Mechanisms by which *Staphylococcus aureus* induces cytokines and cell death in human keratinocytes and mouse fibroblasts

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>AEBSF</td>
<td>Aminoethyl benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>agr</td>
<td>Accessory gene regulator</td>
</tr>
<tr>
<td>AMPs</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>ASMC</td>
<td>Airway bronchial smooth muscle</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>BMMCs</td>
<td>Bone-marrow mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCR CXCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CoNS</td>
<td>Coagulase negative staphylococcal species</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide,</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenyl</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Ecp</td>
<td><em>Staphylococcus epidermidis</em> cysteine proteases</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>Esp</td>
<td><em>Staphylococcus epidermidis</em> serine proteases</td>
</tr>
<tr>
<td>EU</td>
<td>European unit</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FLG</td>
<td>Filaggrin</td>
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<tr>
<td>FnBPs</td>
<td>Fibronectin-binding proteins</td>
</tr>
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<td>FSA</td>
<td>Filtered supernatant from <em>S. aureus</em></td>
</tr>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptor</td>
</tr>
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<td>HDM</td>
<td>House dust mite</td>
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<td>HEp-2</td>
<td>Human laryngeal carcinoma</td>
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<td>HEKa</td>
<td>Human epidermal keratinocyte</td>
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<tr>
<td>HKGS</td>
<td>Human Keratinocyte Growth Supplement</td>
</tr>
<tr>
<td>HKSA</td>
<td>Heat killed <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>HLA</td>
<td>α-hemolysin</td>
</tr>
<tr>
<td>HLPC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HMGBs</td>
<td>High mobility group box proteins</td>
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<tr>
<td>HRP</td>
<td>Horseradish-peroxidase</td>
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<tr>
<td>IDECs</td>
<td>Inflammatory dendritic epidermal cells</td>
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<tr>
<td>ILCs</td>
<td>Innate lymphoid cells</td>
</tr>
<tr>
<td>IRF-3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>ISAAC</td>
<td>International Study of Asthma &amp; Allergies in Childhood</td>
</tr>
<tr>
<td>KLKs</td>
<td>Kallikreins</td>
</tr>
<tr>
<td>KLK5</td>
<td>Kallikrein 5</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LEKT1</td>
<td>Lymphoepithelial Kazal-type 5 serine protease inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LiSA</td>
<td>Live Staphylococcus aureus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic Acid</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>mDCs</td>
<td>Myeloid DCs</td>
</tr>
<tr>
<td>MEFs</td>
<td>Murine embryonic fibroblasts</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSA</td>
<td>Mannitol salt agar</td>
</tr>
<tr>
<td>MSM</td>
<td>Mus musculus molossinus</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient agar</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>NC</td>
<td>Nishiki-nezumi Cinnamon/Nagoya</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaeric Acid</td>
</tr>
<tr>
<td>Nec-1</td>
<td>Necrostatin-1</td>
</tr>
<tr>
<td>NF</td>
<td>Necrotic factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa Beta</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptor protein 3</td>
</tr>
<tr>
<td>NMF</td>
<td>Natural moisturising factors</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide binding oligomerization domain</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicles</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PARs</td>
<td>Protease-activated receptors</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDCs</td>
<td>Plasmacytoid DCs</td>
</tr>
<tr>
<td>PFA</td>
<td>Parafomaldehyde</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PolyI:C</td>
<td>Polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PSMs</td>
<td>Phenol-soluble modulins</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine leukocidin</td>
</tr>
<tr>
<td>RIPK1</td>
<td>Receptor interacting protein kinase-1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rST2</td>
<td>Recombinant sST2</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sarA</td>
<td>staphylococcal accessory regulator</td>
</tr>
<tr>
<td>S.aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SB</td>
<td>Stratum basale</td>
</tr>
<tr>
<td>SC</td>
<td>Stratum corneum</td>
</tr>
<tr>
<td>SCCE</td>
<td>Stratum corneum chymotryptic enzyme</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>ScaA</td>
<td>Staphopin A</td>
</tr>
<tr>
<td>SCTE</td>
<td>Stratum corneum tryptic enzyme</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcus aureus enterotoxin B</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free media</td>
</tr>
<tr>
<td>SFP</td>
<td>Specific free pathogen</td>
</tr>
<tr>
<td>SG</td>
<td>Stratum granulosum</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SMA</td>
<td>Skimmed milk agar</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SpA</td>
<td><em>S. aureus</em> protein A</td>
</tr>
<tr>
<td>SP</td>
<td>Serine protease</td>
</tr>
<tr>
<td>SPI</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SPIK5</td>
<td>Serine protease inhibitor, Kazal type 5 gene</td>
</tr>
<tr>
<td>SS</td>
<td>Stratum spinosum</td>
</tr>
<tr>
<td>SsAgs</td>
<td>Staphylococcal superantigens</td>
</tr>
<tr>
<td>Ssp</td>
<td><em>Staphylococcus aureus</em> serine protease</td>
</tr>
<tr>
<td>SspB</td>
<td>Staphopin B</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>sST2</td>
<td>Soluble ST2</td>
</tr>
<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
</tr>
<tr>
<td>ST2</td>
<td>Suppressor of tumorigenicity-2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline and Tween 20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>Th cells</td>
<td>T helper cells</td>
</tr>
<tr>
<td>Th0</td>
<td>Naïve T cells</td>
</tr>
<tr>
<td>TL</td>
<td>Tethered ligand</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNB</td>
<td>Trinitrobenzene</td>
</tr>
<tr>
<td>TNBSA</td>
<td>Trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T Cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic shock syndrome toxin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia virus</td>
</tr>
<tr>
<td>vWbp</td>
<td>Willebrand factor binding protein</td>
</tr>
</tbody>
</table>
Abstract

Mechanisms by which *Staphylococcus aureus* induces cytokines and cell death in human keratinocytes and mouse fibroblasts

2016

**Background:** *Staphylococcus aureus* is an important trigger of flares in atopic dermatitis. The exact mechanisms by which *S. aureus* induces inflammatory responses and cell death in the skin epithelium is unclear. The aim of this thesis was to elucidate the cellular and molecular mechanisms by which *S. aureus* induces it’s pathogenic effects on keratinocyte and fibroblast cell lines.

**Methods:** Human keratinocytes (HEKa), and mouse embryonic fibroblasts (MEF) from the NC/Nga dermatitis prone mouse strain were used to investigate the induction of Th2-promoting cytokines (IL-33 and TSLP) and cell death by *S. aureus*. Cytokine levels were measured by ELISA and cytotoxicity by flow cytometry.

**Results:** Live, but not killed *S. aureus* or other staphylococcal species, induced release of Th2-promoting cytokines (IL-33 and TSLP) and necrosis in both human and mouse cell lines. Cytokines were not induced by TLR2 ligands, and anti-TLR2 antibodies did not inhibit release, suggesting that the TLR2 pathway was not involved. By contrast, the release of cytokines was induced by a secreted, heat-labile factor/s and could be blocked by protease and PAR2 inhibitors, suggesting that the protease-PAR2 pathway was critical. NC/Nga mouse fibroblasts that lacked soluble IL-33 (sST2) receptor were more sensitive to the effects of *S. aureus* than control MEF.

**Conclusions:** *S. aureus* is unique amongst staphylococcal species in it’s ability to induce an inflammatory response and cytotoxicity in human keratinocytes and mouse fibroblasts. The protease-PAR2 pathway is critical to this bioactivity. Development of specific inhibitors of this pathway may provide novel therapies for treating *S. aureus*-induced eczema flares.
Declaration

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Abdullah Alkahtani
1 Chapter 1: Introduction
1.1 Foreword

Atopic dermatitis (AD) is the most common inflammatory skin condition, particularly in children. *Staphylococcus aureus* (*S. aureus*) is a key trigger of eczematous flares at all ages. The mechanisms by which *S. aureus* interacts with keratinocytes in the superficial epidermis to trigger AD flares is incompletely understood. The aim of this thesis is to more fully elucidate the immune pathways through which this bacterium triggers a local atopic immune response in the skin. Live *S. aureus* and its bacterial components were cultured with human skin cells, as well as mouse embryonic fibroblasts derived from the Japanese NC/Nga mouse, a strain that spontaneously develops eczema after weaning. Th2-promoting cytokine release and cell death pathways induced by *S. aureus* were the main study focus. This Introductory Chapter will provide firstly an overview of what is known about the biological basis of AD; the skin as not only a physical barrier but also an integral part of the innate immune system; and secondly a resume of the immunogenic properties of *S. aureus* and how it might induce a local immune response and cell death in the skin.

1.2 Atopic dermatitis

1.2.1 Epidemiology and clinical overview of the disease in humans

Atopic dermatitis (AD) is a common chronic, recurring, inflammatory skin disease. The clinical features are pruritus, redness and dry skin. Atopy is a term derived from Greek meaning a (no) topos (place) or “out of place”. As well as the relapsing eczematous lesions, the immune response is atypical in that rather than producing IgG and a Th1-type response, 80% of AD patients have elevated serum IgE concentration and Th2-associated allergic hypersensitivity to normally innocuous food and aeroallergens (Ishizaka *et al.* 1967; Donald Y. M. Leung *et al.* 2004; Nutten 2015). AD affects the quality of life of both the patient and their family (McKenna *et al.* 2008). It is often associated with time off school, work and a high economic cost. In Canada, one study estimated the annual cost to be more than a billion dollars (Barbeau *et al.* 2006). In the UK, the annual cost was estimated to be around £465 million in the 1990s, and in 2002, the prescribing cost for corticosteroids alone to treat AD was £11.6 million (Green *et al.* 2004; Herd *et al.* 1996).

AD is much more common in young children (10-20%) and tends to improve as they reach adulthood (1-3%) (Larsen *et al.* 2002; Yu *et al.* 2012). (Figure 1.1) Approximately 85% of patients with AD are between 1-5 years old. Fifty percent of
children with severe AD go on to develop asthma and allergic rhinitis (Bieber 2008; Williams 2000).

Figure 1.1 Prevalence (%) of AD according to age in Korea in 2008 adapted from (Yu et al. 2012).

The prevalence of AD has increased over the last 30 years, especially in industrialised countries (Asher et al. 2006; Kim et al. 2012). Some studies, particularly the International Study of Asthma & Allergies in Childhood (ISAAC), have highlighted the differences in the prevalence of AD across the world. For instance in 1999, the prevalence of AD in Indian children aged 6-7 years was only 1%, while in Ecuador it was 22.5%. In 13-14 year old Chinese children, it was 0.2%, while in Columbia it was 24.6% (Odhiambo et al. 2009). Even within countries, the prevalence of AD has been found to vary. For instance, in the US, the prevalence of AD ranges from 9% to 18%, with higher prevalence in urban population particularly on the East Coast and in people of Afro-Caribbean descent (Shaw et al. 2011) (Figure 1.2).

Figure 1.2 Variation in the prevalence of AD in the United States adapted from (Shaw et al. 2011).
The diagnosis of AD is clinical rather than based on laboratory tests. Standardised clinical diagnostic criteria were initially developed by Hanifin and Rajka in 1980, and these still form the basis of diagnosing the condition worldwide (Hanifin et al. 2004; Watson et al. 2011; Williams et al. 1994). The major criterion is itchy skin (pruritus). There are a number of minor criteria, including a flexural rash and/or history of atopic disease as shown in Table 1.1. The severity of AD is classified by different validated scoring systems such as Scoring AD (SCORAD) and the Eczema Area & Severity Index (EASI) (Stalder et al. 1993; Williams et al. 1994).

**Table 1.1 Diagnostic criteria of atopic dermatitis (Watson et al. 2011).**

<table>
<thead>
<tr>
<th>Major criteria essential</th>
<th>Minor criteria plus three or more of the following</th>
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</thead>
<tbody>
<tr>
<td>• Itchy skin</td>
<td>• visible flexural dermatitis</td>
</tr>
<tr>
<td></td>
<td>• history of eczema in the skin creases (front of elbows, backs of knees, fronts of ankles, neck, around eyes)</td>
</tr>
<tr>
<td></td>
<td>• history of a generally dry skin in the past year</td>
</tr>
<tr>
<td></td>
<td>• personal history of asthma or hay fever</td>
</tr>
</tbody>
</table>

The distribution of eczema varies with the age of the patient, depending on exposure to allergens and scratching. It is scratching of the skin that causes the rash. For instance, in infants, AD tends to be localised to the face, in older children, it is more prominent on the flexural surfaces, and in adults, the hands can be particularly troublesome (Williams et al. 1994). Although laboratory tests are not required to diagnose AD, they may be useful to exclude other skin conditions e.g. skin biopsy for skin lymphoma, patch tests for contact dermatitis, or skin prick tests if there are accompanying type I hypersensitivity reactions (Darsow et al. 2010; Watson et al. 2011).

Management of AD focuses primarily on measures to supplement skin barrier function (moisturisers and cotton or silk garments), but in more severe disease also involves suppressing cutaneous inflammation (topical corticosteroids and calcineurin inhibitors) and eradicating secondary skin infections (antibiotics and antisepsics) (Figure 1.3). Potent topical steroids used long-term are not without local and sometimes systemic side effects such as skin atrophy and systemic toxicity and concern over these side-effects often reduces compliance with treatment (Bieber 2008). As the puritus is
not histamine-mediated, antihistamines do not reduce the itch in eczema, but their sedative properties might promote sleep in the short term.

Figure 1.3 Key steps for treatment of atopic dermatitis (AD) adapted from Bieber 2008

Where topical treatment fails, systemic therapies such as oral cyclosporin (immunosuppressant drug) or UV light therapy may be considered, although fortunately these therapies are needed in only a small proportion of patients. Blue light irradiation is thought to be safer and more effective than UV light, decreasing the severity of pruritus by 50% possibly through it’s effect on local immune responses (Detlef Becker et al. 2011; Kleinpenning et al. 2010). Systemic corticosteroids are effective in the short term, but relapse is common, and thus this treatment should be avoided (Watson et al. 2011).

1.2.2 Japanese NC/Nga mouse eczema model

Many mouse models of AD have been described over the years. Flaky tail mice have a mutation in the filaggrin (FLG) gene and a defect in the skin barrier function, similar to that found in human AD. As such, they spontaneously develop eczema and so are used to explore the pathophysiology of defects in this gene (Fallon et al. 2009). Many other mouse models do not spontaneously develop eczema but require intra-dermal challenge with allergen to illicit the skin lesions.
In 1997, Matsuda and colleagues reported that Nishiki Nezumi NC/Nga Japanese mice in their animal house suffered from severe dermatitis, which was clinically, histologically and immunologically similar to AD (Matsuda 1997). The NC/Nga mouse is a Japanese mouse strain, bred for its attractive cinnamon coat (K. Kondo et al. 1969) (Figure 1.4). Since this discovery these mice have been used to study the biology of AD and use of potential novel therapeutic agents in it’s treatment (Fallon et al. 2009; Mikito Ito et al. 2004; Jung et al. 2011; Kohara et al. 2001; Ogawa et al. 2005; Shah et al. 2010; Takeda et al. 2009; Tanaka et al. 2011; Yamamoto et al. 2007).

![NC/Nga mice with dermatitis. A. Dermatitis on the ears and neck. B. Dermatitis on the face, ears and eyes (Hiromichi et al. 2012)](image)

NC/Nga mice, unlike other mouse strains, spontaneously develop severe dermatitis when housed in conventional environments (Matsuda 1997), although when housed in specific free pathogen (SFP) environments they remain disease free. This suggests that both inherited and environmental factors are involved in the pathogenesis of disease in this model. Similar to human AD symptoms, eczema (itching, erythema, haemorrhage, xerosis and lichenification) typically develops on the face, neck and back of the animals just after weaning (4 - 5 weeks old) (Matsuda 1997). The immunopathology is characterised by infiltration of CD4+ T-cells and increase production of IL-4, IL-5, mast cells and eosinophils (Vestergaard et al. 1999).

However, STAT6 knock-out NC/Nga mice develop dermatitis-like skin lesions with the lack of Th2 cytokines and IgE response; this evidence suggests a role for Th1-induced inflammation (Yagi et al. 2002). Dermatitis can also be induced by application of fur mite antigen or Staphylococcal exotoxin B (SEB) to the skin of the mice (Kang et al. 2003).
Under conventional conditions, NC/Nga mice are often infested with fur mites, which may facilitate the induction of dermatitis (Gao et al. 2004; Heishi et al. 2003). Applying anti-parasitic cream to the skin eradicates the mites, induces wound healing and decreases levels of IgE (Iijima et al. 2000).

To allow for more effective application of potentially medicinal ointments and creams, a hairless NC/Nga mouse strain (NCN24) has recently been generated, by use of diphtheria toxin to delete the gene coding for keratin filament protein (Figure 1.5).

![Figure 1.5 Fourteen days old hairless NCN24 mice next to the NC/Nga parent strain adapted from (Hiromichi et al. 2012)](image)

The NC/Nga strain is derived from Mus musculus molossinus (MSM) mice that live wild in Japan. MSM mice probably originated through cross-breeding between M. m. castaneus, which came to Japan via mainland China and down the North islands, and M. m. musculus which entered Japan via the Korean peninsula (Yonekawa et al. 1988). Although MSM mice do not spontaneously develop eczema (Kohara et al. 2001), they are prone to more exaggerated immune responses to LPS, PGN and LTA compared to BALB/c mice (Conner et al. 2008; Morse Iii 2007; Stephan et al. 2007). A genetic study comparing MSM with NC/Nga mice identified differences within chromosome 9, linked to increased Th2 responses and IgE production (Kohara et al. 2001). However, the specific gene defect leading to a propensity of NC/Nga mice to dermatitis has yet to be identified.

In view of the clinical, histological and immune similarities between the NC/Nga mouse model and human AD, mouse embryonic fibroblasts (MEFs) derived from this strain was used in this thesis. MEFs from Mus musculus molossinus (MSM/Ms) and NIH/3T3 were also produced in our laboratory and used as “healthy” controls.
1.3 The epidermis as a physical barrier

The skin is the first line of defence against the environment, protecting the internal milieu from drying out and preventing infection (Cork et al. 2009). In addition, it is responsible for regulation of body temperature and sensation. It is composed of three layers: the outer epidermis, the middle dermis and the deeper hypodermis. The epidermis consists of keratinocytes which act as the immediate interface between the host and its environment. The dermis is composed of a fibroblast-matrix network which maintains skin integrity and strength. The hypodermis consists of loose fatty connective tissue and acts as a layer of thermal insulation. The skin also contains nerve endings, blood vessels and lymphatics (Avram et al. 2005; Tortora et al. 2011).

1.3.1 Epidermal structure

The epidermis is divided up into four layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Alonso et al. 2003; Simpson et al. 2011). (Figure 1.6) Keratinocytes are responsible for the synthesis and secretion of keratin, the scaffold protein that keeps cornified cell envelope proteins and lipids together in stratum corneum.

![Figure 1.6 Structure of the epidermis.](image)

The deepest layer of the epidermis is the stratum basale, which contains undifferentiated epithelial stem cells that have the ability to regenerate themselves indefinitely. The stratum spinosum is formed as a result of proliferation and
differentiation of these basal cells as they migrate towards the surface. Cells of the stratum spinosum are linked to their neighbour's cells by desmosome tight junctions. The initial innate immune response to environmental allergens and microbes involves activation of these keratinocytes, as well as specialised dendritic cells (Langerhans cells) present in this layer. Keratinocytes continue their transition to the stratum granulosum. This layer expresses filaggrin, a histidine-rich protein, discussed in more detail below. The keratinocytes then become flattened, and their nuclei and cytoplasm are lost to form mature corneocytes of the most superficial layer in direct contact with the air, the stratum corneum (Kanitakis, 2002). Corneocytes are surrounded by lamella, consisting of lipids (ceramides, cholesterol and fatty acid) that give the cells their rigidity and impermeability to water (Cork et al. 2009). Corneocytes are linked together by corneodesmosomes, tight junctions which are a critical part of the skin barrier (Figure 1.7). These corneodesmosomes can be degraded by serine proteases (Cork et al. 2009). In healthy skin, corneocytes on the skin surface are replaced from the deeper layers of the epidermis every 28 days (Cork et al. 2009; Webb et al. 2004).

![Figure 1.7 Schematic showing the structure of the corneodesmosome linking corneocytes in the stratum corneum.](image)

1.3.2 Stratum corneum and its barrier function

As the stratum corneum (SC) is the most superficial layer of the skin structure which is around 15-20µm in thickness and the first line of defence to protect the body from the
external environment. SC is critical for skin barrier function, and its disruption is now thought to lead to AD. The integrity of this layer depends on the balance between proteases and protease inhibitors, which are responsible for the turnover and desquamation of corneocytes (Jonca et al. 2002; Simon et al. 2001). Filaggrin is the main structural protein of the SC (Irvine et al. 2011). The Filaggrin is expressed in an inactive form called profilaggrin and during differentiation, profilaggrin is cleaved by epidermal proteases such as KLKs to produce functional filaggrin (Sandilands et al. 2007). Excessive activity of proteases, particularly kallikreins (KLKs); or inadequate activity of protease inhibitors such as the serine protease inhibitor, Lympho-Epithelial Kazal-Type Inhibitor (LEKTI) in Netherton's syndrome, results in disruption of this superficial skin barrier and severe skin inflammation (Chavanas et al. 2000; Strid et al. 2005). KLKs are serine proteases produced by keratinocytes that degrade corneodesmosomes and promote desquamation (Cork et al. 2006). Increased activity of KLKs accelerates desquamation and leads to a defective skin barrier (Ovaere et al. 2009). AD patients have also been shown to have higher KLKs and lower LEKTI expression in the SC (Komatsu et al. 2007a; Roedl et al. 2009).

Eight different KLKs have been found in SC which are thought to acts as cascade pathways (Komatsu et al. 2005a; Komatsu et al. 2005b; Komatsu et al. 2006a; Komatsu et al. 2006b) KLKs are thought to have a role in activation of SC proteases which lead to degradation of intracellular adhesion molecules and result in desquamation of corneocytes (Caubet et al. 2004; Komatsu et al. 2006b; Simon et al. 2001). KLKs are localised in normal skin in SC and stratum granulosum but also found to be expressed in the lower epidermis of the skin lesions of AD patients (Ekholm et al. 2000; Komatsu et al. 2005b).

There are two main hypotheses regarding how the SC barrier may be disrupted and promote AD (Silverberg et al. 2015).

- **The “outside-in” hypothesis:** the primary problem is an inherent defect in the keratinocyte biology. Defective differentiation or structure of the epidermis leads to a leaky skin barrier, allowing water to evaporate (leading to xerosis) and irritants and allergens to enter triggering an inflammatory response. Furthermore, microbes more easily penetrate the skin barrier leading to secondary skin infections.
• The “inside-out” hypothesis: rather than an inherent defect in the skin, the problem in this model is an unusual or atopic immune response to environmental irritants, allergens or microbes (Van Bever et al. 2011).

In reality, the pathogenesis of AD involves a combination of these two pathways. AD is multi-factorial, where both genetic factors and environmental factors (e.g. detergents and soaps which alter skin barrier function) contribute to the pathology (Table 1.2) (Cork et al. 2009; Van Bever et al. 2011). AD is also considered the initial step in an “allergic march” where many patients not only suffer from AD but subsequently develop asthma, allergic rhinitis and/or food allergies (McKenna et al. 2008; Spergel 2003).

Table 1.2 Contributory factors in the development of AD (Van Bever et al. 2011).

<table>
<thead>
<tr>
<th>Genetic factors</th>
<th>Environmental factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>• gene encoding filaggrin (FLG)</td>
<td>• detergents and soaps</td>
</tr>
<tr>
<td>• genes encoding proteases or serine protease inhibitor Kazal type 5 gene (SPINK5)</td>
<td>• contact with allergens e.g. house dust mite</td>
</tr>
<tr>
<td>• genes encoding tight junction proteins e.g. desmocolin &amp; desmoglein</td>
<td>• food allergens e.g. cow’s milk, egg, wheat, soya</td>
</tr>
<tr>
<td></td>
<td>• colonisation with microbes, particularly S. aureus</td>
</tr>
</tbody>
</table>

1.3.2.1 Genetic factors influencing skin barrier function

AD is more common in monozygotic (85%) than dizygotic (25%) twins (Larsen et al. 2002) It is also more common if one (25%) or both parents (70%) have AD than if there is no family history (10%) (Kulthanan et al. 2011). For some patients with AD of Northern European and Asian descent, the familial association is due to a mutation in FLG gene (Palmer et al. 2006). Filaggrin is expressed in the epidermis and is responsible for aggregation of keratin filaments and skin barrier function (Kim et al. 2012). In natural processes degradation of filaggrin by certain proteases leads to the formation of amino acids such as urocanic acid and alanine, which have a supplementary role in hydration and flexibility of the skin through the production of natural moisturising factors (NMF) (Figure 1.8). Filaggrin deficiency has been shown to lead to a failure in the barrier function of the skin (Cork et al. 2009).
Figure 1.8 The functions of profilaggrin, filaggrin and their final product amino acid in the epidermis adapted from (Brown et al. 2012).

In some European and Asian countries, FLG mutations are found in 25-40% of patients with moderately to severe AD (Barker et al. 2007; Morar et al. 2007; Nomura et al. 2007). Ten to 50% of AD patient in Northern Europe have R510X or 2282del4 FLG mutations (Donald Y. M. Leung 2009; Sandilands et al. 2007; Weidinger et al. 2006). Patients with severe AD are more likely to be homozygous rather than heterozygous for these mutations (Henderson et al. 2008; Nomura et al. 2007; Zhang et al. 2010). The FLG gene is located on chromosome 1q21 and composed of three exons (Figure 1.9). Most mutations are found in exon 3. Close to fifty different FLG mutations have been defined (H. Chen et al. 2011; Irvine et al. 2011) and most are associated with a reduced expression of filaggrin and its breakdown products (NMF) in the skin of AD patients (Cork et al. 2009).
Figure 1.9 Structure of the FLG gene adapted from (Irvine et al. 2011).

Over half of patients with AD do not have FLG mutations. Thus, there must be other factors promoting the development of AD. For instance, in patients suffering from Netherton syndrome, mutations in SPINK5 are associated with severe erythroderma of the skin and inflammation and fluid loss from the gastrointestinal tract. SPINK5 codes for the LymphoEpithelial Kazal-Type 5 serine protease Inhibitor (LEKTI), which is important in skin barrier function (Chavanas et al. 2000). LEKTI suppresses the activity of kallikreins (KLKs) that digest tight junctions and disrupt skin barrier function (Borgono et al. 2006). Other serine proteases involved in regulating the process of desquamation include SC chymotryptic enzyme (SCCE) and SC tryptic enzyme (SCTE). These enzymes are regulated by serine leukoprotease inhibitor and elafin (skin-derived antileukoprotease) (Franzke et al. 1996; Molhuizen et al. 1993). In mice, over expression of the SC chymotryptic enzyme (SCCE) causes dysfunction of the skin barrier and leads to AD-like phenotype (Van Bever et al. 2011).

