Cross talk between stromal cells and immune cells in rheumatoid arthritis: the role of Toll-like receptor activated synovial fibroblasts in B cell autoreactivity

By

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I wouldn’t have made it without you all!
Declaration

I declare that the entire source presented in this thesis is less than 100 000 words and was performed and analysed by the candidate except for the result derived from Toll-like receptor stimulation on synovial fibroblast from osteoarthritis patients and the animal work on collagen-induced arthritis, which were performed by Dr Brentano and Rita Jones, respectively.

The studies presented in this study were conducted in the Centre of Experimental Medicine and Rheumatology at the William Harvey Research Institute.

This thesis has been written by me and the work presented in this manuscript is the result of my investigation. Dr Michele Bombardieri has actively supervised the entire project by providing scientific guidance and advice regarding experimental design and planning as well as in analysing and interpreting the results of this manuscript.

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Professor Constantino Pitzalis
Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the synovial joints leading to cartilage destruction and bone erosion. One of the hallmarks of RA is the presence of circulating autoantibodies and the expansion of autoreactive B cells. Within RA joints, ectopic B cell follicles remain functional and support Immunoglobulin (Ig) somatic hypermutation, class-switching and in situ autoantibody production even in the absence of recirculating cells, as evidenced by the RA-SCID model, suggesting that autocrine mechanisms support ongoing B cell activation within the RA joints.

In this PhD thesis, I investigate the mechanisms regulating RA synovial fibroblasts (SFs) production of B cell survival/proliferation factors BAFF/APRIL, with particular focus on toll-like receptors (TLR) signaling and how these factors in turn regulate B cell activation.

First, I demonstrate that TLR3 stimulation on RASFs led to strong induction of BAFF/APRIL mRNA expression. Resting and TLR3-stimulated RASFs released higher protein levels of BAFF/APRIL as compared to RA dermal fibroblasts (RADF) controls. TLR3 stimulation of RASFs, but not RADF in co-culture with IgD+ B cells strongly enhanced activation induced cytidine deaminase (AID) expression, Iγ-Cμ and Iα-Cμ circular transcripts (CTs) as well as class-switching to IgG/IgA. Notably, TLR3-induced effects were completely abrogated by the combined blockade of BAFFR/BCMA.

Following this, I report a negative feedback mechanism centered on secretory leukocyte protease inhibitor (SLPI) which, is induced in RASFs in response to TLR3 stimulation and modulates RASFs-dependent B cell responses, in part, via inhibition of BAFF production in RASFs but also by direct down-regulation of B cell activation, expression of AID and class-switching to IgG/IgA.
Overall, I highlight a novel and fundamental role for the TLR3-B cell survival factor axis in regulating B cell activation in the RA synovium, which may have profound relevance in response/resistance to biological therapies targeting B cells in RA.
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## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACPA</td>
<td>Anti-citrullinated peptide/protein antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American college of rheumatology</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>Anti-cyclic citrullinated peptide</td>
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<tr>
<td>APRIL</td>
<td>a proliferation inducing ligand;</td>
</tr>
<tr>
<td>Anti-G6PI</td>
<td>Anti-glucose 6 phosphate isomerase</td>
</tr>
<tr>
<td>AID</td>
<td>Activation-induced deoxycytididine deaminase</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor belonging to the TNF family</td>
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<tr>
<td>BAFF-R</td>
<td>BAFF receptor</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BCMA</td>
<td>B cell maturation antigen</td>
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<tr>
<td>BER</td>
<td>Base excision repair</td>
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<tr>
<td>bLP</td>
<td>bacterial lipopeptide</td>
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<tr>
<td>CAIA</td>
<td>Collagen antibody-induced arthritis</td>
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<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>CII</td>
<td>Type II collagen</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining regions</td>
</tr>
<tr>
<td>CMC</td>
<td>Carpometacarpal</td>
</tr>
<tr>
<td>CFA</td>
<td>Freund's Complete Adjuvant</td>
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<tr>
<td>CRP</td>
<td>C reactive protein</td>
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<tr>
<td>CSR</td>
<td>Clss switch recombination</td>
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<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte associated antigen</td>
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<tr>
<td>D</td>
<td>Diversity</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<td>DIP</td>
<td>Distal interphalangeal joints</td>
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<tr>
<td>DMARD</td>
<td>Disease-modifying anti-rheumatic drugs</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<td>dsRNA</td>
<td>double-stranded RNA</td>
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<tr>
<td>EC</td>
<td>Epithelial cell</td>
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<td>EBV</td>
<td>Epstein Barr Virus</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ELS</td>
<td>Ectopic lymphoid structure</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility-shift assay and supershift assay.</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
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<td>FIA</td>
<td>Freund’s incomplete adjuvant</td>
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<tr>
<td>FWR</td>
<td>Framework region</td>
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<td>GC</td>
<td>Germinal centre</td>
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<tr>
<td>GLT</td>
<td>Germline transcripts</td>
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<tr>
<td>HCQ</td>
<td>Hydroxychloroquine</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HRP</td>
<td>Horseradish peroxidise</td>
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<td>heparan sulfate proteoglycan</td>
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<td>Human immunodeficiency virus</td>
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<td>Intercellular adhesion molecules-1</td>
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<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFA</td>
<td>Freund's Incomplete Adjuvant</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IκBβ</td>
<td>Inhibitor of nuclear factor-kappa beta</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>LIGHT</td>
<td>LT-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
</tr>
<tr>
<td>MCP</td>
<td>Metacarpophalangeal</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>MTP</td>
<td>Metatarsophalangea</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messager RNA</td>
</tr>
<tr>
<td>Mal/TIRAP</td>
<td>MyD88 adapter-like/TIR domain-containing adapter protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene (88)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OASF</td>
<td>Osteoarthritis synovial fibroblast</td>
</tr>
<tr>
<td>PADI</td>
<td>Peptidyl arginase deiminase</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal interphalangeal</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RADF</td>
<td>RA dermal fibroblast</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RASFs</td>
<td>RA synovial fibroblasts</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoid-inducible gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>PIC</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α- and armadillo motif containing protein</td>
</tr>
<tr>
<td>SE</td>
<td>Shared epitope</td>
</tr>
<tr>
<td>SLO</td>
<td>Secondary lymphoid organs</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>T1/T2/T3</td>
<td>Transitional type 1/type 2/type 3</td>
</tr>
<tr>
<td>TACI</td>
<td>Transmembrane activator and calcium modulator ligand interactor</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetic acid EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular helper T</td>
</tr>
<tr>
<td>T-I</td>
<td>T cell independent</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor-associated factor.</td>
</tr>
<tr>
<td>TRAM</td>
<td>Toll-receptor-associated molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1.1 Rheumatoid arthritis: general aspects

1.1.1 The immune system and autoimmunity

The survival of human body depends on a continuous fight between the defensive mechanisms of the immune system and various threats arising from the microbial world. Included in the immune system is of a variety of effector cells and accompanying molecules that can be divided into two separate branches: innate and adaptive. Having the ability to fight against a wide range of pathogens, the innate branch rapidly responds and clears most of the harmful substances. On the other hand, the adaptive immune system is more sophisticated, mounting a highly specific response combined with the formation of an immunological memory. An important characteristic of this fight involves the ability of host’s immune system to distinguish unfriendly pathogenic microbial agents from the host’s endogenous tissue. Occasionally, the self-recognition control system fails, which may lead to false recognition of the host and a loss of immune tolerance to self components, allowing an immune response that may eventually lead to autoimmunity. Autoimmunity associated diseases can be characterised by a combination of two arms: organ specific, such as Hashimoto’s thyroiditis, or a more systemic distribution, such as systemic lupus erythematosus (SLE). Rheumatoid arthritis (RA) has features of both organic specific (synovial joints) and systemic autoimmunity the latter being characterised by the involvement of several organs such as the skin, kidney, lung and the cardiovascular system (Figure 1.1).
Figure 1.1 Rheumatoid arthritis and examples of systemic autoimmune disease.

The immune system protects organisms from infection with layered defenses of increasing specificity. Firstly, physical barriers prevent the entry of pathogens into the organism (grey circular ring). If a pathogen breaches these barriers, they activate the innate immune system that provides an immediate, but non-specific/broad-specific response (blue arrows). If pathogens successfully evade the innate response, a second layer of protection, termed the adaptive immune system, will be activated which has a specific and long-lasting response (purple arrow). However, over-activation of immune response, in which the immune system fails to properly distinguish between self and non-self, results in autoimmune disorders (lower left green box). Autoimmune disease could be classified as organ specific or non-organ specific depending on whether the response is primarily against antigen localised to particular organs or against widespread antigen, respectively. (Modified image adapted from Prime to the immune response by Tak Mak and Mary Saunders 2011 Academic Press Chapter 1 “Introduction of the immune response” and Immunology by David Mal, Jonathan Brostoff, David B Roth and Ivan Rott Chapter 20 “Autoimmunity and autoimmune disease”).
1.1.2 History and classification of criteria

RA was first described by Landré-Beauvais in 1800 with the term ‘primary aesthetic gout’ to describe this type of inflammatory arthritis. Later in 1848, a British rheumatologist, Sir Alfred Garrod, discovered excess uric acid in the blood of gouty patients and this allowed him to distinguish gout from what he described in 1859 as “rheumatoid arthritis”. However, at this stage, RA and osteoarthritis (OA), a degenerative joint disease that is often associated with a mild inflammatory reaction, were considered part of the same disease spectrum until the distinction made by his son in 1907 [1]. Originally, RA had no specific medical definition while the discovery of rheumatoid factor (RF) in 1940 [2, 3] allowed for the classification of arthritis as seropositive and, thus, RA was first described as an autoimmune disease. Later in 1987, the American College of Rheumatology (ACR) issued a set of criteria used for differentiating RA from other inflammatory arthritis diseases [4] with a specificity of ~90% (Table 1.1). Those classification criteria were modified further in 2010 by the ACR and the European League Against Rheumatism (EULAR) and now can be used to identify patients with a relatively short duration of symptoms, who may benefit from early establishment of disease-modifying anti-rheumatic drugs (DMARD) (Table 1.2) [5]. This new set of criteria is a score-based algorithm, by which a collective score from four different observations results in a total score of out of 10, where by ≥6 will classify a patient as having RA.
<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Morning stiffness</td>
<td>stiffness of joints lasting at least 1 hr</td>
</tr>
<tr>
<td>2. Arthritis of 3 or more joint areas</td>
<td>Soft tissue joint swelling observed by a physician</td>
</tr>
<tr>
<td>3. Arthritis of hand joints</td>
<td>Arthritis in at least one area of hand/wrist</td>
</tr>
<tr>
<td>4. Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas on both sides of the body</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous non tender nodules in juxtaarticular regions</td>
</tr>
<tr>
<td>6. Serum rheumatic factor</td>
<td>presence of autoantibodies which bind Fc portion of IgG</td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>RA typical changes with erosions or decalcifications in hand or wrist</td>
</tr>
</tbody>
</table>

**Table 1.1. 1987 Criteria for the classification of Rheumatoid Arthritis.**

4 of the 7 criteria have to be fulfilled to be classified as suffering from RA. Points 1 through 4 have to be present for at least 6 weeks. Point 2 and 3 exclude distal interphalangeal joints (DIP). Source adapted from the ACR, 2007, [www.rheumatology.org](http://www.rheumatology.org).[5]
### Classification criteria for RA

Patients should have at least 1 joint with definite clinical synovitis (swelling) and with the synovitis not better explained by another disease.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Joint involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 large joint*</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2-10 large joints</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1-3 small joints #</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>4-10 small joints</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>≥ 10 joints (at least 1 small joint)</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td><strong>B</strong> Serology (at least 1 test result is needed for classification)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative RF and negative ACPA</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Low-positive RF or low-positive ACPA</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>High-positive RF or high-positive ACPA</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>C</strong> Acute-phase reactant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal CRP and normal ESR</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Abnormal CRP or abnormal ESR</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>D</strong> Duration of symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 6 weeks</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>≥ 6 weeks</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Large joint refers to shoulders, elbows, hips, knees and ankles. # Small joint refers to proximal interphalangeal (PIP), thumb IP, metacarpophalangeal (MCP) joints of the hands, 2-5th metatarsophalangeal (MTP) and wrists. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies.

**Table 1.2. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis.**

Score-based algorithm: add score of categories A–D; a score of ≥ 6/10 is needed for classification of a patient as having definite RA. Source adapted from Arthritis Rheum 2010 [5]
1.1.3 Definition and clinical features

RA is a chronic, systemic, autoimmune inflammatory disease resulting in joint deformity, chronic ill-health, losses of mobility and function. If untreated, it can also cause premature mortality due to increased risk of infections in multiple organs as well as cardiovascular diseases. RA can affect individuals of all ages but is most commonly present between the ages of 35 and 45, with maximal onset in women around the menopause. As a very variable disease, RA affects each person in a unique and different way.

The most characteristic feature of RA is a sustained and persistent inflammatory synovitis (swelling), primarily of the peripheral joints in a symmetric distribution. The commonly affected joints involved are the metacarpophalangeal (MCP), proximal interphalangeal (PIP) and metatarsophalangeal (MTP) joints. The finger DIP, the 1st carpometacarpal (CMC) joint of the hand and the 1st MTP joint are generally spared since these joints are typically involved in OA. Although RA develops its pathology within the synovium, many nonarticular organs become involved, particularly in patients with severe joint disease. Fatigue, fever, weight loss, and malaise are the general symptoms that can be associated with variable manifestations of extra-articular involvement (Table 1.3).

<table>
<thead>
<tr>
<th>Affected tissue or organ</th>
<th>Extra-articular manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Pericarditis, endocarditis, myocarditis, myocardial infarction</td>
</tr>
<tr>
<td>Nervous system</td>
<td>Entrapment neuropathies, progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>Skin</td>
<td>Subcutaneous nodules</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>Pulmonary nodules; interstitial lung disease</td>
</tr>
<tr>
<td>Eyes</td>
<td>Scleritis, episcleritis, retinal vasculitis</td>
</tr>
<tr>
<td>Other</td>
<td>Vasculitis, diabetes, gastrointestinal bleeding, cancer (e.g., lymphoma), hematologic abnormalities (e.g. Felty's syndrome)</td>
</tr>
</tbody>
</table>

Table 1.3. List of extra-articular disease of RA.
Although numerous studies have been carried out aiming to dissect RA disease, the poorly defined natural history of this disease as well as the fluctuating clinical course and unpredictable prognosis adds to the difficulty in monitoring RA disease. Moreover, RA is an epidemiologically relevant cause of disability.

1.1.4 Epidemiology

RA is the most common inflammatory arthritis and its prevalence ranges between 0.5% and 1.0% of the adult population. However, the occurrence of RA is not the same throughout the world. Northern European RA prevalence varies between Norway (0.44%), Sweden (0.51%) and the UK (minimum 0.81%), [6-8] and in Europe overall, RA prevalence decreases from north to south. Conversely, native American-Indian populations have the highest recorded occurrence of RA, with prevalence of 5.3% noted for the Pima Indians [9] and 6.8% for the Chippewa Indians [10]. A much lower prevalence has been reported in Southeast Asia [11], including China and Japan [12, 13], which have a very low occurrences of 0.2–0.3%. Importantly, even though no single case has been found in rural Nigeria [14] and rural South Africa [15] prevalence rate rises to nearly 1% in black populations in urban areas, indicating that geographic area of residence may be a strong determinant for disease pathogenesis [16]. On the other hand, it is still unclear whether differences in epidemiological data between countries are solely dependent on environmental factors or if other factors such as genetic origins. As an example, the investigation of a Caribbean population of African origin living in Manchester, UK, showed a low frequency [17], reflecting that the protection to this group was in fact genetically determined. Similarly, a study on a Chinese population living in an urban area in Hong Kong showed the same consistent low occurrence [18].

Furthermore, the incidence of RA is about 3 times higher in women than in men (Table 1.4), possibly due to hormones factor as RA generally develops in women when their hormonal factors are at the highest levels, for instance: after pregnancy or near menopause. This differential ratio between women and men falls with increasing age. Interestingly, studies conducted at various time points within the same geographic regions suggest a fall in the incidence of RA in women as explained by a protective effect of oral contraceptive pill [8].
<table>
<thead>
<tr>
<th>Country</th>
<th>Female (%)</th>
<th>Male (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greece</td>
<td>0.45%</td>
<td>0.19%</td>
</tr>
<tr>
<td>Northern Italy</td>
<td>0.51%</td>
<td>0.13%</td>
</tr>
<tr>
<td>France</td>
<td>0.51%</td>
<td>0.09%</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>1.16%</td>
<td>0.44%</td>
</tr>
<tr>
<td>United States</td>
<td>1.4%</td>
<td>0.74%</td>
</tr>
</tbody>
</table>

Table 1.4. Prevalence of RA by gender in various countries. Source from [19]

1.1.5 Etiology

1.1.5.1 Genetics

It is long recognised that the susceptibility to develop RA is in part heritable. This is supported by evidence that the concordance rate in monozygotic twin is 12-15%, compared to 3.5% in the fraternal ( dizygotic) twin [20, 21]. Although the occurrence of familial clustering of the disease is relatively low, the prevalence of the disease between first-degree relatives is considerably higher than the general population [22]. Below, I discuss the main genetic risk factors believed to influence the development and/or course of RA, including human leukocyte antigen (HLA) and non-HLA susceptibility genes.

1.1.5.1.1 HLA susceptibility genes

The firmest link between a genetic susceptibility factor and RA is the association of the disease with HLA region, which account up to 40-50% of the genetic influence [22, 23]. In human, the class II cluster in HLA region for histocompatibility complex (MHC) class II includes HLA-DP, -DQ, and -DR. It has been reported that RA is associated with the HLA-DR alleles which have similarity on a highly conserved amino acid sequence (QRRAA, RRRAA or QKRAA), extend from position 70 to 74 within the third hypervariable region of the DRβ chains (one of the components of HLA-DR heterodimer), termed the “shared epitope” (SE). This hypothesis provides a strong rationale for adaptive immunity in having a pathogenic role in RA via selectively binding of arthritogenic peptides in SE-HLA-DR molecules, which facilitate the presentation to
auto-reactive T cells. Other prevailing explanations include molecular mimicry with foreign antigens (Ags) [24], T cell repertoire selection [25], and/or SE directly acting as immune-stimulatory ligands unrelated to groove peptides [26].

Most RA patients express particular HLA-DR-alleles, such as HLA-DR4 (DRB1*0401, 0404, 0405, 0408) and HLA-DR14 (DRB*1402), which are more prevalent in RA with the strongest association with the DR4, and HLA-DR10(DRB*1001), in addition to some HLA-DR1β chain (DRB1*0101, and 0102) [27]. The HLA SE alleles are a risk factor for antibodies against cyclic citrullinated peptide (ACPA, as discuss later) and have reported to be a predictor for severity of established RA [28]. However, only 70% of patients with RA display susceptible HLA-DR, suggesting that these alleles are neither necessary nor sufficient for RA expression. Moreover, although the HLA-DR locus has been demonstrated as important in many cohorts [29], the association with DR4 or QKRAA is not as prominent or is not associated with RA in Greeks, Pakistanis, Chileans, and African-Americans [30, 31]. Importantly, the exact nature of the epitope and its role in the diseases are still under debate. For example, the presence of aspartic acid in position 70 alone has been reported to be protective [32], whereas individual with the sequence outside residues 70-74 indeed have much higher incidence and prevalence of RA [33-35].

1.1.5.1.2 Non-HLA susceptibility genes

Protein tyrosine phosphatase non-receptor type 22 (PTPN22), which encodes lymphoid tyrosine phosphatase (Lyp), is a tyrosine phosphatase involved in intracellular T cell signaling [36]. The minor allele of a single nucleotide polymorphism (SNP) in PTPN22 has been found in nearly 15% of patients with seropositive RA [37] and may increase the rate of progression of RA [38]. This SNP directly changes the amino acid sequence of the encoded protein resulting in enhanced regulation of T-cell receptor signaling during thymic selection, allowing autoantigen-specific T cells to escape clonal deletion, thereby predisposing to autoimmunity. PTPN22 polymorphisms have been identified in European populations but are rarely found in Asian populations. In contrast SNPs in the gene encoding for peptidyl arginine deiminase (PADI), an enzyme responsible for the post-
translational modification of arginine to citrulline, have been found more consistently in RA patients from Asian than in European populations [39, 40]. In a Japanese study, a strong association was observed between eight SNPs in the PADI4 genes and susceptibility to RA, as suggested by an extended haplotype in the PADI4 gene that can enhance the stability of PADI4 mRNA and therefore increase the cellular levels of PADI4 protein and citrullinated peptides [41]. Despite this, no consistent association between PADI4 gene and circulating anti-CCP antibodies has so far been demonstrated [42, 43].

A growing list of genetic risk factors for RA has recently been developed using advanced genotyping techniques (GWAS) and increased knowledge of the human genome. Newly recognised RA susceptibility genes include an allele of the Fcγ receptor, a polymorphism marker in the β2-adrenergic receptor; a low-indicible allele of the cytochrome P450 subtype 1A2 [44], nuclear factor κB (NF-κB)-dependent signaling (e.g. TRAF1-C5, TNFAIP3) and genes involved in T-cell activation (e.g. STAT4, CTLA-4) [45, 46], as well as certain polymorphism in cytokine, cytokine-receptor loci, inflammatory mediators and chemokines. However, all newly discovered genetic risk factors are insufficient alone for full expression of the disease and do not explain the major part of heritability of RA as they are also fairly common in healthy individuals. Although the contribution of each gene is relatively small compared with class II MHC, a combination might provide an appropriate genetic background to influence the course of RA.

1.1.6 Environment

1.1.6.1 Environmental factors inducing protein citrullination: Smoking and periodontal disease

Among environmental factors, smoking has by far the strongest association with RA. Cigarette smoking not only increases susceptibility to RA, but is also a risk factor for greater disease severity. Compared to those who had never smoked, patients with a smoking history are more likely to be seropositive, have nodules or radiographically apparent erosions [47, 48]. Multivariate analysis revealed that the duration of use, but not
the number of cigarettes smoked per day, was significantly associated with increased risk [49]. Accordingly, smoking cessation may help prevent RA development. Other potential factors include silica dust, mineral oils, and other airborne exposures. Indeed, in a Swedish study, it was suggested that inhaled components of cigarette smoke, rather than nicotine, may be more important than nicotine itself in etiology [50].

More importantly, in ACPA-positive individuals the combination of the presence of shared epitopes and smoking increases the relative risk of developing RA by 21 folds [51]. It was proposed that long-term smokers have augmented PADI activity in macrophages, which increased the deposition of citrullated proteins in their lungs [52]. The association between RA and periodontal disease has long been postulated [53]. RA patients have significantly higher prevalence of chronic periodontitis compared to normal individuals. However, the potential biological explanation for such association has long been obscure. Recently, evidence that the bacterium responsible for periodontal disease, *porphyromonas gingivalis*, expresses abundant level of PADI and is able to induce protein citrullination [54], has led to the hypothesis that chronic periodontitis may be a fundamental factor for inducing breach of immunological tolerance to citrullinate proteins, leading to humoral autoimmunity in the pre-clinical stage of RA. In keeping with this scenario, a recent study identified a high prevalence of antibodies against citrullinated alpha-enolase in RA patients, which associate with both the SE and smoking [55] and may also cross-react with bacterial enolase, particularly in RA patients with *porphyromonas gingivalis* infection [56].

1.1.6.2 Infectious agents

In addition to *porphyromonas gingivalis*, several microorganisms have been implicated in the development of RA in a genetically susceptible host. Among the bacteria that have been suspected are mycoplasma species [57, 58], and mycobacterium tuberculosis [59]. Among viruses, possible candidates include Epstein-Barr virus (EBV) and parvovirus B19 [60, 61].
EBV is one of the best studies for evaluating the possible association of viruses with RA. Sera of RA patients contain high titres of antibodies to latent and replicative EBV Ags. Interestingly, EBV glycoprotein gp110 contains the same QKRAA sequence of the SE on the HLA-DRB1*0401 allele [24]. Thus, in certain EBV-affected individuals, the mimicry molecules may drive autoimmunity. In addition, EBV is a polyclonal activator for B cells [62] and can stimulate the proliferation of autoantibody producing B cells [63]. EBV DNA has also been detected in higher amounts in the blood, synovial fluid and saliva of patients with RA as compared to healthy individual [61]. There are conflicting data on the remaining organisms, for example parvovirus B19, which has been found in RA synovial tissue and fluid, and has been reported to be capable in transforming normal synoviocytes into an invasive phenotype [64]. Contrary to this though, acute parvovirus B19 arthritis patients show no development of chronic arthritis [65] and there are data demonstrating the presence of its DNA also in the synovial tissue of healthy individuals [66].

