finding is in direct contrast to what was found with \textit{S. aureus}, in that binding led to inhibition of both proliferation and induction of apoptosis. Consistent with our findings \textit{S. epidermidis} induced cell death has not been reported previously (Huang, Liao \textit{et al.} 2000; Nilsdotter-Augustinsson, Wilsson \textit{et al.} 2004; Pharmakakis, Petropoulos \textit{et al.} 2009).

In summary, in this chapter, \textit{S. epidermidis} SdrG was identified as an important factor in mediating attachment to osteoblasts. However, this is not a direct interaction as it appears that SdrG requires fibrinogen to bridge the bacteria to an as yet unidentified integrin expressed on the osteoblast. Furthermore, it was also demonstrated that upon binding to the osteoblast a signal is generated that results in the inhibition of osteoblast proliferation. Interestingly osteoblast death following \textit{S. epidermidis} infection turned out to be strain specific; being verified only after infection with the \textit{S. epidermidis} 9142 strain, and also independent of SdrG expression. Thus it is suggested that SdrG from \textit{S. epidermidis} plays a role in supporting bacterial attachment to osteoblasts and may contribute to bone loss observed in implant infection patients.
Chapter 5

General Discussion
5. General Discussion

Over the last few decades, many investigations have been made to try and understand the development and progression of osteomyelitis. Bacteria of the genus *Staphylococcus* have been identified as major causative agents and numerous antibiotics and surgical therapies are used in its treatment. However, osteomyelitis cases are still increasing, mostly due to the development of antibiotic resistance or infection relapse following surgical therapy. Therefore, understanding the molecular mechanisms underlying staphylococcal induced osteomyelitis remains a challenge. In order to address this, the interactions of *S. aureus* and *S. epidermidis* with osteoblasts were investigated in the hope of identifying new information that may lead to the generation of new therapies to treat this disease.

Osteomyelitis is an infection of the bone cells and tissue associated with abnormal bone remodeling and consequent bone destruction and inflammation (Lew and Waldvogel 2004). Lew and Waldvogel categorized osteomyelitis relying on the different ways by which the infecting microorganisms access the bone. Therefore osteomyelitis can originate secondary to a contiguous-focus of infection (following trauma or surgery), via vascular insufficiency (in immunocompromised patients such as diabetic patients) or by haematogenous source (after a case of bacteraemia) (Lew and Waldvogel 1997; Lew and Waldvogel 2004). Progression of the disease may be acute or develop into a chronic, long lasting infection and may involve different bone structures such as the bone marrow, cortex or surrounding tissue. Different approaches and therapies are administered according to the stage of infection and to the different types of osteomyelitis. The age of the patient is also important as children are more likely to get acute haematogenous osteomyelitis in the long bones, while adults are more likely to get infections following trauma or surgery. In addition, the identification of the infecting microorganisms is crucial for the administration of the correct treatment and may also differ according to the age of the patient, or risk group (*e.g.*: skin infections, immunodeficiency, bone fractures, diabetes).
Although many microorganisms are capable of causing osteomyelitis, *S. aureus* is by far the most prominent pathogen accounting for up to 80% of all osteomyelitis cases (Ellington, Reilly *et al.* 1999). *S. epidermidis* follows *S. aureus*, being the second major cause of osteomyelitis and leading cause of implant-related osteomyelitis (Kloos and Bannerman 1994; Cadorna and Watanakunakorn 1995; Lew and Waldvogel 2004).

**Staphylococci and bone - the first step into infection**

As mentioned in the Introduction chapter, although belonging to the same genus, *S. aureus* and *S. epidermidis* are considerably different in their ability to cause infection. *S. aureus* is considered the most virulent and therefore the major pathogen of the genus staphylococci causing a very broad range of human diseases (Novick 1990; Lowy 1998; Crossley 2009) while *S. epidermidis* requires a predisposed host in order to cause infection, and is therefore defined as an opportunistic pathogen (Vuong and Otto 2002). Comparison of the genomes of *S. aureus* and *S. epidermidis* demonstrated that despite sharing a core genome, *S. aureus* has strain-dependent combinations encoding several virulence factors most of which are absent in *S. epidermidis* (Gill, Fouts *et al.* 2005). This may partially explain the difference in their pathogenicity. In order to identify key mechanisms involved in the initial steps to infection, the different virulence factors of *S. aureus* and *S. epidermidis* and their role in interacting with osteoblasts was investigated.