1.3.2.2 Environmental factors influencing skin barrier function

The increased incidence of AD over the last few decades and the variation in its prevalence in industrialised and non-industrialized countries imply that environmental, as well as genetic factors, are important in its pathogenesis. A number of environmental factors influence skin barrier function, including soaps and detergents and allergens from house dust mite, pet dander and pollen (Van Bever et al. 2011). Washing with soap and detergents thins the SC by dissolving lipids, leading to water loss and an inflammatory response. pH of AD skin has been reported to be generally more alkali than in healthy controls (Eberlein-Konig et al. 2000; Seidenari et al. 1995). Acidic pH helps to maintain barrier function and protects against microbial infection (Cork et al.
Soaps and detergents increase skin pH and can increase the activity of some proteases, e.g., KLK5, leading to increased desquamation (Jang et al. 2016). In addition, house dust mites and their proteases are thought to have a role and affect the skin barrier function (Van Bever et al. 2011).

*S. aureus also* triggers severe AD flares. As the main aim of this project was to study the role of this organism in the pathogenesis of AD, the interaction between this bacteria and the skin is described in detail in section 1.5. An overview of the genetic and environmental factors known to predispose or trigger AD is shown in Figure 1.10.

![Graph showing genetic and environmental factors promoting skin barrier dysfunction](image)

**Figure 1.10 Genetic and environmental factors promoting skin barrier dysfunction adapted from (Gillespie 2015).**

The classical function of the epidermis is a physical barrier, which prevents water loss and the ingress of microbes that could lead to potentially life-threatening infections. Historically, keratinocytes were considered immunologically inert and cutaneous immune responses are thought to be due to the action of bone-marrow derived leukocytes such as skin Langerhans cells and lymphocytes. However, it is now recognised that although bone-marrow derived leukocytes (classical immune response) deal with potentially invasive microbes, the cutaneous atopic/allergic response is usually not initiated by these cells but rather by keratinocytes, now considered part of the innate immune system (Homey et al. 2006). The next section discusses the role of keratinocytes and bone-marrow derived leukocytes in the immune response of the skin.
1.4 Keratinocytes as part of the innate immune system

Besides its physical barrier function, the skin is an integral part of the innate immune system. Innate immunity distinguishes pathogens by a less specificity of signals that does adaptive immunity (De Benedetto et al. 2009). Strong evidence indicates the importance of keratinocytes as inducers of an inflammatory response in AD (Holgate 2007; Wittmann et al. 2006). Keratinocytes play an important role in innate immune responses by expression of TLRs, release of pro-inflammatory cytokines, such as Tumor necrosis factor-alpha (TNF-α), IL-1, GM-CSF (Homey et al. 2006), chemical mediators and by producing antimicrobial peptide (De Benedetto et al. 2009; McGirt et al. 2006). It has been suggested that keratinocytes are the main producer of cytokine and chemokines in the skin (Gröne 2002; Uchi et al. 2000) (Table 1.3).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory cytokines</strong></td>
<td><strong>Cytokine receptors</strong></td>
</tr>
<tr>
<td>IL-1, IL-4, IL-5, TNF-α, &amp; IFNα</td>
<td>IL-1R type 1, 2, IL-4R, IL-10R, IL-13Ra1</td>
</tr>
<tr>
<td><strong>Immunomodulatory</strong></td>
<td><strong>Co-stimulatory receptors</strong></td>
</tr>
<tr>
<td>IL-10, IL-12, IL-18, TGF</td>
<td>MCH class II</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td><strong>Chemokine receptors</strong></td>
</tr>
<tr>
<td>CCL2, CCL3, CCL4, &amp; CCL5</td>
<td>CCR1, CXCR1, CCR3, CXCR2, CCR4, CXCR4, CCR5, CXCR5</td>
</tr>
<tr>
<td><strong>Colony stimulating factors</strong></td>
<td><strong>Pattern recognition receptors</strong></td>
</tr>
<tr>
<td>GM-CSF, SCF</td>
<td>TLR1, TLR5</td>
</tr>
<tr>
<td><strong>Lipid mediators</strong></td>
<td>TLR2, TLR9</td>
</tr>
<tr>
<td>Prostaglandin E2, D2, F2-α</td>
<td>TLR3, TLR4</td>
</tr>
</tbody>
</table>

Pro-inflammatory cytokines activate downstream signals by binding to receptors on the vascular endothelium which promotes local or more generalised influx of leukocytes into the area of inflammation. Keratinocytes also produce antimicrobial peptides (AMPs) such as defensins and cathelicidins (Niyonsaba et al. 2009). AMPs are mostly broad spectrum antimicrobial agents which act against Gram negative and Gram positive bacteria and fungi (Niyonsaba et al. 2009). Some AMPs are specific against certain bacteria such as E. coli (Gläser et al. 2004).
1.4.1 Immune sensory receptors on keratinocytes

1.4.1.1 Toll-like receptors (TLRs)

One system of signal recognition is the pattern recognition receptors (PRR), the most well-characterized of which are Toll-like receptors (TLRs). TLRs are an essential component of innate immunity which recognises different microbial component (Aderem et al. 2000). TLRs are expressed on different cell types either on the cell surface or in endosomes (Figure 1.11). TLRs though are involved in activation of innate and adaptive immune response. Other PRR includes nucleotide binding oligomerization domain (NOD) and RIG-I-like receptors which are intracellular receptor.

TLRs are expressed on keratinocytes and recognise pathogen-associated molecular pattern (PAMPs) such as lipopolysaccharide, lipopeptides, RNA and DNA. There are ten TLRs in humans (Table 1.4) (Kang et al. 2006b). Stimulation of the TLR receptors expressed on keratinocytes and fibroblasts are thought to result in the release of several cytokines and chemokines (Jin et al. 2009; Kollisch et al. 2005). All TLRs have been found to be expressed on human keratinocytes except for TLR7 and TLR8 (Lebre et al. 2007; Miller et al. 2008).

Table 1.4 Human TLRs and their ligands (Kang et al. 2006b).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>triacylated lipopeptides</td>
</tr>
<tr>
<td>TLR2</td>
<td>peptidoglycan, lipoteichoic acid</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>lipopolysaccharide (Gram-negative bacteria), F-protein</td>
</tr>
<tr>
<td>TLR5</td>
<td>flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>peptidoglycan, lipopeptide</td>
</tr>
<tr>
<td>TLR7</td>
<td>single-stranded RNA (ssRNA) / polyuridylic acid, Imiquimod / Gardiquimod</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA / polyuridylic acid</td>
</tr>
<tr>
<td>TLR9</td>
<td>unmethylated CpG DNA</td>
</tr>
<tr>
<td>TLR10</td>
<td>no known ligand, may have TLR2 immunomodulatory properties</td>
</tr>
</tbody>
</table>

TLR2 and TLR4 are the most studied TLRs in AD patients and mouse models. It has been found that activation of TLR2 on monocytes and mast cells of AD patients leads to increased production of different cytokine and chemokines (Kuo et al. 2013; M. Niebuhr
et al. 2008; M. Niebuhr et al. 2009). The mechanisms that control the development of acute and chronic phases of AD is not totally understood. Another study showed that activation of TLR2 by its ligand on DCs leads to Th2 immune response in an AD mouse model (Kaesler et al. 2014). It has been shown that TLR4/− mice develop severe AD and skin inflammation compared with wild-type mice (Brandt et al. 2013). It has been suggested that dysfunction of these receptors could contribute to the inflammation in AD and asthma (Eder et al. 2006; Maintz et al. 2011).

TLR2, 3, 4, 5 and 9 have proved to have a functional role in response to their ligands in keratinocytes (Lebre et al. 2007). It has been suggested that the keratinocytes play a major role in shifting of immune response through the differential activation of TLRs against pathogen as well as function as a link between innate and adaptive immune response (Lebre et al. 2007).

Figure 1.11 Intracellular and extracellular TLRs signalling pathways.

1.4.1.2 Protease-activated receptors (PAR)

Another sensory receptor pathway involves the Protease-Activated Receptors (PARs). These are four trans-membrane receptors belonging to the G-protein-coupled receptor (GPCRs) family (S. R. Coughlin 2005; R. Ramachandran et al. 2009). PARs have been suggested to function as pro-inflammatory or anti-inflammatory receptors, depending on the animal model, species, tissues, or the protease that drives the response (Zhao et al.
PARs are widely expressed in many tissues, including epithelium, fibroblasts, endothelium, smooth muscle cells and bone-marrow derived leukocytes. PAR1, 3 and 4 were first documented as receptors for thrombin, acting together as heterodimers in the coagulation pathway (Coughlin et al. 1998; Shaun R. Coughlin et al. 2000; Kahn et al. 1999; McLaughlin et al. 2007). In contrast, PAR2 is activated by a number of other serine proteases including trypsin, tissue kallikreins, mast cell tryptase and leukocyte protease-3 (PR3) (Y. Kida et al. 2006; Ossovskaya 2004; V. Shpacovitch et al. 2007b; Martin Steinhoff et al. 2005). PAR2 can also be activated by bacterial proteases (Jeong et al. 2008; Kida et al. 2008; Y. Kida et al. 2006; Lourbakos et al. 1998; Sun et al. 2001). PAR2 can be activated by three main mechanisms (Figure 1.12):

- A classical proteolytic pathway, which involves self-activation following cleavage of blocking N-terminal residues of the receptor. Cleavage of this N-terminal peptide results in exposure of a tethered ligand (TL) that then binds to and activates the extracellular domains of the receptor and induce intracellular signalling (Hollenberg 2002; Macfarlane et al. 2001).
- Activation via exogenous peptides that mimic the TL sequence Table 1.5 describes the tethered ligand sequences of PARs in human and mouse
- Biased signalling by activation of PARs at distinct sites and activates distinct signals.

Figure 1.12 Mechanisms of PAR2 activation adapted from (Ramachandran et al. 2012).
Table 1.5 Sequences of Tethered Ligands of Protease-Activated Receptors (PARs) (Hollenberg 2002; Ramachandran et al. 2012).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>PARs tethered ligands sequence</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1</td>
<td>Human</td>
<td>Ser-Phe-Leu-Leu-Arg-Asn</td>
<td>SFLLRNP</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Ser-Phe-Phe-Leu-Arg-Asn</td>
<td>SFFLRNP</td>
</tr>
<tr>
<td>PAR2</td>
<td>Human</td>
<td>Ser-Leu-Ile-Gly-Lys-Val</td>
<td>SLIGKV</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Ser-Leu-Ile-Gly-Arg-Leu</td>
<td>SLIGRL</td>
</tr>
<tr>
<td>PAR3</td>
<td>Human</td>
<td>Thr-Phe-Arg-Gly-Ala-Pro</td>
<td>TFRGAP</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Ser-Phe-Arg-Gly-Gly-Asn</td>
<td>SFNGGP</td>
</tr>
<tr>
<td>PAR4</td>
<td>Human</td>
<td>Gly-Tyr-Pro-Gly-Gln-Val</td>
<td>GYPGQV</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Gly-Tyr-Pro-Gly-Lys-Phe</td>
<td>GYPGKF</td>
</tr>
</tbody>
</table>

In the skin, PAR2 is expressed on keratinocytes, fibroblasts and immune cells such as mast cells, eosinophils, neutrophils, dendritic cells, macrophages and T-lymphocytes (A. Rattenholl et al. 2008; M. Steinhoff et al. 1999). The expression is particularly strong in the stratum granulosum (Hachem et al. 2006; A. Rattenholl et al. 2008) and is seen both in healthy and AD skin (Hachem et al. 2005; M. Steinhoff et al. 1999; M. Steinhoff et al. 2004). KLKs cleave PAR2 and lead to activation of mitogen-activated protein kinase (MAPK), releasing calcium (Gieseler et al. 2013). PAR2 signalling is thought to regulate keratinocytes differentiation in healthy individuals, however in pathological disorders, PAR2 signalling in keratinocytes can induce an immune response (Briot et al. 2009; Demerjian et al. 2008). PAR2 may well play an important role in skin homeostasis (Dery et al. 1998; Rattenholl et al. 2003; Martin Steinhoff et al. 2005), induction of keratinocyte-induced cytokines and subsequent inflammation (Briot et al. 2009). Table 1.6 provides examples of tissue proteases which activate PAR2.

Table 1.6 Tissue proteases activating PAR2 and their target organs.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>tissue kallikreins 5,6 &amp; 14</td>
<td>skin</td>
</tr>
<tr>
<td>mast cell tryptase</td>
<td>most tissues</td>
</tr>
<tr>
<td>trypsin</td>
<td>gastrointestinal tract &amp; pancreas</td>
</tr>
<tr>
<td>transmembrane proteases</td>
<td>skin &amp; gastrointestinal tract</td>
</tr>
<tr>
<td>coagulation factors VIIa</td>
<td>cardiovascular system, skin &amp; innate immune cells</td>
</tr>
</tbody>
</table>
As well as inducing inflammatory responses, PAR2 may also modulate cell death (Chinni et al. 2000; Shpacovitch et al. 2007a). Examples include:

- cell death of colonic epithelial cells induced by IFN-γ and TNF-α is reduced after PAR2 activation by peptide agonists (Iablokov et al. 2014)
- Tryptase reduces apoptosis of glioblastoma cells via PAR2 (Luo et al. 2014).
- Administration of synthetic peptide mimicking tethered ligand of PAR2 reduces neutrophil and astrocyte apoptosis (Shpacovitch et al. 2007a; Wang et al. 2007a).
- *Helicobacter pylori* suppress cell death of gastric epithelial cells after inducing PAR2 expression and signalling via mitogen-activated protein kinases (MAPKs) (Lim et al. 2010).

A number of investigators have documented the importance of PAR2 in allergic skin diseases such as AD and contact dermatitis (Kawagoe et al. 2002; Seeliger 2003; M. Steinhoff et al. 2004). During cutaneous inflammation, mast cells may release tryptase, which upregulates PAR2 expression on keratinocytes (M. Steinhoff et al. 1999). In healthy skin, mast cells are found in the dermis whereas, in AD, mast cells and neutrophils increase in both the dermis and epidermis (M. Steinhoff et al. 1999). Inflammation is suppressed after administration of serine protease or trypsin in PAR2−/− mice (Knecht et al. 2007; Paszcuk et al. 2008). Deletion of PAR2 has also been noted to result in less cutaneous infiltration of T-lymphocytes, macrophages, neutrophils and eosinophils induced by topical application of oxazolone and Picryl Chloride (Kawagoe et al. 2002).

A number of aeroallergens (cockroach and house dust mite faeces,) and microbes (*Pseudomonas aeruginosa, Serratia marcescen, Alternaria alternata*), and even soaps contain proteases that can potentially activate PAR2 and can induce inflammation, at least in the airways (Asokananthan et al. 2002; Boitano et al. 2011; Y. Kida et al. 2006; S. Kondo et al. 2004). It has been demonstrated that *Spink5−/−* epidermis led to hyperactivity of KLK5 and resulted in overexpression of Thymic Stromal Lymphopoietin (TSLP) in human keratinocytes via PAR2 (Briot et al. 2009).
1.4.2 Keratinocyte-initiated immune responses

Cytokines produced by epithelial cells are now recognised to play an important role in initiation of allergic responses and atopic disease (He et al. 2010). Disruption of the SC layer of the epidermis in AD patients results in rapid induction of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α and GM-CSF (Homey et al. 2006). AD patients overexpress Th2 cytokines such as IL-4, IL-5 and IL-13 (Jeong et al. 2003). In this section, epithelial-derived cytokines promoting a Th2 response will be discussed in detail, particularly TSLP, IL-25, IL-33 (Lloyd et al. 2010; Wang et al. 2009).

1.4.2.1 Thymic Stromal Lymphopoietin (TSLP)

TSLP is an IL-7-like cytokine, initially identified in the culture supernatant of a mouse thymic stromal cell line (Friend et al. 1994). TSLP has a distinctive four-helix-bundle structure composed of 140 amino acids and was first cloned in humans in 2001 (Liu et al. 2007; Quentmeier et al. 2001). The TSLP gene is located on the human chromosome 5q22 close to Th2 cytokine genes such as IL-4, IL-5, IL-9, and IL-13 (Al-Shami et al. 2005; Zhou et al. 2005).

TSLP is produced by skin keratinocytes as well as by airway cells and the thymus. Other cells (mast cells, smooth muscle cells, fibroblasts and DCs) may also express this cytokine (Allakhverdi et al. 2007). TSLP activates DCs to drive Th2 responses and induces IL-4, IL-5, and IL-13 production in immune cells. Its expression is upregulated in acute and chronic AD lesions (Allakhverdi et al. 2007; Allakhverdi et al. 2007). It has also been associated with asthma (Allakhverdi et al. 2007; Allakhverdi et al. 2007). The mechanism by which TSLP induces Th2 responses is shown in Figure 1.13.

![Figure 1.13 TSLP-associated Th2 pathway adapted from (He et al. 2010).](image-url)
1.4.2.2 Interleukin-25

IL-25 is composed of 177 amino acids and has a molecular mass of 17 kDa in humans (Hurst et al. 2002). It is also called IL-17E because it has 17% similarity with IL-17A and IL-17B with highly conserved C-terminal regions. It is encoded by chromosome 14q11.1 (Hurst et al. 2002). It binds to IL-17RB and IL-17RA to form a complex. IL-25 is secreted by Th2 cells, mast cells and epithelial cells.

IL-25 induces IL-13, IL-4 and IL-5 (Fort et al. 2001). It may also reduce the synthesis of filaggrin by keratinocytes, resulting in skin barrier dysfunction (Hvid et al. 2011). High levels of IL-25 mRNA has been found in the skin of AD patients and the lungs of asthmatics (Wang et al. 2007b). Normally, high levels are found in the gastrointestinal tract and uterus, with low levels in kidney, brain and lung (Ikeda 2003; Rickel et al. 2008; Wang et al. 2007b).

1.4.2.3 Interleukin-33

IL-33 is a member of the IL-1 family of cytokines, which includes IL-1α, IL-1β, IL-1Ra and IL-18. It is also called IL-1R1 (Arend et al. 2008; Barksby et al. 2007; Dinarello 2009). The IL-33 gene is coded on human chromosome 9p24.1. It is composed of 270 amino acids in humans (30 kDa) and 266 amino acids in mice (Priestle et al. 1988). IL-33 has a highly conserved N-terminal homeodomain-like helix-turn-helix DNA binding site (Baekkevold et al. 2003). IL-33 is expressed in stomach, brain, spleen and by fibroblasts, cardiomyocytes and keratinocytes (Mousson et al. 2008; Priestle et al. 1988). Unlike other interleukins which are localised to the cytoplasm, IL-33 is found in the nucleus (Mousson et al. 2008). It is produced in an active form and is involved in the formation of the inflammasome (specialised pro-inflammatory cellular processing centre). IL-33 is cleaved by caspase-1 at Asp110 in both human and mice to form the inactive form of IL-33 with a molecular weight of 18-20 kDa (Priestle et al. 1988; Talabot-Ayer et al. 2009) (Figure 1.14).

The IL-33 receptor, ST2 (Suppressor of Tumorigenicity-2), was initially identified in fibroblasts and named as serum inducible secreted protein (Cayrol et al. 2009; Miller et al. 2011; Roussel et al. 2008). It is a member of IL-1R family encoded on chromosome 2q12. It has a molecular weight of 60-70 kDa. Four isoforms of ST2 are reported, ST2, ST2L, ST2V and STLV (Bergers et al. 1994). This receptor is expressed in high levels on mast cells, macrophages, natural killer (NK) cells, eosinophils, basophils and fibroblasts (H. Li et al. 2008; Lüthi et al. 2009). It is an important inducer of Th2 cells,
and binding of IL-33 to ST2 leads to the formation of ST2/IL-1 receptor accessory protein (IL-1RACp) and the induction of Th2 cytokines such as IL-5 and IL-13, and also caspase-3 and caspase-7 leading to apoptosis (Moussion et al. 2008).

IL-33 is thought to play a critical role in inflammation and allergy. It has been suggested that IL-33 is a multi-functional protein acting as an alarmin to drive the Th2 immune response and as a transcriptional regulator to induce or inhibit the activity of NF-κB in a gene-dependent manner (Ali et al. 2011; Moussion et al. 2008; Ni et al. 2015; Pichery et al. 2012; Shao et al. 2014). IL-33 can be released upon necrosis, cell damage, and mechanical injury and in response to infection in which case it acts as a danger signal (Cayrol et al. 2009; Chen et al. 2007). During apoptosis, IL-33 is cleaved by caspases and becomes inactive, thus does not influence the immune system, however the opposite is seen in necrotic cell death when full-length active IL-33 is released (Cayrol et al. 2009; Lüthi et al. 2009) (Figure 1.14).

Figure 1.14 The role of IL-33 in homeostatic state, programmed cell death and tissue damage, infection and cellular stress.
Pathogens such as *Alternaria alternata* that produce proteases have been shown to induce the release of IL-33 (Kouzaki *et al.* 2011; Snelgrove *et al.* 2014). In addition, IFN-γ and TNF-α induce expression of IL-33 in human keratinocytes (Taniguchi *et al.* 2013). Mechanisms by which IL-33 causes induction of Th2 cytokines is shown in Figure 1.15

![Figure 1.15 IL-33 signalling adapted from (Borish *et al.* 2010).](image)

The level of IL-33 mRNA is ten-fold higher in the skin of patients with AD than in controls (Pushparaj *et al.* 2009). Recent endobronchial biopsy studies have found an association between IL-33 and severe asthma (Miller *et al.* 2011). In this latter study, IL-33 expression was measured in airway bronchial smooth muscle (ASMC) by a number of methods including PCR, ELISA and Western blotting and was higher in patients with severe asthma than controls (Miller *et al.* 2011) Several studies show that either soluble ST2 (sST2) or anti-ST2 neutralizer antibody could block the activity of IL-33 and consequently inhibit Th2 inflammatory response in mice (Miller *et al.* 2011).
1.4.3 Cell death pathways

Apoptosis and pyroptosis are both pathways of programmed cell death that help to attenuate the inflammatory process in senescent cells. Programmed cell death can also be triggered by pathogenic organisms. Apoptosis and pyroptosis are dependent on caspases.

- **Apoptosis** is characterised morphologically by cell shrinkage, release of cytochromes membrane blebbing, chromatin degradation in the nucleus, nuclear and cell fragmentation and releasing of apoptotic proteins (membrane bound-cell fragments). These fragments are phagocytosed and cleared without inducing an inflammatory response (Chekeni *et al.* 2010; Savill *et al.* 2002). Apoptosis is induced by stimulation of Fas receptors on the host cell surface (extrinsic pathway) or by events inside the host cells (intrinsic pathway) (Duprez *et al.* 2009).

- **Pyroptosis** is another mechanism of cell death induced by infection. Microbial components such as cytosolic flagellin, T3SS rod proteins or crystals initiate the process which results in the formation of pores in the host cell membrane and lysis of infected cells. It is defined as caspase-1-dependent programmed cell death involving activation of inflammasomes such as NLRP3 which leads to release of cytosolic contents and induction of IL-1α, Adenosine triphosphate (ATP) and IL-18 (Kanneganti *et al.* 2006; Kanneganti *et al.* 2007; Mariathasan *et al.* 2006).

- **Necrosis** is another form of cell death which is characterised by disruption of the plasma membrane, the release of cell contents and loss of rigidity (Berghe *et al.* 2009; Krysko *et al.* 2008a; Krysko *et al.* 2008b). It is also known as a harmful process that is associated with uncontrolled cell loss which may promote inflammation. During leakage of the plasma membrane, necrotic cells release different pro-inflammatory factors such as histone proteins, DNA, RNA and high mobility group box proteins (HMGBs) (Zitvogel *et al.* 2010). The different features of cell death are illustrated in Table 1.7 and Figure 1.16.
Table 1.7 Features of different modes of cell death (Miao et al. 2011).

<table>
<thead>
<tr>
<th>Features</th>
<th>Apoptosis</th>
<th>Pyroptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pathway activation</td>
<td>caspase-3/6/7</td>
<td>caspase-1</td>
<td>non-caspase</td>
</tr>
<tr>
<td>lytic / non-lytic</td>
<td>non-lytic</td>
<td>lytic</td>
<td>lytic</td>
</tr>
<tr>
<td>inflammation</td>
<td>non-inflammatory</td>
<td>inflammatory</td>
<td>inflammatory</td>
</tr>
<tr>
<td>cell type</td>
<td>all</td>
<td>macrophage and DC</td>
<td>all</td>
</tr>
</tbody>
</table>

Figure 1.16 Characteristics of different modes of cell death adapted from (Lamkanfi et al. 2010).

1.4.4 Downstream immune cell responses

1.4.4.1 Dendritic cells (DCs)

The keratinocyte-derived cytokines detailed in section 1.4.2 are known to modulate DCs and drive them towards a Th2 phenotype. Two types of DCs are thought to play a role in AD pathogenesis: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (Novak et al. 2007). Myeloid DCs can be subdivided into different subtypes, and two of these are found in the skin lesions of AD patients: Langerhans cells (LCs) and inflammatory dendritic epidermal cells (IDECs). LCs induce Th2 cells to produce IL-4 and initiate the allergic immune response in AD (Novak 2004). LCs also enhance the production of chemotactic factors such as CCL2, CCL22 and CCL17 (Novak et al. 2007). IDECs are present in the chronic phase of AD and found only at sites of inflammation. Activated IDECs promote Th1 and result in production IL-12, and IL-18. The role of LCs and IDECs in AD are illustrated in Figure 1.17.
1.4.4.2 T-lymphocytes

T cells are known to have an important inflammatory role in AD. The immune response in AD is characterised by a biphasic inflammatory T helper cell response. In acute AD, Th2 cells and cytokines such as IL4, IL-5 and IL-13 drive the immune response, while in the chronic stage Th1 responses are dominant with an increase in IFN-γ (Bieber 2008; Grewe et al. 1998; Ong et al. 2006) (Figure 1.18). In both acute and chronic AD lesions, there is high mRNA expression of IL-4, IL-5 and IL-13 compared to that of healthy skin. In chronic AD lesions, the expression of IL-5 and IL-12 mRNA is very high when compared to acute AD lesion, while IL-4 and IL-13 are lower. The high level of IL-5 and IL-12 correlate with the eosinophilic infiltrate (Grewe et al. 1998; Hamid et al. 1996). High expression of IL-13Ra2 has also been observed in AD skin (Lü et al. 2009). The expression of this decoy receptor is regulated in keratinocytes by Th2 cytokines (David et al. 2001). Its depletion increases the risk of cutaneous inflammation, transepidermal water loss and eosinophilic skin infiltration compared to wild-type mice, suggesting that IL-13Ra2 has a role in immunomodulation at the skin surface (Sivaprasad et al. 2010).
Other T-cell subsets, such as Th17, Th19 and Th22, are also represented in AD tissue.