**1.1.7 Current pharmacological therapies of arthritis**

If RA is left untreated or insufficiently treated, joint destruction, subluxation and permanent disability are the likely outcomes. RA patients appear to have at least 2-fold increase in mortality, and this increased risk of mortality is primarily attributed to an increased incidence of cardiovascular disease [67]. Nowadays, there is no known cure for RA but many treatments are able to stop the progression of this chronic illness. Therefore, the goals of treatment are to relieve symptoms of joint pain and swelling, to prevent joint damage and to decrease morbidity and mortality [68]. Treatment of RA has been radically transformed over the last few decades. The first anti-inflammatory agent used for treating RA was acetylsalicylic acid which was introduced later as Aspirin [69]. Subsequently, other substances were developed with similar drug activity and were grouped in a category called non-steroidal anti-inflammatory drugs (NSAIDs). These substances do not inhibit the progress of RA, instead they decrease inflammation and provide fast relieve of pain and stiffness by inhibition of constitutional cyclooxygenases (COX)-1 and induced COX-2, which are enzymes implicated in prostaglandin synthesis.
Another category of drugs is corticosteroids, which have both anti-inflammatory and immunosuppressive activity. Glucocorticoids are used for suppression of more severe symptoms, which act on the inflammatory process by interfering with the cytokine network, inflammatory enzymes, adhesion molecules, permeability factors, cellular function and survival [70, 71]. Treatment with glucocorticoids is proved to be very effective in suppressing the inflammation of RA [72] and has also been reported to reduce the progression of joint destruction in RA [73, 74]. Prolonged treatment with glucocorticoids, however, exerts many serious side effects which are sometimes more severe than RA itself. These include diabetes, hypertension, excess weight gain and osteoporosis [75]. In clinical practice glucocorticoids have traditionally been recommended for short periods during acute phase of disease, in particular, in combination with disease modifying anti-rheumatic drugs (DMARDs) or in the period before DMARDs. Unlike NSAID and corticosteroids, DMARDs are “slow acting” drugs, which are applied for a long period of time. Several “conventional” DMARDs that are currently available for the treatment of RA include cytotoxic drugs such as methotrexate (MTX), leflunomide, cyclosporine, azathioprine, sulphasalazine as well as the anti-malarial drug hydroxychloroquine (HCQ). These drugs are moderately to well tolerated [76] and to certain extent are effective in suppressing the rheumatoid process [77]. DMARD monotherapys are all shown to have beneficial effects on RA activity, but its effect is more pronounced when used either in combination therapy with other DMARDs or in conjunction with biologic agents [78, 79].

In recent years, medications directly targeting specific pathways of the immune system, such as cell surface markers or proinflammatory cytokines that lead to joint inflammation and other abnormalities seen in RA, have been developed and described as biologic DMARDs. These new biologic agents are genetically engineered drugs and because of their expense and side effects, it is recommended that most people with mild to moderate RA be treated with conventional DMARDS before the use of biologic agent unless they are failing to response to the use of combined conventional DMARD therapy.
The first of these biological therapies was the tumor necrosis factor (TNF) blocking agent, Infliximab [80]. Many different anti-TNF antibodies are now used in clinical practice which includes infliximab, adalimumab, etanercept, and the newer golimumab and certolizumab. These medications are effective when used alone, but are often given in conjunction with other DMARD to increase benefit on clinical response, prevent radiographic progression and to reduce the negative effects [81]. Despite the achievement of anti-TNF treatments, up to 40% of patients with RA may not have a clinical response to anti-TNF therapy [82]. Therefore new biologics have emerged. The evidence for the role of B cells in the immunopathogenesis of RA is increasing rapidly. The migration of B cells into inflamed synovium therein: (1) participating in synovial follicular microstructure; (2) performing their classic role in the presentation of antigen to CD4+ T cell; (3) producing auto-antibodies raise its contributing role in the etiology of RA. The clinical impact of B cell targeted therapies includes depleting B cells (an anti-CD20 agent, Rituximab; and an anti-CD22 antibody, Epratuzumab) or targeting B cells survival factors (anti-B cell stimulator protein agent, Belimumab/LymphoStat-B®, a human mAb that specifically recognises and inhibits the biological activity of B cell survival factor of the TNF family, BAFF; Atacicept, a fully human recombinant fusion protein that blocks the activity of BAFF and of a proliferation-inducing ligand, APRIL [83].

Recently, the therapeutic intervention of RA has become more focused on the very beginning of the disease [84]. It has been demonstrated that patients treated in this early period have higher clinical response rates to the mediation than those receiving delayed therapies [85] and they experienced less radiographic damage over time [86]. The intensive treatment in the very brief period when arthritis begins and the development of radiological progression is referring to the so-called “window of opportunity”. This concept asserts that the use of current therapy may not only shorten the effective induction of low disease activity, but also effectively change the clinical course of the disease.
1.2 Synovitis of RA

1.2.1 Normal synovium

Joints that support free mobility of the bone are termed diarthroses. Diarthrodial joints are surrounded by a fibrous membrane capsule that encloses a fluid-containing cavity. It is composed of two distinct layers: an outer layer consisting of a thick, dense connective tissue called the fibrous layer (stratum fibrosum), and an internal one termed the synovial membrane or synovium (Figure 1.2), which helps in mediating nutrient exchange between blood and joint. In the normal state, synovium is divided into two anatomical and functional compartments: the external layer (subintima) and the inner layer (intimal).

![Diagram of diarthrodial joint](image)

**Figure 1.2. Diagram of diarthrodial joint.** Modified image adapted from *Anatomy of the Human Body, Henry Gray, 1918.*

The intimal lining is the most superficial cell layer in contact with the joint cavity. The lining is normally one or two cells thick and composed of type A and type B synoviocytes, representing about one-third and two-thirds of the normal synovial lining cell, respectively (Figure 1.3 and Table 1.5). Synoviocytes are distinguished from other fibroblasts due to their location on the internal connective tissue surface. The synovial lining lacks epithelial cells (EC), tight junctions or desmosomes. Instead, it develops sporadic, discrete and discontinuous regions of contact with wide interceullular spaces. This is in contrast to other serosal surfaces, whose integrity is maintained by densely...
compacted cells that form extensive interactions. Furthermore, the synovial lining lacks a classic basement membrane that underlies most other body lining surfaces. Rather, it is composed of densely packed cells within a meshwork of dense extracellular matrix interspersed with collagen fibrils and other matrix protein. Overall, the synovial lining creates a cellular basement membrane between the synovial fluid compartment and a richly vascularised synovial sublining region [87]. The healthy sublining layer is normally paucicellular and mostly composed of fibroblasts and macrophages and it contains blood vessels, lymphatics, and nerves. Occasionally, lymphocytes, mast cells and adipocytes are also found in the sublining layer.
Figure 1.3. Diagram showing the location and structure of two types of synoviocytes.

Type A cells (A) are located at the superficial layer of the synovial lining and type B cells (B) characterised by a cytoplasm rich in large pale nuclei and cytoplasmic processes, are present at various depths. Frequently found in the deeper layer of the synovial lining. Modified image adapted from Iwanaga et al Arch Histol Cytol 2000;63(1):17-31 [87]

<table>
<thead>
<tr>
<th>Marker</th>
<th>Macrophages-like synoviocytes</th>
<th>Fibroblast-like synoviocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>CD16/CD Immunoglobulin G Fc receptor</td>
<td>Type IV and V collagens</td>
</tr>
<tr>
<td></td>
<td>CD45 Leukocyte common antigen</td>
<td>Structural protein 64</td>
</tr>
<tr>
<td></td>
<td>CD14 LPS/LBP receptor</td>
<td>Vimentin Intermediate filament</td>
</tr>
<tr>
<td></td>
<td>MHC class II Major histocompatibility complex Class II</td>
<td>Thy-1 CD90</td>
</tr>
<tr>
<td></td>
<td>CD11b/CD18 Integrin adhesion molecule and complement receptor ‘Mac-1’</td>
<td>MHC class II Major histocompatibility complex class II</td>
</tr>
<tr>
<td></td>
<td>CD68 Lysosomal glycoprotein</td>
<td>ICAM-1 Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>Relative specific</td>
<td>UDPGD Uridine diphosphoglucose-dehydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCAM-1 Vascular cell adhesion molecule-1, CD106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DAF Decay accelerating- factor, CD55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cadherin-11 Calcium-dependent adhesion molecule-1</td>
<td></td>
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Table 1.5. Selected markers expressed by macrophage-like and fibroblast-like synoviocytes.

Table adapted from Bartok & Firestein Immunological Reviews 2010 Vol. 233: 233–255 [88]
1.2.1.1 Type A synoviocytes

Type A synoviocytes are macrophage-like synovial cells localised in the more superficial regions of the intimal lining. Their belonging to tissue macrophages is evidenced by the fact that they are immunoreactive to several monoclonal antibodies against macrophages or macrophage-derived substances [87]. For instance, they express CD11b, CD68, CD14, CD163 and abundant MHC class II such as HLA-DR and the Fc receptor. Like other tissue macrophages, type A cells are derived from bone marrow and are terminally differentiated cells that have little capacity to proliferate. They likely migrate to the synovium and become resident cells, although it is still under debate whether the differentiation takes place in situ or prior to arrival [89]. Physiologically, type A cells maintain homeostasis of the joint cavity by phagocytosing wastes and cell debris in the synovial fluid and intimal matrix. Other characteristics of these cells include antigen presentation to T cells, the ability to produce prostaglandin as well as in acting as a primary source of several cytokines in the joint, such as interleukin-1 (IL-1), IL-12, IL-15 and tumor necrosis factor-α (TNF-α).

1.2.1.2 Type B synoviocytes

Type B synoviocytes or fibroblast-like synoviocytes show two phenotypes: intimal and subintimal. Currently, it is unknown whether intimal and subintimal fibroblasts arise from independent lineages, and because of the difficulty in determining the source of these fibroblast cells, a general term `synovial fibroblast' (SF) has been applied [87].

One of the major functions of SF in the normal joint is the production of hyaluronan, an important constituent of synovial fluid and extracellular matrix (ECM). Uridine diphosphoglucose dehydrogenase, a rate-limiting enzyme for synthesis of hyaluronan is therefore preferentially expressed by SF [90]. SF also secretes the synovial fluid constituent lubricin, which is critical for joint lubrication [91]. Other specialised products may include vimentin (an intermediate filament that is widely expressed in mesenchymal cells and macrophages) [92], phospholipids [93] and unusual carbohydrate entities [94]. Also, molecules associated with basal laminae, including fibronectin, type IV collagen, laminin, chondroitin-6-sulphate-bearing proteoglycans, tenascin-C (an extracellular
matrix glycoprotein associated with tissue injury and repair), type VI collagen microfibril and fibrillin-1-containing microfibril, are expressed by SF for continuing maintenance of the synovial ECM. Further, SF are also actively involved in collagen synthesis, reflected by the presence of prolyl-4-hydroxylase, procollagen I and III in the cytoplasm of SF [89, 95].

In addition to specialised matrix synthesis, SF expressed a number of cell-surface related molecules. For instance, they constitutively show prominent expression of adhesion molecule vascular adhesion molecule 1 (VCAM-1)/CD106 and decay accelerating factor (DAF). Integrin species (α5β1 and α,β; intergrin receptors), intercellular adhesion molecules (ICAM-1)/CD54, CD55 (complement decay accelerating factor), CD44 and CD90/Thy-1 are also reported to be expressed by SF [96]. Recently, cadherin-11, an integral membrane adhesion molecule, is reported to be abundantly expressed on SF and serves as a major mediator for homotypic aggregation of SF in vitro and in vivo [97]. Its importance in the synovial architecture was confirmed in cadherin-11 deficient mice, in which the synovial intimal lining was virtually nonexistent [97, 98]. Normal SF also synthesises degradative enzymes, such as serine protease, matrix metalloproteases (MMPs) and the membrane type-MMPs (MT-MMP). The distinct features of synoviocytes implicate them not only in the healthy purpose of the joint but also in the abnormal activities that occur during inflammatory disease such as RA. The mechanism by which this appears to occur will be discussed in depth below.

1.2.2 The rheumatoid synovium

Inflammation in the joint first involves the synovial membrane, which is the central site to the pathophysiology of RA. The early changes in rheumatoid synovium tissue are manifested in hyperplasia of synovial lining cells, accompanying injury of microvasculature with vascular congestion, microvascular damage/occlusion and transformation of synovial endothelial cells into high endothelial venules. This inflammatory stage is often associated with edema, vascular proliferation, necrosis and fibrosis of synovial stroma as well as exudation of fibrin characterised by fibrin deposits on the surface of the synovial lining (surface fibrin deposits) and/or within the synovial
stroma itself (stromal fibrin deposits). Angiogenesis is also an early and critical event in the pathogenesis of RA, which promotes ingress of leukocytes and pannus formation [99].

Thickening of the synovium is the hallmark of RA. Within a short period of time the cell lining expands to 10-20 cells thick. The sublining region undergoes striking alternation in both the composition and number of cells, with prominent ingress of T cells and macrophages, along with B cells, plasma cells (PC) and dendritic cells (DC). The role of some of these cells in pathology and joint destruction will be discussed later in this chapter. Besides these cells, polymorphonuclear cells are sometimes present, particularly during acute phase in RA. Natural killer cells, mast cells and neutrophils have also been identified, with the latter appearing in very large numbers. Neutrophils are mainly found in the synovial fluid but they can also be observed close to the synovial-cartilage junction [100].

The synovial lining is often folded into numerous villi that cover the cartilage-bone interface and isolate it from the synovial fluid. This expansion of synovium is termed pannus, which behaves like a locally invasive tumor with cells composed of macrophages, osteoclasts and invasive SFs with relatively few lymphocytes. A formation of resorption pit (Howship’s lacunae) is responsible for the degradation of bone mineral and collagen matrices caused by the activated osteoclasts (Figure 1.4). Subchondral bony erosions are the ultimate characteristic of RA.
Figure 1.4. In RA synovium, SFs interact with diverse cell types promoting inflammation and joint destruction.

In a RA joint, the synovium is swollen due to an infiltrate of immune cells. (1) Pannus tissue, an extension of the hyperplastic synovial lining consisting of both activated fibroblast-like and macrophage-like synoviocytes. (2) SFs actively degrade cartilage by producing matrix degrading enzymes (MMPs and cathepsin). (3) SFs promote osteoclast differentiation and activation via RANKL, leading to bone destruction. Also, SFs may produce osteoblast activity inhibitor (DKK-1). (4) Sublining SF interact with numerous cell types: T cell recruitment and retention by SF-secreted chemokines (SDF-1 and CD40). Although SFs do not normally express MHC its expression is seen on freshly isolated RASFs and can be induced upon T cell cytokine (IFN-γ). RASFs can in turn secret cytokines such as IL-15, which are involved in Th17 differentiation; B cells are similarly recruited and retained via SF-derived chemokines (SDF-1, CXCL13) and via adhesion molecules (VCAM-1). SF-derived IL-6 can in turn promote B cell survival; DCs in the synovium present antigen to T cells and activate T cells, thus providing help to autoreactive B cells; Macrophages are activated to produce many kinds of proinflammatory products partly by immune complexes binding to surface receptors such as Fcγ receptors; SFs can produce proangiogenic factors (VEGF) which can directly promote inflammatory cell recruitment via endothelial cells. Finally, the cell-cell adhesion molecules cadherin-11 links RASFs to other RASFs, which is crucial for synovial lining formation. SFs interact with macrophages via soluble factors (TNF-α, IL-1 and IL-6) or adhesion molecules (ICAM-1 and VCAM-1).
In tissue homeostasis and immunoregulation, cells die by apoptosis and are ingested by macrophages, which concomitantly avoid an inflammatory reaction, compatible with prevention of tissue damage. Upon tissue injury that occurs during hypoxia and acidic conditions, cells die of a necrotic process, releasing their contents and inducing a rapid inflammatory response, which damages the surrounding tissues in the short term but is also a prerequisite for tissue remodeling and repair. Many studies have previously suggested that the rheumatoid synovial microenvironment is relatively ischemic, hypoxic [101, 102] and highly genotoxic (deleterious milieu on a cell's genetic material affecting its integrity). Due to inflammation and expansion of the synovial membrane during RA development there is increased pressure in the synovial cavity, reduced capillary density and increased metabolic needs in the arthritic joints, resulting in abundant reactive nitrogen and oxygen and cellular stress that normally kills cells [103].

Interestingly, RASFs express death receptors, such as Fas/CD95, which when crosslinked by antibodies induce apoptosis [104]. However, RASFs were shown to be relatively resistant to FasL, TNF, and tumor necrosis (TNF)-related apoptosis inducing ligand (TRAIL)-induced apoptosis [105]. This resistance has been related to the high expression of anti-apoptotic molecules such as Fas-associated death domain-like IL1 beta-converting enzyme-inhibitory protein (FLIP), sentrin-1, members of the Bcl-2 family (such as Bcl-2 and Mcl-1), and constitutive activation of Akt (protein kinase B) [88, 106]. In contrast, other Bcl-family members such as Bax, Bak, BH3 interacting domain death agonist (Bid) and Puma are pro-apoptotic and promote mitochondrial membrane permeability [88]. It has been reported that phosphorylation of Akt in RASFs protects against Fas-induced apoptosis through inhibition of Bid cleavage [106].

Cells that enter the rheumatoid joints are exposed to a number of pro-inflammatory cytokines and immune complexes, typically present in the synovial fluid. Indeed, the deposition of immune complexes has been considered to be a major determinant of neutrophil-mediated articular tissue injury, which is responsible for the irreversible anatomical destruction of the normal tissue architecture with consequent articular dysfunction [107].
Recently, Dubikov et al reported that the rate of apoptosis in RA synovial cells was associated with the presence of Bcl-2 and p53, in which their expression level was correlated to disease severity (increased from early to late RA) [108]. It would thus appear that the ratio of anti- and pro-apoptotic molecules within the rheumatoid joint constitutes a rheostat that sets the threshold for susceptibility to activation-induced cell death.

In line with these observations, necrotic cells may also be found in RA joints as a result of inflammatory and destructive processes. Whereas necrotic cells can easily be detected in the synovial fluid, they are not typically present in large quantities in the synovial tissue. However, apoptotic cells in synovial tissue may undergo secondary necrosis [109]. It has been described that the RNA released from necrotic, but not apoptotic, synovial fluid cells may act as an endogenous ligand for toll-like receptor 3 (TLR3) and activates RASFs for the stimulation of proinflammatory gene expression [110]. (Details on TLR will be discussed later in section 1.4.2). Accordingly, it is generally accepted that the presence of necrotic cells, especially neutrophils, is proinflammatory [111]. However, it has also been suggested that not all necrotic cells pose a danger. A study of Miles et at proposed that necrotic human neutrophils exert anti-inflammatory effects via the release of α-defensin, and if a macrophage encounters such a cell its ability to secrete pro-inflammatory cytokines and nitrogen oxide is inhibited, while its ability to phagocytose material is increased [112]. Therefore, neutrophil necrosis may initiate resolution response at sites of inflammation.
1.2.2.1 T cells in RA

T cells constitute the most prominent infiltrating lymphocytes within the sublining region, where they account for 30-50% of all cell types. The activated CD4+ T-helper (Th) 1 cell predominates over Th2 in RA disease. For instance, human rheumatoid synovial membrane and synovial fluid presents large amounts of interferon γ (IFN-γ), a signature Th1 cytokine, but no IL4, a typical Th2 cytokine [113]. Multiple animal models have identified a new subset of Th cell characterised by the production of IL-17A (henceforth called IL-17) by Th17 cells in the RA joint pathology [114]. In human studies, IL-17 has been shown to induce monocyte- and fibroblast-derived proinflammatory cytokines (TNF-α, IL-1β, IL-8), which, mediate of bone and cartilage damage such as MMP and receptor activator of nuclear factor kappa B ligand (RANKL) [115] as well as in induce osteoclastastogenesis [116]. Likewise, CD4+ Th17, but not IL17 producing γδ T cells, drive arthritic bone destruction in mice and human [117]. The importance of CD4+ T cells can also be shown in their transfer to severe combined immunodeficient (SCID) mice engrafted with human synovium, which leads to synovial inflammation by antigenspecific T cell response [118-120]. Conversely, a recent study using the proteoglycan induced arthritis (PGIA) animal model, demonstrated that T cell homing to secondary lymphoid organs (SLOs) is more important than migration to the joint [121, 122].

Costimulation of naïve T cells with CD28 after engagement with its ligand B7-1/CD80 is important for T cells survival and proliferation. To limit uncontrolled T cell activation, stimulated T cells upregulate expression of a second receptor for B7-1, cytotoxic T lymphocyte antigen-4 (CTLA-4), which is an inhibitory receptor and has a greater affinity for B7-1 relative to CD28, thus acting as modulator for T cell activation. In clinical trials, CTLA-4 immunoglobulin fusion protein (Abatacept) yields promising result as a monotherapy, in combination with methotrexate, as well as in patients who are refractory to anti-TNF therapy [123, 124]. These findings highlight the importance of cell-cell interaction mechanisms for mediating T cells activation.

Another CD4+ subset, T regulatory cells (Treg), is also included in the new paradigm for RA based on their participation in regulating autoimmune inflammatory response in vivo. Their role is further strengthened by the observation that RA patients are defective of
CD4^+CD25^+ Treg cells [125]. However, the contribution of Tregs is still controversial as the protective role is dependent on both their number and function. For instance, Treg cells in RA joint fluid failed to inhibit the production of IFN-γ and TNF-α by CD4^+ cells or monocytes. This observation could be explained by an intrinsic inability of RA Tregs in suppressing, or by the fact that the large amount of proinflammatory cytokine secreted in the RA joint fluid influences the sensitivity of effector T cells to be suppressed [126].

Finally, CD8^+ T cells, which are primarily associated with cytotoxic responses towards virally infected cells, can be abundantly expressed within the RA synovium where they may contribute to chronic inflammation via different mechanisms [127, 128]. For the purpose of this thesis, it must be noticed that a subset of non-cytotoxic CD8^+ T cells has been shown to maintain ectopic GC in the RA synovium and are able to modulate antibody production from synovial tissue engrafted into SCID mice [129].

1.2.2.2 B cells in RA

Compared to T cells, the infiltration of B cells is subtle in the pannus lesion where they constitute about 5% of the sublining synovial cells. However, they are a significant and constant population in those RA synovium characterised by the presence of lymphoid follicles in the sublining region [130]. Together with (auto) antibody production, cytokine release and antigen presentation, B cells can also regulate lymphoid tissue architecture and ectopic lymphoid neogenesis, which, as mentioned below, is a common feature in the RA synovial membrane. The role of the interaction between resident stromal microenvironment and B cells in RA pathology will be discussed in later chapter (1.4).