Bacterial binding to the osteoblast surface is the first stage of infection in osteomyelitis disease. A range of different *S. aureus* strains were studied where the Staphylococcal protein A (SpA) was identified as having a role in binding to osteoblasts, and distinguish it from other important *S. aureus* virulence factors such as the capsule, teichoic acid and other surface proteins (e.g. ClfA/B and the Sdr family of proteins). By using a genetically modified strain of *S. aureus* lacking the expression of SpA, a crucial role for SpA in the first step of infection was demonstrated, as the absence of this protein on the bacterial surface
significantly reduced (by approximately 50%) the binding to osteoblasts. SpA is a well known virulence factor widely expressed on the surface of the majority of the *S. aureus* strains. SpA has high affinity to bind important proteins of the immune system such as immunoglobulins. In addition to its ability to mediate *S. aureus* - osteoblast interaction, the importance of SpA in osteoblast inhibition of proliferation was also confirmed. Furthermore SpA seems to be not only important for the first step of infection, but also a mediator of the pathway following infection triggering osteoblast death.

The same line of reasoning as per *S. aureus* - osteoblast interaction, *S. epidermidis* species express SdrG, a well known MSCRAMM highly homologous to clumping factor A and B from *S. aureus* which have been shown to bind fibrinogen in foreign body infections caused by *S. aureus* (McDevitt, Francois et al. 1994; Ni Eidhin, Perkins et al. 1998). In this study a *S. epidermidis* SdrG defective strain and a non-pathogenic *L. lactis* strain expressing SdrG were used to demonstrate that SdrG is important in mediating *S. epidermidis* - osteoblast interaction. However, unlike the *S. aureus* protein A - osteoblast interaction, *S. epidermidis* SdrG- osteoblast binding is not a direct interaction. It was demonstrated that *S. epidermidis* SdrG binds to fibrinogen that is present in the osteoblast external environment, which in turn binds directly to the osteoblast surface via an integrin interaction. This is not a unique interaction since the same mechanism had already been shown in platelets where it stimulates platelet activation and supports platelet binding (Brennan, Loughman et al. 2009). Moreover it was also shown that unlike SpA, SdrG is not required for osteoblast inhibition of proliferation and therefore is not a contributing factor in the death of osteoblast following infection.

These findings clearly demonstrate that *S. aureus* and *S. epidermidis* interact differently with bone. This fact highlights the importance of identifying the infecting microorganism at the time of the diagnosis, so that a different treatment can be administered according to the result obtained. At this point, based in the previous results it would make sense the development of a new
therapy, specifically against SpA when treating infections caused by \textit{S. aureus} and against SdrG in order to block \textit{S. epidermidis} binding to bone. However, as already mentioned, the emergence of antibiotics resistance manifested in the past revokes this hypothetical solution. In addition a surgical procedure in order to remove the affected area might not be the successful solution as it encloses a high risk of relapse.

**Advances in \textit{S. aureus} protein A interaction with bone**

Even though SpA binding is not a unique interaction between \textit{S. aureus} and osteoblast, it seems to account remarkably not only for initiation of infection but also to contribute to the progression of disease. Therefore, identification of the osteoblast receptor for SpA became important in order to understand the role of this interaction in the disease. As previously shown by Gomez \textit{et al} on epithelial cells, SpA binding to TNFR-1 plays an important role in \textit{S. aureus} induced pneumonia (Gomez, Lee \textit{et al.} 2004). In the course of our investigations, following confirmation the expression of TNFR-1 on osteoblast surface, it was demonstrated that using \textit{S. aureus} protein A defective mutants and TNFR-1 blocked and/or silenced osteoblast significantly decreased the binding intensity between bacteria and osteoblast. Moreover, in the absence of both SpA and TNFR-1, bacterial binding to osteoblast was considerably reduced. These results revealed that TNFR-1 is the ligand for SpA in \textit{S. aureus} - osteoblast interaction. Therefore investigating the pathway following bacterial attachment to osteoblast through SpA -TNFR-1 interaction became necessary.