- **Th22 cells:** Higher levels of IL-22 are observed in AD than in other skin inflammatory disease such as psoriasis (Nograles et al. 2009). IL-22 downregulates filaggrin expression in keratinocytes (Gutowska-Owsiak et al. 2011), while Staphylococcal enterotoxin B (SEB) and α-toxin induce IL-22 upregulation (Niebuhr et al. 2010).

- **Th17 cells:** Th17 cells are important for neutrophil recruitment (B. Brandt 2011). Th17 cells are known to produce two different cytokines, IL-17A and IL-17F (Heon Park et al. 2005). The expression of IL-17 is also higher in acute AD lesions than in the chronic stage (Koga et al. 2008; Szegedi et al. 2012). IL-17 may upregulate antimicrobial peptide such as β-defensin (Guttman-Yassky et al. 2008) and thus protect against bacterial infections (B. Brandt 2011).

- **Th9 cells:** Th9 cells are a newly identified subset of T helper cells also expressed in AD. Th9 cells were found to be increased in the skin and serum of AD patients compared to psoriasis or healthy controls (Ciprandi et al. 2012; Ma et al. 2013). IL-9 is expressed mainly by Th9 cells and can recruit mast cells (Dardalhon et al. 2008; Noelle et al. 2010; Veldhoen et al. 2008). Genetic polymorphisms in IL-9 have been associated with an increased risk of AD (Namkung et al. 2011).
- **Treg cells**: Human CD4^+^CD25^+^ Treg (suppressor T) cells help to modulate immune responses in the skin, by producing Transforming Growth Factor-Beta (TGF-β) and IL-10 via CTLA4 (Umetsu 2003). Treg cell numbers seem to be paradoxically increased in the peripheral blood of AD patients compared to healthy controls (Ito *et al.* 2009; Jonuleit *et al.* 2001; Samochocki *et al.* 2012). In contrast, an immunohistochemistry study showed that local infiltration of Treg cells in skin from AD patients is lower than in healthy controls. Superantigens derived from *S. aureus* induce expression of CTLA4 (D. Y. Leung 1995) and block Treg function (Umetsu 2003).

### 1.4.4.3 Innate lymphoid cells

Innate lymphoid cells (ILCs) are another relatively recently identified cell type and are thought to have a role in cutaneous immune responses (Kim *et al.* 2014; Weninger *et al.* 2014). ILCs lack T and B cell receptors and are classified into three main groups: ILC1, ILC2 and ILC3. ILC2 are increased in lesions from AD patients. ILC2s are associated with AD unlike ILC3 which associated with psoriasis (B. S. Kim *et al.* 2013) (Dyring-Andersen *et al.* 2014; Salimi *et al.* 2013). ILC2 like classical Th2 cells are capable of producing IL-4, IL-5 and IL-13 (Mackay *et al.* 2001). Furthermore, their activity is regulated by IL-25, IL-33 and TSLP released by keratinocytes (Monticelli *et al.* 2012). IL-33 produced by ILC2 downregulates the function of mast cells (Roediger *et al.* 2013), and IL-5 enhances the infiltration and expansion of eosinophils (Takatsu *et al.* 2008) (Figure 1.19).

![Figure 1.19 ILC2 in AD adapted from (Roediger *et al.* 2014).](image)
1.4.4.4 Eosinophils

Eosinophils are activated by Th2-lymphocytes via IL-5 and GM-CSF (Bradding et al. 1994; D. Simon et al. 2004). They contain two types of granules in their cytoplasm: primary and specific. Cationic granules have toxic effects against bacteria, parasite and known to induce basophils, mast cells and neutrophils (Kita et al. 1995; Leiferman 1991; Zheutlin et al. 1984). Eosinophilia in AD correlates with the level of IL-5 and eotaxin (Hossny et al. 2001; Jones et al. 1975; Ohman et al. 1974). Th2 cytokines can induce production of IL-12 by eosinophils, which may subsequently have a role in switching AD from an acute (Th2 predominant) to a chronic (Th1 and Th2) phase (Grewe et al. 1998; Kefei Kang et al. 2003).

1.4.4.5 Mast cells

Mast cells in the skin are located in the dermis close to hair follicles, nerves ending, lymphatic and blood vessels. They contain two proteases, tryptase and chymase in their granules. They act as effector cells to induce immune responses against bacterial and parasite infections (Kawakami et al. 2009; Fu-Tong Liu et al. 2011). Mast cells are activated by IgE, which binds to its surface receptor (FcεRI). Sensitization of mast cells leads to release of chemokines, cytokines and growth factors, as well as histamine, heparin, proteases, prostaglandins and leukotrienes (Kawakami et al. 2009). Mast cells induce migration, differentiation and polarisation of T-cells and B cells, as well as regulating IgE production (Frandji et al. 1996; Gauchat et al. 1993; Nakae et al. 2006). They can also act as professional antigen presenting cells (Frandji et al. 1996). Roles of mast cells are summarised in Figure 1.20. In addition, it has been reported that IL-33 can activate mast cells and induce release of IL-6 via ST2 (Enoksson et al. 2011).
In acute AD, Th2 cells predominate and mast cell number may not be increased, but degranulation is seen; whereas in chronic AD, Th1 cells predominate and mast cell numbers are increased (Bieber 2008; Irani et al. 1989; Soter 1989). Mast cell number and degree of degranulation correlates with the severity of AD (Hamid et al. 1996; Horsmanhetmo et al. 1994; Kanbe et al. 2001; Zhao et al. 2006).

1.5 S. aureus, skin and AD

Patients with AD are prone to infection with a variety of skin microbes including bacteria, fungi and viruses such as herpes simplex and vaccinia virus (Lubbe 2003; Vora et al. 2008) Fungi, particularly Malassezia species, may also colonise adult AD patients but are not usually thought to exacerbate the clinical disease.

S. aureus is the main infectious trigger of AD flares. Although only 5% of people with healthy skin are colonized with S. aureus, more than 90% of patients with AD are colonized, suggesting either an enhanced adhesion to the skin or a reduced host immune response unable to clear the bacteria (Leyden et al. 1974; Park et al. 2013; Ring et al. 1992). The number of colonies tends to be higher in more severe AD lesions (Huang et
al. 2009). *S. aureus* also causes a wide range of other diseases, including furuncles, abscesses, pneumonia, osteomyelitis, endocarditis and meningitis. *S. aureus* produces a variety of proteins that contribute to its virulence. Studies linking *S. aureus* cell wall and secreted products to AD are detailed below.

### 1.5.1 S. aureus cell wall and membrane vesicle virulence factors

The *S. aureus* cell wall is composed of different component such as peptidoglycan (PGN) and lipoteichoic acid (LTA) which are recognised by TLR2 (Akira et al. 2006). It has been shown that both PGN and LTA can stimulate the TLR2 pathway, which is thought to have a role in the pathogenesis of AD (Michelsen et al. 2001). One study found that TLR2 may have a role in mediating the host response against *S. aureus* as TLR-/- mice are more susceptible to this pathogen (Strunk et al. 2010). Injection of PGN and LTA induces Th2 cells in the dermis of mice resulting in an increase in gene expression of IL-6, TNF-α and IL-8 cytokines (K. Matsui et al. 2002;Travers et al. 2010).

Both ligands also induce production of IL-5 from peripheral blood mononuclear cells (PBMCs) in AD patients but not from healthy individuals, suggesting an increase in expression or functionality in AD patients (Katsuhioko Matsui et al. 2000). *S. aureus* virulence factors such as staphylococcal protein A (SPA), LTA and α-hemolysin (HLA) have been found to trigger inflammatory responses by epidermal keratinocytes. SPA in particular can induce production of TNF-α and correlates with the severity of AD and production of pro-inflammatory cytokines such as IL-6 and IL-8 (Ezepchuk et al. 1996;Yao et al. 2010). PGN has been demonstrated to induce production of granulocyte macrophage colony-stimulating factor (GM-CSF) via mitogen-activated protein kinases (MAPKs) and IL-8 from keratinocytes via TLR2 (Matsubara et al. 2004;Pivarcsi 2003). Diacylated lipopeptide membrane of *S. aureus* was shown to induce gene expression of TSLP in human keratinocytes via TLR2 and 6 (Vu et al. 2010). In addition, α-hemolysin of *S. aureus* can induce production of IL-1β and IL-6 from human keratinocytes in vitro (Sung-Wook Hong et al. 2014).

Outer membrane vesicles (OMVs) are produced by Gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* (Bomberger et al. 2009;Kesty et al. 2003). OMVs are 20-300 nm in diameter and are membrane fragments consisting of proteins, phospholipids, lipopolysaccharides (LPS), nucleic acid and other virulence factors. They are thought to be cytotoxic to host cells (Beveridge et al. 1996;Eun-Young Lee et al. 2008;Mashburn-Warren et al. 2006). *S. aureus* has been
found to produce extracellular vesicles (EVs) (Eun-Young Lee et al. 2009), with inflammatory and cytotoxic activity (Bhakdi et al. 1991; Burman et al. 2008; Hirasawa et al. 2010; S. W. Hong et al. 2010; Eun-Young Lee et al. 2009; Rollof et al. 1988; Sibbald et al. 2006). (Figure 1.21, arrows)

Figure 1.21 The arrow shows an extracellular vesicle secreted from *S. aureus* using transmission electron microscopy adapted from (Eun-Young Lee et al. 2009).

Soluble virulence factors encased within these EVs include:

- **coagulase** induces local thrombosis, which helps to encase bacteria in a biofilm that protects them from the host immune response
- **α and γ hemolysins**, which lyse erythrocytes
- **proteases**, which promote the spread of bacteria within the extra-cellular space
- **extracellular matrix & plasma-binding protein**, which promote adhesion and internalisation of bacteria in host cells
- **IgG-binding protein**, which protects bacteria from phagocytosis

*S. aureus* EVs may have a role in the induction of AD-like skin inflammation *in vivo*. EVs secreted from *S. aureus* have been found in the skin of AD patients and correlate with the severity of the diseases. These EVs induce Th1, Th17 and Th2 cytokines (S. W. Hong et al. 2010).

In mice, inhalation of EVs secreted from *S. aureus* is thought to trigger inflammation in asthma and chronic obstructive pulmonary disease (COPD) and enhances the production of TNF-α, IL-12, IL-6 and IL-4 (Kim et al. 2012). The severity of asthma correlates to EV-derived peptidoglycan (PGN) and lipoteichoic acid (LTA). Alveolar macrophages stimulated by *S. aureus* EVs, PGN and LTA, release TNF-α and IL-6. Interestingly, respiratory epithelia do not release TNF-α when stimulated by either *S. aureus* EVs, PGN and LTA (Kim et al. 2012).
The possible role of the TLR2 pathway in EV activity was examined using TLR2−/− mice. TLR2−/− mice had a blunted inflammatory response to S. aureus EV (Kim et al. 2012). S. aureus EVs were also cytotoxic, inducing apoptosis of human laryngeal carcinoma (HEp-2) cells, as assessed by Annexin V (AV) and propidium iodide (IP) staining using flow cytometry. EVs bound to the plasma membrane of host cells, before being internalised and releasing their contents into the host cell cytosol. Lysed EVs were not cytotoxic, suggesting that the bioactivity was dependent on soluble toxins such as α-hemolysin (α-toxin) being translocated into the host cells via internalisation of intact EVs (Gurung et al. 2011).

1.5.2 Secreted S. aureus virulence factors

Bacterial proteases may also have a role in the pathogenesis of AD (Hirasawa et al. 2010; Wichmann et al. 2009). Although there is evidence that serine protease (SP) activity is increased in acute skin barrier disruption and topical serine protease inhibitor (SPI) improves the recovery of skin barrier in AD patients (Hachem et al. 2006), it is currently unknown whether staphylococcal proteases can specifically disrupt corneodesmosome function and thus disrupt skin barrier function. It has been shown that serine proteases from S. epidermidis can inhibit biofilm formation of S. aureus (Moon et al. 2001; Wertheim et al. 2005). Another mechanism proposed of how S. epidermidis protects the skin against S. aureus infection is by production of AMPs such as β-defensins in human keratinocytes via the TLR2 pathway (Wanke et al. 2011). In addition, it has been reported that the presence of S. epidermidis mediates the function of T cell effectors in mouse skin (Naik et al. 2012). Examples of proteases secreted by S. aureus and the skin commensal S. epidermidis are shown in Table 1.8.
Table 1.8 Examples of proteases secreted from *S. aureus* and *S. epidermidis* and their functions (Koziel *et al.* 2012).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Protease</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Ssp (V8) serine protease</td>
<td>bacterial adhesion</td>
</tr>
<tr>
<td></td>
<td>Staphopin B (SspB) - cysteine protease</td>
<td>bacterial colonization &amp; digestion of connective tissue</td>
</tr>
<tr>
<td></td>
<td>Staphopin A (ScpA) - cysteine protease</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>cysteine (Ecp) and serine (Esp) proteases</td>
<td>inhibits colonisation of pathogenic bacteria &amp; inactivates adhesion molecules</td>
</tr>
</tbody>
</table>

Another important group of soluble virulence factors secreted by *S. aureus* are exotoxins. Several studies have shown that 60% of *S. aureus* isolated from AD patients secrete exotoxins (T. Werfel *et al.* 1996). Staphylococcal superantigens (SsAgs) include enterotoxins A, B (SEB), C, D, E, G and toxic shock syndrome toxin-1 (TSST-1). They are classically thought of as binding to MHC on antigen presenting cells and T-cell receptors (TCR) leading to polyclonal T-cell activation, *in vivo* leading to severe systemic inflammatory reactions associated with a cytokine storm (Figure 1.22) (Leung *et al.* 1998; Manders 1998). In the skin, Staphylococcus enterotoxin B (SEB) induces monocyte infiltrate and AD flare (Skov *et al.* 2000; Strange 1996). This is associated with the release of TNF-α and GM-CSF (Ezepchuk *et al.* 1996; Girolomoni *et al.* 2001; Matsunaga et al 1996; Pastore *et al.* 1998). It has been shown *in vivo* that Staphylococcus enterotoxin B (SEB) and house dust mite (HDM) were able to induce expression of IL-33 and ST2, in skin of AD patients (Savinko *et al.* 2012). However, a different study suggested that superantigen produced by *S. aureus* does not increase the inflammatory response in the skin of AD patients (Kozman *et al.* 2010). Given the lack of understanding regarding specific receptor and pathways involved, there is currently no consensus on the role of *S. aureus* proteases in AD.
1.5.3 Cytotoxic effects of Staphylococcal virulence factors

Microbial infection can trigger cell death either involving lysis of cell membranes and an inflammatory response, or apoptosis after internalisation of the microbe (Böhme et al. 2009; Grassme et al. 2001; Häcker 2009). Blocking apoptotic signalling may protect the host cells against microbial infections (Ayala et al.; Wesche-Soldato et al. 2007).

*S. aureus* virulence factors, including toxins, proteins and enzymes, are known to have cytotoxic activity in a number of cell types, including keratinocytes, monocytes, endothelial cells, epithelial cells and osteoblasts (M. S. Lee et al. 2001; Menzies et al. 2000; Wesson et al. 2000; Zuba et al. 2004). These cytotoxic effects can be mediated after live bacteria are internalised by the host cells, or as a direct result of secreted factors (Foster 1998). Factors promoting attachment and internalisation of *S. aureus* include staphylococcal protein A, fibronectin-binding proteins (FnBPs), and clumping factors. Staphylococcal α-hemolysin is a recognised soluble factor that can induce cell death in keratinocytes, T-lymphocytes and HeLa cells once released from internalised bacteria by making pores in the cell membrane (Brauweiler et al. 2014; Prince et al. 2012; Thay et al. 2013).

This toxin is the major cytotoxic agent of *S. aureus* which is thought to cause cell lysis and death via binding to the phosphocholine head of sphingomyelin which results in pore formation in the cell membrane (Schwiering et al. 2013; Wardenburg et al. 2007; Wichmann et al. 2009). Filaggrin expressed as a result of keratinocyte differentiation has been found to reduce the toxic effect of α-toxin by secretion of
sphingomyelinase, an enzyme that degrades sphingomyelin on the keratinocyte surface (Brauweiler et al. 2013). It has been approved that filaggrin-deficient keratinocytes are more susceptible to staphylococcal α-toxin more than the keratinocytes from healthy control (Brauweiler et al. 2013).

Another study showed that α-toxin induce more cell death in keratinocytes from AD patients biopsy compared with low toxic effect in healthy differentiated keratinocytes (Brauweiler et al. 2014). Th2 cytokines such as IL-13 and IL-4 have been shown to reduce the formation of ceramides and consequence affect epidermal function (Sawada et al. 2012). The toxic effect of α-toxin is increased in the presence of these Th2 cytokines (Brauweiler et al. 2014). *S. aureus* OMVs can carry active α-toxin protein which can cause apoptosis in T-lymphocytes and HeLa cells (Thay et al. 2013) possibly by inducing cell death via signal transducer and activator of transcription 6 (STAT6) (Brauweiler et al. 2014).

Phenol-soluble modulins (PSMs) are toxins which are produced by some staphylococcal species such as *S. aureus*, *S. epidermidis*, and *S. lugdunensis* (Donvito et al. 1997; Mehlin et al. 1999; Wang et al. 2007a). PSMs including δ-toxin is found to induce the release of inflammatory cytokines such as IL-18, IL-1β from primary human keratinocytes (Syed et al. 2015). Panton-Valentine leukocidin (PVL), is another pore forming exotoxin produced by *S. aureus* and found to cause apoptosis in primary human keratinocytes via a caspase-dependent pathway (Chi et al. 2013).

### 1.6 Synopsis, hypothesis and aims

IL-33 and TSLP are biomarkers of AD, and their expression reflects the severity of the disease. However, the induced release of IL-33 and TSLP in skin cells by *S. aureus* has not been documented. The main aim of this thesis is to study the role of *S. aureus* in the induction of Th2-promoting cytokines associated with atopic inflammation and host cell death in the skin.

The main proposed hypothesis was that *S. aureus* or its secreted factors interact specifically with human and mouse skin cells to induce IL-33 and TSLP release and cell death. Furthermore, the release of IL-33 by dying cells acts as an alarmin to trigger Th2 immune response in AD patients.
Aims

1. To confirm the specificity of *S. aureus* or its secreted factors in induction of pro-inflammatory cytokine (IL-33 and TSLP) release from human keratinocytes and mouse cells.
   a. Human epidermal keratinocyte (HEKa) and mouse embryonic fibroblasts (MEFs) derived from NC/Nga mice (AD model) will be used and compared to MSM/Ms and NIH/3T3 “control MEFs”.
   b. HEKa and MEFs will be stimulated with different staphylococcal species, live *S. aureus* (LiSA), killed *S. aureus* and TLR2 ligands (LTA and PGN). The released IL-33 and TSLP will be measured by ELISA.

2. To investigate the cytotoxic effect of *S. aureus* on the survival of host cells.
   a. HEKa and MEFs will be stimulated with LiSA, different staphylococcal species, TLR2 ligands and extracellular proteases and toxins such as serine protease (V8) and α-hemolysin with different dose and time in order to understand the relation between the release of IL-33 and *S. aureus* growth rates.
   b. The cytotoxicity effect induced will be studied by microscopy and flow cytometry using AV and PI staining.

3. To understand the mechanism by which *S. aureus* derived factors induce the release of pro-inflammatory cytokines and cell death in HEKa and MEFs.
   a. This aim will be carried out by investigation of protease activated receptors (PARs) with appropriate inhibitors and stimuli. The level of IL-33 and TSLP cytokines will be measured by ELISA and cytotoxicity will be assessed by flow cytometry.
2 Chapter 2: Materials and Methods
2.1 Culturing, Freezing and thawing of cell lines

2.1.1 Culture of Mouse Embryonic Fibroblasts (MEFs)

To initiate MEFs culture, stock vials were removed from liquid nitrogen and placed in a water bath (37°C) for 2 minutes until thawed. 1ml from each MEF vial was added to 15ml of Dulbecco's Modified Eagle's Medium (DMEM) complete growth media, (low glucose, 1g/L) (Life Technologies Ltd, Paisley, Scotland) supplemented with 10% (v/v) EC-foetal calf serum (FCS, endotoxin ≤30 European unit (EU)/ml) (PAA, Somerset, UK), 1% of L-glutamine (200 mM), and 1% of penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin per ml) (Sigma-Aldrich, Dorset, England). The cells were centrifuged (400g, 5 minutes, room temperature), resuspended with fresh warm media, counted and plated at 1×10^6 cells/ flask (T-75cm²) and incubated (37°C, 5% CO₂) until 80% confluent (2-3days).

At confluence, cells were washed 3 times with 1X phosphate buffered saline (PBS) without calcium and magnesium (PAA) and detached with 2ml of trypsin/EDTA (0.5 g porcine trypsin and 0.2 g EDTA) (Sigma) for 5 minutes at 37°C. Cell detachment was monitored by light microscopy until around 90% of cells were detached. Then 15ml of complete growth media was added to inhibit the effect of trypsin. Cells were washed, centrifuged (400g, 5 minutes, RT), and cultured again with low endotoxin DMEM media (10% FCS-clone (endotoxin ≤1 European unit (EU)/ml) (PAA)) for 48 hours until the cells were confluent. The cells were then washed again with PBS and detached with trypsin/EDTA as above and counted using a CASYTON “SCHARFE” cell counter. The cells were seeded in 24 well plates at 50 ×10^3 cells/well with 5% low-endotoxin (FCS-clone) complete DMEM media and incubated (37°C, 5% CO₂) for 24 hours before stimulation. Table 2.1 shows a list of reagents and media used for MEFs and human fibroblasts culture.
Table 2.1 Reagents used for culturing MEFs and human fibroblasts in all experiments.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cat No.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM, low glucose, pyruvate</td>
<td>31885-023</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>FCS</td>
<td>B15-106</td>
<td>PAA-Somerest</td>
</tr>
<tr>
<td>FCS-clone (low endotoxin)</td>
<td>A15-102</td>
<td>PAA-Somerest</td>
</tr>
<tr>
<td>1X trypsin/EDTA</td>
<td>T3924</td>
<td>Sigma</td>
</tr>
<tr>
<td>stable L-glutamine</td>
<td>G7513</td>
<td>Sigma</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>P0781</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.1.2 Human epidermal keratinocyte (HEKa) culture

Human epidermal keratinocytes isolated from adult skin were obtained in primary culture stage as recorded by the supplier (Life Technologies Ltd). HEKa were cultivated in basal media (Medium 154, Life Technologies Ltd) as recommended by the manufacturer. This media contains essential and non-essential amino acids, vitamins, organic compounds, trace minerals, and inorganic salts, supplemented with 5ml of Human Keratinocyte Growth Supplement (HKGS, Life Technologies Ltd). Before initiating culture from cryopreserved HEKa, thawed cells were counted, stained with trypan blue to identify the number of viable cells then seeded in a density of 2.5x10^5 viable cells/cm^2. When the HEKa cells reached 80% confluence, they were passaged as follows: adherent cells were washed with 5ml of cold phosphate buffered saline (PBS) without calcium and magnesium at RT. The cells then were detached from the flask surface with 5mL of 2.5% trypsin diluted (1/20) in versene (Life Technologies Ltd), then incubated at 37°C for 5 minutes. A detachment of HEKa cells was followed by 5ml of trypsin neutralised solution (Life Technologies Ltd). Cells were centrifuged (180g, 8 minutes, RT), and re-suspended in the required volume of growth media. Cells were seeded in 24 well plates at 1 x10^5 cells/well and incubated (37°C, 5% CO₂, 24 hours) before co-culture assays. Table 2.2 shows the list of reagents and solutions used for cultivation of HEKa.
Table 2.2 Reagent and solutions used for cultivation of HEKa

<table>
<thead>
<tr>
<th>Cell and Reagent</th>
<th>Cat No.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEKa</td>
<td>C-005-5C</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>Medium 154</td>
<td>M-154-500</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>HKGS</td>
<td>S-001-5</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>Trypsin (2.5%)</td>
<td>15090046</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>Versene Solution</td>
<td>15040033</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>Trypsin Neutralizer Solution</td>
<td>R002100</td>
<td>Life Technologies Ltd</td>
</tr>
</tbody>
</table>

2.1.3 Culture of Human skin fibroblasts

Human fibroblasts were grown up from skin biopsies by Mr William Fergusson and sub cultured by Meena Meenakshi, (both Cell Bank, Molecular Genetics Department, Manchester Royal Children’s Hospital, Manchester). Cells were cultured (T-75 flask) in 15ml of complete low glucose (1g/l) DMEM media (Life Technologies Ltd), which contained; 10% EC-Foetal Calf Serum (FCS, endotoxin ≤30 European unit (EU)/ml) (PPA), 1% of penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin per mL) and 1% of stable L-glutamine (200 mM) (Sigma) at 37°C with 5% CO₂ for 48-72h until confluence was reached. Trypsinization and plating of human fibroblasts prior to stimulation followed the same procedure as described above for MEFs.

2.1.4 Cryopreservation of MEFs, HEKa and human fibroblasts

MEFs and human fibroblasts were stored in 1ml aliquots (5×10⁵ cells/ml) in freezing media; 20% EC-FBS (PAA) in DMEM and 5% DMSO (Sigma). HEKa were stored at 1×10⁶ cells/ml in a serum-free cryopreservation medium (Cryo-SFM, PromoCell, Germany). HEKa and MEFs were frozen first gradually using an isopentane Jacket and stored at -80°C for 24 hours then transferred to liquid nitrogen for long-term storage.

2.2 Preparation of staphylococcal species, their virulence factors and inhibitors

2.2.1 Live *Staphylococcus aureus* (LiSA) and other staphylococcal species

*S. aureus* was a chronic wound isolate provided by Dr Andrew McBain, University of Manchester and the other staphylococcal species (Table 2.3) were obtained from Dr Xia, University of Manchester. Staphylococcal species were stored at −80°C in nutrient broth (NB) media with 10% glycerol. All species were stained by gram stain and observed under a light microscope to confirm their morphological features. All species were grown separately on nutrient agar (NA) and mannitol salt agar (MSA) to distinguish *S. aureus*. For co-culture assays, Staphylococcal species were sub-cultured
on NA and incubated (37°C, 24 hours) to get isolated colonies. 1 colony was then transferred to 15ml of NB and incubated (37°C, 18-24 hours). The resulting bacterial suspension was centrifuged (1600g, 5 minutes) and re-suspended in the required volume of DMEM (fibroblast growth media, see Section 2.1.1) or 154 medium (HEKa media, see Section 2.1.2). The required volume needed for co-culture depended on the colony forming unit (CFU) which was determined by Miles and Misra method (described in Section 2.2.3).

Table 2.3 Staphyloccocal species used in these experiments.