The first evidence for a role of B cells in RA is based on the observation of high levels of circulating autoantibodies in RA patients. Rheumatic factor (RF) is an autoantibody with specificity against the Fc portion of self-IgG and has been shown to fix and activate complement. RF itself has not been recognised as a causative agent in arthritis when transferred into healthy individuals [131], but it is a positive predictor of disease severity and progression in RA [132]. On the other hand, RF is not very specific for RA as it can also be detected in up to 10% healthy individual as well as in other autoimmune conditions including SLE. However, in healthy individuals RF is a low affinity IgM
produced by B1 cells as a natural antibody, whereas RF in RA patients undergoes class-switching as a consequence of B cell-T cell interaction, a phenomenon also observed for anti-CCP antibody. Autoantibodies such as anti-Sa and anti-CCP are dependent on the citrullinated protein. Citrullination is a form of post-translational modification of protein, in which the amino acid arginine is converted into citrulline by peptidylarginine deiminases [133, 134]. This process occurs in the inflamed synovium as well as in other inflamed tissue. In relation to RA, the target antigens include citrullinated fibrinogen, vimentin or α-enolase or type II collagen. A recent study indicated that, autoantibodies against citrullinated protein, though not directly pathogenic, were able to increase arthritis severity in a murine model [135]. This may suggested that that B cells and/or their products are crucial in the exacerbation of RA disease, possibly by amplifying inflammation. Besides producing antibodies, B cells can also act as efficient antigen-presenting cells (APC) to T cells. RF⁺ B cells can take up antigen in a context of IgM-RF-IgG complex via membrane bound Ig receptor, thereafter, they can target for T cell help and activation by several specific foreign antigens. In contrast, nonspecific B cells are unlikely to present antigen to T cells even though they have the ability in binding antigen-antibody complexes [136]. In line with this, T cell activation in the synovial tissue of RA patients is dependent on the presence of B cells. Furthermore, activated effector B cells are able to secrete polarised assays of cytokines.

B cells role in RA disorder has been proven by the effectiveness of B cell depletion therapies. Rituximab is a B-cell-cytolytic chimeric IgG1 CD20-specific monoclonal antibody, which can potently kill B cell subsets from the immature stage (pre-B-cell) up to the memory B cell stage. Alternatively, atacicept, a fusion molecule of the soluble transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor (TACI) receptor and IgG, binds to and sequesters two key B-cell factors involved in RA: the B cell associated survival factors, BAFF and APRIL the B cell associated survival factors, BAFF and APRIL [83]. BAFF and APRIL are involved in B cell survival, differentiation and class-switching during different stage of B cell development. Recently, atacicept has been shown to decrease levels of IgG, IgM and IgA, as well as naive B cells and PC (which are CD20 negative). In Phase II studies in patients
with RA, atacicept 150 mg was associated with median IgG decreases (~30%) [137]. The critical of B cells role and the contribution of survival factors in RA pathophysiology is further discussed in details in section 1.3.
1.2.2.3 Histopathology of synovitis, formation of B cell follicles

Multiple lines of evidence suggest an Ag-dependent immune reaction in a subset of patients with RA, who has the ability to create lymphoid microstructures in a nonlymphoid organ, such as the synovial membrane. There is mounting evidence showing that RA is a heterogeneous entity with three distinct histologically defined phenotypes in the synovial membrane: 1) leukocytes infiltrate into the sublining region of synovium with a diffuse pattern (about 50%); 2) cells cluster as perivascular cuffs forming lymphoid aggregates (about 25%); 3) a high degree of cellular organisation where the leukocytes are organised into secondary follicles with a germinal centre (GC) phenotype, extensively expressed within follicular dendritic cell (FDC) networks accompanied by active proliferating B cells expressing the maturation marker CD20 and proliferation antigens such as Ki67 (about 25%) [138]. The latter can arrange and organise themselves anatomically and functionally as in secondary lymphoid organs (SLO), leading to the de novo formation of B cell follicles and T cells area. This phenomenon has been termed lymphoid neogenesis, or tertiary lymphoid organ (TLO) or ectopic lymphoid structure (ELS). Manzo et al [139] demonstrated that ELS in the rheumatoid synovium consist of a more continuous spectrum, with lymphocytic aggregates displaying different stages of lymphoid neogenesis (Figure 1.5).
Distinct pathways of lymphoid neogenesis can be set in place by an Ag-triggered immune response occurring in extranodal tissue. Selection of the pathway depends on modulating host factors, e.g. aberrant expression of cytokines/chemokines, the attitude of immune system dysregulation and the extent for recruitment of cellular components are all required for determine certain types of lymphoid microstructures. Organisation of the three types of lymphoid infiltrate may suggest RA is a heterogeneous entity or a continuous spectrum that represents different stages of RA. Modified image adapted from Weyand et al Am J PatholVol: 159:3. [140]
Among the chemokines that regulate the compartmentalisation of T cells and B cells, CXCL13 (formerly BLC or BCA-1) and CCL21 (formerly 6Ckine or SLC) seem to be especially important, and their expression in rheumatoid synovium correlates with the presence of ELS [141]. CCL21 is highly expressed on stromal cells within the T cell zone of SLO, endothelial cells of high endothelial venules [142] and lymphatic vessels [143], inflamed synovial specimens of RA [141], fibroblasts from RA synovial membrane and macrophages from RA synovial fluid [144]. CXCL13 is recognised as one of the most important chemoattractors for B cells. Its corresponding receptor, CXCR5, is mainly expressed on B cells [145] and thus CXCL13 has proven to be crucial in the formation of ectopic B cell follicles in RA [146]. In RA, high expression of synovial CXCL13 is shown to be associated with the presence of synovial lymphoid aggregates that resemble GC compared with synovial diffuse infiltration [141].

Takemura et al showed that the architecture of lymphoid structures in rheumatoid synovium is also regulated by members of the tumor necrosis factor (TNF) superfamily, lymphotoxin (LT)-α and LT-β, and these proteins can form two different trimeric molecules; a membrane-bound heterotrimer LTα1β2 (one α chain and two β chains) that binds exclusively to LTβ receptor (LTβ-R), or soluble homotrimer LTα3 that binds to TNF-R1 and TNF-R2. A third related member of this family is called LIGHT (LT-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells). LT-α1β2 and LIGHT, but not LT-α3, are present in RA and have proven to be predisposed for follicular synovitis [146]. Also, LIGHT may activate non-lymphoid cells expressing HVEM or LTβR, such as RASFs, which in turn up-regulate the expression of proteinase MMP-9, increase the expression of adhesion surface molecule CD54 and increase the release of proinflammatory cytokine IL-6 [147].

The development of ELS in rheumatoid synovial tissue, however, has not been reported to confine a specific clinical subset of RA. Thurlings et al demonstrated that lymphoid neogenesis is related to increased expression of systemic inflammatory biomarkers, but not to IgM-RF or ACPA [148], an observation that contradicts with the findings from Klimiuk PA et al [149], and thus, suggesting that the synovial inflammatory responses
varies among patient joints and over time. Of significance, rheumatoid synovium has been regarded as an auto-antibody producing organ as supported by the view that synovial PC are able to produce and secrete RF, ACPA and other auto-antibodies [150, 151]. Our group recently reported that, although the circulating auto-antibodies in RA are not associated with the presence of ELS, the production of auto-antibodies within the synovial tissue does correlate with ELS [152]. Accordingly, ELS+ synovium engrafted into SCID mice was shown to provide elements required for the survival of auto-reactive B cells and the subsequent production of circulating auto-antibodies, such as RF and anti-CCP [152] via local differentiation of autoreactive PC [153].

1.2.2.4 Innate immune cells in RA: macrophages and DC

The myeloid lineage gives rise to several cell types participating in the disease, such as monocytes/macrophages and CD11c+ myeloid DCs. These are only briefly mentioned here.

**Monocytes/macrophages.** Monocytes migrate from the blood into tissue and differentiate into resident macrophages. This takes place via sublining blood vessels in the normal and RA synovium. However, the amount of monocyte infiltrates, and the degree of macrophages accumulates, are markedly increased in RA synovium. The clinical relevance of monocytes/macrophages is not only revealed by the direct correlation between their number/activation with disease activity and radiographic progression of joint damage, but also, by the beneficial effect of therapies such as gold salts, [154], methotrexate [155], anti-TNF [156, 157] and monocyte apheresis [158]. Furthermore, monocytes/macrophages are a major source of cytokines, such as proinflammatory (TNF-α, IL-1) or regulatory cytokines (IL-10), as well as chemokines such as IL-8, MCP-1 and MIP-1α. In line with this, the highest levels of secreted IL-8 are observed in patients with seropositive RA and treatment with recombinant murine IL-8 markedly magnified experimental arthritis, indicating a correlation with activated macrophages in this disorder. Notably, IL-8 exerts a predominant role in angiogenetic processes [159], thus providing a link between macrophage activation and the well-known neovascularisation as observed in RA synovium. Furthermore, it is indicated that
macrophages are responsible for producing reactive oxygen species and degradative enzymes that drive distal joint inflammation and destruction, respectively.

**Dendritic cells.** DCs are the most potent subset of APC with a unique ability to activate naive T cells for helper and cytotoxic function. DCs are distributed in diverse locations such as mucosal and skin surfaces, where the potential for attack by pathogens is high. However, DC populations have also been found in RA joint fluid and synovium. In the latter, perivascular DCs are found tightly associated to T and B cell follicles. The infiltrate of DCs in the synovial tissue and fluid could take-up, process and present antigen locally, contributing to disease perpetuation. The local cytokines in RA synovium in turn promote DC accumulation where they undergo phenotypic and functional differentiation *in situ* that could lead to ongoing autoantigen presentation to T cells [160]. Moreover, animal models and histological evidence showed that DCs drive the formation and maintenance of ectopic lymphoid tissue in inflammatory environments, probably including the synovium [161]. Finally, evidence is accumulating suggesting that DC also play a role in the complications surrounding RA, including atherosclerosis [162].

Transfer of exogenous type II collagen-pulsed mature DCs is sufficient to induce arthritis in DBA/1 mice. More importantly, a model of pre-clinical arthritis demonstrated a regulatory function of plasmacytoid DCs (pDCs) in limiting self-reactivity and subsequent articular pathology [163], whereas conventional DCs (cDCs) are shown to orchestrate the initial breach of self tolerance [164]. Furthermore, immature human DCs may be directly involved in osteoclastogenesis and DC-derived-osteoclasts may promote osteolytic lesions as observed in RA [165].

**1.2.2.5 Synovial fibroblasts in RA, RASFs**

Along with the immune system, the resident cells of joints are also important players in RA pathogenesis, including osteoclasts, osteoblasts, chondrocytes and SF. Regarding SF, there is evidence to suggest that stromal activation within the synovium may be a first step for RA development. Details of RASFs involvement will be discussed in Chapter 1.4.
1.2.3 Cytokine networks in RA

Cytokines bind to receptors on target cells and regulate a vast array of biologic activities such as cell proliferation and release of other cytokines. The expression levels of cytokines exhibit vast heterogeneity between different RA individuals. Although many cytokines do not cause RA per se, they have been identified in the synovium or synovial fluid where they orchestrate the rheumatoid process. More importantly, neutralising antibodies against cytokines is an established treatment for RA, especially with TNF-α-directed approaches. As numerous cytokines have been implicated in RA, the role of IL-1 IL-6, IL-5 and TNF-α superfamily cytokines, and some novel cytokines have been selected to discuss in more detail below.

1.2.3.1 The IL-1 superfamily

IL-1 family cytokines are ubiquitous group of polypeptides with related origin, receptor structure and signaling pathways. They include IL-1α, IL-1β, IL-18, IL-18 binding protein (IL-18BP) and IL-1 receptor antagonist (IL-1Ra). All cytokines are synthesised as inactive precursors and cleaved by enzyme caspase-1 before or during release from the cell. IL-33 is a newly described member of this family due to the requirement of caspase-1 for processing and release.

1.2.3.1.2 Interleukin-1

IL-1α and IL-1β are produced by various cells such as monocytes, macrophages, neutrophils and hepatocytes. In human joint, synovial macrophages are the most abundant source for IL-1, and nearly half of the cells express IL-1β. The release of IL-1 in the synovial lining subsequently can activate SFs for proliferation, stimulate the biosynthesis of IL-6, IL8, and GM-CSF and induce the expression of adhesion molecule such as VCAM-1 and ICAM-1. This cytokine is also believed to mediate most of the articular damage in arthritis, because it induces the degradation of proteoglycan degradation [166], production of metalloprotease and collagenase [167], and enhances bone resorption [168]. Indeed, the level of this cytokine in the synovial fluid correlates with joint inflammatory activity [167].
Animal models show that injection of IL-1α and IL-1β into rabbit and rat knees results in arthritic manifestation similar to RA, which could be blocked by IL-1Ra [169]. In RA, the balance between IL-1 and its physiological inhibitor IL-1Ra is shifted in favour of IL-1. In line with this, IL-1Ra-deficient mice show spontaneous arthritis in an IL-17 dependent manner [170]. More importantly, even though synovial inflammation is developed in TNF-α transgenic mice crossed with IL-1 deficient mice, they had significantly reduced bone erosion and osteoclast formation [171]. Despite the accumulating evidence in favor of a pivotal role for IL-1 in RA pathology, the use of IL-1Ra (anakinra) has failed to produce adequate therapeutic value compared to other biologics, possibly due to its short half-life and the necessitate for very high concentration [172].
1.2.3.1.3 Interleukin-18

IL-18 was originally defined by its ability to bias the immune response towards the Th1 cells, and was described as IFN-\(\gamma\) inducing factor. In the context of RA, IL-18 has been described to be present in serum, synovial fluid and synovium, with significantly higher level than in OA patients [173] [126]. *In vitro* studies indicate that IL-18 induces the production of GM-CSF, nitric oxide, TNF-\(\alpha\), IL-6 and IFN-\(\gamma\) from RA synovial cell cultures. IL-18 and its receptor are abundantly found in RASFs and RA synovial macrophages, and the release of IL-18 by these cells is markedly increased by TNF-\(\alpha\) and IL-1\(\beta\), suggesting a potential role in the RA propagation [174, 175].

In the CIA model, injection of IL-18 increased the development of an erosive, inflammatory arthritis, suggesting that IL-18 can be pro-inflammatory *in vivo* [173]. In contrast, IL-18 deficient mice have reduced frequency and severity of CIA [176], and IL-18 blocking, either with IL-18 binding protein or anti-IL-18 antibodies, reduced the clinical severity of CIA [177]. The same inhibitory effect could be observed in IFN-\(\gamma\) knockout mice, demonstrating that other non-Th1-related activities of IL-18 might be significant. Interestingly, IL-18 knockout mice develop antigen-induced arthritis (AIA) with severity similarly to wildtype controls, suggesting that the contribution of IL-18 in the pathogenesis of arthritis may also be dependent on the model examined [178].

1.2.3.1.4 Interleukin-33

IL-33 was recently identified as ligand for the orphan IL-1 family receptor T1/ST2 with response polarised to the Th2 phenotype. SFs may be the main source of IL-33 [179]. Little or no IL-33 was found in resting RASFs, whereas its expression was markedly upregulated upon the presence of proinflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) [180]. Its expression has been detected in serum and synovial fluid from RA patients [181]. Furthermore, it has been demonstrated that IL-33 enhances CIA, whereas IL-33R knockout mice or mice that were administrated with soluble form of ST2 (a natural antagonist of IL-33) had attenuated disease severity [180]. More interestingly, Mun and colleagues showed that IL-33 can stimulate osteoclast formation and differentiation [182] whereas another study described it as an inhibitor of bone resorption [183].
1.2.3.2 Tumor necrosis factor superfamily

The TNF superfamily is an extensive group of related genes that exert diverse effects by stimulating a variety of cells. At least 19 members of the family have been identified, with TNF-α considered as the central cytokine in RA since its blockade is one of the most effective therapies developed for RA. Several other members (BAFF and APRIL) implicated in synovial inflammation will be discussed later in the relevant section (1.3).

TNF-α forms a membrane bound homodimer and is subsequently cleaved by TNFα-convertase, a member of MMP. TNF-α, similar to IL-1, stimulates collagenase and PGE2 production by human synovial cells [184], and it stimulates proteoglycan resorption and inhibits its synthesis in explant of cartilage [185]. It also has the ability to promote B cells proliferation and differentiation [186], enhance cytokine production, adhesion molecule expression and proliferation and MMP production from culture synoviocytes [88].

In RA, TNF-α is expressed at many sites within the synovium, including the cartilage/pannus junction [187]. Elevated levels of TNF-α, as well as its soluble receptor have been detected in synovial fluid of RA patients [188, 189]. In vivo studies demonstrated that overexpression of TNF-α in transgenic mice leads to development of arthritis [190]. Interestingly, spontaneous arthritis also establishes in transgenic mice that express only the membrane-bound form of TNF-α on T cells [191]. Accordingly, TNF-α blockade is an effective anti-inflammatory agent as shown in the CIA mouse model, though the effect on cartilage and bone damage is less prominent than that with IL-1 inhibitors [192].

Five TNF-blocking agents have been approved in clinical practice since 1998: 1) Infliximab (Remicade), a mouse-human chimeric anti-TNF-α IgG1 antibody; 2) Etanercept (Enbrel), a soluble dimeric fusion protein of human TNF receptor type II-IgG1 (TNFR-Fc); 3) Adalimumab (Humira), a fully human anti-TNF-α IgG1 monoclonal antibody produced by phage display technology.[193] Newer anti-TNFα, biologics have been approved recently, including 4) Certolizumab pegol (Cimzia), a TNFα-specific Fab fragment of a humanised monoclonal antibody and 5) Golimumab (Simponi), a human
monoclonal antibody. Another important member of the TNF superfamily for RA development is receptor activator of NF-κB ligand (RANKL), a type 2 transmembrane cytokine that is produced by bone and lymphoid tissue [194]. Its significant role is in the differentiation and activation of osteoclasts. The expression of RANKL is enhanced by several osteotropic factors/resorption stimuli such as IL-1, parathyroid hormone and 1,25-dihydroxyvitamin D₃ [195, 196]. Subsequently, the binding of RANKL to its receptor RANK induces signal for differentiation into osteoclasts via cell-cell contact with the precursor cells [195], thereafter, promoting osteoclastic bone resorption activity and survival. Osteoprotegerin, on the other hand, acts as a soluble ‘decoy’ receptor for RANKL and blunts osteoclastogenesis [196]. In regard to synovium, RANKL is highly expressed in synovial tissue of RA patients and is mainly produced by SF and T lymphocytes [197]. Cytokines, such as IL-17 [198], as well as TLRs (TLR2, TLR3 and TLR4) [199, 200], have been involved in the production of RANKL by RASFs. Denosumab, a fully human IgG2 monoclonal antibody that binds RANKL, has been approved in 2011 for postmenopausal osteoporosis and limitation for bone loss, and is currently in early phase II clinical trials for its potential in RA [201].

1.2.3.3 Interleukin-6

IL-6 is a protein produced by various cell types such as T cells, monocytes and culture SFs [202]. Originally defined by its B cell stimulating properties, it promotes immunoglobulin synthesis in B cell lines. It also actively participates in the differentiation of cytotoxic T cells, regulation of acute phase response from the liver and induces proliferation of synovial fibroblast-like cells [203].

Regarding RA, IL-6 is elevated in both serum and synovial fluid [204] of patients with RA. Moreover, serum IL-6 activity correlates with serum level of acute phase reactant, such as C-reactive protein, α1-antotrysin, fibrinogen and haptoglobin. It also participates in producing RF from B cells [205] as well as in stimulating osteoclast differentiation in combination with soluble IL-6 receptor α (sIL-6Rα or gp80) [206]. IL-6 blockade has undergone intense investigation for RA, whereby IL-6 knockout mice do not develop AIA [207] and blockade of IL-6 receptor attenuates CIA in mice [208]. More importantly,
a humanised monoclonal antibody against the IL-6R, Tocilizumab (Actemra), is currently approved for use in RA patients who failed anti-TNFα therapy [209].

1.2.3.3 Novel cytokines implicated in RA pathogenesis

Recently, the list of potential contributors to the pathogenesis of RA has been expanded for different cytokines. The most interesting are reported below:

1) IL-17 is produced by CD4+CD45RO+ memory T cells in the RA synovium [210]. In turn, the release of IL-17 triggers the production of other proinflammatory mediators by RASFs, such as IL-6, IL-8, GM-CSF and PGE2 [211]. It could also participate in the process of osteoclast formation [116], up-regulation of NO synthesis in cultured human cartilage [212] and triggering the production of proinflammatory cytokine in human peripheral blood macrophages [213].

2) IL-15 was shown to be increased both in the synovial fluid and serum of RA patients [214, 215]. RASFs were found to spontaneously secrete large amounts of IL-15 that are further upregulated upon TNF-α and IL-1β stimulation. Interestingly, IL-17 production was recently reported to be induced by IL-15 [216]. Moreover, both a soluble fragment of the IL-15Rα and an antagonistic IL-15 mutant/Fcγ2a fusion protein (binds to the receptor but do not undergo signaling), have been reported to have both protective and therapeutic actions in the CIA models [217, 218].

3) IL-23 is able to induce IL-17 expression by Th17 cells and IL-23 deficient mice are resistant to CIA due to their failure to develop IL-17-producing CD4+ cells [219]. In RA, a unique subunit of IL-23 has been abundantly detected in the RA, but not OA synovium even though the levels of bioactive IL-23 remain low [220].

4) IL-32 is a strong inducer of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8. Its expression is elevated in the rheumatoid synovium and correlated with the severity of joint inflammation. Importantly, RASFs have been described as a major
source of IL-32, which is tightly regulated by proinflammatory cytokines and TLR, especially TLR3 [220].
1.3 Role of B cells in RA pathogenesis

1.3.1 Physiological Human B lymphocytes and antibody-mediated immunity

B lymphocytes comprise one of the major arms of the immune system, being responsible for humoral (antibody-mediated) immunity, involving antibody production by B lymphocyte derivatives, PC. Similar to T lymphocytes, B cells are antigen specific and can provide immunological memory, the two hallmarks of the adaptive immune response. B cells bear the surface immunoglobulin on their plasma membrane, known as B cell receptor (BCR), which is functionally related to the T cell receptor (TCR). The BCR is capable of binding and recognising individual antigenic epitopes on soluble molecules. By contrast, TCRs do not recognise antigenic determinants on intact or undenatured molecules. Both BCRs and TCRs are products of site-specific somatic rearrangement, which allow an immense primary diversity of Ag specificities. In addition, B cells are capable of modifying their antibodies in a secondary diversification pathway during an immune response, resulting in a higher specificity for a certain antigen. Following antigenic stimulation, B and T cells divide and develop into specific effector cells (activated) or memory cells (primed). Memory cells become activated only during re-exposure to the same antigen, while effector cells function irrespective of the presence of Ag. Effector cells are generally short lived with a half-life of only a few days in comparison to the long-lived (months to years) memory population.

1.3.1.1 Antibody

As mentioned above, different sIg are continuously produced and inserted into the B cell membrane, where they act as specific Ag receptors. Apart from the transmembrane form, the antibodies can also be expressed in a secreted form, which is solely produced by the end-stage of B cell differentiation, the PC. Antibodies or the so-called immunoglobulins are glycoproteins. Their structure is bilaterally symmetrical, comprising two identical light and two identical heavy polypeptide chains, forming roughly a Y-shaped molecules (Figure 1.6). Each chain can be divided into two distinct regions where one is very varied in sequence (variable region) while the other is relatively constant (constant region). The
site at which the antibody binds antigen is located in the variable domain, where the variable regions of one heavy chain ($V_H$) and one light chain ($V_L$) pair to generate two identical antigen-binding sites which lie at the tips of the arm of the Y. Within the variable regions of both heavy and light chains, some polypeptide segments show exceptional variability within three hypervariable regions in each chain known as complementarity-determining regions (CDR). These segments are responsible for the direct interaction with antigen. The remaining parts of the variable regions are called framework regions (FWR), which determine the fold and ensure that CDRs are maintained close to each other. On the other hand, the carboxy-terminal half of the heavy chain (Fragment crystallisable, Fc fragment) determines the effector function of the molecule. Therefore, while the V region accounts for the specificity of antigen binding, the C regions are responsible for the immunobiological functions of antibodies.