**SpA and TNFR-1 interaction: Pathway into bone inflammation and death**

As mentioned before, the main characteristics of osteomyelitis disease are bone inflammation and bone destruction. During this research it was found that inhibition of osteoblast proliferation was mediated by \textit{S. aureus} protein A interaction with osteoblast. Osteoblast growth was disrupted after infection with \textit{S. aureus} WT while recovered following infection with a SpA knock out \textit{S. aureus}
strain. Moreover the previously mentioned work by Gomez et al in 2004 recognized the ability of SpA to induce TNF-alpha like responses through its binding to TNFR-1 on epithelial cells (Gomez, Lee et al. 2004). Here it is proposed that the same mechanism, SpA binding to TNFR-1, activates TNFR-1 downstream signals such as apoptosis and inflammation. S. aureus mediated apoptosis has already been reported in osteoblasts (Tucker, Reilly et al. 2000). Another study involving S. aureus induced apoptosis on epithelial cells demonstrated the importance of caspase 8 and 3 in the programmed cell death mechanism (Wesson, Deringer et al. 2000). In this study it was demonstrated the activation of caspase 3 following S. aureus infection and its inhibition when using a S. aureus strain defective in SpA. It seems therefore that the TNFR-1 downstream pathway leads to osteoblast death and consequent restriction of proliferation following SpA binding (Figure 5-1). Inflammation processes in response to bacteria can be mediated by the activation of NFkB which regulates the expression of cytokines and chemokynes which in turn recruit immune cells such as leukocytes and neutrophils responsible for clearing infection. Gomez et al have also shown that SpA-TNFR-1 pathway triggers inflammation on epithelial cells whereas SpA induced NFkB activity through its binding to TNFR-1 (Gomez, Lee et al. 2004). More recently another study confirmed that S. aureus binding was required for NFkB activation but also for IL-6 release in human osteoblasts (Ning, Zhang et al. 2010). Additionally this work demonstrates that following SpA binding to TNFR-1, IkB degradation is induced which is associated with NFkB activation and consequent stimulation of IL-6 release (Figure 5-1). In addition, the release of this cytokine from osteoblasts has been shown to further increase bone resorption following S. aureus infection (Bost, Ramp et al. 1999). Recent work in our laboratory demonstrated that the up-regulation of IL-6 expression following SpA binding to TNFR-1 on osteoblasts induces osteoclast differentiation and consequently bone destruction. Furthermore, stimulation of osteoclastogenesis might be related to an increase in the expression of RANK-L following SpA-TNFR-1 interaction while SpA deletion prevents RANK-L expression (Claro, Widaa et al. 2011).
Taken all together *S. aureus* protein A binding to the osteoblast TNFR-1 induces receptor activation triggering osteoblast inhibition of proliferation, osteoblast death via apoptosis and osteoblast inflammation through the activation of NFkB and IL-6 release (Figure 5-1).

![Figure 5-1 - Model of infection.](image)

**(A)** - **Internalization of *S. aureus* by osteoblast:** mediated by FnBP binding to fibronectin which binds to the osteoblast α5β1 integrin. This interaction promotes caspase-8 activation with consequent osteoblasts apoptosis. Interaction of SpA and TNFR-1 promotes the expression of RANKL, inducing osteoclastogenesis. Following osteoblasts apoptosis and the activation of osteoclasts, bone destruction ensues. Adapted from (Montanaro, Testoni et al. 2011).

**(B)** - **TNFR-1 signalling pathway:** *S. aureus* protein A binds to TNFR-1 on osteoblasts surface. Following this, two distinct pathways can be activated. The apoptotic pathway by means of caspase-3 activation together with membrane blebbing and finally cell death and consequent bone destruction; or the NFkB pathway, activated via IκB degradation, with induction of IL-6 release and cell inflammation.
Anti-TNFR-1 therapy

In the last decade, two different therapies of anti-TNF alpha signalling have became commercially available and proven to be effective for the treatment of rheumatoid arthritis: infliximab, a chimeric TNF-alpha monoclonal antibody and etanercept, a soluble TNF receptor (Hurlimann, Forster et al. 2002; Jacobsson, Turesson et al. 2005). However, TNF-alpha functions are not only regulated by the TNFR-1 but also by TNFR-2 which plays an important role in the biophylactic system (Grell 1995; Grell, Douni et al. 1995). Therefore, complete inhibition of the TNF-alpha signaling still compromises the risk of infection as demonstrated by Bongartz et al (Bongartz, Sutton et al. 2006).

The present study shows the need to develop a new treatment for osteomyelitis using the TNFR-1 as a specific therapeutic target. Nevertheless recently, Nomura et al created a unique TNFR1-selective antagonist capable of inhibiting TNFR-1 function without jeopardizing TNFR-2 function in host defence (Nomura, Abe et al. 2010). Moreover, in August 2011, Kitagaki and co-workers have demonstrated that the use of a selective TNFR-1 antagonist suppressed arterial inflammation by inhibiting the TNFR-1 signaling following a cuff injury in mice with excessive post-injury inflammation (Kitagaki, Isoda et al. 2011).