<table>
<thead>
<tr>
<th>No.</th>
<th>Staphyloccocal species</th>
<th>Coagulase</th>
<th>pathogenicity</th>
<th>Colony appearance</th>
<th>hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. aureus</td>
<td>+ve</td>
<td>Highly pathogen</td>
<td>Gray-beige</td>
<td>β-hemolysis</td>
</tr>
<tr>
<td>2</td>
<td>S. epidermidis</td>
<td>-ve</td>
<td>“accidental” pathogen</td>
<td>Gray-white</td>
<td>Non-hemolytic</td>
</tr>
<tr>
<td>3</td>
<td>S. capitis</td>
<td>-ve</td>
<td>Less pathogenic</td>
<td>White-creamy</td>
<td>Non-hemolytic</td>
</tr>
<tr>
<td>4</td>
<td>S. cohnii</td>
<td>-ve</td>
<td>Less pathogenic</td>
<td>White-creamy</td>
<td>Non-hemolytic</td>
</tr>
<tr>
<td>5</td>
<td>S. carnosus</td>
<td>-ve</td>
<td>Non pathogenic No toxin</td>
<td>Gray-white</td>
<td>Non-hemolytic</td>
</tr>
<tr>
<td>6</td>
<td>S. lentus</td>
<td>-ve</td>
<td>Non pathogenic No toxin</td>
<td>white o-creamy</td>
<td>α-hemolysis</td>
</tr>
<tr>
<td>7</td>
<td>S. lugdunensis</td>
<td>-ve</td>
<td>Less pathogen</td>
<td>white o-creamy</td>
<td>Non-hemolytic</td>
</tr>
<tr>
<td>8</td>
<td>S. haemolyticus</td>
<td>-ve</td>
<td>opportunistic pathogen</td>
<td>Gray-white</td>
<td>β-hemolysis</td>
</tr>
</tbody>
</table>

2.2.2 Establishment of Staphyloccocal species growth curve

A growth curve was established to define the different growth phases of Staphyloccocal species during bacterial replication. All species were inoculated in NB as detailed in the text above and incubated (37°C, 4-24h, depending on species). Then, each species was diluted 1:100 in 96 well plate in triplicate and incubated overnight. The optical density (OD) of each well was measured every hour at 600nm. Each well was analysed using Gen5 Software (Biotek, Bedfordshire, UK) and a Powerwave XS Microplate Spectrophotometer (Biotek). The main purpose of this step was to identify the time needed to reach the stationary phase, thought to be the time at which the bacteria release most of its protein virulence factors.
2.2.3 Quantification of bacterial culture by Miles Misra method

Miles and Misra was used to enumerate the cell titre (CFU) of each staphylococcal species. All species were cultured in 10ml NB overnight at 37°C, then diluted using 1 in 10 serial dilution (10^(-1) to 10^(-9)). 50μl of each dilution was inoculated in triplicate on NA, spreading gently with a sterile plastic spreader, and incubated at 37°C overnight. After incubation, the number of colonies was counted; fewer than 30 colonies and more than 180 colonies were ignored. The number of bacterial CFU was identified by the following formula as described by (Aneja et al, 2003).

\[
\text{Number of bacteria CFU} = \frac{\text{number of colonies}}{\text{inoculated volume}} \times \text{dilution factor}
\]

Different CFU of staphylococcal species were used for stimulation of HEKα and MEFs for various time points as described in the text.

2.2.4 Collection of filtered bacterial supernatant of S. aureus and S. epidermidis culture

*S. aureus* and *S. epidermidis* were grown in 15ml NB (37°C, overnight), then washed and harvested by centrifugation (1600g, 5 minutes, RT). 10ml of 10^8 CFU of each species were inoculated in 30ml of conditioned growth media (DMEM or 154 media), and incubated in a shaking incubator (37°C, 2-10 hours, 2 hours interval time). After each time point, the bacteria were centrifuged (1600g, 5 minutes, RT). 4ml of resulting supernatant was collected and filtered through a 0.22μm pore size filter from Millipore (Bedford, MA, USA). The filtered supernatant of *S. aureus* (FSA) was heated at 95°C, frozen and thawed for 5 cycles or treated with penicillin/streptomycin (P/S) then stored at -80°C until needed for stimulation of HEKα and MEFs.

2.2.5 Inactivation of LiSA

*S. aureus* was grown in NB overnight at 37°C then collected by centrifugation (1600g, 5 minutes, RT) and washed with PBS. The pellet was re-suspended in growth media (DMEM or 154 media) then bacterial cells were killed by either sonication with high ultrasound (5micron, 5 minutes), antibiotics (1% penicillin/streptomycin) or high temperature (95°C) for 5 minutes. The viability of *S. aureus* was examined by culturing on nutrient agar for 24 hours. Killed SA were used for stimulation of HEKα and MEFs.
2.2.6 Preparation of TLR2 antagonist, TLR2 ligands and *S. aureus* virulence factors

Commercial *S. aureus* peptidoglycan (PGN-SA, 1-20μg/ml), lipoteichoic acid (LTA-SA, 1-20μg/ml), heat-killed *Staphylococcus aureus* (HKSA, 10^8 CFU), monoclonal antibody for mouse TLR2 (mTLR2, T2.5 clone), monoclonal antibody for human TLR2 (anti-hTLR2, B4H2 clone, 1-10μg/ml) were obtained from InvivoGen, (San Diego, CA, USA) and α-Hemolysin (α-toxin, 1-20μg/ml), was provided by Sigma. Human and mouse isotype controls were included as recommended by the supplier. Lipopolysaccharide (LPS, 50ng/ml) (Sigma) was prepared as recommended by the manufacturer and stored in aliquots at -20 °C until needed.

2.2.7 Preparation of PAR1, PAR2 agonist, NDGA, KLK5, V8 serine protease of *S. aureus*, different proteases inhibitors, necrostatin-1 and general caspases inhibitor

All inhibitors and agonists were prepared as recommended by the manufacturer and used at concentrations detailed in the text. PAR1 agonist peptide, (TFLLR-NH2, 5mg/ml), PAR2 agonist peptide (SLIGRL-NH2, 1mg/ml) and nordihydroguaiaretic Acid (NDGA) were purchased from Abcam (Cambridge, UK) and stored in aliquots at -20 °C until needed. Additional reagents were purchased as follows: V8 serine protease of *S. aureus* (1mg/ml) (MP Biomedical, Santa Ana, USA); recombinant mouse kallikrein 5 (KLK5) (10μg/ml) (R&D System, Abingdon, UK); 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, serine protease inhibitor), E-64 (cysteine protease inhibitor) and Ilomastat (il-o), (R)-N4-Hydroxy-N1-[(S)-2-(1H-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide, metalloproteinases (MMP) inhibitor, (all Sigma); Necrostatin-1 (Nec-1) (Sigma); general caspases inhibitor (Z-VAD-FMK, 1mg/ml) (R & D System); recombinant ST2/IL1R4 (R & D).

2.3 Co-cultures of MEFs and HEKα

2.3.1 Stimulation of HEKα and MEFs with different staphylococcal species, *S. aureus* filtered supernatant, killed *S. aureus* and TLR2 ligands

HEKα and MEFs were grown until 80% confluent as described in Section 2.1.1 and 2.1.2. Both cell lines were seeded in 24-well tissue culture plate (Costar, Corp, Cambridge, MA) at a density of 10^5 cells/well (HEKα) or 50 x10^3 cells/well (MEFs) and incubated (37° C, 5%CO₂, 24h). HEKα and MEFs were stimulated with 10^7 CFU of Live *Staphylococcus aureus* (LiSA), staphylococcal species (Table 2.3), HKSA (10^8 CFU), LPS (50ng), killed *S. aureus* as described in section 2.2.5 or with different
concentrations of TLR2 ligands (PGN and LTA) and α-hemolysin for 6 hours at 37°C and 5% CO₂. To determine dose-response, HEKα and MEFs were stimulated with different CFU of LiSA (10⁶, 10⁷, 10⁸ CFU) for 2, 4 and 6 hours. To study the effect of TLR2 in induction of pro-inflammatory cytokines, both cell types were stimulated with 10⁷ CFU LiSA in the absence and presence of different concentrations of mouse or human anti TLR2. Separately, *S. aureus* was separated from the cell culture by transwell (Trans) system with pore size 0.4µm to prevent direct cell-cell contact with MEFs and HEKα.

After incubation of all conditions for 6 hours, the co-culture supernatant was removed, collected after centrifugation (10000g, 15 minutes, RT) and stored at -20°C for cytokine analysis. To investigate the cytotoxicity of all conditions, cells were washed, stained with AV and PI and analysed by flow cytometry as described in section 2.4.3.1.

### 2.3.2 Stimulation of mast cells by IL-33 of MEFs co-culture

Murine bone-marrow mast cells (BMMCs) were kindly gifted by Rajia Bahiri, Institute of Inflammation and Repair, University of Manchester. BMMCs were cultivated in RPMI complete media with 10% FCS (life Technologies Ltd), 10ng/ml IL-3 (rIL-3) and 50ng/ml stem cell factor (SCF) (Peprotec, London, UK). After 5 weeks of culture, BMMCs were cultured at a concentration of 1.5x10⁵ cells/well in 96 well round bottom plate. BMMCs were stimulated with supernatant of 3T3-MEF co-culture or recombinant murine IL-33 (rIL-33, positive control) (10ng/ml, Peprotec) in the absence and presence of monoclonal IL-33 antibody (Nessy-1, 100µg/ml) (Enzo Life Sciences, Farmingdale, USA). Isotypes or medium alone were used as a control. Each sample was carried out in duplicate or triplicate and incubated (37°C, 5% CO₂) for 24-48 hours. In a separate experiment, BMMCs were pre-treated overnight with 1µg/ml of IgE (SP7, Sigma) and then the IgE pre-sensitized BMMCs and cultured in the presence of antigen 200ng/ml dinitrophenyl (DNP) (Sigma) prior to incubation with the co-culture supernatant. Cells were washed, fixed and intracellular IL-6 measured by flow cytometry (as described in section 2.4.2.4). All isotypes controls were as recommended and the data analysed by flow Jo (Version 10).
2.3.3 T cell polarisation

48 well tissue culture plates were pre-coated with 5µg/ml of anti-CD3 antibody (clone: 17A) (eBioscience, Hatfield, UK) 24h before the experiment. Splenic T cells were collected from C57BL/6 mice and seeded at a density of 2×10^6 cells/well. Different dilutions of 3T3MEF co-culture supernatant (correlating with concentrations of IL-33 as determined by ELISA 12.5, 25 and 50pg/ml) were used to activate whole splenic cell populations in vitro. For Th0 conditions: 50µg/ml of anti IFN γ (clone:AN-18), 20µg/ml of anti-IL-4 (clone:11B11), 5µg/ml of anti CD28 (clone:37.51). For Th2 conditions: 50ng/ml of recombinant mouse IL-4 and 5µg/ml of anti CD28 (clone: 37.51) (all provided from eBioscience) were used in this experiment. 50pg/ml of monoclonal IL-33 antibody (Nessy-1, 100µg/ml) (Enzo Life Sciences) was used to block IL-33 activity in the supernatant. The plate was incubated (37°C, 2-3 days) after which the culture supernatants were collected and frozen (–20 °C). IFN-γ (Th1) and IL-13 (Th2) cytokines were quantified in supernatants from co-culture by enzyme-linked immunosorbent assay (ELISA) (R&D System).

2.4 Assays and Analysis

2.4.1 Quantification of pro-inflammatory cytokines (TNF- α, TSLP, IL-33 and ST2) by ELISA

TNF-α, TSLP, IL-33 and ST2 DuoSet® ELISA (R&D System) and mouse ST2 ELISA kit (eBioscience) were used to measure the production of these different cytokines by MEFs and HEKa stimulated with Staphylococcus aureus and other conditions. ELISA was performed according to the manufacturer’s instruction. The concentrations of capture, detection antibodies and standards were prepared as in Table 2.1 and stored at -80 °C. Briefly, 96-well flat bottom ELISA plates (Dynatech) were coated with anti-mouse/anti-human specific antibody for each cytokine (capture antibody) and left sealed, overnight at RT or 4°C as recommended. The plates were washed with washing buffer (0.05% Tween20 in PBS), (Sigma) using Skatron Scan Washer 400 (3 washes, no aspiration). After washing, the remaining liquid was removed from wells by inverting the plate. Then each well was blocked with 1% Bovine Serum Albumin (BSA) (PAA) (reagent diluent in 1x PBS, 1 hour, RT). After washing and aspiration, 50µl of undiluted samples and serial diluted standard were added to each well, and the plate incubated at RT for 2h. After incubation, the plates were washed as before, and 50µl of detection antibody was added to each well and incubated again for 2h at RT. After washing, the plate was incubated with 50µl of Streptavidin-horseradish peroxidase
(HRP) (R&D System) for 20 minutes at RT. The ELISA plate was washed and aspirated again, 50µl of substrate solution, Tetramethylbenzidine (TMB) (R&D System) was added to each well. After incubation for 20 minutes at RT in the dark, 25µl of stop solution (2N H₂SO₄) was added to each well to stop the reaction and the colour of the standards and positive samples change to yellow (Acidic). The optical density of each well was measured by a Dynex MRX11 microplate Reader (Dynex Technologies, Berlin, Germany) set to 450 with 570nm reference filter. The concentrations of the antibodies and the standard are illustrated in Table 2.4.

**Table 2.4 shows the concentrations of the antibodies and standard used for ELISA**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Antibody/Standard</th>
<th>Concentrations</th>
<th>Kit number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSLP</td>
<td>Capture antibody</td>
<td>1.0µg/ml</td>
<td>DY555</td>
</tr>
<tr>
<td></td>
<td>Detection antibody</td>
<td>200ng/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>1000pg/ml</td>
<td></td>
</tr>
<tr>
<td>IL-33</td>
<td>Capture antibody</td>
<td>800ng/ml</td>
<td>DY3620</td>
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<tr>
<td></td>
<td>Detection antibody</td>
<td>75ng/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>1000pg/ml</td>
<td></td>
</tr>
<tr>
<td>TNF- α</td>
<td>Capture antibody</td>
<td>800ng/ml</td>
<td>DY410</td>
</tr>
<tr>
<td></td>
<td>Detection antibody</td>
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</tr>
<tr>
<td></td>
<td>Standard</td>
<td>2000pg/ml</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>Capture antibody</td>
<td>4.0 µg/ml</td>
<td>DY413</td>
</tr>
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<td></td>
<td>Detection antibody</td>
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<td>Standard</td>
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</tr>
<tr>
<td>IFNγ</td>
<td>Capture antibody</td>
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<td>Standard</td>
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<tr>
<td><strong>Human</strong></td>
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<td>Standard</td>
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<td></td>
<td>Standard</td>
<td>1500pg/ml</td>
<td></td>
</tr>
<tr>
<td>TNF- α</td>
<td>Capture antibody</td>
<td>4.0 µg/ml</td>
<td>DY210</td>
</tr>
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<td></td>
<td>Detection antibody</td>
<td>350ng/ml</td>
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<td></td>
<td>Standard</td>
<td>1000pg/ml</td>
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<tr>
<td><strong>eBioscience ELISA kit</strong></td>
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<tr>
<td><strong>Mouse</strong></td>
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<tr>
<td>sST2</td>
<td>Capture antibody</td>
<td>Diluted as stated</td>
<td>88-9334-22</td>
</tr>
<tr>
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<td>Diluted as stated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>10000pg/ml</td>
<td></td>
</tr>
</tbody>
</table>
2.4.2 Fluorescence activated cell sorting (FACS) assays

2.4.2.1 Summary of FACS protocol

After stimulation of MEFs or HEKa with different conditions, cells were washed and transferred to a 96 well round bottomed plate, washed with PBS and centrifuged (400g, 5 minutes, 4°C). Cells were resuspended in FACS buffer (PBS, 2% FCS, 0.2 mM EDTA). For toxicity assays, cells were stained with Annexin-V (AV) and propidium iodide (PI) as described in section 2.4.3.1. For surface staining cells were washed, centrifuged and stained either with anti-human or anti-mouse PAR-2 antibody as detailed in section 2.4.2.3.

Separately, unstimulated and stimulated MEFs were stained with anti-mouse ST2L as described in section 2.4.2.3. For intracellular staining of IL-6, BMMCs were washed with FACS buffer then the FC receptor blocked with anti-FcεRI (eBioscience). Then the surface marker of BMMCs and intracellular IL-6 were stained and determined as detailed in section 2.4.2.4.

The isotypes were included for each stain as recommended by the manufacturer. After staining cells were resuspended in 200µl FACS buffer and carried out on LSR-II, using BD FACS Diva software and analysed by flowJo.

2.4.2.2 Surface expression of ST2 and PAR2 expression

To determine the expression of membrane bound ST2L and PAR2 in HEKa and MEFs, confluent cells were stimulated with LiSA for 6 hours (37°C with 5% CO₂); cells were collected, washed, centrifuged (400rcf/ 5 minutes/4°C) and resuspended in 200µl cold PBS. Then blocked with Rat IgG diluted in FACS buffer (10µg/ml, Sigma) and incubated on ice in the dark for 20 minutes. Unstimulated and stimulated MEFs were centrifuged again and washed, then single stained with either monoclonal anti mouse ST2 antibody conjugated with PerCP-eFluor 710 (RMST2-2, eBioscience) diluted in FACS buffer (10µg/ml), the recommended isotype control (Rat IgG2a K isotype control PerCP-eFluor 710 (eBioscience), anti-human PAR-2 antibody (344222, R and D system) or anti-mouse PAR-2 antibody (SAM11, Santa Cruz Biotechnology, Texas U.S.A).

Samples were incubated on ice in the dark for 20 minutes again, then washed and centrifuged as described above. The isotypes were included for each stain as recommended. Cells were resuspended in 200µl FACS buffer, viewed on LSR-II using BD FACS Diva software and analysed by flowJo.
2.4.2.3 Detection of intracellular IL-6

After 24h stimulation of mast cells, cells were washed with FACS buffer (PBS1X + 2% FCS + 0.2 Mm EDTA) and centrifuged (300rcf, 5 minutes, 4 °C). Cells were re-suspended in 50µL of FACS buffer containing FC receptor block (anti-CD16/CD32 eBioscience). Mast cell surface markers anti-FcεRI-FITC (93, 1:200, dilution) and anti-CD117-Percp-Cy5 (2B8, 1:200, dilution) and IL-33 receptor anti-ST2 - APC (RMST2-2, 1:200, dilution) were all provided by eBioscience. After incubation at 4 °C for 20 minutes, cells were washed with 200μL PBS and 50μl of live/dead blue solution (1:1000, dilution) were added and incubated in dark (room temperature, 10 minutes). The cells were washed with PBS and fixed with intracellular fixation buffer (eBioscience) for 20 minutes at 4 °C. After incubation, cells were washed with permeabilization buffer and stained with FC block and anti-IL6 (1:100) (eBioscience) or isotype control, diluted in permeabilization buffer and incubated at room temperature for 1 hour. Cells were then washed with 200μl of permeabilization buffer and re-suspended in FACS buffer. Protein expression was measured by flow cytometry (LSRII). The data were analysed with flowJo software.

2.4.2.4 FACS analysis

For cell death assays carried out by FACS, positive and negative controls were included. The analysis was carried out on gated live cell population which had the same size and granularity as the unstimulated control then applied to the stimulated cells. The percentage of positive single staining of all tested samples was determined by comparison with the unstimulated control. The results were represented as a dot plot with four quadrants (as stated in Chapter 4) or as a histogram. For protein expression, isotype controls were included as a control. The percentage of positive single staining was determined by overlaying the histogram of isotype controls to the tested samples.

2.4.3 Cell death assays

2.4.3.1 Detection of cell death by AV and PI

Assessment of cell death in MEFs and HEKα was determined by staining with Annexin-V APC (AV-APC) and PI cell death detection kit (eBioscience) and analysed by FACS. Stimulated cells were washed twice with cold PBS and detached by trypsin/EDTA as described above (Section 2.1.1 and 2.1.2). Cells were transferred to 96 well round bottom plates, washed with FACS buffer and centrifuged (400rcf, 5 minutes, 4 °C). Cells
were resuspended with 200µl of diluted AV-APC (1:100) in AV binding buffer (eBioscience) and incubated on ice for 20 minutes. After centrifugation as above, 200µl of diluted PI (1:100) was added in AV binding buffer. Then immediately, all samples were analysed by FACS (LSR-II). Live cells do not stain for either of these markers; cells undergoing cell death where the membrane remains intact will stain for AV but not PI; if there is cell membrane disruption cells stain with PI (Figure 2.1).

![Figure 2.1](image)

**Figure 2.1** Cells were analysed by flow cytometry, after being stained with AV and PI. The percentage of cells positive for PI and/or AV is reported inside the quadrants.

### 2.4.3.2 Detection of cell death by trypan blue

After stimulation of HEKa or MEFs in 24 well plates, cells were washed twice with PBS and detached and collected as described. Cell aliquots were mixed briefly with 0.4% Trypan blue solution (Invitrogen). 20µl of the suspension was counted using a haemocytometer. The viable cells did not take up the stain whilst the dead cells stained blue. The percentage of viable MEFs or HEKa were identified using the following calculation:

\[
\text{Percentage of viability} = \frac{\text{Viable count}}{\text{Total count}} \times 100
\]

### 2.4.4 Immunohistochemistry of IL-33

HEKa, 3T3-MEFs and human fibroblast were grown as described until 80% confluent (T-75 flask). Sterile coverslips (13mm diameter) were placed in each well of 24 well tissue culture plates, and 1x10^5 HEKa, 5x10^4 of 3T3-MEFs or human fibroblasts were seeded in each well. Cells were incubated at 37°C in humid conditions with 5% CO₂.
until confluent. All cells were then co-cultured with $1 \times 10^7$ CFU of LiSA for 1 hour and incubated. After incubation, cells were washed twice (PBS) to remove the bacteria, and the supernatant replaced with complete media containing 1% of penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin per ml) (Sigma) to kill the extracellular bacteria. After washing, cells were fixed with 4% paraformaldehyde (PFA, 500µl) and incubated (20 minutes, RT). Cells were washed again twice with PBS and permeabilized with 1% Triton (500µl, 15 minutes, RT). After washing, cells were incubated with 100-300µL of trinitrobenzene (TNB, NaCl 150 mmol/L, Tris 100mmol/) Blocking Buffer in a humidified condition for 30 minutes at RT. Then cells were incubated with anti-IL33 monoclonal antibody (Enzo Life Sciences) diluted 1:200 in TNB and incubated overnight at 4°C. Cover slips were then washed twice with PBS for 15 minutes and incubated (30 minutes, RT) with biotinylated anti-mouse antibody (500µl, diluted 1:200, Vectastatin ABC kit (Vector Laboratories, USA), followed by washing. Streptavidin Cy3 (500µl, diluted 1:100) (Sigma) was added and incubated (30 minutes, RT). Cells were washed as above and incubated (4°C, overnight) with anti-keratin-14 polyclonal antibody (500ul, diluted 1:200, Convance, USA). After washing again, cells were incubated (30 minutes RT) with goat anti-rabbit IgG conjugated with Alexa Fluor® 488 (Invitrogen). The coverslips were removed from the well and placed on a drop of mounting media on clean slide and left to dry at 4°C overnight. Images were acquired using a Fluorescence Microscope (Olympus, BX51, and 20X) with Cy3 and FITC filter in the Bio-Imaging Facility Unit, Faculty of Life Sciences, University of Manchester.

2.4.5 Western blotting

The expression of IL-33 and soluble ST2 was determined by western blotting. After co-culture or stimulation, supernatants were collected in 1.5ml Eppendorf tubes then centrifuged (10000rcf, 15 minutes, RT) and stored in 200µl aliquots at -20°C until needed. 6µl of each sample was mixed with 6µl of Laemmli sample buffer and 2µl betamercaptoethanol (Bio Rad, Hercules, USA). All mixtures were incubated at 95°C for 5 minutes (denaturation step). Then 20µl of each sample or 6µl of the leader, (Precision polyprotein standard, Bio Rad) were loaded into the wells of the Nu PAGE 4-12% Bis-Tris gel (life Technologies Ltd) and run for 1-2 hours at 100V in running buffer (MES SDS Running Buffer, life Technologies Ltd). The gel was activated with 3ml of methanol for 1 minute then transferred to polyvinylidene difluoride (PVDF) membrane in transferring buffer (700ml dH₂O + 200ml methanol + 100ml of 10X TGS) at 70V for 1-2hours. The membrane was incubated with blocking buffer (50ml of Tris-Buffered
Saline and Tween 20 (TBST) 1X +2.5g dry milk) to prevent non-specific binding (1 hour, RT), followed by incubation with anti-IL-33 polyclonal antibody (2μg/ml, Thermo Fisher Scientific, Rockford, USA) or anti-ST2 antibody (polyclonal, 1 - 2 μg/ml, Abcam or R and D systems, diluted 1:1000 in blocking buffer) and incubated overnight at 4°C. Incubation membrane was then washed 4 times with washing buffer (PBS + 1% tween 20, 30 minutes, RT), followed by incubation with secondary antibody, horseradish-peroxidase (HRP)-conjugated with goat anti-rabbit (1:5,000 in TBS Tween, Cell signalling) for 1 hour at RT. Finally, the membrane was washed again with TBS Tween 1% 4 times (30 minutes, RT), then the membrane was covered and scanned by Odyssey Imagers. The reagents and solutions used in this assay are listed in Table 2.5.

### Table 2.5 List of reagents used in western blotting

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Catalogue number</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Laemmli sample buffer</td>
<td>161-0737</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>beta-mercaptoethanol</td>
<td>161-0710</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Precision polyprotein standard</td>
<td>161-0373</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>Running buffer (MES SDS Running Buffer)</td>
<td>NP0002</td>
<td>Life Technologies Ltd</td>
</tr>
<tr>
<td>Transferring buffer (700ml dH₂O + 200ML Methanol + 100ml of 10X TGS)</td>
<td>161-0732</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>polyvinylidene difluoride (PVDF)</td>
<td>162-0177</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>Blocking buffer (50ml of TBST 1X +2.5g dry milk)</td>
<td>Prepared in the lab.</td>
<td>Prepared in the lab.</td>
</tr>
<tr>
<td>Primary antibody Anti-ST2</td>
<td>ab25877</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mouse ST2/IL-1 R4 Biotinylated Antibody</td>
<td>BAF1004</td>
<td>R and D system</td>
</tr>
<tr>
<td>Primary anti IL-33 antibody</td>
<td>PA5-20398</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Nu PAGE 4-12% Bis-Tris gel</td>
<td>NP0321</td>
<td>Life Technologies Ltd</td>
</tr>
</tbody>
</table>

### 2.4.6 Detection of protease genes in *S. aureus* by PCR

DNA was extracted from *S. aureus* strain by puregene yeast/bact kit B (Qiagen, City, UK) according to manufacturer’s instructions. *S. aureus* was grown overnight in NB for 18-24 hours. 500μl of the bacterial culture was collected in a 1.5 ml micro-centrifuge tube then centrifuged (13000-16000rcf) to pellet the bacteria. The supernatant was discarded, and the bacteria were resuspended in 300μl cell suspension solution, and 1.5μl of lytic enzyme was added, mixed well and incubated at 37°C for 30 minutes. Samples were centrifuged again (13000-16000rcf), the supernatant discarded and the bacteria were resuspended in 300μl lysis solution and 1.5μl Rnase A solution. The
samples were mixed by inverting and incubated at 37°C for 15-60 minutes. Then the samples were centrifuged again, and 300μl of isopropanol was added to the supernatant, mixed gently and centrifuged again to end with a visible small white pellet (DNA). The upper layer was discarded and the DNA washed with 300μl of 70% ethanol by inverting several times. After centrifugation, the pellet was left to dry for 5 minutes at room temperature, then 100μl of DNA hydration solution was added and incubated at 65°C for 1 hour to dissolve the DNA. DNA was be stored at 15-25°C overnight with gentile shaking then stored at 4°C until the PCR. The PCR steps are illustrated in Table 2.7.