**Figure 1.6. Basic structure of an antibody.**

Schematic drawing of an antibody illustrates its bilaterally symmetrical Y-shaped structure composed of two identical heavy and light chain polypeptides. The variable region (shown in grey) of the both heavy and light chain form the antigen binding site, whereas the constant regions of the antibody (shown in blue) determine the specific immune effector response.
1.3.1.2 Antibody classes

There are five antibody classes in humans; IgA, IgD, IgE, IgG and IgM, and the heavy chains that define these different isotypes are designated by the lower case Greek letter, α, δ, ε, γ, μ. There are four main ways in which antibodies contribute to immunity:

(1) Inhibiting binding of bacterial exotoxins to their receptors on target cells or binding to cell attachment sites on viruses, a process used for neutralising pathogen;

(2) Coating the surface of a pathogen that certain antibodies are specific for (opsinisation), where the bound antibodies are then recognised by specific Fc receptors (FcR) on the surface of phagocytic cells. Alternatively, antibodies bind to complement receptors on phagocytes (complement fixation), which enhance the opsonisation and promote direct killing of bacteria cells;

(3) Formation of an immune complex (integral binding of an antibody to a soluble antigen) triggers the activation of the complement classical pathway and thus contributes to the direct lysis of microorganisms by forming a pore in their cell membrane;

(4) Sensitisation for extracellular killing by natural killer (NK) cells via a process called antibody-dependent cellular cytotoxicity (ADCC), in which NK cells express the FcR and recognise and kill target cells that are coated with antibodies.

IgM

All mature naïve B cells constitutively express cell-surface IgM (mIgM) as an antigen-specific receptor, yet IgM comprises less than 10% of the immunoglobulin found in plasma, suggesting that most antibodies are produced by B cells that have undergone isotype switching. IgM is secreted as a pentamer (sIgM) and is largely confined to the intravascular pool. On initiation of a primary immune response, IgM is the first antibody class to be produced and is often of low affinity. When bound to antigen with repeating identical epitopes, pentamer sIgMs undergo dislocation to form a “staple” structure,
which is able to initiate neutralisation and acts as a potent activator of the classical complement system.

**IgD**
Together with IgM, IgD is expressed on naïve mature B cells (mIgD) that are yet to encounter antigen. This antibody class accounts for less than 1% of the serum immunoglobulin pool and has no known effector functions.

**IgG**
IgG equilibrates between the intravascular and extravascular pools and is the predominant immunoglobulin of the secondary immune response. In human, this class of antibody is subdivided into four subclasses, IgG1, IgG2, IgG3 and IgG4. IgG1 is the most abundant antibody in internal body fluids and accounts for 70-75% of the total serum immunoglobulin pool. It is secreted as monomers that have relatively higher affinities for antigen compared to IgM antibodies. The four IgG subclasses are highly homologous in structure, but each has a unique profile of effector functions. All subclasses, except IgG4, are able to activate complement. IgG1 and IgG3 are able to enhance phagocytosis by opsonisation. Of note, IgG2 and IgG4 are the only isotypes for placental transfer.

**IgA**
IgA is present in serum as a monomer of the basic four-chain structure and can be subdivided into two subclasses IgA1 and IgA2. It is the predominant antibody isotype in seromucous secretion, such as saliva, colostrum, milk and trachebroncual and genitourinary secretions. In secretions, IgA exits in a dimeric form (sIgA) in association with a protein chain termed the secretory component. The serum concentration of sIgA is relatively low, but can appear to be very high in intestinal secretions. Available effector functions are opsonisation, activation of the complement system as well as in protection of the respiratory, gastrointestinal and reproductive tracts.
IgE
Serum IgE antibodies are produced in very low amounts (<0.05 μg/ml) relative to the other immunoglobulin isotypes. However, in response to antigen, IgE triggers the release of inflammatory mediators from basophils and mast cells and plays an important role in protection against parasitic worms and in allergy.

1.3.2 Human B cell development and differentiation

B cells are generated from hematopoietic stem cells (HSC) originated in the yolk sac from a precursor called hemangioblasts. During fetal to postnatal life, this pool of cells progressively migrates from yolk sac to a primary lymphoid organ, fetal liver, and finally to the bone marrow (BM) during the adult's life where lymphopoiesis occurs. The development of functional mature B lymphocytes takes place in two stages which are localised in different tissues: (i) the differentiation from HSC through multipotent progenitors (MPPs), then through common lymphoid progenitor (CLP) to distinctive B cell lineage restricted-cells in BM niches, and (ii) the maturation from immature BM-derived B cells to mature pre-effector (plasmablast)/effector/memory B cells in the spleen, where, mature B cells may stay and recirculate within the site or migrate repeatedly through the blood and the lymph to B cell areas of other SLO, like lymph nodes (LN), muscosa-associated lymphoid tissue (MALT), which includes Peyer’s patches (PP) and the tonsil. Of note, out of 10–20 million immature B cells are generated daily in the BM, where only 10% reach the periphery; of these, only 10–30% join the mature B cell pool. Quiescent memory B-cells remain recirculating in the PB or they migrate to Ag-draining tissues. In the meantime, plasmablasts look for survival niches in the BM or the MALT to complete their differentiation to PC (Figure 1.7).

1.3.2.1 Early development steps in bone marrow

BM stromal cells form specific niches and provide microenvironmental conditions for the development of B cell subsets via specific cell-cell contact and soluble cytokines. The early-pro B cell binds to hyaluronic acid and VCAM-1 on stromal cells via CD44 and VLA-4 respectively, and these initial contacts promote the binding of the surface c-kit tyrosine kinase on early pro-B cell to stem-cell factor (SCF) on the stromal cell surface.
At the late pro-B cell stage, a second growth factor receptor, IL-7R (CD127) appears on the surface, which promotes the production of several transcription factors such as Pax5 and PU.1. These transcriptional factors induce the synthesis of the terminal deoxynucleotidyl transferase (TdT) and the recombination activating gene (RAG) products, rag-1 and rag-2 [221]. The recombinase proteins are required for DNA rearrangement between the D and J gene segment at the Ig heavy chain (IgH) locus, and the enzyme TdT catalyses the insertion of N-nucleotides to the joining exons. Later, the genetic recombination of germline IgH chain is completed by joining one \( V_H \) gene segment to the preformed DJ\(_H\) complex. Meanwhile, the expression of Pax5 promotes the commitment to pre-B1 cells where CD19 is first detected [222]. Upon the generation of a functional VD\(_J\)H exon, the heavy chain gene is spliced to the constant Ig\( \mu \) exons, generating an Ig\( \mu \) heavy chain in the cytoplasm and in the cell surface, classified as pre-B II cells. The \( \mu \)H chains will associate with surrogate light (SL) chains consisting of VpreB and \( \lambda 5 \) and signaling proteins composed of Ig\( \alpha \) and Ig\( \beta \) (CD79a and CD79b), forming the pre-B cell receptor (pre-BCR). Pre-BCR signaling induces allelic exclusion, cellular proliferation and Ig light chain (IgL) gene rearrangement, which increases the diversity of the antibody molecules generated. After successful rearrangement of both IgH and IgL loci, B cells are able to express IgM, which is transported to the cell surface as BCRs. At this stage, cells expressing only IgM, as well as pan B cell markers (e.g. CD20 and CD22), can be classified as immature B cells [223]. The final stage of development in the BM includes loss of CXCR4 (C-X-C chemokine receptor-4), the receptor for CXCL12, resulting in loss of B cell retention in the BM [224].

**1.3.2.2 Late development steps in peripheral lymphoid organs**

All newly formed immature Ig\( M^+ \) B cells that have left the BM but not yet matured into naïve Ig\( M^+ \)Ig\( D^+ \) B cells are called transitional (T) B cells, and they can be distinguished from mature B cells by a series of cell surface markers, such as CD24/CD38 (these markers are downregulated as cells become more mature). Recent studies show that human splenic B cells can be subdivided into three short-lived T subsets, T1/T2/T3. Early T1 can differentiate into T2, which in turn are thought to serve as the precursor to either subsequent transitional pre-marginal zone (T2-preMZ) or mature follicular (FoBI and
FoBII) B cells. The T2-preMZ cells likely give rise to MZ B cells. The recently described T3 cells represent mature anergic cells that are not able to give rise to mature B cells. Since the fraction of immature B cells that survive the transition to the mature naïve stage is small, the development stage of transitional cells is widely believed to harbour checkpoints for removing autoreactive B cells.

The mechanisms that govern the selection for the long-lived mature B cell compartment are poorly understood, but the strength of BCR signaling and self-antigen specificity appear to be crucial determinants in this transition [225-227]. Moreover, emerging evidence suggests that trophic or environmental signals can influence these outcomes significantly, and inappropriate effects may lead to the development of autoimmune diseases [228, 229]. BAFF is one of the most important of these trophic factors (see below), and BCR and BAFF receptors are functionally linked in the regulation of B cell maturation and the subsequent maintenance of mature B cells [230, 231].
Figure 1.7. Schematic representation of B cell differentiation stages in bone marrow and peripheral lymphoid organs.

Successful completion of eleven important processes is required to meet the subsequent checkpoints as are indicated by letters A through K. The development of B cells is characterised by changes in surface receptor expression and Ig gene rearrangement which allows the identification of different developmental stages. The first stage identified in the B cell lineage is the pre-pro-B cell at which rearrangement of the heavy chain is first induced, but functional heavy chain is not expressed until the pre-B cell stage. Rearranged μ heavy chain is expressed on the surface in combination with λ5 and VpreB to yield the pre-BCR, which is associated with Igα and Igβ (CD79a/b) on the cell surface and initiated with light chain rearrangement. Surface expression of functional μ heavy and light chain complexes (IgM) is a characteristic feature of the immature B cell. Immature B cells, which survive from negative selection, are released from the bone marrow into the circulation and migrate to the SLO. The initiation of IgD expression on B cells indicates their development into transitional B cells that require both BCR and BAFF dependent signaling for development into mature naïve B cells. An activated mature B cell can subsequently branch into two main developmental possibilities; becoming an antibody secreting PC or a memory B cell. Image derived from Berkowska et al [232].
1.3.2.3 B cell subsets in peripheral blood/secondary lymphoid organs

The identification of the selective expression of B cell markers in different B cell subpopulations has allowed their differentiation in the peripheral blood and SLO of human adults. Memory B cells were historically identified by the loss of IgD together with CD38: memory (IgD⁻CD38⁻), naïve (IgD⁺CD38⁻) and GC (IgD⁻CD38⁺) B cells (Figure 1.8). Most studies now include IgM and/or CD27 expression to improve the discriminatory power for different human B cells subsets: naïve (IgM⁺IgD⁺⁺CD19⁺CD38⁻CD27⁻), pre-GC/GC founder (IgM⁺IgD̺⁺ CD19⁺CD38⁺CD27⁻), switched memory (IgD⁻CD19⁺CD38⁻CD27⁺) and late plasmablast (IgD⁻CD19⁺CD38⁺⁺ CD27⁺). CD27⁻ and CD27⁺ B cells were found to have distinct responses to stimulation, for instance CD27⁺ B cells produce 5- to 100-fold more Ig in vitro than CD27⁻ B cells [233-235]. Indeed, using CD27 as a marker for the identification of memory B cells led to the discovery of a new memory B cell population, the IgM pre-switched memory (IgM⁺IgD̺⁺⁺CD27⁺) [236] that contains comparable mutated Ig sequences as IgD⁻CD27⁺ memory cells [237]. This B cell subset could be driven to differentiate into antibody secreting cells under appropriate stimulation, and it is still uncertain whether such response is dependent on T cell participation.
Figure 1.8. Scheme of the differentiation stages of mature B cell development in human tonsil.

The B cell subsets isolated from tonsil samples of young children were identified by indicated markers. Image adapted from Jackson et al [233].
1.3.3 The germinal centre reaction

The immune system requires the production of high affinity antibodies of different subclasses to accomplish its many effector functions. The GC is a microenvironment in peripheral lymphoid organs where specific steps occur leading to the maturation of high affinity humoral responses. A critical role in the GC reaction is played by FDCs which are radiation-resistant stromal cells that form a network able to capture and present large amounts of antigen in the form of immune complexes in highly ordered units termed iccosomes [238, 239]. Upon stimulation of Th cells by the APC migrating in the subcapsular sinus, a subset of antigen specific Th cells known as T follicular helper (TFh) cells undergo clonal expansion within the T cell zone [240], migrate into the boundary between B cell follicles and the T cell zone and later to the B cell follicle via CXCL13 and its receptor CXCR5 where they contribute to initiating a GC response via cognate B cell help and cytokine production (mostly IL-21) [241, 242]. Under T cell help (through cell-to-cell interactions, T-cell-CD40L engagement of CD40 on B cells and costimulatory-signal, CD28-CD86) and the regulation of other cytokines, including IL4, IL10, and transforming growth factor (TGF)-β [243, 244], activated B cells either recirculate to other lymphoid follicles or migrate to extrafollicular sites. In extrafollicular areas, activated B cells undergo terminal differentiation into short-lived PC [245]. GCs display anatomically distinct dark and light zones as defined on the basis of their histological appearance. The dark zone is demarcated by Ki-67+ proliferating centroblasts that actively mutate their antibody genes via somatic hypermutation (described in details in 1.3.4). CXCR4 is required for GC B cell positioning in the dark zone where its ligand, CXCL12, is locally produced by stromal cells. Centroblasts subsequently exit the light zone as non-dividing centrocytes via the CXCL13-CXCR5 axis. Centrocytes rely on antigen-antibody immune complexes on FDCs and CD4+ T cells to receive survival signals [246] that are dependent on antibody affinity. Inadequate survival signal and T cell help leads to GC B cell apoptosis with subsequent uptake by tingible body macrophages [247] (Figure 1.9).
Figure 1.9. The germinal centre reaction.

Mature B cells are activated when they encounter cognate antigen. When T cell help is available, B cell activation takes place in the T cell zone of lymphoid organs, e.g., lymph node, termed T cell-dependent response. Afterwards, naïve mature B cells will migrate from the T cell rich areas into B cell follicles called centroblasts. The centroblasts downregulate their surface Ig expression and undergo rapid proliferation and somatic hypermutation in which the latter generates mutations at a high rate in the V region genes, thereby antibody variants are generated (1). After having mutated their Ig, centroblasts stop dividing and then migrate into the light zone following a chemokine gradient and then differentiate into resting centrocytes that are selected for high affinity of their BCR for the cognate antigen. FDCs in the light zone retain Ag and present it to all B cells, but only centrocytes with high affinity receptors are selected and allowed to survive, whereas low affinity or self-reactive BCR-expressing cells undergo apoptosis. The positively selected centrocytes interact with T cells thereby receiving CD40-mediated signals and resulting in isotype class-switching that replaces the originally expressed immunoglobulin heavy-chain constant region genes by those of another class. Finally, selected GC B cells develop into either memory B cells or PC and leave the GC (2).
Together with SHM, another fundamental process involving somatic DNA modification occurs in this unique anatomic location, termed class switched recombination (CSR). Both SHM and CSR require the presence of activation-induced cytidine deaminase AID, an enzyme that is only expressed in GC B cells [248] and in B cells undergoing CSR or SHM in vitro [249]. AID deficiency leads to impaired CSR and SHM in both mice [248] and humans [250]. Moreover, AID has been shown to be the only B-cell-specific protein required for CSR and SHM, because ectopic expression of this enzyme alone is sufficient to trigger SHM and CSR in mammalian (non-B) cells [251] and even in *Escherichia coli* [252]. Of note, although CSR and SHM can occur together in the GC upon BCR/CD40 activation, normally SHM is initiated before CSR [253]. However, neither CSR nor SHM are prerequisite of the other because not all IgG or IgA carry hypermutated sequences, whereas some IgM can display mutations, such as in unswitched memory B cells.

A substantial proportion of B cells which complete affinity maturation become memory B cells that are programmed to recognise and respond to Ags on repeat exposure or become long-lived PC that are programmed to migrate to the BM (upon surface expression of CD44, VLA-4 and CXCR4) where they secret large quantities of specific antibodies. Syndecan-1 (CD138) positive PC are end-stage cells as highlighted by the expression of a transcriptional repressor called B lymphocyte-induced maturation protein-1 (Blimp-1) and X-box-binding protein-1 (XBP-1) which result in the inhibition of proliferation and of Pax-5 expression [254].
1.3.4 Somatic hypermutation

In activated B cells, SHM introduces point mutations in the V region of heavy and light chain genes, specifically at the hypervariable regions (CDRs) that encode the Ag-binding site [255]. The first stage of SHM requires the intervention of AID. AID deaminates cytidine (C) residues in the DNA, converting them to uridine (U) residues. The resulting U:G mispair can subsequently process in different ways (Figure 1.10): U can: 1) simply undergo replication, where DNA polymerases insert an A nucleotide opposite the U, ultimately producing C to T (G to A on the other strand) transition mutations (phase 1a), 2) be deglycosylated by the uracil DNA glycosylase (UNG) (phase 1b, mismatch repair, MMR), yielding a nick by UNG and the mismatch repair machinery can produce an abasic site that can be bypassed by error-prone DNA polymerases to generate transition and transversion mutations, 3) be recognised by the component of mismatch recognition and repair machinery (phase 2, base excision repair pathway, BER), where U:G mismatch is excised and resynthesised by error-prone polymerase that will create additional mutations including mutation at A:T base pairs near the initiating U:G lesion [256]. SHM occurs at the centroblast stage of B cell maturation where B cells expressing Ig with a high affinity to given Ags are selected to receive signal for survival, while those that have lost functional Ig, or acquired only low-affinity Ig after SHM, die by apoptosis. Therefore, after repeated cycles of GC, B cells are selectively expanded with high affinity antibody, which in turn exit the GC and then programme to secret massive amount of specific antibodies.
Figure 1.10. Model of somatic hypermutation.

After AID catalysed deamination of cytosine (C) to uracil (U), the DNA may be subjected to three different modification pathways as shown, leading to mutation in the variable region of an antibody. The mechanism is described in text. MMR: mismatch repair; BER: base excision repair, MSH2, MSH6, example of component of mismatch repair machinery (MSH).
1.3.5 Class switch recombination

By substituting the H chain C region of IgM with that of IgG, IgA, or IgE, class-switching enables antibodies to acquire new effector functions that are crucial for the neutralisation of invading pathogens. CSR involves an exchange of upstream Ig heavy constant region C\( \mu \) with downstream targeted C\( \gamma \), C\( \alpha \), C\( \varepsilon \) genes through a recombination process involving a switch (S) region that is located before each C gene segment (except C\( \delta \)). Each S region is preceded by a short intronic (I) exon and a promoter that initiates the expression of germline transcripts (GLTs). It has been generally believed that CSR is regulated by two major activatory signals. The first is when IgD\(^+\) naive B cells are exposed to specific activating cytokines, among which IL-4 induces switching to IgG and IgE [257, 258], IL-10 to IgG and IgA [259, 260] and TGF-\( \beta \) to IgA [261, 262]. The stimulatory signal activates the I\( H \) gene promoter located at the 5' end of each S region and triggers NF\( \kappa \)B-dependent transcriptional activation for initiating GL transcription. The resulting GL transcripts are thought to be crucial to elicit CSR as they render the S region suitable for AID binding [263, 264] (Figure 1.11).

Accessibility to AID is achieved when a primary transcript is spliced out to form a non-coding GLT. At this stage, the primary transcript is physically associated with the template strand of DNA, inducing chromatin opening and allowing the access for AID. The second signal is delivered by either: (1) cellular interactions, such as CD40/CD40L; or (2) complex viral/bacterial proteins, such as LPS; or (3) soluble factors, such as B cell survival factors, BAFF and APRIL [263-265].

AID initiates CSR by deamination of C residues in both strands of the S DNA region thus creating U and multiple DNA lesions which ultimately lead to DNA double strand break (DSB). Thereafter, CSR is processed by looping-out deletion of the DNA segment between recombined S regions with generation of: (1) an extrachromosomal reciprocal switch DNA product, known as switch circle (SC), which contains the I\( H \) promoter of the targeted S region, (2) the deleted DNA fragment, C\( \mu \) and, (3) a chromosomal sequence, termed mature transcript, which encodes the class-switched protein. Under the influence
of AID and the I_{H} promoter, the SC is transcribed into chimeric I-C_{\mu} product, known as a circular transcript (CT). Since CT are rapidly degraded by nucleases (within 48h), CT constitutes specific molecular markers of ongoing CSR [263].
Figure 1.11. Model of Class Switch Recombination (CSR) and the generation of circular transcript (CT).

The human IgH chain locus and the molecular events involved in switching from Cµ to Cε are shown schematically on the left. Ovals indicate switch regions (S), and the rectangles before and after S regions are I_H exons and constant region exons. Location of the IgH chain intronic enhancer (iEµ) and 3' IgH chain enhancers (triangles) are shown. The IgE CSR product is shown at the bottom. The V-shaped lines representing splicing; arrowheads are the positions and directions of the primers used to amplify SCs, CTs, germline transcripts, and mature transcripts. Modified from Cerutti *et al* J Immunol 169:11 [264]. Right: Flowchat illustration of CSR pathway.
1.3.6 T-cell-independent B cell activation

B cell responses are classified as T-D or T-I depending on whether T cell help is required to induce antibody production. Most Ags elicit a T-D antibody response within the GC following their processing and presentation to cognate helper T cells in the context of MHC class II molecules. It has been demonstrated that CSR is highly dependent on CD40 ligation as secondary isotype production is dramatically impaired in hyper-IgM syndrome where CD40L is mutated [266, 267]. In contrast, T-I Ags are typically polysaccharides that cannot be presented via MHC molecules and are of two main types distinguished by their requirement for BCR recognition. T-I type 1 Ags are mitogenic stimuli such as LPS or CpG that activate TLR to induce non-specific or polyclonal B cell activation. T-I type 2 Ags are large molecules with highly repetitive structures, such as capsular polysaccharides from bacterial cell walls or repetitive antigenic epitopes from viral particles. As these Ags do not function as B cell mitogens, they can only stimulate mature B cells and simultaneously crosslink a number of B cell receptors for activation. This process mainly occurs in the extrafollicular splenic marginal zone of the spleen and intestinal lamina propria which provide prompt protection against invading pathogens by actively participating in IgG and IgA production [268]. Although the initiation of CD40-independent CSR remains obscure, growing evidence indicates that DC, epithelial cells or stromal cells play an important role in such response. IgA CSR can be induced by release of APRIL by gut ECs and DCs after recognition of intestinal bacteria [244]. Accordingly, IgA production is near normal in the absence of CD40, but is dramatically impaired in APRIL- or BAFF-deficient mice [269-271]. Both human [259, 272] and mouse [265, 269] naïve B cells are reported to be able to undergo T-I Ig class-switching and antibody production upon exposure to BAFF or APRIL in the presence of appropriate cytokines. Of note, although CD40 deficiency impairs T-D but not T-I IgG and IgA responses [273], BAFF deficiency impairs both T-D and T-I IgG and IgA synthesis [274]. Similar observations can be obtained by neutralising BAFF and APRIL with respective decoy receptors (as discussed below) [275-278]. Alternatively, when overexpressing these two cytokines in mice, Ig production is increased and an autoimmune-like phenotype is observed, suggesting their crucial roles in the activation of
B cells. I shall discuss these two members of the TNF superfamily, BAFF and APRIL in more detail below.
1.3.6.1 Classical B cell regulatory factors

1.3.6.1.1 BAFF (B cells activating factor of the TNF-family)

BAFF was discovered by several groups utilising genomic data to uncover novel TNF family members. It is also known as BLyS (B lymphocyte stimulator), TALL-1 (TNF- and ApoL-related leucocyte-expressed ligand 1), THANK (TNF homologue that activates apoptosis, NF-B, and JNK) and zTNF4. BAFF is a type II transmembrane protein of the TNF ligand superfamily (TNFSF13b) and it is closely related to APRIL (a proliferation inducing ligand), another member of the TNF ligand family (discussed in detail in 1.3.6.1.2 below). BAFF and APRIL share about 50% sequence homology within the TNF homology domain (THD), which is a C-terminal domain characteristic of TNFSF. BAFF exists in two biologically active forms, a 285-amino-acid transmembrane protein (31.2 kDa) and as a soluble protein (152 amino-acids, 17 kDa) after release from the membrane by a furin-like protease [259, 279, 280]. Furthermore, a shorter isoform termed deltaBAFF (ΔBAFF) exists, which is an alternatively spliced form of BAFF. ΔBAFF is also a transmembrane protein, but it lacks the furin protease recognition motif and thus is insufficiently cleaved from the cell surface. ΔBAFF acts in a dominant negative fashion and has suppressive effects on BAFF activity as demonstrated in vivo in ΔBAFF transgenic mice [281]. All forms of BAFF adopt a trimeric structure, while the soluble homotrimer BAFF can also assemble as an ordered symmetric structure comprising 20 trimers. Heterotrimers with ΔBAFF or APRIL have also been described, are biologically active, and have been found in patients with autoimmune diseases [282] (Figure 1.12).
Figure 1.12. Interaction and expression of ligands and receptors in the BAFF/APRIL system.