Combined model of bone infection

In this thesis it is described the primary interaction between bacteria and osteoblast leading to infection and consequent cell death and inflammation. In addition, previous studies showed staphylococcal internalization and persistence inside the host on bone cells. The ability of S. aureus and S. epidermidis to invade cultured osteoblasts has been published by several research groups (Hudson, Ramp et al. 1995; Ellington, Reilly et al. 1999; Jevon, Guo et al. 1999; Reilly, Hudson et al. 2000; Khalil, Williams et al. 2007). The internalization process in S. aureus but not S. epidermidis (Khalil, Williams et al. 2007) occurs via Fnbps binding to fibronectin which serves as a bridge to the integrin α5β1 acting as a “phagocytic” receptor (Sinha, Francois et al. 1999) (Figure 5-1). Furthermore other studies demonstrated that S. aureus- infected osteoblasts induce caspase-8 triggering apoptosis (Alexander, Rivera et al. 2003; Mahalingam, Szegezdi et al. 2003).
2009) (Figure 5-1). These studies may help to explain the persistence of osteomyelitis cases where the bacteria are protected inside the host cell, evading from the immune system and from the action of antibiotics (Henderson and Nair 2003). In addition osteoblast apoptosis following *S. aureus* infections is most likely contributing to the bone loss characteristic of osteomyelitis patients (Henderson and Nair 2003).

Even though there is still much work to do to completely unravel the mechanisms underlying osteomyelitis, this study together with the recent findings in the anti- TNFR-1 therapy area, provide an attractive approach for the development of an effective drug to treat and prevent osteomyelitis and other inflammatory diseases.

### 5.1. Future work

The knowledge obtained in the present study answers some of the questions about *S. aureus* and *S. epidermidis* bone infections. However, as already mentioned, additional studies are necessary to fully understand the molecular mechanisms underlying Staphylococcal induced bone disease. Therefore, suggestions to continue with this investigation are described below:

**S. aureus** induced osteomyelitis

- Identify the exact binding site on SpA interacting with the TNFR-1 on osteoblast. Taking into account the high homology between SpA and Sbi, and the fact that only SpA is involved in binding osteoblasts, examination and comparison of both sequences may be the key to identify the exact SpA binding site to osteoblast;
- Study of additional mediators of the *S. aureus* – osteoblast interaction. As demonstrated in this study, SpA binding comprises 50% of the *S. aureus* interaction with osteoblasts. Therefore the remaining 50% of this interaction...
may be due to additional *S. aureus* proteins or specific sugars on the *S. aureus* cell wall in the binding to osteoblast;

- Further investigate SpA and TNFR-1 interaction using *in vivo* models of osteomyelitis, in order to develop a new therapy for osteomyelitis patients.

**S. epidermidis induced osteomyelitis**

- Identify the integrin receptor for SdrG that mediates the binding to osteoblasts via fibrinogen;
- Investigate further interactions between *S. epidermidis* and osteoblasts. Look for the role of other bacterial cell wall components such as sugars and surface proteins;
- Explore the pathway following infection leading to inflammation (looking for cytokines release) and bone destruction;
- Studies using medical implant material will certainly contribute to a better understanding of the *S. epidermidis* infections;
- Further investigate the role of *S. epidermidis* SdrG in bone infections using *in vivo* models of disease.
VI. APPENDIX
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i. Oral presentations

Bioengineering In Ireland Conference, 22-24 January 2009
ISBN no. 978-0-9548583-3-9

RAMI Biomedical Sciences Section Meeting, 23-24 June 2010

Society for General Microbiology, 6-9 September 2010 Autumn Conference

Research Day, 6th April 2011, Royal College of Surgeons in Ireland
Claro T, Widaa A, Miajlovic H, Foster T, O’Brien FJ, Kerrigan SW (2010). New insights into the death and inflammatory pathways associated with bone infection-

RAMI Biomedical Sciences Section Meeting, 22nd June 2011
ii. Poster presentations

RAMI Biomedical Sciences Section Meeting, 2009

Bioengineering In Ireland Conference, 22-24 January, 2010
ISBN no. 978-0-9548583-3-9

110th ASM- General Meeting, May 23-27, 2010

RAMI Biomedical Sciences Section Meeting, 23-24 June 2010

RAMI Biomedical Sciences Section Meeting, 2011
iii. Publications


2. Widaa A, **Claro T**, Foster TJ, O’Brien FJ, Kerrigan SW. Multiple signals induced by *S. aureus* Protein A results in bone loss and bone destruction. *Submitted PLoS ONE 2012*
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Chassaingac, E. (1852). De l'ostéomylite chronique.


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