Protease genes of *S. aureus* were determined by PCR using primers listed in Table 2.6 (Karlsson *et al.* 2002). PCR fragments were purified with QiaQuick PCR purification kit (Qiagen) and analysed on 1.5 % agar gel (Sigma) together with a 1-kb plus size marker (life technologies Ltd). All primers were diluted 1:10 in nuclease free water as recommended by the supplier in a final concentration of 10µmol (Eurofins Genomic Services Ltd, Wolverhampton, UK) and stored at -20°C. Each sample contained 3µl of the extracted DNA, 1µl of each of the primers, 12.5µl of My Taq red mix (Bioline Reagents Ltd, London, UK) and 7.5µl of DNA free water. The PCR steps were carried out as illustrated in Table 2.7.

**Table 2.6 Primers used for detection of *S. aureus* protease genes by PCR (Karlsson *et al.* 2002)**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sspA V8</em> forward</td>
<td>5'-GCC ACA CTT GTG AGT TCT CCA GC-3</td>
</tr>
<tr>
<td><em>sspA V8</em> reverse</td>
<td>5'-GTT TTA AGA AGT TGC GTA CAT TTT C-3</td>
</tr>
<tr>
<td><em>sspA</em> forward</td>
<td>5'-GAC AAC AGC GAC ACT TGT GA-3</td>
</tr>
<tr>
<td><em>sspA</em> reverse</td>
<td>5'-AGT ATC TTT ACC TAC AAC TAC A-3</td>
</tr>
<tr>
<td><em>sspB</em> forward</td>
<td>5'-TGA AGA AGA TGGCAA AGT TAG-3</td>
</tr>
<tr>
<td><em>sspB</em> reverse</td>
<td>5'-TTG AGA TAC ACT TTG TGC AAG-3</td>
</tr>
<tr>
<td><em>aur</em> forward</td>
<td>5'-TAG TAG CAC ACAG AAT TAA CAC AC-3</td>
</tr>
<tr>
<td><em>aur</em> reverse</td>
<td>5'-TTC CCT ATT GCT TGA ATC ACG-3</td>
</tr>
</tbody>
</table>
Table 2.7 PCR steps for protease genes of *S. aureus*

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
<th>Temperature</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3 minute</td>
<td>95°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 minute</td>
<td>95°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 minute</td>
<td>50°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>1 minute</td>
<td>72°C</td>
<td>30 cycles</td>
</tr>
</tbody>
</table>

2.4.7 Protease activity assays

The activity of proteases produced by *S. aureus* was investigated using different methods as below.

2.4.7.1 Skimmed milk agar

The activity of proteases produced by *S. aureus* was investigated by digestion of skimmed milk agar (SMA). Skim milk agar medium was prepared by adding of 20g of skim milk powder to 1000ml distilled water and left to stand at RT for 20 minutes, then dissolved by heating in a water bath. The mixture was autoclaved at 121°C for 15 minutes, left to cool to 50°C then poured into sterile Petri dishes. The plates were kept at RT to solidify then stored at 4°C until required. *S. aureus* was inoculated on NA for 18-24 hours, then on SMA and incubated at 37°C for 24-48 hours. The presence of a clear zone of hydrolysis reflected the functional activity of proteases.

2.4.7.2 Colorimetric Protease Assay

The Protease activity of *S. aureus* was determined by colorimetric protease assay (Pierce protease assay kit, Thermo Scientific). In this assay, succinylated casein was used as a substrate which is capable of cleavage by all proteases. Cleavage of this substrate by proteases in specific peptide bond resulted in the exposure of new amino acids which react with trinitrobenzene sulfonic acid (TNBSA) and produce an orange-yellow product. In this experiment, all reagents and materials were prepared as recommended by the manufacturer. The protease activity assay was carried out in 96 microtiter plate. In each well 100µl of succinylated casein, 100 µl of the assay buffer and 50µl of the *S. aureus* supernatant or the standard were added and incubated at RT for 20 minutes. Addition of substrate, assay buffer and clean growth media served as a blank for the assay. The standard used in this assay was trypsin which was diluted serially (1:2) and treated the same as the samples. After incubation, 50µl of TNBSA was added to all wells and incubated again for 20 minutes at RT. The absorbance of all
wells was determined in a plate reader set at 450nm. The standard curve of the standard was blotted and used to assess the activity of *S. aureus* proteases.

### 2.4.8 Mass spectrometry (MS) for filtered supernatant of *S. aureus*

Mass spectrometry (Thermo Velos) was used to identify the extracellular proteins produced by *S. aureus*. The analysis of bacterial supernatant was performed in the Faculty of Life Science, Biomolecular Analysis Unit, University of Manchester by Dr David Knight using an inhouse protocol. *S. aureus* was inoculated on NB for 18-24h. Then 10ml of LiSA $10^8$ CFU was diluted with 30ml of growth media (DMEM) and incubated for 6 hours in a shaking incubator at 37°C. The bacteria were pelleted by centrifugation at 1600rcf for 5 minutes, and the supernatant was collected and filtered at 0.22µm to remove the remaining bacteria. Before MS analysis, 20µl of the bacterial supernatant proteins were separated on a gel electrophoresis for 5 minutes at 180V and stained with instant blue to visualise the protein bands. Proteins were identified based on MASCOT database search engine (http://www.matrixscience.com/). The analysis of the data and the list of proteins in the bacterial supernatant were provided by Dr David Knight. This molecular technique was able to identify proteins which had a molecular size of between 5 and 500 kDa.

### 2.4.9 Statistical analysis

All experiments were carried out at least two times with triplicate samples (as detailed in results chapters). The ELISA results were analysed by Prism 6 using one or two-way ANOVA between the treated samples and the controls. Data was presented as standard error of the mean (SEM). The P value was calculated and considered statistically significant if $p<0.05$. The FACS data were analysed by FlowJo and represented as mean +/-SEM, a histogram or a representative dot plot.
Chapter 3: *S. aureus*-induced release of the Th2-promoting cytokines IL-33 and TSLP by mouse embryonic fibroblasts and human keratinocytes
3.1 Foreword

Compared with healthy individuals, the skin of patients with AD is heavily colonised with *S. aureus* (Huang et al. 2009). Furthermore, the severity of AD correlates with the degree of *S. aureus* colonisation, and antimicrobials that reduce colonisation also reduce eczema severity (Breuer et al. 2002; Gong et al. 2006; Huang et al. 2009), suggesting a key role of these bacteria in driving clinical disease. The precise mechanisms by which *S. aureus* drives inflammation is however still unclear.

Both acute and chronic AD are associated with increased activity of Th2 cytokines such as IL-13 and IL-4 (Hamid et al. 1996). TSLP (Sano et al. 2013) and IL-33 (Savinko et al. 2012; Tamagawa-Mineoka et al. 2014) produced by keratinocytes and fibroblasts are known to induce and promote a Th2 cytokine response. It has previously been shown that *Staphylococcus* enterotoxin B (SEB), house dust mite (HDM) and diacylated lipopeptide of *S. aureus* can induce expression of IL-33 and its receptor (ST2) in skin of AD patients (Savinko et al. 2012), as well as induce TSLP from keratinocytes via TLR2 and TLR6 pathways (Vu et al. 2010). However, another study suggested that the superantigen SEB produced by *S. aureus* cannot increase the inflammatory responses in the skin of AD patients (Kozman et al. 2010). Therefore it is not clear whether *S. aureus* or it’s secreted products can induce the release of Th2-promoting cytokines from keratinocytes.

This chapter sets out to investigate the ability of *S. aureus* to induce Th2-promoting cytokines (IL-33 and TSLP) by human keratinocytes and mouse embryonic fibroblasts (MEFs) *in vitro*.

3.2 Growth curves of *S. aureus* and other staphylococcal species in culture medium

The growth rate of *S. aureus* was measured in order to determine optimal timing for harvesting bacteria for subsequent *in vitro* experiments. Figure 3.1 shows the growth of *S. aureus* in nutrient broth over a 24 hour period. After an initial lag phase of 2 hours (A), *S. aureus* entered an exponential growth phase (B) and finally a stationary phase at 18 hours (C). Based on these data, *S. aureus* was harvested at 6 hours in subsequent experiments.
The growth rate of *S. aureus* was compared with that of other staphylococcal species. *S. aureus* and *S. lugdunensis* showed higher growth rates than other staphylococcal species tested (Figure 3.2).

[Figure 3.1] Growth curve of *S. aureus* grown at 37°C in nutrient broth. (A) Lag phase; (B) log phase; (C) stationary phase.

[Figure 3.2] Growth curves of staphylococci species in nutrient broth over 3 hours. Optical density was measured at 600nm.
3.3 *S. aureus* but not other *Staphylococcus* species induced IL-33 and TSLP release by MEFs and human keratinocytes

The pro-inflammatory effects of different staphylococcal species were studied in both MEFs and human keratinocytes. Live *S. aureus* (LiSA), but none of the other seven staphylococcal species tested, induced release of IL-33 and TSLP from 3T3-MEFs and the human keratinocyte cell line (HEKa) (Figure 3.3). More TSLP was released by HEKa cells than by MEFs, but both released similar amounts of IL-33.

![Figure 3.3](image)

**Figure 3.3 S. aureus but none of the other seven staphylococcal species induced IL-33 and TSLP release by 3T3-MEFs and HEKa.** (A) IL-33 and TSLP release from 3T3-MEFs, (B) IL-33 and TSLP release from HEKa. MEFs and HEKa were stimulated with $10^7$ CFU/ml of LiSA or other *Staphylococcus* species for 6 hours. C = negative control supernatant of untreated cells. Data are representative of duplicate experiments. Green bars = 3T3-MEFs, Blue bars = HEKa. Analysis by one-way ANOVA, ****p < 0.0001.

3.4 Live *S. aureus* (LiSA) but not heat-killed *S. aureus* (HKSA) or lipopolysaccharide (LPS) induced release of IL-33 and TSLP by MEFs and HEKa

The ability of LiSA, commercially available HKSA and LPS to induce TSLP, IL-33 and TNF-α release were studied in MEFs from dermatitis-prone (NC/Nga) and control (3T3 & MSM) mice. LiSA, but not HKSA or LPS, induced significant release of IL-33 from all three MEF cell lines to a similar level (Figure 3.4). There was also a small but statistically significant increase in TSLP release by all three lines. None of the MEFs
produced TNF-α (Figure 3.4). The results indicated that live but not killed *S. aureus* were able to induce the Th2-promoting cytokines, IL-33 and TSLP, by MEFs.

Figure 3.4 Cytokine release induced by HKSA, LPS and LiSA from MEFs. (A) IL-33, (B) TSLP and (C) TNF-α release from MEFs 6 hours after stimulation with 108 CFU/ml HKSA, 50ng/ml LPS or 10⁷ CFU/ml LiSA. Data are from five separate experiments. Green = 3T3, Blue = MSM, Red = NC/Nga. One-way ANOVA was used to assess differences between groups, ***p < 0.001.
The ability of LiSA, HKSA and LPS to induce TSLP, IL-33 and TNF-α release was studied in human primary keratinocytes derived from non-atopic healthy children and those with AD. Keratinocytes from both non-atopic children and those with severe AD released significant levels of TSLP, IL-33 and TNF-α after stimulation with LiSA but not HKSA or LPS. There were no significant differences in the amount of these cytokines produced by cells from healthy and AD children (Figure 3.5). Human fibroblasts from non-atopic and AD children were also stimulated, and no IL-33 or TSLP cytokines was released into the supernatant, (data not shown).

Figure 3.5 Cytokine release by human primary keratinocytes derived from non-atopic and AD children stimulated with HKSA, LPS and LiSA for 6 hours. (A) IL-33, (B) TSLP and (C) TNF-α after stimulation with $10^8$ CFU/ml HKSA, 50ng/ml of LPS and $10^7$ CFU/ml LiSA. Data are representative of three experiments. Blue: non-atopic, Red: AD. Data were analysis by one-way ANOVA, *p<0.05, ***p < 0.001
In conclusion, LiSA but not HKSA or LPS induced Th2-promoting cytokines by both MEFs and human keratinocytes. No significant differences were found in the quantity of cytokines released from control (MSM and 3T3) and the dermatitis model (NC) MEFs, or between human non-atopic and AD keratinocytes.

3.5 Killing of *S. aureus* with heat, sonication and antibiotics abrogates their inflammatory effects on MEFs and HEKα

To further study the effects of different methods of bacterial killing on the pro-inflammatory activity of *S. aureus* bacteria were subjected to degrees of heat-inactivation as well as sonication and addition of antibiotics. Results showed that heating of bacteria to over 60°C completely inhibited bacterial growth. After exposure to temperatures between 50 and 65°C, the bacteria remained viable and able to grow, albeit more slowly (Figure 3.6A). Sonication and treatment with antibiotics also completely abrogated bacterial growth (Figure 3.6B).

![Graph showing viability of *S. aureus*](image)

**Figure 3.6 Viability of *S. aureus* on nutrient agar after exposure to temperatures ranging from 37 to 70°C, sonication and treatment with antibiotics.** (A) No growth was seen in bacteria grown on nutrient agar after exposure to temperatures above 60°C; (B) sonication or treatment with antibiotics (P/S). Data are representative of two separate experiments. The analysis was by one-way ANOVA, **p < 0.01.
3T3 MEFs were co-cultured with LiSA and heat-inactivated to various degrees of *S. aureus* to examine whether these heat-inactivated *S. aureus* were still able to induce production of IL-33 and TSLP (Figure 3.7).

![Figure 3.7 Cytokines released by 3T3 MEFs after stimulation with LiSA exposed to different temperatures](image)

**(A)** IL-33 and **(B)** TSLP released from 3T3 MEFs after stimulation with 10⁷ CFU/ml *S. aureus* exposed to temperatures ranging from 37°C to 70°C for 5 min. Untreated cells were used as negative control. Data are representative of two separate experiments. Analysis was by one-way ANOVA, **p < 0.01, ***p < 0.001.

*S. aureus* grown at temperatures of up to 60°C induced release of IL-33 and TSLP. However when the bacteria were exposed to higher temperatures as previously described in section 3.4, they did not induce cytokine production (Figure 3.7). These results indicated that live but not dead bacteria could induce Th2-promoting cytokines by 3T3-fibroblasts.

3T3-MEFs and HEKa were then stimulated with LiSA, sonicated LiSA, HKSA and *S. aureus* treated with penicillin/streptomycin for 6 hours (Figure 3.8). There was no release of IL-33 or TSLP after 3T3-MEFs were exposed to *S. aureus* killed by sonication, antibiotics or high temperature. Furthermore, neither cytokine was detected when HEKa were stimulated with *S. aureus* treated with antibiotics, sonication, or high temperature. In conclusion, live but not dead *S. aureus* induced TSLP and IL-33 release by 3T3-MEFs and HEKa.
Figure 3.8 IL-33 and TSLP release by 3T3-MEFs and HEKas after stimulation with LiSA, or LiSA treated with antibiotics, sonication or heat. (A) IL-33 and TSLP released from 3T3-MEFs, or (B) IL-33 and TSLP released from HEKas after stimulation for 6 hours with $10^7$ CFU/ml LiSA. P/S: LiSA treated with penicillin and streptomycin for 30 min, sonic: sonication or exposure of the bacteria to $95\degree$C for 5 minutes. Data are representative of three separate experiments. Green bars = 3T3, Blue bars = HEKas. Analysis was by one-way ANOVA, *$p < 0.05$, **$p < 0.001$.

3.6 Time course of IL-33 and TSLP release by MEFs and HEKas

To examine the kinetics of IL-33 and TSLP release by MEFs and HEKas after stimulation with LiSA, they were co-cultured with $10^7$ CFU/ml LiSA for 1 to 8 hours (Figure 3.9). IL-33 release by MEFs was detectable from 2 hours and peaked at 5 hours in all mouse fibroblasts. There were no significant differences between the release of IL-33 by 3T3, MSM and NC/Nga MEFs. There was little or no TSLP release by MEFs. HEKas released both IL-33 and TSLP and levels were higher than in MEFs, still increasing after 8 hours of co-culture with LiSA. The number of MEFs and HEKas used per cell was identical.
Figure 3.9 Time-course of IL-33 and TSLP released by MEFs and HEKa after stimulation with $10^7$ CFU/ml LiSA. (A) IL-33 and TSLP release by MEFs. (B) IL-33 and TSLP release by HEKa. LiSA were added to HEKa and MEFs and incubated for 1 – 8 hours. Results are the mean of triplicates experiments. Left hand panels A: Green = 3T3, Blue = MSM, Red = NC. Right-hand panels = HEKa.

3.7 Dose-dependent release of IL-33 and TSLP by MEFs and HEKa after stimulation with LiSA

The effect of different quantities of LiSA on cytokine release by MEFs and HEKa were investigated. Cells were co-cultured with $10^5$, $10^6$ & $10^7$ CFU/ml LiSA for 6 hours (Figure 3.10).

Cytokine release (IL-33 in MEFs; IL-33 and TSLP in HEKa) was highest when both types of cells were stimulated with $10^7$ CFU/ml of LiSA. Low concentrations of cytokines were observed when MEFs and HEKa were co-cultured with $10^6$ CFU/ml of LiSA and little or no cytokine production was observed with $10^5$ CFU/ml of LiSA. Higher concentrations of IL-33 and TSLP were released from HEKa than MEFs. TSLP released from MEFs was only just above baseline, even with $10^7$ CFU/ml of LiSA. In conclusion, induction of IL-33 and TSLP is dose and time dependent in both MEFs and HEKa.
3.8 Effect of bacteria cell wall components on the release of IL-33 and TSLP by MEFs and HEKa

TLR2 is thought to be a key cell surface receptor through which bacteria stimulate inflammatory responses. Bacterial ligands for this receptor include lipoteichoic acid (LTA) and peptidoglycan (PGN). Experiments were undertaken to study the importance of this pathway to S. aureus-induced cytokine production in MEFs and HEKa. Stimulation of both mouse and human cell lines with different concentrations of staphylococcal LTA and PGN did not induce IL-33 or TSLP release (Figure 3.11).
Figure 3.11 Cytokine released by LiSA and *S. aureus* components by MEFs. (A) IL-33, (B) TSLP. IL-33 and TSLP released by MEFs after 6 hours of stimulation with $10^7$ CFU/ml LiSA, and *S. aureus* cell wall components (peptidoglycan (PGN) and lipoteichoic acid (LTA)). Data are representative of two separate experiments. Green bars = 3T3, Blue bars = MSM, Red bars = NC. Analysis was by one-way ANOVA, ****$p < 0.0001$.

Cytokine released by HEKα after stimulation with *S. aureus* cell wall components/TLR2 ligands were also tested. No IL-33 or TSLP were released by HEKα by either LTA or PGN (Figure 3.12).

In conclusion, LTA and PGN did not induce either IL-33 or TSLP release from either MEFs or HEKα. Thus, induction of these cytokines seems to require live *S. aureus*. Even LiSA was not able to induce the release of IL-33 or TSLP by human skin fibroblasts derived from either non-atopic or AD children suggesting that the effects of live *S. aureus* are specific for keratinocytes and not fibroblasts in humans in contrast to mice.
Figure 3.12 IL-33 and TSLP released by HEKa after stimulation with LiSA and *S. aureus* cell wall components. (A) IL-33 and (B) TSLP. IL-33 and TSLP release by HEKa after 6 hours of stimulation with 10^7 CFU/ml LiSA, 1, 10 and 20µg/ml of *S. aureus* peptidoglycan (PGN) and lipoteichoic acid (LTA). Data are representative of two separate experiments. Analysis was by one-way ANOVA, ****p < 0.0001.

3.9 Effect of neutralising anti-TLR2 antibodies on the release of IL-33 and TSLP from MEFs and HEKa

Experiments were conducted to look more directly as to whether TLR2 is involved in driving cytokine release by HEKas and MEFs *in vitro*. Cells were pre-incubated with anti-TLR2 antibodies for one hour before being exposed to 10^7 CFU/ml LiSA for 6 hours. Untreated cells and cells incubated with isotypes antibodies were used as negative controls (Figure 3.13). There was no suppression of IL-33 or TSLP released from HEKas or 3T3-MEFs by anti-TLR2 antibodies.
In conclusion, the induction of IL-33 and TSLP by 3T3-MEFs and HEKα does not appear to be mediated through the TLR2 pathway.

3.10 LiSA is a more potent inducer of IL-33 in MEFs and HEKα in vitro than mechanical injury

IL-33 is an alarmin which promotes an inflammatory response through binding to its membrane receptor ST2L after release by cell damage or necrosis. The release of IL-33 induced by LiSA on 3T3-MEFs and HEKα was compared with that triggered by mechanical damage (scraping by cell scrapers or 5 cycles of freeze-thawing). IL-33 release after LiSA co-culture with 3T3-MEFs and HEKα was significantly higher when compared to cells damaged by scraping or freeze-thawing (Figure 3.14).
Figure 3.14 IL-33 released by 3T3-MEFs and HEKa after exposure to LiSA or mechanical injury. (A) 3T3-MEFs, (B) HEKa. Both cell types were exposed to $10^7$ CFU/ml for 6 hours, scraping (Scrape) or 5 cycles of freeze-thawing (F/T). Experiments were performed in duplicate. Statistical analysis was by one-way ANOVA, ****$p < 0.0001$.

3.11 Trans-well and filtered supernatant of *S. aureus* (FSA) induced release of cytokines by MEFs and HEKa

To study whether direct contact of *S. aureus* and mammalian cells is essential to induce the release of pro-inflammatory cytokines by 3T3-MEFs, a Trans-well system was used to separate the bacteria and cells. LiSA were co-cultured with MEFs for 6 hours then the released cytokines were measured by ELISA.

A soluble product from the bacteria side of the Trans-well was able to induce IL-33 release by 3T3-MEFs although not as well as LiSA. There was no significant release of TSLP (Figure 3.15). To rule out the possibility that live bacteria were getting across the trans-well system, the supernatant was taken from the non-bacterial side and cultured on mannitol salt agar (MSA). No growth was found on the MEFs side, but a heavy growth was observed from the bacterial side of the trans-well system (Figure 3.16), indicating that live *S. aureus* did not pass through the pores (0.22µm) of the Trans-well. In conclusion, these experiments suggest that release of cytokines does not require direct contact with live *S. aureus* and MEFs, but that a soluble factor or factors secreted by the live bacteria were sufficient to induce the cytokine production.
Figure 3.15 IL-33 and TSLP release by 3T3-MEFs after stimulation for 6 hours with $10^7$ CFU/ml LiSA directly or via a Trans-well system (Trans). (A) IL-33, (B) TSLP. Untreated cells were used as a negative control (C). Data shows the mean (SEM) of two separate experiments. Trans: transwell; LiSA: live S. aureus. Statistical analysis was by one-way ANOVA, **$p < 0.01$ and ****$p < 0.0001$.

Figure 3.16 Bacterial growth after trans-well culture (A) heavy growth of S. aureus from the bacterial side of trans-well system (B) no growth seen from the cell side of the Trans-well.

S. aureus was then grown in different growth media (nutrient broth, conditioned media (DMEM: MEF growth media) and (154: HEKa growth media). The supernatant from S. aureus grown in these media for 6 hours was collected, filtered and added to MEFs and human cells. As shown in Figure 3.17, IL-33 was released from both 3T3-MEFs and HEKa stimulated with supernatant from S. aureus ($10^7$ CFU/ml) grown in growth media (GM) but not nutrient broth (NB).
Figure 3.17 IL-33 released from 3T3-MEFs and HEKa after stimulation for 6 hours with filtered supernatants (FSA) derived from *S. aureus*. (A) 3T3-MEFs, (B) HEKa. Live unfiltered 10⁷ CFU/ml LiSA was used as a positive control. Data are representative of three separate experiments (n=3). Analysis was by one-way ANOVA, **p < 0.01, ****p < 0.0001.

Further experiments were performed in which 3T3-MEFs and HEKa were exposed to *S. aureus* filtrate extracted and filtered at different time points. *S. aureus* used in these experiments were grown in 154 keratinocytes media for HEKa and DMEM growth media for 3T3-MEFs for 2 to 10 hours, then the supernatant was filtered and sub-cultured on NA to confirm the absence of bacterial colonies before adding to mammalian cells (Figure 3.18).

Figure 3.18 IL-33 and TSLP released by 3T3-MEFs and HEKa after stimulation for 6 hours with filtered supernatant of *S. aureus* (FSA). FSA was extracted from *S. aureus* after different lengths of time ranging from 2 to 10 hours. Data are representative of two separate experiments (n=3). Green bars = 3T3, Blue bars = HEKa. Analysis was by one-way ANOVA, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The sterile filtrates obtained from *S. aureus* induced the release of IL-33 but not TSLP from 3T3-MEFs, while both IL-33 and TSLP were detected from HEKa. The pro-inflammatory effect could be titrated depending on the time before collection of the supernatant.

In conclusion, both LiSA and *S. aureus* supernatants are able to induce cytokines from both mouse and human cell lines, although higher levels were produced from HEKα than 3T3-MEFs. The production of these cytokines by *S. aureus* supernatants was time and dose dependent.
3.12 Expression of IL-33 by HEKa, human fibroblasts and MEFs

Comparative studies were conducted to determine where IL-33 was localised in MEFs and human skin cells, as the short time course of IL-33 release suggested that this cytokine is pre-formed rather than dependent on *de novo* synthesis. IL-33 is known to be stored in the nucleus and released into the extracellular space from damaged or necrotic cells i.e. as an alarmin or damage associated molecular pattern (DAMP) (Haraldsen *et al.* 2009). HEKα were stimulated with LiSA for 1 or 6 hours. Stimulated and unstimulated cells (controls) were then stained with an anti-IL-33 monoclonal antibody (clone Nessy-1) and keratin 14 (cytoskeleton stain of epithelial cells). Immunohistochemistry showed clear expression of IL-33 in the nuclei of unstimulated HEKα (Figure 3.19A), but reduced staining after stimulation for one hour (Figure 3.19B), and loss of nuclear staining and translocation of the cytokine to a cytoplasmic or extracellular space after 6 hours (Figure 3.19C).