The top part of the figure shows the different types of ligands. From left to right: (1) a heterotrimer with ΔBAFF with red road signs indicating the impossibility of this ligand to be cleaved; (2) membrane BAFF homotrimers; (3) membrane BAFF homotrimers with a cutter indicating potential cleavage into (4) soluble BAFF homotrimer which can also form (5) a capsid-like assembly of 20 trimers (60-mer); (6) APRIL cleaved from the golgi then released as (7) soluble ligand or (8) the TWE–PRIL fusion protein. Additional forms of BAFF/APRIL heterotrimers (9) have also been described. In the middle part of the figure, receptors are represented from left to right: BAFF-R, brown; TACI, pink; BCMA, blue and heparin sulfate proteoglycans (HSPG), green. Blue solid arrows represent strong interactions of BAFF with respective receptors while broken lines represent weak interactions. Green solid or broken lines applied to the interaction of APRIL with its respective receptors. The lower part of the figure demonstrates a summary of each of the BAFF/APRIL receptors function in human. Details are decribed in the text.
The expression of BAFF protein was thought to be mainly restricted to cells of myeloid origin, such as monocytes, macrophages, monocyte-derived DC and leukemia myeloid cell lines (HL-60, U937, and THP-1) which produce both the membrane-bound and the soluble forms [279]. However, activated T cells, malignant B cells or B cells activated with LPS and CpG can also upregulate BAFF expression and secretion [283]. Whether BAFF is produced by normal B cells is uncertain, but most reports agree that normal B cells do not express BAFF [284] apart from a report from Kern C et al [285].

In humans, BAFF is produced by monocytes, macrophages and DCs and is upregulated upon stimulation with IFN-α, IFN-γ, CD40L or LPS [259]. Recently, neutrophils have been shown to synthesise and secrete the highest levels of BAFF, particularly upon activation with IFN-γ or G-CSF [280]. However, unlike the classic cleavage mechanism as reported in myeloid cells, BAFF undergoes intracellular processing before being released by neutrophils [280]. Recent studies also identify some non-haematopoietic cells that express BAFF, particularly during chronic inflammatory/autoimmune conditions. These include epithelial cells from airway [286], salivary gland of Sjogren’s syndrome patients [287], astrocytes of multiple sclerosis patients [288], VCAM-1 positive stromal cells from human BM [289], CD105 and CD166 positive stromal BM cells from patient with RA and OA [290], SFs of RA patients [291-293] and osteoclasts in patients with multiple myeloma [294].

BAFF binds to three known receptors, transmembrane activator and CAML interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R or BR3). While BAFF-R specifically binds BAFF, TACI and BCMA are shared by both BAFF and APRIL. Moreover, TACI has been shown to be activated by the membrane bound form of these ligands, BAFF/APRIL heterotrimers [282] or oligomeric BAFF [295], but not by the soluble forms of these ligands. All three receptors are mainly expressed on B cells [296]. In humans, TACI is predominantly expressed on CD27+ memory B cells, by PC and by certain subsets of naïve and activated B cells. BCMA is highly expressed by PC, plasmablasts, tonsillar memory and GC B cells. BAFF-R is expressed by all peripheral B cells and its expression is down-regulated in GC B cells and absent in BM PC [297].
All BAFF receptors belong to a subgroup of the TNFRSF that triggers intracellular signaling events via engaging at least one of the six known TNF receptor associated factors (TRAFs). BAFF-R was found to interact only with TRAF3 and activate the alternative NF-κB pathway. In the absence of BAFF-R signaling, TRAF3 is distributed in the cytoplasm and forms a complex with TRAF2 and with NF-κB inducing kinase (NIK), thus inducing proteasome-mediated degradation of NIK, and resulting in the inhibition of the alternative NF-κB2 pathway. Following BAFF binding to BAFF-R, TRAF3 is recruited to the receptor and then degraded by TRAF2 dependent proteolysis, thereby allowing NIK to activate the IκB kinase α (IKKα), which results in phosphorylation of the NF-κB2 p100 precursor protein to p52. Along with its heterodimeric partner, RelB, p52/RelB dimer translocates to the nucleus and up-regulates anti-apoptotic proteins. Furthermore, BAFF-R signaling is also reported to inhibit the activation of the pro-apoptotic protein kinase Cδ (PKCδ), a process that is highly dependent on TRAF3. TACI and BCMA signal through the classic NFkB pathway, as well as through the Mek pathway to counteract apoptosis and to drive class-switching (JNK/p38) [230].

Initially, BAFF was described solely as a co-stimulatory or growth factor for B cells based on its ability to augment anti-IgM mediated B cell proliferation and Ig secretion. However, recent data indicates that:

1) **BAFF is a potent survival factor for B cells and essential for the production of functional mature B cells.** B cell numbers are dramatically reduced in BAFF-deficient mice [276] whereas overexpression of BAFF in BAFF Tg mice leads to the opposite phenotype [298]. Most of the T1 B cells are eliminated via signals through BCR, which trigger apoptosis of immature and T1 B cells. Only those B cells that receive additional signal are allowed to survive and such additional signal is delivered by BAFF, since B cells mostly remain at the T1 stage in BAFF-deficient mice. Both *in vivo* and *in vitro* studies have demonstrated that BAFF-signaling induces expression of anti-apoptotic molecules such as Bcl-2 in B cells, which appear to be required to keep B cells from undergoing apoptosis once they mature beyond the T1 stage [299]. Moreover, BCR signaling strength controls the amount of BAFF signal as it generates p100, a component
of the alternative NFkB pathway. Indeed, BAFF-R expression is upregulated by BCR ligation, thus promoting an increased sensitivity to BAFF-mediated survival signals as B cells mature [300]. Noteworthy, even in the absence of BCR stimulation, BAFF significantly increases splenic B cell survival \textit{in vitro} resulting in the expansion of peripheral B cells [301]. A recent study, however, showed that even though memory B cells express BAFF-R, the survival and reactivation of these class switched memory B cells are independent of BAFF under normal physiologic conditions [302]. BAFF can, on the other hand, work together with inflammatory cytokines like IL-21 and IL-17 for reactivating memory B cells and their differentiation to PC [303, 304]. The overall role of BAFF at different stages of B cell development and differentiation is summarised below (Figure 1.13).
Figure 1.13. Expression of BAFF receptor during human B cell development and differentiation.

Within the BAFF network, the expression of the receptors and ligands is spatially, as well as temporally, highly regulated at various stages of B cell development and function. The expression of BAFF-R, BCMA and TACI at different stages of B cell development (immature $\rightarrow$ transitional) and differentiation (mature B cell $\rightarrow$ memory B cell/PC) is summarised above. The parentheses indicate differences in expression of BAFF receptor between murine and human B cell. Image adopted from Tangye et al Semin Immunol Vol18:5 [305]
(2) **BAFF can also drive MZ B cell differentiation.** Tardivel *et al* [306] suggested that BAFF is a critical differentiation factor for MZ B cells, and Hase *et al* [307] showed that BAFF could upregulate Pax5 activity and its downstream target, CD19. Moreover, a recent analysis demonstrated the persistence of B cells expressing IgD (a hallmark of T2 and mature B cells) in BAFF-deficient mice [308]. Also, BAFF-deficient mice have shown to retain the ability to mount antigen-specific antibody responses, albeit in a much reduced magnitude [274].

(3) **BAFF is important for the maintenance of stable GC reactions and antibody production.** Studies in murine models demonstrated that while the initiation and formation of GC was BAFF-independent, the longevity of the GCs was dependent on BAFF [277]. Consistent with this observation, a short-lived but not sustained GC reaction can be induced in both BAFF-R mutant and BAFF-deficient mice [309]. Of interest, BAFF-R deficient mice also display impaired development of the mature FDC within the GCs, along with diminished IgG and secondary response [309]. Also, T-I type 2 and T-D IgM responses have been shown to require the interaction of BAFF with TACI, and BAFF transgenic mice exhibit an increased level of serum Ig of all isotypes and IgG subclasses [230]. In addition, *in vitro* experiments demonstrated that human B cells undergo class-switching towards IgG and IgA when stimulated with BAFF alone, and this was greatly increased in combination with IL-4 or IL-10. BAFF may further contribute to Ig isotype switching by inducing B cells to secret IL-10, a known switch factor for the secretion of IgG and IgA by human B cells. The crucial role of BAFF in CSR is also evident by the finding that Ig isotype switching can be inhibited by the presence of neutralising antibody against BAFF. Also, as mentioned above, the ability of EBV to induce the production of BAFF by human B cells may explain how EBV can activate CSR, since EBV-mediated switching from IgM to IgG, IgA and IgE has been shown to be dependent on endogeneous BAFF production [244]. Although Litinskiy *et al* [259] claimed that secretion of the switched isotype antibodies requires an additional signal, such as cross-linking of the BCR, recent evidence [286] demonstrated that EC-derived BAFF together with IL-10 induced class-switching to IgG and IgA in the absence
of a BCR trigger. Of note, the production of these switched isotype antibodies can be further increased upon BCR engagement.

(4) **BAFF production in response to innate immune stimuli.** BAFF is induced in myeloid DC by type I interferons (IFNs) [310] and collaborates with IL-6 and TLR signals to promote Ig class-switching and PC differentiation [311, 312]. Activation of intracellular TLRs in murine B cells by immune complexes or apoptotic material containing nucleic acids increased expression of TACI [312] and induced class-switching of these cells. On the other hand, activation of B cells via TLR4 up-regulates BAFF-R which renders the cells sensitive to Fas-mediated apoptosis [313]. Therefore, there is compelling evidence that TLRs, type I IFNs and BAFF may combine to create an amplification loop that promotes B cell differentiation and the secretion of antibodies in the absence of T cell help.

1.3.6.1.2 **A proliferation-inducing ligand, APRIL**

APRIL (also known as TALL-2, TRDL-1 and TNFSF13a) is expressed by monocytes, macrophages, DC and at lower levels by T cells [259], and as recently reported in this thesis and by others, by RASFs [291, 314]. Several cytokines such as IFN-γ, IFN-α, IL-10, GM-CSF, CD40L as well as LPS and peptidoglycan can activate APRIL expression in macrophages [259]. Similar to BAFF, APRIL has also been reported to be expressed by neoplastic B cells [315, 316]. However, unlike BAFF, the transmembrane APRIL (27 kDa) is not functional and is processed and cleaved in the Golgi apparatus by a furin-convertase enzyme before its secretion. Therefore, APRIL only exists and functions as a secreted soluble ligand (17 kDa) [317]. Due to alternative splicing in the TWEAK/APRIL locus, APRIL can be expressed as a cell surface fusion protein with TWEAK, termed TWE-PRIL, which has been detected in human primary T cell and monocytic cell lines [318]. In addition, a ΔAPRIL variant has also recently been reported but not yet characterised as a protein [319].

APRIL binds TACI and BCMA with high affinity, but not at all to BAFF-R. Whereas BCMA is a high-affinity receptor for APRIL, TACI can be activated by multimerised
BAFF, membrane bound BAFF and multimerised APRIL (apart from homotrimers). Also, human BCMA has been shown to bind BAFF with low affinity, while only mouse BCMA is APRIL-specific [320]. Interestingly, a recent study demonstrated that a short variant form of mouse APRIL exhibits weak but detectable binding to mouse BAFF-receptor [321]. In addition, APRIL also has a private receptor, expressed on both B cells and non-B cells, termed heparan-sulfate proteoglycan (HSPG) [322] (Figure 1.12). Regarding B cells, APRIL binds preferentially to HSPG at the surface of syndecan-1+ (CD138+) PC to induce the expression of pro-survival factors resulting in the establishment of long-lived PC.

While BAFF-BAFFR interactions control peripheral B cell survival and homeostasis, BCMA function seems limited to the survival of long-lived bone marrow PC. The functional activity of the third receptor TACI is, however, ambiguous and has been suggested to serve as a positive or negative regulator for B cell responses depending on conditions. Sakurai et al firstly proposed that TACI exerted a negative regulation in human peripheral blood B cells showing that B cell proliferation, CSR and IgG production induced by BAFF and CD40 were inhibited via TACI [323].

In contrast to BAFF, the role of APRIL in immune regulation is less well defined, but it is most likely described as a positive regulator of tumor growth or survival in vitro and in vivo [324]. To date, its biological role is more elusive due to the conflicting results emerged from different studies. One showed that APRIL-deficient mice display normal T and B cell development and normal in vitro functions [325], while another study demonstrated a significant decline of IgA basal serum levels with larger GC and increased numbers of effector T cells [269]. Furthermore, in vitro data using human cells suggestd that APRIL plays a role in IgA class-switching after antigenic challenge [259]. Xu et al recently reported that APRIL and BAFF are responsible for class-switching to both IgG and IgA via BCMA in pre-switched IgD+ B cells in a T-I manner [286]. Finally, APRIL, as compared to BAFF, has a marginal effect on the survival of peripheral B cells; accordingly APRIL-deficient mice do not show any major alteration in peripheral B-cell subsets.
1.3.6.1.3 Role of BAFF/APRIL in promoting autoimmunity in RA

In general, BAFF and APRIL are thought to promote autoimmunity due to their ability in promoting the inappropriate survival of autoreactive B cells and PC. A recent study demonstrated increased BAFF and APRIL levels in very early RA (VERA) as compared to healthy controls as well as in patients with established RA [326]. Importantly, BAFF/APRIL levels were observed to be higher in synovial fluid compared to sera, suggesting a local up-regulation in the synovium. APRIL has been demonstrated to promote IgA class-switching [269] and the differentiation/survival of PC [327], suggesting its involvement in maintaining the presence of autoreactive B cells in the joints. Accordingly, Dong et al [328] demonstrated a significant association between the infiltration of PC and synovial fluid levels of APRIL from RA patients. Furthermore, an in vivo experiment demonstrated a close association between BAFF and anti-collagen type II antibodies in collagen-induced arthritis (CIA) [329] (an animal model of RA, see supplementary information/data, Chapter 8, for further information). Lastly, both BAFF and APRIL can also be produced by resident synovial cells [291-293, 314] as discussed in detail in Section 1.4. Collectively, increased synovial levels of APRIL and BAFF are likely to influence autoreactive B cell activation in the RA joint over and above circulating levels.

Finally taken together all the above evidences indicate that BAFF and APRIL possess antibody-inducing and antibody-diversifying functions that become essential in the context of TI B-cell responses.
1.3.6.2 A Novel factor involved in B cell regulation, secretory leukocyte protease inhibitor (SLPI)

1.3.6.2.1 Biology of SLPI

Human SLPI is a 12kDa non-glycosylated single chain protein, which is constitutively derived from the mucosae, in particular from ECs and cells lining body cavities, and can be found in the fluids lining these surfaces, such as saliva, seminal fluid, and cervical, nasal, bronchial mucus [330] as well as in blood [331]. It was first named by its immunologically activity related to the regulation/inhibition of the activity of serine proteases released by leukocytes at site of infection, thus ensuring a tissue protective effect by preventing degradation by proteolytic enzymes produced during local inflammatory reactions. SLPI is composed by two highly homologous cysteine-rich domains termed whey acid protein (WAP). The C-terminal domain contains the antiprotease activity whereas the N-terminal domain displays anti-bacterial functions and/or the later described immunomodulating function. Apart from acting as a natural epithelial product, it has been recently documented that SLPI can also be also produced and secreted by a number cells of the immune system, including mast cells, neutrophils, B lymphocytes, DC, macrophages (mouse only) [332] as well as by non-immune cells such as human chondrocytes [333], keratinocytes (Jacobsen et al 2008) in addition to neurons and astrocytes in ischaemic brain tissue [334].

SLPI exerts its inhibitory activity mainly against serine proteases such as elastase, cathepsin G and trypsin from neutrophils or chymase and trypase from mast cells [332]. In normal physiological conditions, these proteases offer regulatory effects towards fundamental cellular processes such as cell proliferation/growth, wound healing, innate immune response in addition to tissue remodelling (Figure 1.14 and Table 1.6). However, sustained expression of these otherwise temporary protective processes have considerable potential to harm the host tissue, as observed in the RA joints where degradation of proteoglycans and collagen are mediated by excess of proteases and metalloproteinases. Moreover, SLPI is well recognised as an antimicrobial factor. Due to the fact that SLPI is a cationic protein, and it has been postulated that SLPI can disrupt microbial cell
membrane, and can therefore inhibit pathogens such as *S. aureus*, *Staphylococcus epidermidis*, *Ps. aeruginosa* and *Candida albicans*. Lastly, SLPI displayed antivial activity which has been reported to inhibit human immunodeficiency virus (HIV) replication in monocytes and can interfere with HIV infection of macrophages via binding to a phospholipid-binding protein, annexin II [335] (Figure 1.14 and Table 1.6).

1.3.6.2.2 Immumodulatory role of SLPI

Apart from acting as an anti-protease, SLPI can act as an “alarm” inhibitor and thus it is inducible at the site of injury in response to primary cytokine production, such as IL-1 and TNF. LPS-stimulated monocytes express SLPI, which acts by reducing inflammation by suppressing the production of pro-inflammatory molecules via interfering with the interaction between CD14 and LPS [336]. Although LPS can induce SLPI expression in macrophages directly or by way of IL-1β, TNF-α, or anti-inflammatory cytokines such as IL-6 and IL-10, SLPI can in return up-regulate macrophage production of the anti-inflammatory/repair-type cytokines TGF-β and IL-10 [337, 338]. Notably, SLPI production can be down regulated by TGF-β in ECs in vitro [339]. In this regard, it is worth to note that SLPI can exert its biological inhibitory activity either as extracellularly secreted molecules (as mentioned above) or gain access to intracellular compartments to alter transcriptional events for inflammatory mediators by suppressing NF-κB activation. Taggart *et al* reported that SLPI could be rapidly internalised by monocytic cells and translocated into the nucleus in vitro. Overall, inhibition of NFκB activation has been suggested to occur via two possible mechanisms: firstly, SLPI can prevent the proteolytic degradation of IRAK-1, IκBβ and IκBα, and secondly, SLPI can bind directly to the p65 subunit of NF-kB and therefore compete with p65 for occupancy of the promoters of NFκB-regulated genes, leading to the reduced expression of pro-inflammatory cytokines, such as TNF-α [340] (Table 1.6).

In addition to the direct down-regulating effects on innate immune cells as discussed above, it has been suggested that SLPI may be involved in the adaptive immunity through the maintenance of a mucosal tolerance threshold. Regarding B cell regulation, in vitro studies have shown that SLPI can penetrate the cytoplasm and the nucleus of
intraepithelial IgD⁺ B cells [286]. SLPI can then interfere with the activation of NF-kB in B cells and the class-switching to IgG and IgA. Interestingly, the expression of nuclear Oct-1, a ubiquitous nuclear transcription factor that regulates Ig gene expression is not affected by SLPI [286]. In agreement with the postulated inhibitory role of SLPI in B cell activation, Nakamura et al report that B cells from SLPI-deficient mice (SLPI⁻/⁻) display increased proliferation and IgM production after LPS treatment compare to SLPI⁺/+ B cells [341].

Only limited information is available on the role of SLPI in the joints during arthritis. SLPI has been shown to be released by RASFs in response to activation with microparticles [342]. Of note, treatment with recombinant SLPI has been shown to attenuate inflammation in a model of bacterial cell wall-induced arthritis [343] (Figure 1.14). Thus, at present, the relevance and biological properties of SLPI in modulating inflammatory responses and B cell activation in RA joints are yet to be defined.
Figure 1.14 A scheme of the reported biological functions of SLPI.

The top part of the figure shows that each of the two domains of SLPI (COOH terminal and NH₂ terminal) have distinct enzyme activities. The bottom part of the figure summarised the main biological properties of SLPI. From right to left, SLPI: (1) is a potent anti-protease that prevents the activation of complement cascade from protease cleavage; (2) protects ECs from deleterious effects exerted by proteases or MMPs; (3) decreases the production of prostaglandin, e.g. prostaglandin E2, by monocytes; (4) protects against infection such as prevention of HIV replication; (5) is involved in tissue repairing such as wound healing; (6) ameliorates joint inflammation and destruction; (7) is a key inducer of anti-inflammatory mediators such as IL-10 and TGF-β; (8) antagonises the pro-inflammatory activity of bacterial LPS.
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<td>• Suppression of epithelin</td>
<td>Epithelin</td>
<td>Dependent of antiprotease activity</td>
</tr>
<tr>
<td>• Decrease TNF α, MCP1, IL6 expression</td>
<td>TNF α, MCP1, IL6</td>
<td>Dependent of antiprotease activity</td>
</tr>
<tr>
<td>• Increase HGF expression</td>
<td>HGF</td>
<td>Dependent of antiprotease activity</td>
</tr>
<tr>
<td>• Suppression of PGE₂, MMP1, MMP9</td>
<td>PGE₂, MMP1, MMP9</td>
<td>Independent of antiprotease activity</td>
</tr>
<tr>
<td>• Decrease TGF β activity</td>
<td>TGF β</td>
<td>Regulating gene expression/ Dependent of antiprotease activity?</td>
</tr>
<tr>
<td>• Increase TGF β, IL10</td>
<td>TGF β, IL10</td>
<td>Unknown mechanism</td>
</tr>
<tr>
<td>• Suppression of class switch</td>
<td>AID?</td>
<td>Inhibition of AID induction</td>
</tr>
<tr>
<td>• Suppression of NFkB activity</td>
<td>IκB β, NFkB</td>
<td>Inhibition of IκB β degradation/inhibition of NFkB and DNA binding?</td>
</tr>
<tr>
<td><strong>Cell proliferation</strong></td>
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<tr>
<td>• Increase cyclin D expression</td>
<td>Cyclin D</td>
<td>Unknown mechanism</td>
</tr>
<tr>
<td><strong>Anti-infection</strong></td>
<td></td>
<td></td>
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<tr>
<td>Anti-HIV infection</td>
<td>Annexin II</td>
<td>Binding to annexin II</td>
</tr>
<tr>
<td>Anti-microbial infection</td>
<td>Unknown</td>
<td>Unknown/ Independent of antiprotease activity</td>
</tr>
</tbody>
</table>
Table 1.6. Biological functions of SLPI.

AID, activation-induced cytidine deaminase; HGF, hepatocyte growth factor; HIV, human immunodeficiency virus; MCP, monocyte chemoattractant protein 1; MMP, matrix-metalloprotease; NF, nuclear factor; PGE, prostaglandin E; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

Table adapted from Nukiwa et al Cancer Sci Vol 99:5 [344]
1.3.7 Importance of B cells in RA pathogenesis

The critical relevance of B cells in the pathogenesis of RA has been clearly demonstrated by the clinical effectiveness of B cell depletion using the anti-CD20 monoclonal antibody Rituximab. It is now recognised that the role of B cells in RA can be subdivided into two processes: autoantibody dependent or independent pathways.

1.3.7.1 Autoantibody production

The persistent presence of high affinity autoantibodies is thought to be dependent on a chronic antigen-driven activation of B cells that prompt self-reactive B proliferation/survival via ongoing CSR and SHM. These antigen-driven processes have long been presumed to exclusively occur within the GC of SLO, such as LN. However, accumulating observations suggest that CSR and low-level SHM can still occur outside GCs at the extrafollicular area of the spleen in autoimmune prone mice, and in humans the gut or in follicular structures of chronically inflamed tissue of autoimmune diseases with ectopic GC-like structures, such as the salivary gland in Sjögren’s syndrome patients. Similarly, a recent report from our lab showed that lymphoid structures invariably expressed AID within the FDC network in the RA synovium, at both protein and RNA level, while auto-antibodies producing cells surround to these ectopic structures within the same tissue [152, 345]. Additional support for the notion that auto-antibodies are produced locally in the synovial compartment comes from the observation that these antibodies constitute higher levels in RA synovial tissue than in paired serum samples [151]. The targets of these autoantibodies are quite diverse and include collagen, cartilage linked proteins, heat shock proteins (hsp), enzymes, nuclear proteins and most importantly, citrullinated proteins (Table 1.7).
Table 1.7. Autoantigens of potential pathogenetic relevance in rheumatoid arthritis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>AntiAntibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin G</td>
<td>Rheumatoid factors</td>
</tr>
<tr>
<td>Citrullinated proteins</td>
<td>ACPA, anti-CCP</td>
</tr>
<tr>
<td>(fibrin, vimentin, filaggrin)</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein A2 (RA33)</td>
<td>anti-RA33</td>
</tr>
<tr>
<td>Collagen II</td>
<td>Anti-collagen</td>
</tr>
<tr>
<td>Stress proteins</td>
<td>Anti-BiP, anti-hsp90</td>
</tr>
<tr>
<td>Glucose-6 phosphate isomerase (GPI)</td>
<td>Anti-GPI</td>
</tr>
</tbody>
</table>

Among the various autoantibodies described in RA, only RF, ACPA, and to a lesser extent, anti-RA33, have so far proven to be useful for diagnostic purposes. In particular, ACPA shows high specificity for RA and considerable prognostic value since their presence is significantly associated with the development of bone erosions. As mentioned above, their detection years prior the onset of RA disease suggests that alterations in the B cell compartment take place at an early stage of RA development [346]. In light of the effect of biologic treatments in RA, and particularly of B cell depletion, these autoantibodies have acquired a further pathogenetic role. Efficacy of anti-CD20 Rituximab has firstly been proven in RF⁺ patients and shown to parallel a substantial and sustained reduction of IgM-, IgG- and IgA-RF levels [347]. Also, the levels RF and ACPA levels reduce upon treatment with Rituximab, where RF production is lowered prior to ACPA suggesting that RF (IgM) are preferentially produced by short-lived PC, whereas ACPA (IgG) are predominantly produced by long-lived PC.

Although the pathogenic relevance of these antibodies in human RA is still to be determined, recent evidence in animal models demonstrate that antibodies with specificity for a commonly expressed enzyme of the glycolytic pathway, the glucose-6-phosphate isomerase (GPI), are pathogenic and can directly induce experimental arthritis.
It has been shown that GPI is present on the cartilage surface, thus, the anti-GPI autoantibody can bind to the articular surface [348] and form immobilised Ag-antibody complexes which are able to activate the complement cascade, enhance local inflammation and promote tissue destruction. Furthermore, B cells with specificity for self-Ig can indeed bind and internalise Ag-antibody complexes. Therefore, a much broader spectrum of peptides is generated for enhancing Ag presenting function. This will support further activation and maturation of self-reactive B cells and thereby increase the production of high-affinity antibodies with RF specificity [345].

1.3.7.2 Autoantibody-independent proinflammatory role of B cells in RA

(1) Production of soluble mediators
B cells can produce a large series of cytokines that are able to regulate innate and adaptive immune responses. In the context of ectopic lymphoid follicles, B cells play a fundamental role by producing pro-inflammatory cytokines, such as TNF-α and LT-β, which are essential for lymphoid neogenesis [146]. Furthermore, there is increasing evidence that a novel subset of B cells, regulatory B cells (Breg), also play an immunoregulatory function via production of IL-10 a pivotal anti-inflammatory cytokine [349]. Although evidence of Breg in the RA joints is lacking, in human RA, follicular synovitis expresses higher levels of IL-10 mRNA compared to the diffuse pattern [350]. Therefore, B cells in the RA joints can actively contribute to the production of factors that orchestrate synovial lymphoid neogenesis and promote the organisation of GCs in inflammatory lymphoid tissues [345].

(2) Antigen presentation for T cell activation
B-cells are well known for their capacity to present antigens to T cells in a very efficient manner [351]. In the context of follicular synovitis Takemura and colleagues provide solid evidence that B cells have the ability to regulate T cell response. Treatment with human anti-CD20 monoclonal antibodies in severe combined immunodeficient (SCID) mice transplanted with GC+ RA synovial tissues led to disruption of GCs, loss of FDC networks and impairment of T cell activation, with a fall in the production of T cell-derived cytokines, such as IFN-γ and IL-1β [352]. This observation indicates that B cells
are directly involved in maintaining inflammation within the synovial membrane, and that T-cell activation can be B-cell dependent.

1.4 Role of fibroblast-like synoviocytes in RA pathogenesis

1.4.1 Activation of RASFs in RA

Although SF in the normal joint are the main cells that contribute to joint homeostasis, in RA, activation of SF by a wide range of soluble factors and cell surface interactions overrides this homeostatic function, and in turn RASFs express a broad assay of destructive enzymes and proinflammatory mediators as reported in Table 1.8 below.

<table>
<thead>
<tr>
<th>Class of activator/stimuli</th>
<th>RASF activators</th>
<th>RASF stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td>TNF-α, IL-1β, IL-6, IL-17, IFN-γ, IL-18</td>
<td>IL-6, IL-15, IL-23, type IFNs, IL-1β, TNF-α</td>
</tr>
<tr>
<td>Growth factors</td>
<td>FGF, PDGF, TGF-β</td>
<td>GMCSF, VEGF, TGF-β, PDGF, SCF</td>
</tr>
<tr>
<td>Chemokines</td>
<td>CCL2, CCL5, CCL13, CXCL12, CXCL1</td>
<td>CXCL1,5,6,8,9,10,11,12,13, CCL2,3,5, CX3CL1</td>
</tr>
<tr>
<td>Bioactive lipids</td>
<td>Proteoglandins, leukotrienes</td>
<td>Proteoglandins</td>
</tr>
<tr>
<td>Cell surface ligand interactions</td>
<td>CD40/CD40L, VLA-4/VCAM-1, LFA-1/ICAM-1</td>
<td>CD40, VCAM-1, ICAM-1, TLR2, integrins, cytokines and chemokine receptors</td>
</tr>
<tr>
<td>Tissue degradation products</td>
<td>Endogenous TLR ligands, fibronectin and thrombin fragments, microparticles</td>
<td>MMPs, cathepsins</td>
</tr>
</tbody>
</table>

Table 1.8. Representative list of RASF’s stimuli and products after respective stimulation.

Table adapted from Noss and Brenner, Immunol Rev, Vol223 [353]
Already in 1997, Smith et al suggested SFs could be sentinel cells, based on the observation that fibroblasts do not just function as a structural element, but also have immuno-regulatory functions, such as synthesis of numerous growth factors, chemokines, cytokines and cell adhesion molecules that help in leukocyte recruitment, retention, survival and differentiation and the subsequent maintenance of immune responses within the joint microenvironment [354].

1.4.1.1 Morphology and altered behaviour of RASFs

It should be noted that RASFs differ considerably from fibroblasts of healthy joints in terms of the distinctive morphological features and stromal abnormalities as described by Fassbender earlier in the 1980s [355]. RASFs can proliferate in an anchorage-independent manner and exhibit defective contact inhibition in vitro [355], with expression of a variety of transcription factors such as c-myc that promote cell cycling control and metabolic upregulation of MMPs [356]; thus they have been considered as a tumor-like cell (also described as 'mesenchymal transformation') [357] (Figure 1.15). The intrinsic aggressive and destructive behaviours were convincingly demonstrated in SCID mice engrafted with synovium and cartilage, where RA, but not OA, synovium deeply invaded cartilage matrix and expressed MMP mRNA at the site of destruction. Accordingly, isolated lining RASFs, but not normal SFs (or dermal fibroblast) or SFs from OA patients (OASFs), can maintain the invasive and destructive tendencies towards healthy human cartilage after multiple passages in cell culture or when engrafted under the renal capsule of SCID mice [358]. These data provide strong evidence that SF are irreversibly altered in RA allowing them to remain activated even after removal from the articular inflammatory milieu or in the absence of help from other cytokine-producing cells. Indeed a recent study demonstrated that RASFs are able to spread the pathology by invading unaffected joints, an ability seen lacking in non-RASFs [359].
A working hypothesis is illustrated in the upper part of the diagram postulating that aggressive RASFs emerge as a consequence of different stages of regulation. SF originate from mesenchymal stem cells, which are undifferentiated, multipotent cells capable of rapidly mobilising, proliferating, and differentiating into appropriate cell types in response to certain environmental signals. In the lower part of the diagram, SF are shown to translate from a relatively quiescent state into (1) aggressive phenotype of RASFs, with the potential of (2) producing matrix degrading enzymes, (3) expressing adhesion molecules and facilitating cartilage damage and (4) producing cytokines for the recruitment of inflammatory cells to the site of tissue injury.
Under the influence of inflammatory cytokines, RASFs produce prodigious quantities of enzymes that degrade the extracellular matrix such as serine protease, cathepsins and MMPs. MMPs are probably the most important as collagenases (MMP-1, MMP-13) and stromelysins (MMP-3), which are especially relevant in RA. Their synthesis and activation is induced by various factors including proinflammatory cytokines, growth factors as well as TLR ligands. TLR3 and TLR4 stimulation increases MMP production in RASFs [110, 360]. Lining layer hyperplasia is characterised by increased numbers of RASFs due to increased proliferation and reduced apoptosis. An indication of the rapid proliferative capacity of RASFs is provided by the constitutively elevated expression of transcription factors such as NF-kB and AP-1, and proto-oncogenes which may subsequently alter the expression and function of different tumour suppressor genes (i.e. somatic mutation of p53 in RASFs as described by Firestein and colleagues [361]). Furthermore, several soluble factors present in the inflamed joint such as platelet derived growth factor (PDGF), TGF-β, IL-1 and TNF-α have been implicated in enhancing RASFs proliferation. Similarly, resistance to apoptosis contributes significantly to the synovial hyperplasia in RA. Several studies suggest that RASFs are resistant to Fas-ligand (FasL)-induced apoptosis [362]. Moreover, two specific anti-apoptotic molecules, termed FLICE inhibitory protein (FLIP) and small ubiquitin-like modified 1 (SUMO-1), which can inhibit Fas-mediated apoptosis are markedly expressed in the RA synovium, in particular along the synovial lining layer and at the site of cartilage invasion [88].
1.4.1.2 Involvement of RASFs as stromal cells that support B cells survival

Evidence suggests a critical involvement of RASFs in regulating B cell survival. *In vitro* studies showed that cell-cell contact between SF and B cells is required to promote B cell proliferation and differentiation of CD20$^+$ tonsilar B cells into CD20-CD38$^+$ cells, consistent with PC morphology [363] (Figure 1.16). Furthermore, synovial nurse-like cells have been reported to enhance B cell viability via adhesion molecules such as CD106 (VCAM-1) in addition to increase B cell Ig production. Similarly, Reparon-Schuijt and colleagues showed that RASFs can protect synovial fluid B cells from undergoing apoptosis [364]. Moreover, RASFs engagement of the chemokine stromal-derived factor-1 (SDF-1/CXCL12) and the adhesion molecule VCAM-1, with their respective ligands on B cells, CXCR4 and VLA-4, support B cell pseudoemperipolesis (migration under RASFs monolayers) [365].

It is highly relevant to this PhD thesis that blockage of the above interactions (chemokines, adhesion molecules) can only partially inhibit the downstream effects on B cell survival/activation/differentiation, suggesting that additional and yet undefined mechanisms regulate the crosstalk between RASFs and B cells.
Figure 1.16. Representative examples of RASFs in supporting B cell survival and differentiation.

Resting fibroblasts differentiate into aggressive spindle-like RASFs during inflammation and exert FDC-like functions (i.e., the capacity to bind GC B cells and to rescue them from apoptosis). Representative phase contrast photomicrographs demonstrating (1a) pseudoemperipolesis of B cell lines cultured for 2h on RASFs (Linhdout et al [96]) and that RASFs can (1b) bind and rescue purified tonsillar GC B cells from apoptosis by adhering along the RASFs surface (Burger et al [365]). (2) Co-culture of CD20⁺ tonsillar B cells with RASFs demonstrates terminal differentiation of B cells into PC, illustrated by Giemsa staining showing one RASF cell surrounded by plasma cells and by FACs analysis showing that tonsillar B cells lose their CD20 expression and acquire CD38 expression (Dechanet et al [363]).
1.4.2 Role of Toll-like receptors in RASFs activation

1.4.2.1 Toll like receptors and the innate immune system

As RA joints are characterised by chronic inflammation of the synovium, initiation of inflammatory arthritis requires a “pre-prime” or “induction” phase that precedes the clinically obvious synovitis. It is now believed that regulators of the innate immune system play a significant role in this phase. Aberrant TLR signaling recognising pathogen-associated molecular patterns (PAMP) is speculated to be involved in the initiation and perpetuation of synovitis and the regulation of innate and adaptive immune responses within the RA synovium.

TLRs are the best characterised pattern-recognition receptors (PRRs). They were discovered on account of their homology with fruit fly Drosophila toll protein (Toll) [366]. All TLRs are type 1 integral membrane family glycoproteins and belong to the IL-1R/TLR superfamily of proteins which all contain a conserved cytoplasmic motif, the Toll/IL-1R (TIR) domain. To date, 13 members of the TLR family are known in mammals, 10 of which are present in humans. TLRs can be classified into two subfamilies according to their cellular localisation, TLRs 1, 2, 4, 5, 6 and 10 are expressed on the cell surface while TLR3, 7, 8, and 9 are found on intracellular compartments, such as endosomes and the endoplasmic reticulum. Each TLR has been shown to recognise specific conserved components of pathogens and trigger a variety of defense mechanisms depending on the receptor and cell type. This includes the internalisation of microbes by phagocytic cells, antimicrobial killing mechanisms such as production of reactive oxygen species, the production of inflammatory cytokines, chemokines and the expression of costimulatory molecules. In addition to direct detection of pathogens via the recognition of microbial components, TLRs are also critical for linking the innate and adaptive immunity, in particular in the activation of DCs and macrophages, for the generation of T and B lymphocyte responses. Therefore, the activation of TLRs is highly likely to be critical also in the initiation of autoimmunity [367].
1.4.2.2 TLR ligands and their signaling

TLRs are type I transmembrane receptor with extracellular leucine repeats and a carboxy terminal intracellular tail containing a conserved region called Toll/interleukin1 receptor (TIR) homology domain. The extracellular domain is involved in ligand binding but is also necessary for dimerisation. A number of ligands have been identified through in vitro systems or knockout mice. TLR can recognise natural and synthetic exogenous and endogenous ligands in such a way that each TLR, alone, or by dimerisation, can recognise different bacterial, fungal and viral PAMPs as well as host-derived components [368]. Affinity between TLRs and their ligands appears lower than that between cytokines and their receptors. In spite of their low affinity, TLRs seem to recognise their ligands specifically. This has been demonstrated by analysing species-specific responses to TLR ligands. Generally, two main groups can be identified according to their corresponding ligands: 1) cell surface TLRs (except TLR5 and TLR10) recognise lipid-based PAMPs that are on the surface of microbes; 2) endosomal TLRs recognise nucleic-acid based PAMPs (double stranded RNA, dsRNA, single stranded RNA, ssRNA and CpG DNA). TLR5 and TLR10 do not correspond to either group; TLR5 represents the receptor for flagellin, the major structural protein of the flagella of gram-negative bacteria, whereas a specific ligand(s) for TLR10 is still unknown (Table 1.9).
<table>
<thead>
<tr>
<th>TLR</th>
<th>Adaptors</th>
<th>Pathogen activator</th>
<th>Exogenous</th>
<th>Class of activator</th>
<th>Endogenous</th>
</tr>
</thead>
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<tr>
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<td>MyD88/TIRAP</td>
<td>Bacteria, mycobacteria</td>
<td>Triacyl lipopeptides</td>
<td>Protein/peptides</td>
<td>βdefensin3</td>
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<td></td>
<td>Gram' bacteria</td>
<td>Lipoproteins</td>
<td>Protein/peptides</td>
<td>HSP 60,70, gp96, HMGB1, HMGBl-nucleosome</td>
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<td>PGN, LTA</td>
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<td>complexes, β-defensin 3, Hyaluronan, Surfactant</td>
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<td>Lipoproteins, lipopeptides</td>
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<td>PIC</td>
<td>Nucleic acids/ protein–nucleic acids</td>
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<td>Sulphate,</td>
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<td>Internal Ligands</td>
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<tr>
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<td>Bacteria</td>
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<td>Diacyl lipopeptides</td>
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<td>MyD88</td>
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<td></td>
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</table>

HMGB-1: high-mobility group box 1 protein; CEP: omega-(2-carboxyethyl)pyrrole; IgG: immunoglobulin G; PGN: Peptidoglycans; LTA: Lipoteichoic acid; aPL: antiphospholipid; LDL: low-density lipoprotein; NE: neutrophil elastase; PAUF: pancreatic adenocarcinoma up-regulated factor; ssRNA: single-stranded RNA.

Table 1.9. Human TLR, adapters and the relevant external and internal ligands [369, 370].
1.4.2.2.1 TLRs and lipid-based PAMPs

TLRs 2 and 4 have been studied most extensively. In 1998, genetic analysis revealed that TLR4 is a critical signal transducer for lipopolysaccharide (LPS), a glycolipid component of the outer membrane of gram-negative bacteria [371]. LPS not only can provoke a variety of immunostimulatory responses, but also cause a clinically life-threatening condition called endotoxin shock. Apart from lipids, other molecules recognised by TLR4 include proteins (F protein) and diterpene (Taxol, the LPS mimetic drug) as well as myobacterial components. However, recent evidence suggests that TLR4 also responds not only to exogenous but also endogenous factors produced by stress or cell damaging, such as heat shock proteins (HSP) [372]. HSPs are released from necrotic cells in certain pathological conditions, thereby inducing DC maturation by the activation of NF-κB pathway [373]. Such immune activation may provide a molecular basis for the danger theory of immune activation as previously proposed by Matzinger et al [374]. According to this theory, the immune system does not discriminate between self and nonself per se, but rather responds to antigens that are associated with danger signals released from damaged or stressed cells. During inflammation or tissue injury degraded extracellular matrix components, produced by proteases such as fibronectin [375] and soluble hyaluronan [376], can also exert their proinflammatory activity by binding to TLR4.

Microbial recognition by TLR4 can be modulated by other factors. As an example, a glycosylphosphatidylinositol (GPI)-anchoring protein, CD14, was identified as a facilitator of LPS signaling by binding and retaining LPS on the cell surface. Another small secreted molecule, MD-2, has been shown to associate with the extracellular domain of TLR4 and evoke a cellular response upon the interaction of TLR4 with LPS [377, 378].

TLR2 ligands recognise the widest repertoire of PAMPs due to the heterodimerisation of TLR2 with other TLRs (in particular TLR1 or TLR6) in order to optimally detect these ligands preceding cellular signaling. TLR2 was found to play a central role in recognising ligands associated with Gram-positive bacteria, mycobacteria, protozoan parasites, as
well as microbial lipoproteins, glycoproteins, glycolipids and nonenteric LPS. TLR2 interacts with TLR1 to recognise native mycobacterial lipoprotein and several triacylated bacterial lipoprotein such as the synthetic structure S-(2,3-bis(palmitoyloxy)-2-(RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH trihydrochloride (Pam3Cys) and meningococcal lipoprotein [379]. In contrast, a complex of TLR2 and TLR6 is needed for the detection of yeast zymosan and diacylated bacterial components including mycoplasma lipopeptide (MALP) and peptidoglycan (PGN) [380].

Recently, endogenous TLR2 ligands have also been identified. Necrotic but not apoptotic cells activate fibroblasts and macrophages via TLR2 [381]. This could be mediated by uptake of intracellular proteins such as HSP70 which induce IL-12 production via TLR2 [382]. Therefore the recognition of molecules that are expressed during cellular injury may modulate the function of non-immune cells.
1.4.2.2 Nucleic acid based TLR ligands

Generally TLRs recognising nucleic acid based PAMPs are localised as intracellular receptors, resident on the plasma membranes of endosomal vesicles. The signal propagation through these receptors depends on internalisation of the ligand to an acidic intracellular compartment. As a result, several studies have shown that inhibitors of endosomal/lysosome acidification, such as chloroquine and bafilomycin A1, can block the activation process through TLR3, TLR7 and TLR9, indicating that these signaling pathways require acidification and maturation in the endosome [383-385].

The ligand for TLR3 was identified as double stranded RNA (dsRNA) [386] whilst TLR7 and TLR8 recognise single stranded RNA (ssRNA) [387, 388]. Viral replication within infected cells results in generation of dsRNA that can provoke antiviral defense, suggesting that TLR3 fight against viral infection. Upon recognition of dsRNA, TLR3 transmits signals via the adaptor protein TIR-domain-containing adaptor inducing IFN-β, TRIF. This activates the transcription factors interferon regulatory 3 (IRF3), NF-KB and AP-1, leading to the induction of type 1 interferon (especially IFN-β) and proinflammatory cytokines. Type 1 interferon in turn activates interferon stimulated genes and production of anti-viral proteins, thus amplifying the anti-viral immune response. Also, TLR3 can potentially be activated by endogenous host single stranded mRNA and by the presence of secondary structures within the mRNA, such as hairpins which contain short segments of dsRNA [389]. Conversely, modification of RNA, such as methylation, can reduce the signaling through TLR3 compared to minimally modified viral nucleic acid [390]. At present, it is generally accepted that RNA from various sources can activate TLR3, as long as the RNA displays a secondary structure containing double-stranded regions, provided that the RNA is present in the appropriate cellular vesicles. TLR3 and TLR7 have been reported to localise in the same intracellular compartments that are often found adjacent to phagosomes containing apoptotic cell particles, suggesting that TLR3 and TLR7 can be triggered by nucleic acid from apoptototic cells during viral infection [391]. In experimental models, among all the synthetic analogs of viral dsRNAs, polyriboinosinic:polyribocytidylic acid (PIC) was
found to be the most potent IFN inducer [392] and is therefore utilised as a ligand for TLR3 to mimic viral infection. A study on the unmodified nature of PIC strengthened the evidence that TLR3 recognises the RNA complex rather than any modification or other structures of dsRNA [393]. In contrast to cofactor requirement for TLR4 activation, TLR3 simply interacts with dsRNA through the cationic surface of the receptor, while the receptor dimer is held in place by intermolecular forces within TLR3 [394].

Briefly, mouse TLR7, human TLR8, and to a lesser extent human TLR7, recognise ssRNA viruses such as the influenza, Sendai and Coxsackie B viruses. In addition to viral ssRNA and GU-rich ssRNAs, low molecular anti-viral compounds activate TLR7 and TLR8. TLR7 is involved in the recognition of imidazoquinoline compounds (imiquimod [R837] and resiquimod [R848]) [395]. TLR8 also recognises R848 in humans but not in mice, suggesting that TLR8 is nonfunctional in mice [396]. Furthermore, Lee et al. reported that guanosine analogs (Loxobribin) activate immune cells via TLR7 but not TLR8 [397].

TLR9 is the main receptor for unmethylated cytosin-guanosin dinucleotide (CpG)-DNA, a dinucleotide sequence that has been identified as a stimulatory motif of bacterial and viral DNA [398]. CpG motifs are more commonly found in DNA of bacteria and viruses rather than vertebrate, in which DNA is usually methylated. CpG rich DNA and oligodeoxynuclctides carrying the CpG motif induce specific immunostimulatory activities on B cells and DC via TLR9, as TLR9-deficient mice lack these responses [399]. TLR9 activation on B cells not only induces their proliferation, the crosslinking of the surface B cell receptors with TLR9 by immunocomplexes that contain chromatin can also triggers T-I secretion of IgG and IgM autoantibodies [400].
1.4.2.3 Distribution and expression of TLRs

TLRs are differentially expressed by a range of cellular subsets in human peripheral blood as well as in non-immune cells. A breakdown of each member of TLR mRNA expression is listed below [401-404] (Table 1.10):

<table>
<thead>
<tr>
<th>TLR</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Ubiquitously expressed on leukocyte population. Predominantly found on monocytes, macrophages, B cells, T cells, DCs, PMN, NK cells; non-immune cells (fibroblasts, astrocytes, ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR2</td>
<td>Leukocyte population: monocytes, macrophages, DCs, PMN; non-immune cells (fibroblasts, astrocytes, ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR3</td>
<td>Leukocyte population: DCs, macrophages, mast cells, NK cells, mucosal B cells; non-immune cells (fibroblasts, astrocytes, epithelial cells, keratinocytes)</td>
</tr>
<tr>
<td>TLR4</td>
<td>Leukocyte population: Monocytes, macrophages, DCs, PMN; non-immune cells (fibroblasts, astrocytes, ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Leukocyte population: Monocytes, macrophages, T cells, DCs, PMN; non-immune cells (fibroblasts, astrocytes, ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR6</td>
<td>Leukocyte population: Monocytes, macrophages, B cells, T cells, DCs, PMN, NK cells; non-immune cells (fibroblasts, astrocytes, ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR7</td>
<td>Leukocyte population: B cells, plasmacytoid DCs</td>
</tr>
<tr>
<td>TLR8</td>
<td>Leukocyte population: Monocytes, myeloid DCs</td>
</tr>
<tr>
<td>TLR9</td>
<td>Leukocyte population: B cells, plasmacytoid DCs; non-immune cells (GI ECs, keratinocytes)</td>
</tr>
<tr>
<td>TLR10</td>
<td>Leukocyte population: B cells (highest), plasmacytoid DCs</td>
</tr>
</tbody>
</table>

Table 1.10. Expression of human TLRs in different cell types.