![Localization of IL-33 in unstimulated HEKα and after stimulation with LiSA for 1 and 6 hours (20X magnification)](image)

**Figure 3.19** Localization of IL-33 in unstimulated HEKα and after stimulation with LiSA for 1 and 6 hours (20X magnification). (A) Unstimulated, (B) stimulated for 1 hour, (C) stimulated for 6 hours. HEKα were grown until confluent on a cover slip and immunostained for IL-33 (red) and keratin 14 (green).
Figure 3.20 Translocation of intracellular IL-33 between HEKa cells through microtubule (arrow) (100X magnification). HEKa cells were grown on cover slip in 24 well plates until confluent, then stimulated with $10^7$ CFU/ml LiSA for 1 hour. Immunostaining of HEKa with IL-33 (red) and keratin 14 (green).

Figure 3.20 shows a higher power image, clearly demonstrating the nuclear localization of IL-33 in HEKa cells. Early phase of translocation of intracellular IL-33 was observed after stimulation with LiSA for 1 hour. It seems that a microtubule network between HEKa cells acts to translocate IL-33 and may have a role in transferring the alarmin to neighbouring cells.

There was no detectable nuclear IL-33 immunostaining in human fibroblasts (Figure 3.21). These data suggest that human fibroblasts do not inherently store nuclear IL-33, in keeping with the lack of IL-33 detected by ELISA after stimulation with LiSA. The red spots on images of cultures after 6 hours for both HEKa and fibroblasts suggested that the fluorescent marker may also be non-specifically binding to *S. aureus*. 
Figure 3.21 Intracellular IL-33 in unstimulated human skin fibroblasts, and after stimulation with LiSA for 1 and 6 hours (20X magnification). (A) Unstimulated (B) stimulated for 1 hour and (C) stimulated for 6 hours. Human fibroblasts were grown on cover slip until confluent and then stained with IL-33 (red) and keratin 14 (green).

Intracellular IL-33 immunostaining in 3T3-MEFs at baseline was minimal (Figure 3.22A). After stimulation with LiSA for 6 hours, red spots were observed (Figure 3.22B). These may relate to the fluorescent marker non-specifically binding to aggregated bacteria.
In conclusion, these data suggest that IL-33 is expressed in the nuclei of unstimulated HEKα, less in 3T3-MEFs and none in human fibroblasts. Live *S. aureus* is a strong inducer of IL-33 release and results in translocation of nuclear IL-33 to the cytoplasmic and extracellular space of infected keratinocytes.

### 3.13 T cell polarisation by IL-33 in the supernatant of 3T3-MEFs after stimulation with LiSA

Western blotting was used to confirm the size of the IL-33 protein released into the supernatant from MEFs and HEKα after stimulation with LiSA (Figures 3.23 & 3.24). Anti-IL-33 polyclonal antibody that cross-reacts with human and mouse was used to show the expression of IL-33. The size of the protein was just below 50 kDa as expected from that quoted by the supplier.

Figure 3.23 Expression of IL-33 cytokine produced from stimulated and unstimulated HEKα and 3T3-MEFs. IL-33 protein was detected in the native and diluted (1:2) supernatant of HEKα and 3T3-MEFs stimulated *in vitro* with FSA, 10^7 CFU/ml LiSA and unstimulated cells (negative control).
Figure 3.24 Expression of IL-33 cytokine released into the supernatant of stimulated and unstimulated MEFs. IL-33 protein expression was detected by Western blotting in the supernatant of different MEFs cell lines. MEFs were stimulated in vitro with $10^7$ CFU/ml LiSA (SA) and unstimulated cells used as a negative control (C).

To determine if the IL-33 was bioactive, *S. aureus* supernatants were added to T-cells obtained from spleens of C57BL/6 mice and also to bone marrow-derived mast cells. Th0 and Th2 murine T-cells were cultured with supernatant, with or within neutralising anti-CD3 antibody for 3 days. Th2 T-cells produced significantly higher concentrations of IL-13 when stimulated with a *S. aureus* supernatant compared with cells cultured without the supernatant. The IL-13-inducing effect of the supernatant on IL-33 was inhibited by anti-IL-33 antibody (Nessy-1) (Figure 3.25).

Figure 3.25 MEF supernatant-derived IL-33 induces IL-13 by Th2-cells of C57BL/6 mice. (A) Naïve T cells (Th0) and (B) Th2 cells with SA-MEF supernatant (sup) in the presence and absence of anti-IL-33 for 3 days. Data are representative of two separate experiments (n=3). Analysis was by one-way ANOVA, **$p < 0.01$, ***$p < 0.001$. 

IL-33 is also known to stimulate the production of IL-6 from mast cells (Tung et al., 2014) and so the ability of SA-MEF supernatant to induce intracellular expression of IL-6 by murine bone-marrow derived mast cells was studied. Mast cells were stimulated with MEF supernatant for 24h in the presence and absence of anti-IL-33 specific antibody.

Recombinant IL-33 (rIL-33) was used as a positive control to determine how much IL-33 was required to induce IL-6 expression. After incubation, intra-cellular expression of IL-6 was measured in mast cells by flow cytometry. High concentrations of rIL-33 (10ng/ml) were required to induce expression of intracellular IL-6 in mast cells, and anti-IL33 antibody (Nessy-1 antibody) blocked this activity. This result suggested that 100-fold higher concentrations of IL-33 are required to induce IL-6 intracellular expression from mast cells than present in the supernatant from SA-MEFs (100pg/ml).

Activation of mast cells induces the release of mediators such as cytokines and chemokines. The strongest activating stimulus is sensitization with IgE antibody (Hsu et al., 2010; Gilfillan et al., 2006). Mast cells were therefore sensitised with IgE antibody 24 hours and dinitrophenyl (DNP) prior to incubation with SA-MEF- supernatant and IL-6 expression were assessed by flow cytometry. SA-MEF supernatant was able to induce detectable expression of intracellular IL-6 from these IgE-sensitized mast cells (Figure 3.27 A and 3.27B).

![Figure 3.26](image_url)

**Figure 3.26 rIL-33 induced production of intracellular IL-6 by mast cells.** Mast cells were stimulated with rIL-33 (1 and 10ng/ml) for 24h. Cells were then stained with anti-IL-6 antibody and examined by flow cytometry. Data represent mean (SEM) of triplicate samples. Analysis was by one-way ANOVA, ****p < 0.0001.
Figure 3.27 Supernatant of SA-MEF induces expression of intracellular IL-6 in IgE sensitised mast cells. Sensitised and unsensitized mast cells were stimulated with *S. aureus* supernatant for 24 hours. (A) Histogram and (B) bar chart shows the percentage of intracellular IL-6 expressed. The analysis was by one-way ANOVA, *p* < 0.05.

### 3.14 Summary

*Staphylococcus aureus* but not other staphylococcal species induce the release of Th2-promoting cytokines (TSLP and IL-33) by cell lines. Killing *S. aureus* by heating above 60°C, sonication or with antibiotics completely abrogated the pro-inflammatory activity of *S. aureus* in both mouse and human cell lines. There was no significant difference in the amount of either IL-33 or TSLP released by the different MEF strains, or from primary skin keratinocytes derived from non-atopic and AD children. Cell wall components, LTA or PGN, had no bioactivity, and the pro-inflammatory effect of LiSA was not blocked by neutralising anti-TLR2 antibodies, suggesting that the TLR2 pathway is not involved.

By separating *S. aureus* from cells using a Trans-well system, it could be demonstrated that direct contact between the bacteria and the mammalian cells was not essential for cytokine release, but that bioactive soluble factor(s) were present in the filtered supernatant of *S. aureus*. IL-33 but not TSLP was released from MEFs treated with filtered supernatant, while both cytokines were released by human keratinocytes. After stimulation with LiSA, IL-33 was released from nuclear stores of keratinocytes rather than requiring *de novo* synthesis. The IL-33 in co-culture supernatant was bioactive and able to induce IL-13 from mouse T-cells and intracellular IL-6 from sensitised mast cells (Figure 3.28).
Figure 3.28 Th2-promoting effects of *S. aureus* on human keratinocytes, fibroblasts and MEFs.
4 Chapter 4: Cytotoxic effects of *S. aureus* on mouse embryonic fibroblasts and human keratinocytes
4.1 Foreword

Bacteria induce cell death through a number of pathways which either (i) leave the cell membrane intact e.g. apoptosis, or (ii) disrupt the cell membrane e.g. necrosis/necroptosis. Unlike cell death pathways that leave the cell membrane intact, pathways that disrupt the cell membrane induce inflammatory response through release of endogenous danger signals and pro-inflammatory cytokines.

*S. aureus* is known to secret a variety of lytic virulence factors including α-toxin. This toxin also known as α-hemolysin is the most studied toxic agent of *S. aureus* (Wardenburg *et al.* 2007). α-toxin is known to cause cell death by forming pores on the target cells by binding to the phosphocholine head of sphingomyelin (Schwiering *et al.* 2013; Wichmann *et al.* 2009). Cytotoxic effects of α-toxin are increased in filagrin-deficient keratinocytes and in the presence Th2 cytokines such as IL-13 and IL-4, known to reduce expression of filaggrin (Brauweiler *et al.* 2013; Brauweiler *et al.* 2014). Thus these cytokines have been shown to be less marked on healthy keratinocytes than those from AD patients (Brauweiler *et al.* 2013).

The previous chapter showed that live *S. aureus* is able to induce the release of not only TSLP but also IL-33 by keratinocytes. As IL-33 release is thought to be dependent on cell membrane disruption secondary to cell death (Cayrol *et al.* 2009), the cytotoxic effects of LiSA and its sterile filtrate on MEFs and human epidermal keratinocytes (HEKa) were studied in detail in this chapter. Cell death was measured using Annexin-V (AV) (assessing apoptosis) and propidium iodide (PI) (assessing necrosis) by flow cytometry.

4.2 *S. aureus* has a greater cytotoxic effect on 3T3-MEFs than other staphylococcal species

The cytotoxic effects of eight staphylococcal species on 3T3-MEFs were assessed *in vitro* by light microscopy and flow cytometry.

Dot plots in Figure 4.1 show that after incubation with *S. aureus*, 18% of 3T3 cells stained with PI (cytotoxicity with cell membrane disruption) and 8% stained with AV (cytotoxicity without cell membrane disruption), compared with a mean of 2% in controls. Other staphylococcal species induced cytotoxicity of a similar magnitude to unstimulated controls. Results are summarised in Figure 4.2.
Figure 4.1 *S. aureus* had a greater cytotoxic effect than other staphylococcal species. 3T3-MEFs were incubated for 6 hours with $10^7$ CFU/ml *S. aureus* or other staphylococcal species. AV and PI staining was used to assess the degree of cytotoxicity. The numbers inside the quadrants represent the percentage of cells stained with AV, PI, both or none. Data are representative of two separate experiments.

Figure 4.2 *S. aureus* induced greater 3T3 cytotoxicity associated with cell membrane disruption than other staphylococcal species. (A) PI staining and (B) AV staining. Data are representative of two separate experiments.
4.3 Heat-killed *S. aureus* and LPS do not induce cytotoxicity in 3T3-MEFs or HEKa

Results shown from Chapter 3 demonstrated that LiSA but not commercially available HKSA or LPS induced release of IL-33 in MEFs and HEKa. The cytotoxic effects of live *S. aureus* (LiSA), HKSA and LPS were now studied in 3T3-MEFs and HEKa. In all experiments, LiSA or bacterial components were co-cultured with cells for 6 hours. Cytotoxicity was assessed by staining with AV and PI. 43% of 3T3-MEFs and 29% of HEKa stained with PI after incubation with LiSA (Figure 4.3 & 4.4). In contrast, little or no cytotoxicity was observed when 3T3-MEFs and HEKa were exposed to Gram positive (HKSA) and Gram negative (LPS) bacterial cell wall components.

![Figure 4.3 HKSA and LPS are not cytotoxic to 3T3-MEF or HEKa.](image)

(A) 3T3-MEFs, (B) HEKa. 3T3-MEFs and HEKa were exposed to $10^7$ CFU/ml LiSA, $10^8$ CFU/ml HKSA or 50ng/ml LPS for 6 hours and then stained with AV and PI. The numbers inside quadrants represent the percentage of cells stained with AV, PI, both or none. Data are representative of two separate experiments.
Figure 4.4 Summary of cytotoxic effects of live LiSA and bacterial cell wall components on 3T3-MEFs and HEKa. Cell death was assessed by AV and PI staining. PI positive cells and AV positive of (A) 3T3-MEFs, (B) HEKa. Data are representative of two separate experiments.

The cytotoxic effects of live and killed *S. aureus* on 3T3-MEFs and HEKa were further compared. 3T3-MEFs and HEKa were grown until 80% confluent before subculturing in 24 well plates for 24 hours. *S. aureus* was killed by sonication or heating to 95°C for 10 minutes. The killed *S. aureus* was co-cultured with 3T3-MEFs and HEKa for 6 hours and cytotoxicity assessed by staining with AV and PI analysed using flow cytometry (Figure 4.5).

Figure 4.5 Killing of LiSA abrogates its cytotoxic effect on 3T3-MEFs and HEKa. 3T3-MEFs and HEKa were exposed to 10⁷ CFU/ml of LiSA, *S. aureus* killed with sonication (Sonic) and heating to 95°C (5 min). Unstimulated cells were used as a negative control (C). Data are representative of two separate experiments.
Killing of *S. aureus* by sonication or heating destroyed the bacteria’s cytotoxic activity (Figure 4.5). Figure 4.6 summarises the percentage of AV and PI-positive staining.

![Figure 4.6](image)

**Figure 4.6** LiSA, but not sonicated or heat-killed *S. aureus* induced cell death of 3T3-MEFs and HEKa. PI positive and AV positive staining of (A) 3T3-MEFs, (B) HEKa. Data are representative of two separate experiments.

### 4.4 Filtered supernatant from *S. aureus* (FSA) induced cytotoxicity with cell membrane disruption in 3T3-MEFs and HEKa

Experiments were performed to study whether soluble factor(s) produced by live *S. aureus* not only have pro-inflammatory but also the cytotoxic effect on MEFs and HEKa. 3T3-MEFs and HEKa were grown until confluent, then sub-cultured in 24 well plates for 24h before stimulation. *S. aureus* was cultured in growth media (DMEM) or HEKa growth media (medium 154) for 2 to 8 hours, then centrifuged. The supernatant was filtered through a 0.22 μm pore size filter to remove any remaining *S. aureus*. 3T3-MEFs and HEKa were then co-cultured with FSA extracted at different time points. After 6 hours of incubation, cells were stained with PI and AV to examine the cytotoxic effects of the FSA on MEFs and HEKa. Cells co-cultured with LiSA were used as a positive control and unstimulated cells used as a negative control. FSA extracted at 4 and 6 hours induced significant PI but not AV staining of 3T3-MEFs, peaking at 4 - 6 hours after co-culture (Figures 4.7 and 4.8).
Figure 4.7 Filtered supernatant of *S. aureus* (FSA) induced disruption of 3T3-MEFs cell membrane (PI positive). Dot plots are shown of untreated cells (C) (negative control), cells exposed to live *S. aureus* (SA, positive control) and cells incubated with FSA extracted at different time points. After culture for 6 hours, cells were stained with PI and AV to assess the cytotoxicity of FSA on 3T3-MEFs.

![Dot plots of untreated cells (C) and cells exposed to live S. aureus (SA).](image)

**Figure 4.8** Filtered supernatant of *S. aureus* (FSA) induced disruption of 3T3-MEFs cell membrane (PI positive) over 6 hours. (A) Percentage of PI positive and (B) AV positive. FSA extracted at different time points (2, 4, 6 and 8 hours), then incubated with 3T3-MEFs for 6 hours. After incubation, the cells were stained with PI and AV to assess cytotoxicity.

Co-incubation of HEKa with FSA also induced cytotoxicity, which became apparent only at 8 hours (Figure 4.9 & 4.10). No apoptosis was detected in HEKa incubated with FSA.
Figure 4.9 Filtered supernatant of *S. aureus* (FSA) induced disruption of HEKa cell membrane (PI positive) after 6 hours. Dot plots of untreated cells (C) (negative control) and cells incubated with FSA extracted at different time points (2, 4, 6 and 8 hours). After co-culture for 6 hours, cells were stained with PI and AV to assess the cytotoxicity of FSA on HEKas.

Figure 4.10 Filtered supernatant of *S. aureus* (FSA) induced disruption of HEKa cell membrane (PI positive) over 6 hours. (A) PI positive, (B) AV positive. FSA was collected at different time points (2-8 hours) before adding to HEKas. The cytotoxic effects were assessed by staining with AV and PI and analysed by flow cytometry.

4.5 Cytotoxic effect of live *S. aureus* on 3T3-MEFs and HEKas is dose and time dependent

The cytotoxic effects of LiSA on 3T3-MEFs and HEKas were investigated in more detail. Cells were grown until confluent, then exposed to 10^6, 10^7 or 10^8 CFU/ml of LiSA for 2, 4 and 6 hours.
3T3-MEFs Cytotoxicity was observed from 4 - 6 hours of co-culture with $10^6$ or $10^7$ CFU/ml *S. aureus*, although some cytotoxicity was observed at 2 hours when 3T3 were co-cultured with $10^8$ CFU/ml, (Figure 4.11 & 4.12). The cytotoxic effects were time and dose dependent. $10^8$ CFU/ml of LiSA also led to more noticeable AV staining compared with lower CFU/ml (16-31% for $10^8$ CFU/ml compared with 6-9% for $10^6$-$10^7$ CFU/ml).

Figure 4.11 LiSA-induced cytotoxic effects on 3T3-MEFs were dose and time-dependent. 3T3-MEFs were stimulated with $10^6$, $10^7$ and $10^8$ CFU/ml of LiSA CFU/ml for 2, 4 and 6 hours. After stimulation, cells were stained with AV and PI and examined by flow cytometry. Each quadrant shows different staining as described in previous figures. Data represent three separate experiments.
Figure 4.12 LiSA-induced cytotoxic effects of 3T3-MEFs was dose- and time-
dependent. (A) PI staining, (B) AV staining. 3T3-MEFs were exposed to $10^6$, $10^7$ and $10^8$ CFU/ml of LiSA for 2, 4 and 6 hours. Cytotoxic effects were assessed by PI and AV analysed by flow cytometry. Data represent three independent experiments.

**HEKa** LiSA had less of a cytotoxic effect on HEKa than on MEFs. $10^7$ CFU/ml *S. aureus* induced PI staining of HEKa after 4 hours (10%) of stimulation with and peaked after 6 hours (38%) (Figures 4.13 & 4.14). Lower concentrations of LiSA were not cytotoxic in this time period.

Figure 4.13 Cytotoxicity of HEKa induced by LiSA is dose and time-dependent. HEKa were stimulated with $10^6$ and $10^7$ CFU/ml of LiSA for 2, 4 and 6 hours. Cytotoxicity was examined by staining with AV and PI and analysed by flow cytometry. Data are representative of two separate experiments (n=2).
LiSA-induced cell membrane disruption (PI positive) of HEKα is dose and time dependent. (A) PI positive, (B) AV positive. HEKα were exposed to $10^6$ and $10^7$ CFU/ml LiSA for 2, 4 and 6 hours. Cytotoxic effect was assessed by AV and PI analysed by flow cytometry. Data represent two independent experiments.

4.6 Cytotoxic effects of LiSA on 3T3-MEFs and HEKα assessed by trypan blue staining

Further experiments were carried out to show the cytotoxic effect of LiSA on 3T3-MEFs and HEKα using trypan blue staining. Both cells were stimulated with $10^7$ LiSA for 6 hours and the cytotoxic effects examined by trypan blue staining and morphological changes observed by light microscopy. 25% of 3T3-MEFs and approximately 20% of HEKα were dead after stimulation with LiSA for 6 hours. *S. aureus* clumped around MEFs and HEKα cells as observed by microscopy (Figure 4.15)
Figure 4.15  Effect of LiSA on viability of 3T3-MEFs and HEKa. (A) 3T3-MEFs, (B) HEKa (10X magnification, light microscopy) and (C) percentage of viable cells of both 3T3-MEFs (green) and HEKa (blue) stained with trypan blue. Data represent triplicate from one experiment. Each group are displayed as the mean (SEM). **p< 0.01.

4.7 The effect of RIPK1-inhibitor (necrostatin-1) and caspase inhibitor on the cytotoxic effects of LiSA on 3T3-MEFs

Necrostatins are a family of compounds that block necroptotic cell death. Necrostatin-1 (Nec-1) is an allosteric inhibitor of RIPK1 kinase. The effect of Nec-1 on cell death induced by LiSA in 3T3-MEFs was examined. MEFs were stimulated with $10^7$ CFU/ml of LiSA in the presence or absence of different concentrations of Nec-1. The cytotoxic effects were assessed by staining with AV and PI analysed by flow cytometry (Figure 4.16).
S. aureus induced necrosis (26%, PI positive) of 3T3-MEFs after 6 hours of incubation, which was not inhibited by Nec-1. Thus RIPK1 does not appear to mediate the cytotoxic effect of LiSA on 3T3-MEFs (Figure 4.17).

**Figure 4.16** Nec-1 does not inhibit cytotoxicity of 3T3-MEFs induced by live S. aureus. 3T3-MEFs were stimulated with LiSA with and without Nec-1 (20, 40, 60µM). After 6 hours incubation, the cytotoxic effects were assessed by AV and PI analysed by flow cytometry. Data represent two separate experiments of duplicate wells.

**Figure 4.17** Nec-1 did not inhibit the cytotoxic effect of 3T3-MEFs induced by LiSA. Percentage of cell stained (A) PI positive and (B) AV positive. Data represent two separate experiments of duplicate wells.
To investigate the possible role of caspases in cell death induced by *S. aureus* on 3T3-MEFs, a general-caspase inhibitor (Z-VAD-FMK) which predominantly inhibits caspase-1 and 3 was used. 3T3-MEFs were co-cultured with LiSA for 6 hours in presence and absence of Z-VAD-FMK. Cell death was assessed by AV and PI analysed by flow cytometry. Stimulation of 3T3-MEFs with LiSA in the presence of different concentrations of Z-VAD-FMK did not prevent toxicity of SA. This result suggests that caspases are not involved in cell death pathway induced by LiSA (data not shown).

4.8 TLR2 ligands and α- hemolysin are not involved in the induction of 3T3-MEF cell death *in vitro*

TLRs and α- hemolysin are thought to be important in innate immune responses to microbes and have a role in the induction of cell death. To study whether cell wall components and their TLR2 ligands or α- hemolysin were able to induce cell death, peptidoglycan (PGN), lipoteichoic acid (LTA) as well as α-hemolysin (α-hemo) were added to 3T3-MEFs for 6 hours. LiSA was used as a positive control and untreated cells as a negative control. After incubation, all cells were washed and stained with AV and PI to assess cytotoxicity.

LiSA induced necrotic cell death of 3T3-MEFs (PI staining 43%) at 6 hours, but little apoptosis (Figure 4.18 & 4.19). PGN, LTA and α-hemolysin did not induce any cell death in 3T3-MEFs after co-culture for 6 hours.
Figure 4.18 *S. aureus* but not PGN or LTA induced disruption of 3T3-MEFs cell membranes. LiSA (10^7 CFU/ml), 20, 30, 40µg/ml of PGN, 5, 15, 20µg/ml of LTA and 5, 10, 20 µg/ml of α-hemolysin were added to 3T3-MEFs for 6 hours. AV and PI were used to assessed cell death and the stained cells processed by flow cytometry. Data is representative of two separate experiments.
Figure 4.19 Percentage of PI and AV positive cells after co-incubation with LiSA or different concentrations of PGN and LTA. (A) PI positive, (B) AV positive. LiSA (10^7 CFU/ml), different concentrations of PGN (20, 30, 40µg/ml), LTA (5, 15, 20µg/ml) and α-hemolysin (5, 10, 20 µg/ml) were used to stimulate 3T3-MEFs for 6 hours. Data is representative of two separate experiments.

4.9 NC/Nga -MEFs are more susceptible to the cytotoxic effects of S. aureus than 3T3 and MSM-MEFs

Experiments were designed to study the possible differences in LiSA cytotoxicity on 3T3 (Swiss mouse line), MSM (wild-type Japanese mouse) and NC-MEFs (an inbred strain of Japanese mouse which spontaneously develops dermatitis when housed in conventional environments). All MEFs were grown until confluent in 75cm² flask then sub-cultured in 24 well plates for 24h. Cell lines were then co-cultured with 10^7 CFU/ml of LiSA for 2, 4 and 6 hours. After each time point, cells were collected and stained with AV and PI and examined by flow cytometry to assess toxicity.
After exposure to LiSA for 2h, a significantly higher percentage of NC-MEFs were PI positive (14%), compared with 3T3-MEFs (3%) and MSM-MEFs (6%). These differences were more marked at 4h (NC-MEFs 41%, 3T3-MEFs 8%, MSM-MEFs 14%) and 6 h (NC-MEFs 50%, 3T3-MEFs 16% and MSM-MEFs 17%) (Figure 4.20). In contrast, there was little AV positive staining after co-culture of MEFs with LiSA and no difference between the three MEF strains. In conclusion, NC-MEFs are more sensitive than 3T3 and MSM to the cytotoxic effects of LiSA (Figure 4.21).

Figure 4.20 Cytotoxic effects of LiSA on NC, 3T3 and MSM-MEFs. MEFs were stimulated with $10^7$ CFU/ml of LiSA for 2, 4 and 6 hours, before staining with AV and PI. Untreated cells were used as a control (C). Each quadrant means different result as described in previous figures. Data are representative of three separate experiments.
Figure 4.21 Cytotoxic effects of LiSA on 3T3, MSM and NC-MEFs. (A) PI positive and (B) AV positive. MEFs were exposed to $10^7$ CFU/ml of LiSA for 2, 4 and 6 hours, then stained with AV and PI to assess cytotoxicity. Data representative of three separate experiments. Green bars: 3T3-MEFs; Blue bars: MSM-MEFs; Red bars: NC-MEFs. **p<0.01 and ****p<0.0001; two-way ANOVA.

4.10 Expression of membrane-bound IL-33 receptor ST2 (ST2L) and soluble ST2 decoy

The biological effects of IL-33 are mediated after binding to the membrane ST2 receptor expressed on the surface of most innate immune cells. Two isoforms of ST2 exist, a transmembrane receptor (ST2L) and a soluble decoy receptor (sST2) that has a regulatory function. Binding of IL-33 to ST2L leads to the formation of ST2/IL-1RAcP and caspase-3 and caspase-7 leading to apoptosis. To investigate possible reason for the higher cytotoxicity induced by LiSA on NC compared with 3T3 and MSM-MEFs, experiments were carried out to examine the expression of ST2L and sST2 in these MEFs lines. ST2L were assessed by flow cytometry and sST2 by ELISA in the supernatant of co-culture.

In unstimulated MEFs, 3T3 and MSM, ST2L was expressed at high levels (72%), but after stimulation with LiSA for 6 hours, the level decreased to baseline. This may have been due to internalisation of ST2L after conjugation with IL-33 produced by MEFs, which acts as paracrine signal for neighbouring cells, or otherwise release of bound ST2L from the cell surface after stimulation. Interesting, the expression of ST2L on NC-MEFs was significantly lower than MSM and 3T3-MEFs (Figure 4.22A, 22B & 4.23).
Figure 4.22 Expression of ST2L on 3T3, MSM and NC-MEFs. (A) Unstimulated MEFs and (B) MEFs stimulated with LiSA. MEFs were grown in 24 well plate and stimulated with LiSA (10^7 CFU/ml), stained with anti-mouse ST2 antibody and assessed by flow cytometry. Data is representative of two separate experiments (n=3). An isotype control antibody was used as a negative control (grey shaded area). Green: 3T3-MEFs; Blue: MSM-MEFs; Red: NC-MEFs.

Figure 4.23 Membrane-bound ST2L expression in unstimulated and stimulated MEFs. MEFs were grown in 24 well plates. Unstimulated cells and cells stimulated with LiSA (10^7 CFU/ml) were stained with anti-mouse ST2 antibody and assessed by flow cytometry. Data is representative of two separate experiments (n=3). 3T3 = green, MSM = blue and NC = red. **p < 0.01; one-way ANOVA and ****p < 0.0001; two-way ANOVA.
The expression of sST2 was assayed in the supernatant of unstimulated and LiSA-stimulated MEFs. Although 3T3 and MSM MEFs expressed high levels of sST2, MEFs from dermatitis prone NC mice produced no sST2, at least when assessed by ELISA (Figure 4.24). In contrast, sST2 protein was seen in all the MEFs by Western blotting, including NC-MEFs after stimulation with LiSA but not in unstimulated cells (Figure 4.25). The size of the protein was 40 kDa as expected from the size quoted by the supplier.

**Figure 4.24** Soluble ST2 (sST2) expression on 3T3, MSM and NC MEFs. MEFs were grown in 24 well plates then stimulated with different CFU/ml (10⁶, 10⁷ and 10⁸) of LiSA for 6 hours. The level of sST2 was assayed in co-culture supernatant by ELISA. Untreated cells were used as a control for each cell line. Data representative of three separate experiments. 3T3 = green bars, MSM = blue bars and NC = red bars. ****p<0.0001; two-way ANOVA.

**Figure 4.25** sST2 protein detection by Western blotting in unstimulated and stimulated MEFs. All MEFs were stimulated with LiSA (10⁷ CFU/ml) for 6 hours. sST2 was detected in the supernatant of co-cultured MEFs. Anti–ST2 polyclonal antibody was used as a primary antibody.

As levels of sST2 detected by ELISA in NC-MEFs were low and NC-MEFs were more susceptible to LiSA than other control MEFs, experiments were carried out to determine if the addition of exogenous recombinant sST2 (rST2) reduced the cytotoxic effects of LiSA on NC-MEFs. NC-MEFs were grown in growth media for 24 h, then co-cultured with LiSA for 6 h in the presence and absence of different concentrations of rST2. Cells
were stained with AV and PI and cytotoxic effects assessed by flow cytometry. The percentage of cells stained positive with PI and AV are shown in Figures 4.26 & 4.27.

The addition of 10µg/ml of rST2 reduced PI staining of NC-MEFs after stimulation with LiSA from 37% to 28% (Figure 4.26 & 4.27). These data suggested that rST2 reduced the cytotoxic effects of LiSA on NC-MEFs.

**Figure 4.26** Effect of recombinant sST2 on PI staining of NC-MEFs after coculture with SA. NC-MEFs were stimulated with LiSA for 6 hours in the absence and presence of different concentrations of rST2 (1, 3 and 10µg/ml). Cells were then stained with PI and AV to examine the cytotoxic effect as analysed by flow cytometry. Data is representative of two separate experiments.

**Figure 4.27** Effect of recombinant sST2 on PI staining of NC-MEFs stimulated by LiSA. (A) PI positive, (B) AV positive. NC-MEFs were stimulated with LiSA in the presence and absence of different concentrations of rST2 (1, 3 and 10µg/ml). PI and AV staining was used to assess the cytotoxic effect and analysed by flow cytometry. Data is representative of two separate experiments (n=3). *p< 0.05 and **p<0.01; two-way ANOVA.
In conclusion, NC-MEFs were more sensitive than 3T3 or MSM-MEFs to the cytotoxic effects of LiSA. sST2 was detected by western blotting but not by ELISA in NC-MEFs compared to 3T3 and MSM-MEFs. The addition of rST2 to NC-MEFs reduced the cytotoxic effects induced by LiSA.

4.11 Summary

The results in this chapter demonstrate that *S. aureus* but not other staphylococcal species, or cell wall components (LTA, PGN) or α-hemolysin, induce cytotoxicity associated with disruption of the cell membrane (necrosis rather than apoptosis) in both MEFs and HEKα. MEFs are more susceptible to the cytotoxic effects of LiSA than HEKα. Of the MEFs tested, NC-MEFs are significantly more susceptible to the cytotoxic effects of *S. aureus* than 3T3-MEFs, with MSM-MEFs falling somewhere in between. Expression of the transmembrane receptor (ST2L) of IL-33 is slightly lower on the surface of NC-MEFs compared to 3T3 and MSM-NEFs, but most interestingly the soluble decoy receptor (sST2) is not detected by ELISA from NC-MEFs, but can be detected by Western blot. Adding of recombinant sST2 reduces the cytotoxic effect of LiSA on NC-MEFs.

Soluble factor(s) released by *S. aureus* into the filtrate (FSA) are sufficient to induce cytotoxic activity. Killing the bacteria by heating, sonication or antibiotics destroy the cytotoxic activity. These cytotoxic effects are dose and time dependent.

The cytotoxic effects are associated with disruption of the cell membrane and PI staining indicative of necrosis. AV staining is low or absent, and pan-caspase inhibitors do not block the cytotoxicity, suggesting that the apoptotic pathway was not activated. The RIPK1 inhibitor necrostatin-1 has no effect of the cytotoxic effect of LiSA suggesting that the necroptotic pathway is also not involved.

Further experiments to elucidate the nature of the bioactive factor released by SA to induce cytokine release and cytotoxicity and alternative pathways of *S. aureus* interacting with MEFs and HEKα are investigated in the next chapter.
Chapter 5: Possible role of *S. aureus* proteases inducing PAR pathways on MEFs and HEKs as an explanation for their pro-inflammatory and cytotoxic activity.
5.1 Foreword

Proteases and their inhibitors naturally present in the skin are key players in epidermal homeostasis and maintaining skin barrier function (Komatsu et al. 2007a). Endogenously produced kallikreins (KLK-5, 7, 8 and 14) are present in the stratum corneum (SC) (Kishibe et al. 2006; Komatsu et al. 2007a; Komatsu et al. 2007b; Komatsu et al. 2006a; Voegeli et al. 2009). They mediate skin inflammation by stimulating Protease-Activated Receptors (PARs) expressed on keratinocytes (Hansen et al. 2008; Rattenholl et al. 2003; A. Rattenholl et al. 2008). PAR2 expression is increased in eczematous skin (Hachem et al. 2005; M. Steinhoff et al. 1999; M. Steinhoff et al. 2004).

Exogenous proteases present in house dust mite faeces, pollen and microorganisms can also activate PAR2 and induce inflammation (Asokananthan et al. 2002; Boitano et al. 2011; S. Kondo et al. 2004). In NC mice, activity of KLKs and keratinocyte PAR2 activity are known to lead to TSLP release (Komatsu et al. 2007a; Komatsu et al. 2005b; Voegeli et al. 2009). In Chapter 3 of this thesis, S. aureus and it’s supernatant were found to induce release of IL-33 and TSLP from both MEFs and HEKa. Chapter 4 showed that this bacterium and the soluble factors are also cytotoxic in 3T3-MEFs and HEKa. The current chapter examines the possibility that the inflammatory effects of S. aureus are via a protease-PAR2-IL-33/TSLP pathway.

5.2 Heating filtered supernatant of S. aureus (FSA), but not freezing or treating with antibiotics, reduces its cytotoxic effect on HEKa

As demonstrated in Chapters 3 and 4, FSA induces production of IL-33 and TSLP, and disrupts the cell membrane of 3T3-MEFs and HEKa leading to increased PI staining. Further experiments were designed to investigate whether the activity of FSA is heat-labile, as would be expected if the activity was due to a protease enzyme. FSA was co-cultured with HEKa before and after heating to 95°C, as well as freezing to -80°C and thawing and co-culturing in the presence of Penicillin/Streptomycin (P/S), before assessing its bioactivity. In HEKa, the release of IL-33 and TSLP was completely abrogated by heating but not freeze-thawing or addition of antibiotics (Figure 5.1).
Figure 5.1 Effect of heat-treatment, freeze-thawing and addition of antibiotics on the pro-inflammatory cytokines released of HEKa. (A) IL-33, (B) TSLP. HEKa were co-cultured with filtered *S. aureus* supernatant (FSA), freeze-thawed FSA, FSA with added penicillin/streptomycin (P/S), or FSA heated at 95°C for 5 min. Data is representative of two separate experiments (n=3). ****p< 0.0001; one-way ANOVA.

Heating FSA abrogated cytotoxic effects on cells, whilst freeze-thawing, or addition of antibiotics had no effect (Figure 5.2). Cytotoxic activity of FSA is, therefore, heat-labile.

Figure 5.2 Effect of heat-treatment, freeze-thawing or addition of antibiotics on cytotoxicity of FSA on HEKa. (A) PI staining, (B) AV staining. HEKa were co-cultured with filtered *S. aureus* supernatant (FSA), freeze-thawed FSA, FSA with added penicillin/streptomycin (P/S), or FSA heated at 95°C for 5 min Data is representative of two separate experiments (n=3). ***p< 0.001; one-way ANOVA.

In conclusion, cytokine production and disruption of HEKa cell membrane (PI positive) caused by FSA is abrogated by heating to 95°C, indicating the bioactivity is heat-labile.
5.3 Effect of protease inhibitors on release of IL-33 and TSLP, and cytotoxicity induced by LiSA on 3T3-MEFs and HEKa

The heat-labile nature of the bioactive factor produced by S. aureus is in keeping with it being an enzyme. The possibility that it is a protease was investigated more directly by addition of protease inhibitors to the experimental systems. Serine but not metallo- or cysteine protease inhibitors blocked the release of IL-33 by 3T3-MEFs in a dose-dependent manner (Figure 5.3A). All three groups of protease inhibitors blocked the release of both IL-33 and TSLP by HEKa in a dose-dependent manner (Figure 5.3B).

![Figure 5.3](image.png)

**Figure 5.3 Effect of protease inhibitors on cytokines release induced by LiSA on 3T3-MEFs and HEKa.** (A) IL-33 produced by 3T3-MEFs, (B) IL-33 and TSLP produced of HEKa. Cells were stimulated with LiSA in the presence and absence of different proteases inhibitors, serine proteases inhibitor (SPI), metalloprotease inhibitor (IL-O) and cysteine protease inhibitor (E64) for 6 hours. Data representative of two separate experiments (n=3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; by one-way ANOVA.

The cytotoxic effects of LiSA were also inhibited by a specific serine protease inhibitor in both 3T3-MEFs (Figure 5.4) and HEKa (Figure 5.5).
5.4 LiSA induced expression of PAR2 on 3T3-MEFs and HEKa in vitro

PAR2 is upregulated in the skin of AD patients and thought to have a role in cell death and proliferation. The expression of PAR2 on 3T3-MEFs and HEKa was therefore investigated. Cells were grown until confluent in 24 well plates then stimulated with $10^7$ LiSA for 6 hours. The stimulated, and unstimulated cells were stained with PAR2 antibody. Isotype controls were included as a negative control and the data analysed by flow cytometry.
In unstimulated 3T3-MEFs, expression of PAR2 was low (3%), but increased (34%) after stimulation with LiSA for 6 hours (Figure 5.6). The expression of PAR2 was also low in unstimulated HEKa (9%) and increased after incubation with LiSA (55%) (Figure 5.7).

**Figure 5.6 LiSA upregulated PAR2 on 3T3-MEFs.** (A) Histogram shows PAR2 expression and (B) the percentage of PAR2 positive population in unstimulated and stimulated 3T3-MEFs. 3T3-MEFs were stimulated with $10^7$ CFU/ml LiSA for 6 hours then stained with anti-PAR2 mouse antibody. Data represent two separate experiments of triplicates samples. An isotype control antibody was used as a negative control. **p < 0.01; one-way ANOVA.

**Figure 5.7 LiSA upregulated PAR2 on HEKa.** (A) Histogram shows PAR2 expression and (B) the percentage of PAR2 positive population in unstimulated and stimulated HEKa. HEKa were stimulated with $10^7$ CFU/ml LiSA for 6 hours then stained with anti-PAR2 human antibody. Data represent one experiment of triplicates samples. An isotype control antibody was used as a negative control. **p < 0.01; one-way ANOVA.
5.5 Analysing proteases from *S. aureus*

*S. aureus* expresses a number of different proteases (Karlsson *et al.* 2002). Experiments were designed to study protease gene expression by the *S. aureus* strain used in this project. *S. aureus* was grown overnight in NB for 18-24 hours. The DNA is extracted and analysed by PCR and the produced run on gel electrophoresis as described in chapter 2. The major protease genes expressed were identified as V8, SPA (serine protease), and SPB (cysteine protease), and all expressed at high concentration (Figure 5.8), while, AUR (aureolysin) and SCP (Staphopain) were also expressed but at low levels.

![Agarose gel electrophoresis](image.jpg)

**Figure 5.8 Agarose (1.5%) gel electrophoresis of *S. aureus* PCR products for known genes.** V8 and SPA (serine protease gene), SPB (cysteine protease gene), AUR (aureolysin) and SCP (Staphopain).

Proteins secreted in FSA were determined by mass spectroscopy. After inoculation of *S. aureus* in growth media for 6 hours, the FSA was submitted to the mass-spectroscopy core facility for analysis. The proteins were identified according to their mass and compared with the database by the Mascot program. The most abundant proteins identified in the filtered supernatants of *S. aureus* are listed in Table 5.1.
Table 5.1 Proteins identified from filtered supernatant of *S. aureus* by mass spectroscopy (MS)

<table>
<thead>
<tr>
<th>Identified Proteins</th>
<th>Name of the proteins</th>
<th>M.Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Staphopain A OS</td>
<td>cysteine protease</td>
</tr>
<tr>
<td>2</td>
<td>Enterotoxin type B OS</td>
<td>Staphylococcal enterotoxin B (SEB)</td>
</tr>
<tr>
<td>3</td>
<td>Enolase OS</td>
<td>laminin-binding protein</td>
</tr>
<tr>
<td>4</td>
<td>Bifunctional acetyltransferase OS</td>
<td>Antibiotic resistance enzyme</td>
</tr>
<tr>
<td>5</td>
<td>Formate acetyltransferase OS</td>
<td>Pyruvate formate-lyase</td>
</tr>
</tbody>
</table>

To examine protease activity of the *S. aureus* strain used in this project, the bacteria or its supernatant was inoculated on casein agar medium and incubated at 37°C for 24-48 hours. The presence of a zone of proteolysis would indicate protease activity on casein agar plates. Surprisingly, no protease activity was detected on casein agar medium even after 48 hours of inoculation of *S. aureus* (data not shown). Possible protease activity of the filtered supernatant of *S. aureus* (FSA) was also tested using a colorimetric protease assay kit to measure total protease activity using trypsin as a positive control. The substrate used in this assay was succinylated casein, known to be cleaved by serine proteases (e.g. trypsin). Again no protease activity could be detected by this method. Standard curves for trypsin and V8 assays are shown in figure 5.9 and 5.10.

**Figure 5.9 Standard curve of trypsin, and protease activity of FSA.** Trypsin and FSA were diluted 1:2 then incubated with succinylated casein for 20 min at 37°C. 50µl of trinitrobenzene sulfonic acid was then added to each well and incubated again at room temperature for 20 min. The optical density of the standard, unknown and blank were measured at 450nm.
Figure 5.10  **Standard curve of *S. aureus* V8 protease.** V8 was serially diluted then incubated with succinylated casein for 20 min at 37°C. 50µl of trinitrobenzene sulfonic acid was then added to each well and incubated again at room temperature for 20 min. The optical density of the standard, unknown and the blank was measured at 450nm.

5.6 **KLK5 and *S. aureus* V8 protease have no pro-inflammatory or cytotoxic activity on 3T3-MEFs**

KLK5 is one of the most important serine proteases expressed in healthy skin (Eissa et al. 2008; Lundwall et al. 2008). V8 is the most studied serine protease and important in survival of SA and activation of other SA proteases (Massimi et al. 2002; Rice et al. 2001). The pro-inflammatory and cytotoxic effects of commercial KLK5 and V8 were examined on MEFs and HEKα. MEFs and HEKα were co-cultured with LiSA, V8 or KLK5 for 6 h. After incubation, cytokine production and cytotoxicity were assessed.

No IL-33 or TSLP were detected after stimulation of the either MEFs or HEKα with V8 (Figure 5.11) or KLK5 (Figure 5.12) Neither *S. aureus* V8 protease nor KLK5 had any cytotoxic effects on MEFs (Figures 5.13 & 5.14).
Figure 5.11 Effect of *S. aureus* V8 protease on the release of IL-33 and TSLP from 3T3-MEFs and HEKa. 3T3 (A) and HEKa (B) were co-cultured with $10^7$ CFU/ml LiSA or V8 (1, 2, 10µg/ml) for 6 hours. The supernatants were collected and the level of IL-33 and TSLP determined by ELISA. Data is representative of two separate experiments (n=3). ***$p<0.001$; one-way ANOVA.

Figure 5.12 Effect of KLK5 on the release of IL-33 and TSLP from 3T3-MEFs and HEKa. 3T3 (A) and HEKa (B) were exposed to $10^7$ CFU/ml LiSA and different concentrations of KLK5 (10, 50, 100ng/ml) for 6 hours. The supernatant were collected and the level of IL-33 and TSLP was determined by ELISA. Data represent two separate experiments. ***$p<0.001$; one-way ANOVA.
Figure 5.13 V8 does not induce cell death in 3T3-MEFs. (A) Percentage of PI positive stain and (B) percentage of AV positive stain. 3T3-MEFs were exposed to LiSA (10^7 CFU/ml) and different concentrations of V8 (1, 2, 10µg/ml) for 6 hours. Data represent two separate experiments.

Figure 5.14 KLK5 does not induce cell death in 3T3-MEFs. (A) Percentage of PI positive stain and (B) percentage of AV positive stain. 3T3-MEFs were exposed to LiSA (10^7 CFU/ml) and different concentrations of KLK5 (10, 50, 100ng/ml) for 6 hours. Data represent two separate experiments.

In conclusion, V8 and KLK5 alone do not induce production of IL-33 and TSLP or any cytotoxic effects in the mouse or human cell lines used.
The expression of PAR2 in unstimulated MEFs and HEKα was low as shown in Figure 5.6 and 5.7. Therefore further experiments were designed to induce expression of PAR2 before administration of V8 and KLK5. HEKα were incubated with LiSA for 1 hour then washed and different concentration of V8 and KLK5 added for 6 hours, before IL-33 and TSLP determined by ELISA. The level of IL-33 and TSLP were reduced in the presence of high concentration of V8, but not KLK5. In addition, the level of IL-33 is increased with 10ng KLK5 compared with SA (Figure 5.15A & 5.15B).

**Figure 5.15** V8 but not KLK-5 reduced the level of IL-33 and TSLP induced by LiSA in HEKα. (A) IL-33 and TSLP after administration of V8; (B) IL-33 and TSLP after adding of KLK5. HEKα were stimulated with LiSA (pSA) for 1 hour, then washed and different concentration of V8 (1, 5, 10µg/ml) or KLK5 (10ng, 40ng) for 6 hours. The supernatant were collected and the level of IL-33 and TSLP was determined by ELISA. Data represent one experiment done in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA.

A further experiment was carried out to study the effect of *S. aureus* serine protease (V8), trypsin and FSA on the stability of exogenous IL-33 and TSLP standard. V8, trypsin and FSA were mixed with IL-33 (1500pg/ml) or TSLP (2000pg/ml) for 2h hrs during assaying cytokine levels remaining by ELISA IL-33 standard was degraded by...
V8, trypsin and whereas TSLP was degraded by trypsin only but not by V8 or FSA (Figure 5.16). This result suggests that IL-33 may have cleavage sites that are recognised by V8 and the soluble factors of *S. aureus* filtrate (FSA) but TSLP only has the cleavage site recognised by trypsin.

![Figure 5.16](image)

**Figure 5.16** V8, Trypsin and FSA breakdown IL-33 but not TSLP top standard. (A) Human IL-33 and (B) TSLP standards (STD) in the absence and presence of different concentrations of V8 (1, 10µg/ml), trypsin (0.125, 0.05%) and FSA. Data represent one experiment (n=3). *p* < 0.05, **p** < 0.001, ***p*** < 0.0001; one-way ANOVA.

### 5.7 Effect of the PAR2 inhibitor nordihydroguaiaretic acid (NDGA) on the production of cytokines and the cytotoxic effects induced by SA on 3T3-MEFs and HEKas

Experiments were performed to investigate the possible role of PAR2 in pro-inflammatory and cytotoxic effects of SA on 3T3-MEFs and HEKas. Nordihydroguaiaretic acid (NDGA) is an antioxidant extracted from natural herbal and found in *Larrea tridentate* (He. *et al.* 2004). This compound is an anti-tumor agent (Lu *et al.*, 2010) and inhibits Ca\(^{2+}\) intracellular signalling. In addition, NDGA is thought to
have antibiotic activity against *S. aureus* (Ooi et al., 2015). HEKa and 3T3-MEFs were stimulated with LiSA in the absence and presence of different concentrations of NDGA. NDGA inhibited the release of IL-33 by 3T3-MEFs and more completely inhibited IL-33 and TSLP release by HEKa (Figure 5.17). There was also a dose-dependent suppression of cytotoxic effect of LiSA on 3T3-MEFs (Figure 5.18) and HEKa (Figure 5.19).

Figure 5.17 NDGA reduced LiSA-induced IL-33 and TSLP release by 3T3-MEFs and HEKa. (A) IL-33 released by 3T3-MEFs; (B) IL-33 and TSLP released by HEKa. Both cells were stimulated with LiSA in the presence of NDGA and incubated for 6 hours. The supernatants were collected and the level of IL-33 and TSLP cytokines measured by ELISA. Data are representative of two separate experiments (n=3). 3T3-MEFs (Green) and HEKa (Blue). The analysis was by one-way ANOVA, ****p< 0.0001.

Figure 5.18 Nordihydroguaiaretic acid (NDGA) blocks LiSA-induced cytotoxicity of 3T3-MEFs. (A) Percentage of PI positive cells; (B) AV positive cells. Data represent duplicate wells of two separate experiments. ***p< 0.001 and ****p< 0.0001; one-way ANOVA.
Figure 5.19 Effect of nordihydroguaiaretic acid (NDGA) on the cytotoxic effect of LiSA on HEKa. (A) PI positive; (B) AV positive. Data represent duplicate wells of two separate experiments. *p<0.05, **p<0.01 and ***p<0.001; one-way ANOVA.

To study the possible anti-microbial effect of NDGA on viability of *S. aureus*, the bacteria were inoculated in growth media for 6 h in the presence and absence of NDGA. After incubation, the CFU was assessed by Miles and Misra method. The viability of SA was reduced when a high concentration of NDGA (135-200µM/ml) was added (Figure 5.20), however there was little effect at lower concentrations (66µM of NDGA).

Figure 5.20 Effect of NDGA on the viability of LiSA. SA was grown the presence and absence of NDGA (66, 135, 200 µM/ml) for 6 hours. CFU was determined by Miles and Misra method.

In conclusion, NDGA blocked the cytotoxic effects of LiSA on 3T3-MEFs and HEKa. High concentrations of NDGA paradoxically induced apoptosis of 3T3-MEFs, but not HEKa in the presence of LiSA. The antibiotic properties of NDGA could not account for its suppression of IL-33 and TSLP release.
5.8 Effect of trypsin on cytokine release and cytotoxicity induced by LiSA on 3T3-MEFs and HEKa

PAR2 is classically activated by trypsin. It is not known whether activation of PAR2 by trypsin may affect the cytokine production and cytotoxicity induced by *S. aureus* on 3T3-MEFs and HEKa. Both cells were stimulated with LiSA in the absence and presence of different concentrations of trypsin for 6 hours. Trypsin suppressed LiSA-induced IL-33 and TSLP release by 3T3-MEFs and more completely by HEKa (Figure 5.21).

![Figure 5.21](image)

**Figure 5.21** Effect of trypsin on LiSA-induced IL-33 and TSLP release by 3T3-MEFs and HEKa. (A) 3T3-MEFs and (B) HEKa were co-cultured with LiSA in the presence and absence of trypsin for 6 hours. Data are representative of three separate experiments done in triplicate. **p < 0.01 and ****p < 0.0001; one-way ANOVA.

Trypsin had a slight but statistically significant inhibitory effect on the cytotoxic effect of LiSA on 3T3 MEFs (Figure 5.22) and HEKa (Figure 5.23).
Figure 5.22 Effect of trypsin on the cytotoxic effect of LiSA on 3T3-MEFs. PI positive staining. Data are representative of two separate experiments. *p < 0.05 by one-way ANOVA.

Figure 5.23 Effects of trypsin on cytotoxicity of HEKa induced by live S. aureus. PI positive staining. Data are representative of two separate experiments. *p < 0.05 and by one-way ANOVA.

5.9 Effect of PAR1 and PAR2 tethered-ligand peptides on cytokine production and cytotoxic effects induced by LiSA on 3T3-MEFs and HEKa

In Chapter 5 of this thesis, LiSA was found to induce expression of PAR2 in 3T3-MEFs and HEKas. To study the effects of synthetic peptides corresponding to the tethered ligand domains of PAR1 and PAR2, 3T3-MEFs and HEKas were stimulated with LiSA in the presence of PAR1 (TFLLR-NH₂) or PAR2 (SLIGRL-NH₂) peptides. After 6 hours of incubation, the produced cytokines were determined in the supernatants of co-
culture and the cells were stained with AV and PI to assess the cytotoxic effects by flow cytometry.

PAR1 and PAR2 tethered-ligand peptides had little effect on LiSA-induced IL-33 release by 3T3-MEFs. In contrast, they completely blocked both IL-33 and TSLP release by HEKa (Figure 5.24).