Modified from [405].
1.4.2.4 TLR signaling

Once the TLRs encounter their ligand, TLR usually dimerise (homo- or hetero-) and interact with the TIR domain of adapter proteins. Five adaptor molecules interact via TIR domains to transmit TLR signaling: (1) MyD88 (myeloid differentiation factor 88); (2) Mal/TIRAP (MyD88 adapter-like/TIR domain-containing adapter protein); (3) TRIF (TIR domain-containing adaptor-inducing IFN-β); (4) TRAM (Toll-receptor-associated molecule); and (5) SARM (sterile α- and armadillo motif containing protein) (Figure 1.17). Among these, MyD88 is the universal adaptor essential for all TLRs with the exception of TLR3. In fact, the signaling pathways activated by TLRs are classified into MyD88-dependent and MyD88-independent pathways. The latter pathway starts with TRIF and is therefore also called the TRIF-dependent pathway. TRIF is the sole adaptor for TLR3 and with the bridging adapter TRAM by TLR4. It signalises in the following ways: (1) receptor-interacting protein 1 (RIP1) recruited with TNF receptor associated factor 6 (TRAF6) are able to switch on the proinflammatory cytokine pathway, NF-kB; (2) RIP1 can also recruits interleukin-1 receptor-associated kinase 1 (IRAK1) leading to activation of both the NFkB and activator protein-1 (AP-1) pathways; (3) the TNF receptor associated factor 3 (TRAF3) activates interferon regulatory factors (IRFs) and thus shifts to the type I IFN pathway for initiation of an antiviral defense.

For the MyD88-dependent pathway, the adaptor activates its own TIR domain or activation occurs via TIRAP, where it then recruits IRAK4 and IRAK1 which bind to TRAF6, resulting in activation of (1) transcription factor NFkB via IKB kinase (IKK) activation and (2) transcription factor AP-1 via activation of mitogen-activated protein kinases (MAPKs), including Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK).
Figure 1.17. TLR-mediated MyD88-dependent and independent signaling pathway.

Both superficial and endosomal signaling are shown. Following activation of extra- and intracellular TLRs by various PAMPs or DAMPs, TLRs recruit the adaptor proteins MyD88, TRIF, TIRAP, TRAM and SARM as indicated in blue. These then initiate signaling cascades involving IL-1R-associated kinase ( IRAK ) and TNFR-associated factor ( TRAF ) proteins, which finally converge at the activation of the IkB kinase ( IKK ) family members ( IKKa, IKKβ and IKKγ/NEMO  [used by MyD88 pathway]; IKKe and TBK1 [used by TRIF pathway]). The functions of SARM may be species-dependent. In humans, SARM could not activate NF-κB or IRF3 but has been identified as an inhibitor of TRIF signaling. As it contains a TIR domain, it binds directly to the TIR domain of TRIF, thereby blocking the interaction of TRIF with TLR3 or TRAM with TLR4 signaling. This interaction prevents the activation of IRF3, IRF7, NF-κB and apoptosis by TRIF[406]. NESCA is newly decribed as a NEMO-interacting protein and implicated in the NEMO-mediated NFkB activation pathway. All TLRs activate NK-κB and AP-1, inducing the proinflammmatory cytokines. Some TLRs can also activate IRK3, IRF5 and/or IRF7, leading to the production of type I interferon. Modified from image adapted from Shotorbani et al World J Biol Chem Vol 2:7 [407].
1.4.2.5 The role of TLRs in RA

1.4.2.5.1 TLR signaling in animal models of arthritis

A number of animal models of arthritis support the involvement of TLRs in chronic arthritis. Classical animal models of arthritis such as streptococcal cell wall (SCW) arthritis are dependent on the activation of TLR ligand, as TLR2 deficient animals exhibit a significantly reduced severity of arthritis. Similarly, no SCW-arthritis develops in mice that lack the adaptor molecule MyD88 [408]. Moreover, injection of staphylococcal peptidoglycan, CpG DNA or dsRNA into joints of mice results in a self-limited form arthritis [409, 410]. Furthermore, arthritis may also develop upon transfer of serum-containing antibodies to glucose-6-phosphate isomerase (GPI) in K/BxN mice. In this model, antibody deposits at the intra-articular space and provokes immune complex-mediated arthritis, which is ameliorated in TLR4–/– mice [411]. In a humanised model in which intact RA synovial explants were transplanted into SCID mice, spontaneous cytokine production is markedly suppressed upon specific blockade of TLR4 with similar efficacy compared to adalimumab treatment, an anti-TNF monoclonal antibody. In addition, TLR4 contributes to the development of the highly pathogenic Th17 cells and IL-17 production that promotes severe autoimmune spontaneous arthritis in mice [411]. Another recent study proposed TLR3 to act as the central player in the pristane-induced arthritis rat model where TLR3 expression was up-regulated during early disease stages, and aggravated disease severity upon TLR3 stimulation. Alternatively, small interfering RNA (siRNA) targeting TLR3 in vivo reduced disease severity [412]. These studies suggest that: (1) the availability of TLR ligands might be sufficient to initiate arthritis in a susceptible host; (2) endogenous TLR ligands contribute to arthritis persistence and destruction.

1.4.2.5.2 Local expression of TLR ligands in the rheumatic joint

Apart from the TLR ligands of microbial origin (cell wall fragment, e.g. peptidoglycan or bacterial DNA e.g. dsDNA) that have been detected in joint of RA patients, host derived endogenous ligand or damaged “self” molecules known as damage-associated molecular pattern (DAMP) might locally activate TLRs in driving inflammation during RA.
DAMPs are endogenous molecules that are immunologically silent in healthy tissues but become active and expressed within cells upon inflammation. These molecules can also be released: (1) under environmental stress even in the absence of apoptosis or necrosis; or (2) represent extracellular matrix (ECM) components that may be created by tissue damage or proteolysis and (3) ECM molecules that are specifically expressed as a result of tissue injury. In normal circumstances they serve as danger signals that alert the host to tissue injury and initiate the process of tissue repair. In addition to such a physiological role, there is evidence suggesting that DAMPs are also implicated in the pathogenesis of many inflammatory and autoimmune diseases characterised by aberrant TLR activation including RA.

ECM components including fibrinogen, fibronectin extra-domain A (EDA) [413], biglycan [414] and tenacin-C [415] are specifically up-regulated in response to tissue injury, and low molecular weight fragments of the ECM, such as hyaluronic acid [376] and heparin sulphate might act as endogenous TLR2 and TLR4 ligands within the RA joint (Figure 1.18). Furthermore, extravascular deposition of fibrinogen and fibrin clots might further trigger the TLR signaling cascade via TLR4. Extracellular HSPs, such as HSP60, 70 and HMGB1, have been reported to be released by activated/necrotic cells and are implicated in RA pathogenesis [416, 417]. However, it should be noted that in order to activate cell surface TLR2 and TLR4 within the joint, endogenous TLR ligands must be present locally in the inflamed joint and gain access to the extracellular milieu for subsequent activation. Accordingly, among DAMPs, EDA fibronectin, tenacin-C, serum amyloid A, gp96 and HMGB1 have been identified in the synovial fluid. However, although significant levels of HSP70 and biglycan were readily detected in the lysates of cells from rheumatoid joint, these molecules were not found in synovial fluid. Therefore, the molecular mechanism by which these potential DAMPs bind and activate TLRs remains to be determined.

Recently, TLR8 has also been shown to be important for the production of TNF-α within RA synovium since inhibition of TLR8 prevents “spontaneous”cytokine release by synovial tissue [418]. Therefore, stimulation of TLR8 by unknown ssRNA containing
components in the RA synovium leads to the expression of pro-inflammatory cytokines. Moreover, blood serum DNA in RA patients is considerably enriched with fragments of ribosome repeats containing immunostimulatory CpG-motifs, suggesting that endogenous TLR9 ligands may be increased in RA disease [419]. Notably, the above-mentioned studies emphasise the contribution of endosomal TLRs in RA. Accordingly, chloroquine, which prevents intracellular TLR function by impairing endosomal acidification, reduces cytokine release in synovial cells [418]. The anti-depressant drugs of selective serotonin reuptake inhibitors, fluoxetine and citalopram, and the anti-depressant small molecule mianserin, can also inhibit TLR3, 7, 8 and 9 activities and are therefore reported to be involved in inhibiting synovial cell cytokine release [420] as well as in improving symptoms in RA patients [421]. Overall, these studies suggest a significant role for TLR2 and 4 as well as the endosomal TLRs 3 and 8 in human RA disease.
Figure 1.18. Representative molecules derived from injured tissue, blood vessels, and necrotic cells that activate TLRs and induce inflammatory response.

Following tissue and cell injury, endogenous ligands of TLRs are generated and/or released. Extracellular matrix components (hyaluronic acid, fibronectin, heparan sulfate) released from damaged tissue; fibrin and fibrinogen released from injured blood vessels and heat shock proteins (HSP) released from necrotic cells, can bind to TLR4. RNA and chromatin-associated DNA released from necrotic cells can bind and activate TLR3 and TLR9, respectively.
1.4.2.6 Expression and function of TLRs on RASFs

Among the 10 human TLRs, expression of functional TLR2 was first detected in RA synovial tissue, and shown to be induced by IL-1 and TNF-α, resulting in the translocation of NF-kB and secretion of proinflammatory cytokines and MMPs. Likewise, a variety of chemokines found in the synovial fluid of RA patients was found to be secreted by TLR2-treated RASFs [422, 423]. Besides TLR2, enhanced expression of TLR3, TLR4 and TLR7 in RA synovial tissue has also been demonstrated. In vitro stimulation of TLR3 and TLR7 in RASFs resulted in significant up-regulation of chemokines, cytokines, metalloproteinases and type I IFNs [110, 360, 424]. More recently, Kim and colleagues suggested that TLR2, TLR3 and TLR4 signaling could induce RANKL expression on RASFs and promote the subsequent osteoclastogenesis [199, 200]. Notably, stimulation of TLR3 and TLR8, but not TLR7 or TLR9, increased TNF-α release in RA membrane cultures [418]. Conversely, disruption of downstream adaptors for TLR such as MyD88 and TIRAP, reduced cytokine synthesis in cultured RA synovial tissue, further strengthening the role of TLRs in RA pathogenesis [425].

Recently, functional TLR1-6, but not TLR7-10, have been shown to be expressed on RASFs, with TLR3 being the highest. Accordingly, stimulation of cultured RASFs in vitro with TLR2, TLR3 and TLR4 ligands leads to to up-regulated IL-6 expression as well as MMPs 1&3, with TLR3 ligand, PIC, be the most effective stimulaton [110, 360]. Similarly, activation of TLR3 by dsRNA released from synovial necrotic cells has been described, and injection of dsRNA into mice resulted in self-limited arthritis, suggesting an arthritogenic role of viral dsRNA. Interestingly, the dsRNA released from necrotic cells was demonstrated as an endogenous ligand for the stimulation of proinflammatory cytokines and chemokines in RASFs. Since these endogenous ligands are abundant in the synovial fluid of RA patients, TLR expression levels and the corresponding responsiveness, particularly for TLR3, might be critical factors for the perpetuation of RA [110].
1.4.2.7 Role of TLR-activated RASFs, as part of an innate immune system, in B cells differentiation and survival

Most studies have focused on the role of cytokines or classic proinflammatory mediators on B cell survival and maturation by RASFs. However, one should not exclude the fact that complex networks involving innate immunity and cytokines may also account for these processes. Previous studies suggested that innate immune responses mediated by TLR recognition of bacterial DNA could participate in triggering B cells to secret auto-antibodies in a TI manner. A recent study from Messer and colleagues demonstrated that ligands for TLR2, TLR4 or TLR9 cannot induce the de novo synthesis and release of BAFF by RASFs. Moreover, they found that exposure of TLR2, 4 and 9 with ligand to their respective ligands exert a negative regulation of BAFF synthesis in IFN-γ-primed RASFs [293]. However, their study did not include the influence on BAFF in response of the most prominent receptor, TLR3. Therefore, in my PhD project, I aim to dissect the role of TLR2, 4, as well as TLR3 on B cell activation/survival within the synovial microenvironment.
CHAPTER 2

Hypothesis and aims of the project

Several lines of evidence suggest that humoral autoimmunity within the rheumatoid joint is critically regulated by local interactions between infiltrating B cells and resident stromal cells. However, the mechanisms regulating such processes are largely unknown. In particular, the following evidence directly raises various questions, which I aim to address in this thesis:

(1) *In situ* B cell activation and humoral immunity towards RA-associated auto-antigens are retained for several weeks when RA synovium containing B cell follicles is engrafted into SCID mice, suggesting that resident cells are required for regulating B cells survival and activation in the ectopic lymphoid structures within the RA synovium.

(2) RASFs are critical candidates to regulate such interactions as they share properties of FDCs in rescuing B cells from apoptosis and promoting their proliferation and differentiation into antibody-producing cells. However, the mechanisms regulating the crosstalk between RASFs and B cells are unclear.

(3) Recent evidence demonstrated that B cells can be directly activated to undergo Ig class-switching in the presence of BAFF and APRIL released at mucosal sites by ECs sensing pathogens via toll-like receptors (TLRs), particularly TLR3.

(4) Accordingly, RASFs can express BAFF and APRIL, although the regulation of these factors in RASFs and their effective capacity in supporting AID expression and ongoing CSR in B cells is unclear. In particular, although accumulating evidence suggests that the activation state of RASFs is critically dependent on the expression of TLRs, the relationship between TLRs and BAFF/APRIL expression in RASFs is controversial.
(5) In addition to B cell pro-survival factors, TLR stimulation can also induce counter-immunoregulatory molecules, which negatively affect B cell activation and proliferation. In particular, a critical role for TLR3-induced endogenous SLPI in down-modulating B cell function has been suggested in airway ECs, but its expression/function in RASFs is unknown.

Thus, on the basis of the above, the main hypothesis of this PhD thesis is that RASFs play a fundamental role in modulating B cell activation and differentiation in the synovial microenvironment via a mechanism regulated by TLR stimulation and involving the release of pro (i.e. BAFF/APRIL) and anti-inflammatory factors (i.e. SLPI).

In order to investigate this aspect, in Chapter 4, I examined the effect of TLR2/3/4 ligands in modulating BAFF/APRIL expression in RASFs and their role in promoting AID expression and CSR in unswitched IgD⁺ B cells. In particular my aims were as follows:

- To investigate the expression of B cell survival factors (BAFF and APRIL) in RASFs/RADFs/OASFs
- To examine whether the overexpression of B-cell survival factors in RASFs are modulated upon TLR stimulation
- To assess whether these factors were functional in terms of the promotion of AID expression and the production of class-switched antibodies in unswitched IgD⁺ B cells
- To gather direct evidence that the stimulating capacity of RASFs in promoting B cell activation is dependent on BAFF and/or APRIL =
This hypothesis is summarised in Figure 2.1 below:

**Figure 2.1. Hypothesis I.**

Cross talk between stromal cells and B cells is dependent on the release of BAFF/APRIL by RASFs and is regulated by TLR stimulation.

The results of the experimental work which I carried out to test this hypothesis are presented in Chapter 4 and have been published in Bombardieri, M*, Kam, NW*, Brentano, F, *et al* (2011). A BAFF/APRIL-dependent TLR3-stimulated pathway enhances the capacity of rheumatoid synovial fibroblasts to induce AID expression and Ig class-switching in B cells. (*joint first author*) *Ann Rheum Dis* 10.1136/ard.2011.150219.
In the second part of my experimental work, presented in Chapter 5, I investigated the effect of TLR2/3/4 ligands in modulating SLPI expression in RASFs and their subsequent role in B cell activation. In particular I aimed to investigate:

- The expression of SLPI in RASFs vs. RADFs
- Whether SLPI in RASFs was modulated upon TLR stimulation
- The direct functional impact of SLPI in B cell activation in terms of modulation of AID expression, CSR and production of class-switched Ig in mature unswitched IgD\(^+\) B cells (*route 1, Figure 2.2*)
- The role of SLPI in modulating the capacity of RASFs to release B cell survival factors in response to TLR stimulation (BAFF/APRIL) (*route 2a, Figure 2.2*) and to influence the downstream B cell activation and proliferation (*route 2b, Figure 2.2*)
- To examine *in vivo* in the collagen-induced arthritis model the therapeutic potential of SLPI (data presented in Chapter 7, supplementary results).
Figure 2.2. Hypothesis II.

The cross talk between stromal cells and B cells is negatively regulated by RASFs-released SLPI via direct inhibition of B cell activation (route 1) and/or via down-regulation of BAFF released by RASFs (route 2).

The results of the experimental work related to this part of my PhD project are presented in Chapter 5 and are being finalised for publication.
CHAPTER 3
Materials and methods

3.1 Synovial fibroblasts isolation and culture

3.1.1 Synovial tissue collection

Synovial tissues were obtained from patients undergoing total knee/hip replacement. A total of 6 RA and 8 OA samples were available at QMUL and used for isolation of synovial fibroblasts (SFs). Samples were collected after informed consent (LREC 05/Q0703/198). Matched synovial and skin fibroblasts from 5 RA patients undergoing total knee/hip replacement were obtained from the University of Birmingham (LREC 5735), as previously reported [426]. All RA patients (10F/1M, mean age 64.1 yrs, 82% RF positive) fulfilled the American College of Rheumatology criteria for the classification of RA [4] and had long-standing disease requiring joint replacement. All patients had received DMARDs (in monotherapy or combination) and 2 patients received anti-TNF therapy. Patients had discontinued treatment before surgery for an appropriate washout period. Patients with OA had advanced disease and were all diagnosed with primary OA.

3.1.2 Patients samples and preparation

Synovial tissues obtained from patients were used for isolation of SF. After discarding fat and dense fibrous tissues, the synovium was dilacerated with tweezers, cut into small pieces with blade and gently mashed under 70-μm nylon mesh cell strainer (Becton Dickinson, Oxford, UK). Specimen pieces were collected and digested with 1.5mg/ml Dispase II (Gibco/Invitrogen, Paisley, UK) at 37°C in complete cell culture medium made of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin-streptomycin, 10mM HEPES buffer (all from
(Gibco/Invitrogen) overnight under rotation. Following enzymatic digestion, the remaining finely minced synovial tissues and detached cell-suspension were passed through a cell strainer and washed with DMEM complete medium by centrifugation at 400 x g for 10 min. The pellet containing synovial cells was collected, re-suspended in DMEM complete culture medium and plated into 75 cm$^3$ tissue-culture T75 flasks in a 5% CO$_2$-humidified incubator at 37°C.

3.1.3 Generation of fibroblast-like synoviocytes from RA and OA patients

RASFs and OASFs were obtained as previously described [110]. Synovial cells were left in culture flask for 7 days including removal of non-adherent cells every other day. Although there was variation from line to line, in general, culture medium was replenished once weekly. When adherent cells reached 90% confluency, they were passaged at dilution 1/3 into fresh T75 culture flasks by 0.25% trypsin/EDTA (Sigma, Poole, UK) treatment. The latter agent, EDTA, is a chelator of divalent cations with gentle dissociative properties. It acts to increase trypsin efficiency by neutralizing calcium and magnesium ions. These two ions are typically found in the extracellular matrix for cell-cell adhesion and normally obstruct the peptide bonds where trypsin acts on. After gentle trypsinization, FCS was added to the cells for inactivation of trypsin and cells were centrifuged for 10 min at 400 × g in order to remove the remaining trypsin. Synoviocytes were used between passages 4-8; at this stage the cell culture is devoid of contaminating lymphocytes and macrophages [427] (Figure 3.1). Paired control fibroblast cultures obtained from the skin of RA subjects undergoing total arthroplasty were kindly provided by Dr Andy Filer at University of Birmingham (as mentioned above). Similarly to RASFs, the RA dermal fibroblasts (RADFs) were also grown in complete medium, maintained in monolayer culture and used between passage 4 and 8 as described above.
Figure 3.1 Human synovial fibroblasts from RA patients (RASFs).

At passage 4-8, cells appeared to be a homogeneous population with large, flat, spindle-shaped or stellate-shaped cells, a typical fibroblast-like morphology.

3.1.4 TLR stimulation of RASFs, OASFs and RADFs

In study I (Chapter 4), all experiments were carried out with fibroblast cultures at ~90% confluence before stimulation. Fibroblasts were seeded into 24-well plates at 4x10^4 cells/well and stimulated with optimal concentrations of TLR2-4 ligands [360] [300ng/ml of bacterial lipopeptide (bLP), 10µg/ml of polyinosinic:polycytidylic acid (PIC) (InvivoGen, Toulouse, France), 100ng/ml of lipopolysaccharide (LPS) from Escherichia coli J5 (Sigma)] or left untreated for 4, 8, 24, 48 and 72h. Supernatants were harvested after the allocated time period and stored at -20°C until tested in ELISA. For RNA extraction, adherent fibroblasts were first rinsed with phosphate buffered saline (PBS) and then lysed in RLT buffer (Qiagen) under vigorous pipetting/vortex-mixing in order to lyse the cells thoroughly (see below section of RNA isolation). Lysed cells in RLT buffer were kept at -20°C until tested. Stimulation scheme is illustrated in Figure 3.2.
Figure 3.2. Diagram of the experimental design used to study the interaction between TLR-activated RASFs/RADFs/OASFs, B cell survival factors release and B cell activation.

Bacterial lipopeptide (bLP) is a TLR2-specific ligand (concentration: 300ng/ml); polyinosinic: polycytidylic acid (PIC) is a TLR3-specific ligand (concentration: 10µg/ml); lipopolysaccharide (LPS) is a TLR4-specific ligand (concentration used: 100ng/ml). The untreated RASFs/RADFs/OASFs were used to assess the basal expression of B cell survival factors.

In study II (Chapter 5), culture conditions were similar with the exception that fibroblasts were pre-treated: (a) with or without recombinant human SLPI (R&D 1274-PI, at a concentration of 50 and 500ng/ml); (b) with or without goat anti-human SLPI (R&D AB-260-NA)/goat anti rabbit isotype control (absorbed against human and mouse immunoglobulin, Southern Biotechnologies, Birmingham, Alabama, USA), both at a concentration of 10µg/ml. After 1h of pre-incubation, cells were stimulated with PIC at 10µg/ml for 4, 8, 24, 48 and 72h and harvested as mentioned above. A relevant isotype control goat anti- rabbit IgG (absorbed against human and mouse immunoglobulins) was used.

3.2 Human B cells isolation and culture

3.2.1 Purification of unswitched IgD+ human B cells

Human B cells were immunomagnetically selected from tonsils obtained following routine tonsillectomy. The tonsil specimens were obtained from children attending the weekly ENT tonsillectomy Day Surgery Clinic at Barts and the London NHS Trust and
kindly provided by the Human Tissue Resource Centre. Briefly, tonsil tissues were washed in a petri dish with chilled (4°C) MACS buffer [PBS, 0.5% FCS and 2 mM EDTA]. Specimens were cut into smaller pieces followed by gentle mashing through a 70-μm nylon mesh cell strainer (Becton Dickinson) by the rubber end of a 5ml syringe plunger, creating a tonsillar mononuclear cell suspension in the MACS buffer. The suspension of dispersed cells was filtrated through the cell strainer, washed and centrifuged at 400 x g for 10mins at 4°C.