**Figure 5.24 LiSA-induced IL-33 and TSLP release by 3T3-MEFs and HEKa in the presence of PAR1 and PAR2 tethered-ligand peptides.** (A) IL-33 released by 3T3-MEFs and (B) IL-33 and TSLP released by HEKa. Both cells were stimulated with SA in the presence of PAR peptides for 6 hours. The supernatants were collected, IL-33 and TSLP cytokines measured by ELISA. Data are representative of two separate experiments, performed in triplicate. Green = 3T3-MEFs; Blue = HEKa. Analysis was by one-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

PAR1 and PAR2 peptides induced a slight and variable inhibitory effect on the cytotoxic effect of LiSA on 3T3-MEFs (Figure 5.25) and HEKa (Figure 5.26).
Figure 5.25 Effect of PAR1 and 2 tethered-ligand peptides on the cytotoxic effects of LiSA on 3T3-MEFs. PI positive staining in the presence of (A) PAR1 peptide, (B) PAR2 peptide. Data represent of two separate experiments. *p < 0.05 and **p < 0.01 by one-way ANOVA.

Figure 5.26 Effect of PAR1 and PAR2 tethered-ligand peptides reduced the cytotoxic effect of HEKa induced by SA. (A) PI positive staining in the presence of PAR1 agonist and (B) PAR2 agonist. Data represent of two separate experiments. **p < 0.01 and ***p < 0.001 by one-way ANOVA.
5.10 Summary

Data shows that *S. aureus* bioactivity observed in Chapters 3 and 4 was heat labile and blocked by protease inhibitors, suggesting that it was a protease. However although protease genes and proteins could be detected in the *S. aureus* strain used in this thesis, no protease activity was found, either from LiSA or FSA, both of which had bioactivity. Furthermore, neither KLK-5 and commercial staphylococcal protease V8 had any stimulatory effects on cytokine production or cytotoxicity in MEFs or HEKa.

In HEKa, the inhibitory effects of the protease inhibitors were noticeably greater on the pro-inflammatory rather than on the cytotoxic activity of LiSA, while in MEFs it was reversed with a greater inhibitory effect on cytotoxic rather than pro-inflammatory activity. Similar trends, particularly in relation to inhibition of cytokine release, were noted with the PAR2 inhibitor NDGA, the canonical PAR2 activator trypsin, and PAR1 and PAR2 peptides. A summary is shown in Table 5.2 below.

<table>
<thead>
<tr>
<th>condition</th>
<th>MEFs</th>
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<tr>
<td>Protease inhibitors</td>
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<tr>
<td>serine</td>
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<tr>
<td>cysteine</td>
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<tr>
<td>metallo</td>
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<tr>
<td>Heat-treated FSA</td>
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<tr>
<td>trypsin</td>
<td>↓ ↓</td>
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<tr>
<td>KLK5</td>
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<tr>
<td><em>S. aureus</em> V8</td>
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</tr>
<tr>
<td>PAR2 inhibitor (NDGA)</td>
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<td>PAR1 peptide</td>
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<td>PAR2 peptide</td>
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6 Chapter 6: Discussion
Three major conclusions can be drawn from this thesis regarding the pro-inflammatory and cytotoxic effects of *S. aureus* on mouse embryonic fibroblasts and human keratinocytes:

1. **Pro-inflammatory effects:** Live *S. aureus* and factors secreted by this bacteria but not bacteria killed by heating, sonication or addition of antibiotics, induces the release of IL-33 by MEFs and HEKa. Bacteria induced keratinocytes to release large amounts of TSLP, while MEFs produced little of this cytokine. The pro-inflammatory effects appear to be specific for *S. aureus* and are not found to be induced by the other staphylococcal species tested. The release of IL-33 and TSLP does not appear to be mediated via a TLR2 pathway, as classical TLR2 ligands (heat-killed *S. aureus*, *S. aureus*-derived peptidoglycan and lipoteichoic acid) did not stimulate cytokine release and neutralizing anti-TLR2 antibodies did not block pro-inflammatory activity. The released IL-33 is bioactive in that it can induce mouse spleen Th2 cells to produce IL-13 and bone marrow-derived mast cells to express IL-6.

2. **Cytotoxic effects:** Live *S. aureus* and factors secreted by this bacteria were also able to induced cell toxicity and death associated with PI staining (necrosis). Cytotoxicity was not blocked by the caspase inhibitor Z-VAD-FMK or the RIPK1 inhibitor necrostatin-1, suggesting that the bacteria did not induce other forms of cytotoxicity namely apoptosis and necroptosis. The cytotoxic effect was dose and time dependent and most marked for *S. aureus*, with other staphylococcal species having relatively slight cytotoxic effects. MEFs of dermatitis-prone NC/Nga mice were three to five times more sensitive to the cytotoxic effects of *S. aureus* than the MEFs of non-dermatitis prone mice, such as Swiss albino mice (3T3) and MSM. Although IL-33 was released in similar concentrations in all mouse strains, there was a striking difference in the lack of detectable soluble ST2 decoy receptor detected by ELISA in the NC/Nga mice compared to the other two mouse strains. sST2 was however detected in the supernatant of NC mice by Western blot, suggesting a subtle difference in the protein structure in this strain. Addition of recombinant sST2 to *S. aureus*-NC/Nga co-culture partly inhibited the observed cytotoxicity, suggesting that the lack of sST2 activity in this strain may have a role in causing the observed heightened cytotoxic effects.
Involvement of a protease-PAR2 receptor pathway in mediating the bioactivity of *S. aureus* in this experimental system: *S. aureus* induced cell surface expression of PAR2 on both MEFs and HEKa. The pro-inflammatory (IL-33 and TSLP release) and cytotoxic activities induced by *S. aureus* were heat labile, and blocked by protease inhibitors as well as by PAR2 inhibitors in both MEFs and HEKa, providing evidence for the involvement of the protease-PAR2 pathway in mediating bioactivity of *S. aureus* in this experimental system. The inhibitory effects of protease inhibitors and PAR2 inhibitors were more marked on cytokine production than on cytotoxicity. A number of protease genes and proteins were detected from the *S. aureus* strain used in this project, but functional protease activity could not be found either in *S. aureus* or its filtrate. The most studied *S. aureus* protease V8, and the commercial KLK5 did not induce any pro-inflammatory response or cytotoxic effect on MEFs and HEKa. Bacterial protease V8, but not KLK-5, reduced detectable IL-33 but not TSLP released by HEKa, raising the question as to whether V8 directly or indirectly results in proteolytic cleavage of IL-33.

The hallmark of AD is the infiltration of Th2-cells into inflamed skin, induced by cytokines such as IL-33 and TSLP (Savinko *et al.* 2012; Zhu *et al.* 2011). IL-33 and TSLP are also markers of disease severity and response to the treatment (Nygaard *et al.* 2016). The mechanism of induction of these cytokines is not fully understood. TSLP is expressed in lesional but not non-lesional skin of patients with AD (Soumelis *et al.* 2002; Takai 2012). IL-33 is expressed in the nucleus of resting keratinocytes, but during inflammation, translocates to the cytoplasm and extracellular space. Many studies have been carried out to understand how IL-33 is processed and activated. The full length IL-33 (30 kDa) is released during injury or necrosis, while during apoptosis it is inactivated by caspase-3 and 7, but not caspase-1 (Sjöberg *et al.* 2015). The released full length IL-33 is thought to alert the immune cells via its receptor (ST2).

In this thesis, IL-33 localised to the nucleus of unstimulated human keratinocytes but not human fibroblasts. Translocation of IL-33 to the cytoplasm and extracellular space was observed in HEKa after stimulation with LiSA. IL-33 has previously been detected in human keratinocytes (mRNA) after stimulation with TNF-α and IFN-γ, but not in cell culture supernatant (Meephansan *et al.* 2012; Taniguchi *et al.* 2013). Increased
expression of IL-33 has also been demonstrated in the skin of AD patients after exposure to SEB and house dust mite antigen (Savinko et al. 2012).

IL-33 through binding to its receptor (ST2) on epithelial and immune cells, induces cytokines and chemokines involved in innate and adaptive immune responses in the skin (Liew et al. 2010; Pushparaj et al. 2009). In addition, IL-33 has an important role in linking and communication of adaptive and immune response via innate lymphoid cells (ILCs) (Saglani 2011). In this thesis, the expression and cellular localization of IL-33 was studied in HEKa and MEFs cell culture. It would be interesting to examine how the distribution and expression of IL-33 differ through the epidermis of AD patients and healthy controls. Auto-regulatory mechanism of IL-33 has been reported in mouse skin macrophages and dendritic cells (Li et al. 2014; Su et al. 2013). The expression of mRNA in mouse macrophages and embryonic fibroblasts was controlled by transcription Interferon regulatory factor 3 (IRF-3) and cAMP response element-binding protein (CREB) (Polumuri et al. 2012). Another experiment proposed that IFN-γ control the degradation of IL-33 in mouse fibroblasts via activation of STAT1 (Kopach et al. 2014). In further experiments it would be interesting to understand the exact mechanism of IL-33 and TSLP induction in vitro or in vivo.

The data from this thesis to some extent conflicts with that from previous studies, which suggest that S. aureus cell wall components such as LTA, PGN and protein A are able to induce inflammatory responses in human keratinocytes. For instance:

- LTA has previously been shown to induce expression of TNF-α, IL-6 and IL-8 (Travers et al. 2010),
- PGN led to production of GM-CSF via MAPKs and IL-8 from keratinocytes via TLR2 (Matsubara et al. 2004; Pivarcsi 2003),
- S. aureus-derived diacylated lipoproteins and lipopeptides induced expression and release of TSLP by human keratinocytes via TLR2-TLR6 heterodimer (Vu et al. 2010).

Possible reasons for the conflicting results are either that the growth media used in this thesis was very low endotoxin media, the virulence factors associated cell wall such as PGN and LTA simply do not induce IL-33 or TSLP, or otherwise that co-stimulatory factors such as IFN-γ and TNF-α are required. Challenging skin cells from TLR2−/− mice
with LiSA and its filtrate would be a more direct way of proving whether or not the TLR2 pathway is involved.

If as the data from this thesis suggests, bacterial cell wall components are not important for the bioactivity of *S. aureus* and secreted factors are, then what are the possible candidates? *S. aureus* is known to secrete a number of lytic factors including α-toxin (α-hemolysin), Panton-Valentine leukocidin (PVL) and Phenol soluble modulins (PSMs). *S. aureus* induces cell death of different cells, including keratinocytes.

- **α-toxin (α-hemolysin)**, is a major cytotoxic virulence factor produced by *S. aureus*. It binds to the phosphocholine head of sphingomyelin and results in pores within the cell membrane (Schwiering *et al.* 2013; Wardenburg *et al.* 2007; Wichmann *et al.* 2009). Addition of sphingomyelinase protects keratinocytes from the toxic effect induced by α-toxin (Brauweiler *et al.* 2013). α-toxin also induces IL-1β and IL-6 in human keratinocytes (Sung-Wook Hong *et al.* 2014). EV-associated α-toxin induces necrosis, but soluble α-toxin induces apoptosis of human keratinocytes (Sung-Wook Hong *et al.* 2014). In activation of α-toxin function in H35A mutated alpha-toxin expression strains blocked the cytotoxicity of alpha-toxin on lung epithelial cells (Liang *et al.* 2008). However, α -toxin is unlikely to be the bioactive factor in our experimental model, as commercially available α -toxin was not found to induce pro-inflammatory bioactivity in either MEFs or HEKa. However, to more directly disprove the role of this toxin, the cytotoxic effect *S. aureus* with mutations in *hla* gene, which encodes α-hemolysin could be studied (Liang *et al.* 2006; Luong *et al.* 2003). The cytotoxic effects of α-toxin are augmented by Th2 cytokines such as IL-13 and IL-4 (Brauweiler *et al.* 2014) , and thus a role for this toxin in other experimental models cannot be excluded.

- **Panton-Valentine leukocidin** (PVL) of *S. aureus* mediates apoptosis of human keratinocytes via caspases dependent pathway, as zVAD-FMK (pan-caspase-inhibitor) was able to block this process (Chi *et al.* 2013). The fact that zVAD-FMK had no effect on the bioactivity of LiSA and FSA in this thesis, suggests that it is also not the key factor involved in the observed bioactivity.

- **Phenol soluble modulins** (PSMs) are produced by different *Staphylococcus* species, not only *S. aureus* (Donvito *et al.* 1997; Mehlin *et al.* 1999; Wang *et al.* 2007a). PSM acts like surfactant and causes pore formation and lysis of keratinocytes independently of caspase 1 (Essmann *et al.* 2003; Syed *et al.*
In the present study, the cytotoxic effects of LiSA on HEKa and MEFs were caspase-independent, as a general caspase inhibitor did not block the cytotoxic effects.

Given that Staphylococcal cell wall antigens did not induce IL-33 we were interested to know if *S. aureus* was unique amongst Staphylococcal species in being able to induce cytotoxicity and IL-33 release. It has previously been proposed that various staphylococcal species can act as nosocomial or opportunistic pathogens e.g., *S. aureus, S. haemolyticus,* and *S. epidermidis* although the associated immune response is not well defined. The most abundant skin commensal is *S. epidermidis* which induces production of anti-microbial peptides (AMPs) that reduce colonisation and survival of microbes (Duckney *et al.* 2013; Gallo *et al.* 2012; Iwase *et al.* 2010). Most of coagulate negative staphylococcal species (CoNS) produce fewer virulence factors than *S. aureus* (Becker *et al.* 2014). In comparing to *S. aureus*, little is known about the virulence factors of CoNS and their mode of regulatory control in colonisation and pathogenicity.

We have demonstrated the species specificity of IL-33 and TSLP responses by human keratinocytes or mouse fibroblasts. We have also demonstrated the unique cytotoxic effect of *S. aureus* on HEKa and MEFs. The question arises as to why the bioactivity of *S. aureus* is so different to other staphylococcal species. Possibilities include:

- **Coagulase** activity is the primary readout for diagnosis of *S. aureus* infection since all other staphylococcal species used in this thesis are coagulate negative and known as CoNS (Becker *et al.* 2014). Coagulase and Willebrand factor binding protein (vWbp) activate the coagulation cascade by converting fibrinogen to fibrin (McAdow *et al.* 2012). The resultant fibrin clot encases the staphylococcal colonies and allows the bacteria to evade host immunity. Once bacteria have proliferated sufficiently, this fibrin mesh is degraded by staphylokinase, allowing the dissemination of bacteria throughout the vasculature (Loof *et al.* 2015). Experiments using a coagulase-deficient *S. aureus* mutant strain or a coagulase inhibitor to study the direct or indirect effect of coagulase in the induction of inflammatory response and cell death *in vivo* or *in vitro* system would be interesting.

- **Proteases**, the protease genes among most clinical isolates are conserved, but the functional activity of proteases is different from one isolate to another (Karlsson *et al.* 2002). The most studied proteases are those produced by *S. aureus* and *S. epidermidis* and their role in biofilm formation. It has been
demonstrated that *S. epidermidis* serine protease has 59% identical sequence of (V8) serine protease of *S. aureus* (Dubin *et al.* 2001). It has been described that serine proteases of *S. epidermidis* are able to inhibit the biofilm formation of *S. aureus* (Iwase *et al.* 2010).

Proteases produced by pathogens have been shown to induce cytokines. For instance, *Serratia marcescens* (serralysin) induced IL-6 and IL-8 mRNA expression in a human lung cell line (Y. Kida *et al.* 2006). It has been shown that a pan serine protease inhibitor can reduce airway inflammation in mouse allergic airway disease model (Saw *et al.* 2012). Here we have shown that cytokine release by filtered supernatant of *S. aureus* was inhibited by heat and addition of proteases inhibitors both in HEKa and MEFs. This is the first report to show that serine protease inhibitors block IL-33, TSLP and cytotoxicity in HEKa and 3T3-MEFs induced by LiSA.

The production of extracellular proteases varies amongst different *S. aureus* isolates (Karlsson *et al.* 2002). The major proteases produced by *S. aureus* are serine glutamyl Endopeptidase (serine protease), metalloproteinase (aureolysin) and cysteine proteinase. Serine protease is produced in an inactive form and thought to be cleaved by aureolysin to be mature and functionally active (Shaw *et al.* 2011). In this thesis, different protease genes including V8 were detected in this *S. aureus* isolate by PCR. However, no serine protease activity was detected in the bacterial supernatant and no activity observed on casein agar plate. This may be due to variation in protease activity between different *S. aureus* strains (Karlsson *et al.* 2002). Some virulence factors of *S. aureus* such as proteases are produced once *S. aureus* has colonised a surface (Peterson *et al.* 1977). The commercial V8 serine protease and KLK5 did not induce the release of pro-inflammatory cytokines, and no cytotoxicity effects were observed. V8 but not KLK5 was able to reduce the level of IL-33 and TSLP released from HEKa after stimulation with LiSA by its direct proteolytic effect on IL-33.

Further work is required to inactivate the aureolysin gene which is thought to have a role in activation of serine proteases. A comparative study is also recommended between different strains of *S. aureus* to show the level of expression of *sarA* and *agr* which thought to reflect the activity of proteases. Rather than a candidate protein approach, experiments could be conducted to identify the soluble bioactive factors that induce the release of pro-inflammatory cytokines from HEKa and MEFs by fractionating the supernatant by either HLPC or Reverse Phase Liquid Chromatography. Composition of fractions could be determined by one or two-dimensional SDS-PAGE.
Expression of staphylococcal virulence factors including proteases is controlled by the accessory gene regulator (agr) and staphylococcal accessory regulator (sarA) (Björklind et al. 1980; Lindsay et al. 1999). During the post-exponential growth phase, Agr is expressed, leading to the suppression of cell surface-related proteins and activation of extracellular proteins such as proteases and toxins (Oscarsson et al. 2006).

The agr gene in CoNS such as S. epidermidis and S. lugdunensis has been described before (Van Wamel et al. 1998; Vandenesch et al. 1993). It has been shown that the agr in S. lugdunensis is a homologue, like that of S. aureus and is transcribed actively during the post-exponential phase. The sarA gene is highly conserved between S. aureus and S. epidermidis also (85% homology) (Fluckiger et al. 1998). Significant differences have been described at the nucleotide level and is apparent in the region of the sarA coding region (Fluckiger et al. 1998). Therefore, a comparative study is recommended between different strains of S. aureus to show the level of expression of sarA and agr which thought to reflect the activity of proteases. Experiments to knock-out the Agr and SarA gene would be interesting to see what effects these genes have in the bioactivity observed in this thesis. It would also be interesting to characterise proteases expressed from S. aureus and other staphylococci at different time points in their growth curves by quantitative real-time PCR.

Having shown that S. aureus and its supernatant are able to induce the release of IL-33 and TSLP from HEKa and MEFs, we next needed to understand the biochemical pathway involved. A few studies have previously reported the induction of inflammatory responses by fungal, bacterial and allergen proteases via PAR2. Proteases such as trypsin and papain, as well as Alternaria extracts also induced expression of TSLP mRNA from airway epithelial cells via PAR2 (Kouzaki et al. 2009). In addition, Alternaria alternata induced the release of IL-33 via PAR2 (Snelgrove et al. 2014). PAR2 has previously been shown to be expressed at high levels in skin lesions of AD patients, and these findings suggested that PAR2 may have an important role in the inflammatory response in AD (Komatsu et al. 2007a; Komatsu et al. 2006a). In normal skin turnover, epidermal proteases and their inhibitors have a role in desquamation of human keratinocytes (Zeeuwen 2004). The SPINK5 gene encodes the serine protease inhibitor LEKTI, the natural inhibitor of KLK5 (Chavanas et al. 2000). Unopposed activity of KLK5 results in PAR2 overexpression and the severe dermatitis characteristic of Netherton syndrome (Briot et al. 2009). In our study, PAR2 expression
on HEKa and 3T3-MEFs increased after exposure to LiSA. However, in the in vitro model used here, recombinant KLK5 did not induce cytokine release or cytotoxicity, which may be because the expression of PAR2 in unstimulated HEKa and MEFs were low.

NDGA is a PAR2 pathway inhibitor which is thought to have a role in the recovery of the skin barrier and reduce the inflammatory response in human keratinocytes (Kim et al. 2012). In our study, NDGA was found to reduce the level of IL-33, TSLP and cytotoxicity of HEKa and 3T3-MEFs induced by LiSA. NDGA has been shown to have anti-staphylococcal activity (Ooi et al. 2014), but the viability of S. aureus in our hands was not significantly reduced by the concentrations of NDGA that induced cytokines and cytotoxicity in these experiments. This result suggests that NDGA may act as an antagonist and protect HEKa from the inflammatory response and toxicity induced by S. aureus. This work therefore confirms the importance of the PAR2 pathway in induction of IL-33. Activation of PAR2 has been shown to reduce cell death of colonic epithelial cells (Iablokov et al. 2014). In addition, activation of PAR2 reduced production of IFN-γ and IL-2 in allergic airways sensitised rabbits (D'Agostino et al. 2007). Furthermore, it has been demonstrated that PAR-2 deficient mice failed to develop airway inflammation when challenged with house dust mite antigen (Davidson et al. 2013). Surprisingly, PAR2 and PAR1 "agonists" reduced the level of pro-inflammatory cytokines (IL-33 and TSLP) in this study as well as the cytotoxic effects mediated by LiSA especially on HEKa. It seems that activation of PAR2 by a peptide agonist can inhibit release of IL-33 and TSLP from HEKa more than from 3T3-MEFs and slightly reduce the toxic effect induced by S. aureus. It has been shown that activation of PAR2 by the same activation peptide (agonist) can reduce cytokine-induced apoptosis in colonic epithelial cells (Iablokov et al. 2014). It would be interesting to do knockdown of PAR2 in human keratinocytes using the PAR siRNA as an alternative method to NDGA to understand the role of PAR2 in IL-33, TSLP and cell death induced by S. aureus.

Finally, we have demonstrated a correlation between the IL-33 pathway and cytotoxicity in a mouse model of atopic dermatitis. Naruto Research Institute Otsuka Atrichia (NOA) mice, a dermatitis mouse model of human AD, have previously been found to be sensitive to the cytotoxic effects of S. aureus (Kondo et al. 2006), but ours is the first study to compare the toxic effect of S. aureus in the NC/Nga mouse model of dermatitis. As a result of disruption of the cellular membrane of affected MEFs, IL-33 is released and binds to its membrane-bound receptor (ST2L), with IL-1RAcP (co-
receptor) (Moussion et al. 2008). Binding of IL-33 to ST2L induces production of pro-inflammatory cytokines via the NF-κB signalling pathway (Kurowska-Stolarska et al. 2008; Schmitz et al. 2005). sST2 is decoy receptor and acts a natural antagonist for IL-33 (Liew et al. 2010). It acts as negative regulator of Th2 cytokines by stopping signalling of IL-33 and reducing production of IL-4, IL-5 and IL-13, demonstrated in a murine model of asthma (Hayakawa et al. 2007). Data from this thesis showed that sST2 was absent in unstimulated or stimulated NC-MEFs by ELISA, but not by western blotting. It is possible that the anti-ST2 antibodies used in Western blot and ELISA recognise different epitopes of the sST2. It is possible that a mutation leading to a lack of function but some expression of sST2 contributes to the reason for the NC/Nga mouse being prone to dermatitis. Different single nucleotide polymorphisms (SNPs) have been documented in the ST2 distal region, which are thought to have a negative effect on the activation of Th2 cells (Shimizu 2005). It has previously been shown that the stimulatory effect of IL-33 on DCs can be blocked by neutralising antibody for ST2 or using of soluble ST2 (Su et al. 2013). Interestingly in this thesis, addition of sST2 reduced the toxicity effects of LiSA on NC/Nga MEFs. Further experiments would be helpful to show the difference between the transmembrane ST2 and the soluble ST2 for instance by full sequencing of the receptor in NC/Nga and control MSM-MEFs.

Limitations:

- Experiments were carried out in HEKa and MEFs, to determine differences in immunological responses between human and mouse (Mestas et al. 2004). Mice have limitations as models of human disease, partly because the networks relating genes to disease may differ between these two species even though the pathways involved may be the same. The use of mice in biomedical research needs to take account of the differences as well as the similarities between mice and humans (Perlman 2016). It would be useful to confirm similar observations made here with primary human keratinocytes as well as in skin tissue sections where the stratum corneum remains intact.

- The finding from in vitro studies may not reflect the clinical response, as the human skin contains other cell types such as dermal fibroblasts, melanocytes, Langerhans cells, dermal dendritic cells, resident T cells, and endothelial cells. All these are thought to have active functions that influence the normal physiological responses in the skin (De Wever et al. 2015).
Induction of IL-33 and TSLP by *S. aureus* but not by other staphylococcal species was an interesting result as no previous work has studied the effect of different staphylococcal species on pro-inflammatory cytokines released from human keratinocytes. However this experiment was carried *in vitro*, so it would be interesting to repeat *in vivo* using superficial epidermal inoculation of different staphylococcal species or *ex vivo* using a 3D organotypic human skin tissue model (Popov *et al.* 2014).

Another limitation is that the level of IL-33 and TSLP after stimulation in all conditions was detected by ELISA. Use of an alternative molecular technique as another parameter to support these data, such as real-time PCR, would support increased expression of the proteins. This would be interesting as our data suggest that IL-33 is not made *de novo* on stimulation with *S. aureus*, but is released directly from the nucleus.

IL-33 and TSLP were the main readout cytokines of this project, and it would be useful to include other cytokines and chemokines such as IL-4, IL-5, IL-6, IL-13 TNF-α, and IL-25 to study their contributions in inflammatory responses of the skin cells and the functionality of IL-33 as Th2 cytokines mediator. Furthermore, detection of IL-33 was carried out by ELISA which recognises both the active and inactive form. Use of a new ELISA kit that recognises only the active form would help to understand the role of IL-33 (Eunsom Kim *et al.* 2016).

The cytotoxic effect of *S. aureus* was assessed by AV and PI staining and flow cytometry. Use of another technique to demonstrate the type of cell death either by electron microscopy to show cellular fragmentation, nuclear and cytoplasmic condensation or use of different histological stains or fluorescent dyes (Fink *et al.* 2005) would be helpful to understand the mechanism of toxic effect induced by *S. aureus*.
**Conclusion:**

The role of *S. aureus* and pathogenesis of AD is an important area of study and potentially may lead to the development of novel therapeutic agents. Colonisation of *S. aureus* has been correlated with the severity of AD and a reduction in colonisation is thought to improve the signs and symptoms of this disease. Current treatments of skin-colonised *S. aureus* with antibiotics alone cannot improve allergic skin inflammation and does not prevent relapse. Furthermore, prolonged treatment of *S. aureus* with topical or systemic antibiotics may cause the development of antibiotic resistance strains of *S. aureus* (Arkwright *et al.* 2002). The main purpose of this study was to understand which component(s) of *S. aureus* are responsible for induction of pro-inflammatory cytokines and cell death in skin cells. Understanding the mechanism by which this pathogen induces inflammation is important for therapeutic design in order to reduce inflammation, colonisation and patient outcome.
7 Chapter 7: References


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