After counting, aliquots of tonsillar mononuclear cells were collected as pre-sorted (PS) fraction for subsequent purity analysis (Figure 3.3). IgD⁺ unswitched B cells were magnetically sorted by incubating 3x10⁸ tonsillar mononuclear cells with 1 μl of anti-IgD biotinylated monoclonal antibodies (2032-08; Southern Biotechnologies, 0.5 μg/10⁶ cells) for 30mins on ice to avoid capping of antibodies on the cell surface during the labeling process. The amount of biontinylated antibodies used was optimised for least background staining. Cells were then washed, centrifuged at 300g for 10mins at 4°C, resuspended in MACS buffer and incubated with anti-biotin MicroBeads (130-090-485; Miltenyi Biotec, Bisley, UK, 80μl per 10⁷ cells) for 15mins at 4°C. After washing, cells were subsequently passed through magnetic separation columns (MS; Miltenyi Biotec) with a magentic cell separator. The effluent was collected as flow through (FT) fractions. The column was then removed from the separator and the bead-bound cells [positive selected, after-sorted (AS) fraction] retained in the columns were eluted and collected as enriched, positively selected IgD⁺ unswitched B cells fractions. All sorting procedures were performed on ice and under sterile conditions. The purity of IgD⁺ unswitched B cell isolation was checked by fluorescence-activated cell sorting (FACS; Figure 3.3).
Figure 3.3. Isolation of IgD+ unswitched B cell from human tonsils using MACS B cells enrichment kit.

Tonsillar B cells were labeled with anti-IgD biotinylated antibodies and anti-biotin MicroBeads. With the use of magnetic separation columns, labelled cells were eluted and collected as IgD+ B cells. Aliquots of pre-sorted (PS), flow though (FT) and after-sorted (AS) fractions were harvested for purity check by flow cytometry.
3.2.2 Flow cytometry analysis for B cell surface markers

Flow cytometry is a technique that uses the principles of light scattering, light excitation and emission of fluorochrome molecules to generate specific multi-parameter data from cells as they flow in a fluid stream through a beam of light.

The presence of forward scatter channel (proportional to cell-surface area or size), side scatter channel (proportional to cell granularity or internal complexity) and separate fluorescent channels allow the collection of light reflected by the cells. The combination of optical signals (emitted from fluorochrome-conjugated antibody targeting on an epitope of interest) derived from different detectors produce electronic signals which define multiple phenotypical, biochemical or molecular characteristics of the cells. The data can be displayed as single parameter histogram, two parameter dot plots with a quadrant marker, a two parameter dot plot with regions and three-dimensional plots.

In this part of my PhD work, flow cytometry was used to confirm purity and quality of the B cell separation procedure prior to downstream experiments with isolated IgD⁺ B cells.

Aliquots of PS, AS and FT cell fractions were collected, washed and resuspended in chilled fluorescence-activated cell sorter (FACS) buffer (PBS supplemented with 0.5% FCS). All subsequent processes were performed on ice in order to prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity. Cells were counted and added into 96-well round-bottom plate (0.5 x 10⁶ cells in 200µl FACS buffer/well) and washed twice by centrifugation at 2,000 rpm at 4°C for 3mins. After washing, cells were resuspended in 20µl (0.5 x 10⁶ cells) FACS buffer and stained with flurochrome conjugated antibodies (see Table 3.1) on ice for 30mins, unless stated otherwise.

The identification of B cell subpopulations was based on the expression of defined surface markers CD20, CD27 and IgD, whereby unswitched naïve B cells are
CD20+/IgD+/CD27+, unswitched memory B cells are CD20+/IgD+/CD27+ and switched memory B cells are CD20+ IgD−/CD27+ (as mentioned in the Introduction). A summary of staining scheme in each cell fraction is demonstrated in Table 3.1. Firstly, the B cells lineage was identified based on CD20 expression by incubating each cells fraction (PS/AS/FT) with directly conjugated R-phycoerythrin (RPE)-labelled mouse anti-human CD20 antibodies (2µl/0.5 x 10^6 cells in 20µl total FACS buffer; Clone B-Ly1; R7014; DAKO, Tube 3).

Additional classification was based on the expression of defined surface markers along B cells developmental pathway: (1) unswitched B cell were identified by staining the PS cell fraction with anti-IgD biotinylated monoclonal antibodies for 15mins and the biotinylated antibodies were detected by using Streptavidin-Flurescein isothiocyanate (FITC) (0.5µg/0.5 x 10^6 cells in 20µl total FACS buffer; 554060; BD Pharmingen, Tube 2); (2) unswitched B cells were also identified by staining the AS/FT cell fractions with Streptavidin–FITC (Tube 2); (3) memory B cells were identified by staining each cell fraction (PS/AS/FT) with direct conjugated PE-labelled mouse anti-human CD27 antibodies (25ng/0.5 x 10^6 cells in 20µl total FACS buffer; 555441; Clone M-T271; BD Pharmingen, Tube 4); (4) to further confirm that the positively selected IgD+ unswitched B cells expressed the pan B cells marker, CD20, each fraction (PS/AS/FT) was co-stained with CD20 and IgD (Tube 5); (5) to confirm that most of the memory B cells were eliminated after MACS enrichment for IgD+ B cells, each fraction (PS/AS/FT) was co-stained with CD27 and IgD (Tube 6). This staining approach allows us to determinate the efficiency of isolation technique in sorted unswitched B cells (IgD^hi, CD27^neg/low, CD20^+).
<table>
<thead>
<tr>
<th>Tubes</th>
<th>1 (control)</th>
<th>2 (IgD)</th>
<th>3 (CD20)</th>
<th>4 (CD27)</th>
<th>5 (IgD/CD20)</th>
<th>6 (IgD/CD27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Pre-sort fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin-IgD</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Strep-FITC</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>After-sorted fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin-IgD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strep-FITC</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Flow through fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin-IgD</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Strep-FITC</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.1. Scheme for purity check by FACS analysis on three different cell fractions.
After incubation with the relative antibody combinations as in Table 3.1, cells were washed twice in FACS buffer and stained cells were resuspended in 500µl of chilled FACS buffer and placed into Eppendorf tubes. In order to preserve light scattering signal and fluorescence intensity as well as maintain sufficient antigenicity for FACS, 2% paraformaldehyde (PFA) was added to the cells suspension. PFA is an aldehyde fixative which creates crosslinks between amino acid residues or between proteins. Addition of PFA was performed on ice followed by vortexing to prevent cells clumping. Fixed cells were stored in the dark at 4°C overnight prior to flow cytometric analysis. On the day of analysis, fixed cells were transferred into 5ml polypropylene round-bottom tubes (Becton Dickinson) and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences) and FlowJo Cytometry Analysis Software. Initial gating was set according to forward and side light scatter to exclude dead cells from the analysis and at least 10,000 events were counted for each sample. Spectral overlap between the fluorochromes was compensated electronically by single colour control samples. The purity of the isolated B cells was ≥97% IgD⁺, and was used for subsequent in vitro work.

3.2.3 Stimulation of IgD⁺ B cells

In preliminary experiments, increasing density (1-2 x 10^6 cell/ml per well) of unswitched B cells (IgD^{hi}, CD27^{neg/low}, CD20⁺) were seeded into 24-well plate and cultured in DMEM supplemented with 10% FCS, 50 IU/ml penicillin-streptomycin, 10mM HEPES buffer. Cells were stimulated with a combination of 1µg/ml recombinant human BAFF (310-13; Peprotech) and 100ng/ml recombinant human IL-4 (554605; BD Pharmingen). After 24, 48 and 72h of stimulation, cell culture supernatants were collected into Eppendorf tubes, and pelleted at 2,000 rpm for 7mins on a benchtop centrifuge. Supernatants were then collected for subsequent ELISA analyses (i.e. secreted Ig subclasses) while pelleted cells were lysed in RLT buffer for studying AID expression. The cell density of 1 x 10^6 cell/ml/well was chosen for subsequent culture or co-culture experiments as an adequate amount of Ig and transcripts were observed at this cell density, allowing meaningful comparisons.
For inhibition experiments using SLPI, each well containing 1 x 10^6 IgD^+ B cells/ml per well was incubated with increasing concentrations of recombinant SLPI at 10, 50 or 500ng/ml (R&D 1274-PI) for 1h. B cells were subsequently treated with or without 1µg/ml of BAFF and/or 100ng/ml IL-4. After 72h of incubation, cells were harvested in RLT buffer, supernatants collected for ELISA experiments, and mRNA extracted (for real-time PCR).

3.3 Co-cultures of fibroblast with IgD^+ B cells

3.3.1 Co-culture of RASFs, OASFs and RADFs with B cells

In study 1 (Chapter 4), all co-culture experiments were performed on ~90% confluent monolayers of fibroblasts. Cultured fibroblasts were detached from culture flask with 0.25% Trysin-EDTA and re-suspended in DMEM complete culture medium. A cell density of 4 x 10^4 cells/ml/well was seeded into 24-well plate at least 24h prior contact. On the day of co-cultures, medium was replenished with 700µl DMEM complete culture medium.

Sorted unswitched B cells (IgD^hi, CD27^neg/low, CD20^+) were washed twice in DMEM complete culture medium and added into 24-well plate with or without (control) fibroblast. In the case of studying direct cell-to-cell interactions, B cells (1x10^6 cells/300µl/well) were re-suspended in DMEM complete culture medium and added onto the fibroblast monolayers cultured in the presence or absence of 10µg/ml PIC. In the case of transwell studies, microporous PET cell culture inserts (1-µm pore, Beckon Dickinson) were employed. B cells were seeded in the cell culture insert and PIC treated or untreated fibroblasts were grown on the bottom of the culture well. The two separate populations were kept physically separated while allowing passage of macromolecules via the porous membrane of the cell insert. The co-culture experiments are outlined in Figure 3.4. B cells alone in the absence of fibroblasts were used as control to investigate the baseline expression of Ig and mRNA transcripts in the isolated IgD^+ B cells. Cell-free supernatants and B cells were collected after 24h, 72h or 8 days. For gene expression profiling, B cells were pelleted and lysed in RLT buffer (Qiagen).
Figure 3.4 Diagram showing different conditions for RASF/B cell co-cultures.

Co-cultures inserts are constructed of plastic sides with a microporous membrane on the base. The interaction between B cells and TLR-activated RASFs/RADFs/OASFs was studied either by: (1) direct cell-cell contact or (2) separating them via a transwell. B cells cultured alone and stimulated with PIC or BAFF/IL4 served as a positive control.

In study II (Chapter 5), co-culture experiments were performed in cell-cell contact manner. Fibroblasts (4 x 10⁴ cells/well) were seeded into 24-well plates and pre-treated for 1h with or without recombinant human SLPI (at a concentration of 50 and 500ng/ml) in the presence or absence of 10µg/ml PIC. After 1h of pre-incubation, B cells (1x10⁶ cells/well) were added onto the fibroblast monolayer. Control co-cultures received no fibroblasts. Cell-free supernatants and B cells were harvested after 72h of co-cultures. As above, for gene expression profiling, pelleted B cells were lysed in RLT buffer (Qiagen).
3.3.2 Blockade of BAFF/APRIL signaling on B cells with anti-BCMA and anti-BAFFR neutralizing antibodies

BAFF/APRIL dependent signaling in B cells was blocked using two different neutralizing antibodies: polyclonal goat anti-human BAFFR for neutralizing the interaction of BAFF to its specific receptor, and/or goat anti-human BCMA for neutralizing the interaction of both BAFF and APRIL to BCMA. The neutralizing antibodies (used alone or in combination) and their relevant goat IgG isotype control (all from R&D Systems) were used at 10µg/ml and incubated with B cells for 30mins before co-culture with RASFs and then throughout the experiment. This concentration of neutralizing antibodies was chosen based on the optimal response observed in preliminary experiments whereby BAFF/IL4 stimulated IgD⁺ B cells were seeded into a 24-well plate and incubated with increasing concentrations of anti-BAFFR or anti-BCMA antibodies at 3µg/ml, 5µg/ml or 10µg/ml (data not shown). After 72h and 8 days of co-culture, supernatants and cells were harvested as described above.

3.4 mRNA analysis

3.4.1 RNA isolation

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to manufacturer’s instruction. General aseptic techniques were employed when handling RNA. Briefly, cells were disrupted with RLT buffer containing 350µl of guanidine isothiocyanate with an addition of 10µl of fresh β-mercaptopethanol (M7522; Sigma) per ml of RLT buffer. The suspension (cells in lysis buffer) was transferred into Eppendorf tubes and placed on benchtop (room temperature) for 5mins with occasional vortex. This step ensures a complete disruption of cell walls and plasma membranes of cells and organelles which promotes the release of RNA contained in the sample. The endogenous RNases can be inactivated by the β-mercaptopethanol.
An equal volume of 70% ethanol solution (in RNase-free Baxter water) as the lysis buffer was then added to the cell lysate and mixed thoroughly by pipetting several times in order to precipitate the RNA from the aqueous phase. An aliquot of sample (total 700μl) was added to the RNeasy mini column placed in a 2ml collection tube and centrifuged at 10,000 rpm for 15sec. This step promotes selective RNA binding onto the silica-based membrane in the spin column. Afterwards, the flow through was discarded and the column was washed once with 700μl RW1 buffer, and twice with 500μl RPE buffer. After each wash tubes were centrifuged at 10,000 rpm for 15sec and the flow through was discarded. The tube was then centrifuged again for 2mins at 10,000 rpm in order to dry the RNeasy silica membrane followed by another centrifugation at 14,000 rpm for 1min to avoid ethanol carry over. For elution, the RNeasy column was transferred to a new RNase free 1.5ml collection tube and the RNA was eluted with 30μl of RNase-free dH2O, centrifuged at 10,000 rpm for 1min. Extracted RNA was immediately placed on ice for subsequent purity assessment and stored at -20°C until used.
3.4.2 Quantification of total RNA

RNA samples can be contaminated with chemicals, proteins, and other molecules that can inhibit or interfere with downstream applications such as reverse transcription polymerase chain reaction (RT-PCR). Spectrophotometric measurement of the amount of ultraviolet light absorbed by nucleic acids at particular wavelength provides a simple, but accurate assay for determining the yield of RNA and the degree of salt, solvent or protein contamination during the process of RNA extraction. To assess RNA yield and purity, after elution, an aliquot of each RNA sample was placed onto a Nanodrop-1000 v. 3.2.1 Spectrophotometer (Thermo Scientific) and absorbance was read at 260nm (RNA has its absorption peak at 260nm). The quantification of RNA content per sample is determined on the basis of optical density (OD) with 1 unit at 260nm corresponding to approximately 40µg/ml of single-stranded DNA and RNA. For quality assessment, samples are read at 280nm (proteins have an absorbance peak at 280 nm, mostly due to the presence of tryptophan residues). Pure preparations of RNA have a 260/280 OD ratio between 1.8 to 2.

3.4.3 Reverse transcription of RNA

To generate complementary DNA (cDNA), reverse transcription reaction was performed using the Thermoscript RT-PCR System for first-strand cDNA synthesis kit (Invitrogen, 11146-016). Generally, 9µl of the extracted total RNA was mixed with 2µl of 10mM dNTP Mix and 1µl of 50µM oligo (dT)$_{20}$ first strand primer and brought to a final volume of 12µl with Baxter water in 0.2ml thin walled-nuclease-free PCR tubes. The 1X first-strand PCR buffer mix was briefly mixed by centrifugation at 2,000 rpm for 1min before placing into a thermal cycle (GeneAmp PCR system 9700, Applied Biosystems) and heated at 65°C for 5mins to disrupt the secondary structure. Next, the reaction mixture was promptly chilled on ice to maintain RNA and oligos annealing. Subsequently, reverse transcription of RNA was performed in a total volume of 20µl reaction mix by adding 8µl of reverse transcriptase cocktail containing 4µl of 5X cDNA synthesis buffer, 1µl of DTT (0.1M), 1µl of DEPC treated water, 1µl of Thermoscript Reverse
transcriptase (15U/µl) and 1µl of RNaseOUT™ ribonuclease inhibitor (40U/µl). Samples were briefly mixed by centrifugation before incubating at 50°C for 1h in the thermal cycle. The reactions were terminated at 85°C for 5mins in order to inactivate the reverse transcriptase. Because the presence of RNA can interfere with the quantitative real-time PCR analysis, samples that contained more than 500ng/µl of total RNA underwent RNA digestion with 1µl of E-coli RNaseH (2U/ µl) at 37°C for 20mins. Finally, the completed strand of cDNA was diluted with RNase-free Baxter water at a final concentration of 10ng/µl of cDNA. All cDNA samples were stored at -20°C until used.

3.4.4 Detection of circular transcripts (CTs) by semiquantitative PCR

Detection of Iγ1/2-Cµ and Iα-Cµ CTs were performed as previously described with minor modifications [152]. Briefly, 20ng cDNA (10ng/µl) were mixed with 0.5U Taq DNA Polymerase buffer (MP biomedicals), 200μM dNTP mix, 1.5mM MgCl, 5pmol/ml of the reverse (Cµ) primer, 5pmol/ml of forward Iγ1/2 primer or 2.5pmol/ml of forward Iα primer (all from Invitrogen) and the final volume of each reaction was adjusted to 20µl with sterile Baxter water. Each reaction was mixed thoroughly before transferring into the thermal cycle. The standard PCR condition consists of an initial denaturation step at 94°C for 5mins, followed by three steps amplification cycle consisting of denaturing/annealing/extension at temperature varying among the different set of primers used. Primers sequences and PCR conditions are summarised in Table 3.2 and Figure 3.5 and 3.6. After the final cycle of PCR amplification the reaction mixes underwent a final extension at 72°C for 5mins before chilling on ice.

PCR products were analysed by agarose gel electrophoresis..Agarose gel (1%) in 1X Tris Acetic acid EDTA (TAE) buffer was prepared by boiling in microwave oven for 1.5 minutes, followed by addition of 0.5μg/ml ethidium bromide (a dye that binds to nucleic acid and emits fluorescent light upon UV stimulation). The solution was then poured into a minigel tray where a well-comb was inserted. After polymerization, the gel was placed into a minigel tank soaked with 1X TAE buffer. Samples were first mixed with blue loading dye (dilution of 1:6) to monitor the migration of DNA bands and then were loaded into appropriate wells and run at 120V for 30mins. 0.3μg PCR marker (N324,
New England Biolab) was mixed with loading dye (1:1) and loaded into one well of the gel in order to estimate the molecular weight of DNA bands. DNA bands were visualised under UV light and photographed with Bio-Rad Gel 1000.
Figure 3.5. Diagram showed an optimised cycling number for CT Iγ\(^{1/2}\)-Cμ (indicating ongoing class-switching from IgM to IgG1/2).

Tonsil, but not RASFs RNA extracts (negative control) expressed CT Iγ\(^{1/2}\)-Cμ. Highlighted bands indicate CT Iγ\(^{1/2}\)-Cμ (500 bp) amplified from total tonsillar cells at 40 cycle which appeared as 2 strong and specific bands representing alternative CSR to IgG1 or IgG2.

Figure 3.6 Diagram showed an optimised cycling number for CT Iα-Cγ (indicating ongoing class-switching from IgG1/2 to IgA).

Tonsil, but not RASFs expressed CT Iα-Cγ. Highlighted band indicates CT Iα-Cγ (502 bp) amplified from tonsil total RNA extracts after 33 cycles of amplification and provided the most specific PCR product compared to 35 and 37 cycles of amplification. RASFs did not show any specific band.
Table 3.2. List of primers and PCR conditions used to detect circular transcripts.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer forward and reverse sequence</th>
<th>PCR conditions (°C)</th>
<th>PCR conditions (cycles)</th>
<th>Amplicon size (bp)</th>
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<tr>
<td></td>
<td></td>
<td>denaturing/annealing/extension</td>
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</tr>
<tr>
<td>Cμ (reverse)</td>
<td>5’-GTTGCCGTTGGGTGCTGGAC-3’</td>
<td>94/60/72</td>
<td>40 cycles</td>
<td>502</td>
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<tr>
<td>Iγ1/2 (forward)</td>
<td>5’-GGGCTTCCAAGCCAACAGGACAGGACA-3</td>
<td>94°C, 1 min</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C, 1 min</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 1 min</td>
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</tr>
<tr>
<td>Iα (forward)</td>
<td>5’-CAGCAGCCCTCTTGAGACAGCCAGC-3’</td>
<td>94/58/72</td>
<td>3 cycles</td>
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<td>58°C, 1 min</td>
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<tr>
<td></td>
<td></td>
<td>72°C, 1 min</td>
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</tbody>
</table>
3.4.5 Gene expression analysis by quantitative real-time PCR (QT-PCR)

To determine the relative expression levels of a specific gene product, quantitative real-time PCR (QT-PCR) was performed on cDNAs obtained as in 3.4.3. According to the content of RNA obtained, 10ng of cDNA (but sometimes varying depending on the gene expression level of the target gene) was used in each PCR reaction to determine the levels of BAFF, BAFFR, APRIL, SLPI, IL-10, AID and TLR3 (Table 3.3). The gene expression profiling of QT-PCR was performed using 20x sequence-specific Taqman primers and probes diluted in 2x AmpliGold PCR master mix (Applied Biosystems, cat no: 4304437). PCR was performed in 384-well plates (Applied Biosystems) and for each well the final volume was adjusted to 10µl with sterile Baxter water. The PCR plate was sealed and centrifuged for 2min at 1,000 rpm to remove any air contamination. Afterwards, real-time PCR (Taqman) assay was performed in an ABI PRISM 7900HT Sequence Detection System Version 2.3 (SDS 2.3).
Table 3.3. List of Taqman primers and probes used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
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<th>ABI Gene expression Assay ID</th>
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<tbody>
<tr>
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<td>NM_020661</td>
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<tr>
<td>APRIL</td>
<td>NM_003808</td>
<td>Hs00182565_m1</td>
</tr>
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<td>NM_052945</td>
<td>Hs00606874_g1</td>
</tr>
<tr>
<td>BCMA</td>
<td>NM_001192</td>
<td>Hs00171292_m1</td>
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<td>NM_003265</td>
<td>Hs00152933_m1</td>
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<td>X03205.1</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Human β actin</td>
<td>NM_001101</td>
<td>Hs99999903_m1</td>
</tr>
</tbody>
</table>
The Taqman PCR reaction exploits a 5’ nuclease activity of the DNA polymerase which binds with the primer, resulting in cleavage of the Taqman MGB (minor groove binder) probe. Within the probe, a FAM reporter dye is conjugated at the 5’ end and linked to a non-fluorescent quencher at the 3’ end. During the reaction, cleavage of the probe allows the separation of the reporter dye from the quencher, resulting in increased fluorescence signal. Therefore, the fluorescence emitted during the reaction is directly related to the amount of specific amplicon generated during each cycle (in real time). This approach is opposed to the endpoint detection by the conventional quantitative PCR methods. The result generated from the real-time reaction is based on the detection and quantification of a fluorescence reporter where the increased fluorescence signal is directly proportional to the amount of PCR produced in the reaction. The thermal cycling condition consists of an initial UNG activation at 50°C for 2min and a Taq polymerase enzyme activation step at 95°C for 10min, followed by 45 amplification cycles consisting of denaturation at 95°C for 15sec and primer annealing and extension at 60°C for 60sec with fluorescence acquisition during each annealing and extension stage.

Where appropriate, the constitutively expressed human housekeeping genes 18S and beta-actin were used as internal controls for normalization. Tonsil cDNA was used as positive controls in each experiment, while RNAase-free dH₂O was used as negative control. A threshold cycle (Cₜ value) was obtained from each amplification curve for relative quantification (Comparative Cₜ Method). Relative mRNA expression analysis (calculations of the fold difference of a gene-of-interest) was conducted where the amount of target, normalised to an endogenous reference gene (18S/beta actin) and relative to the control sample (usually untreated sample in each different time point), was given by 2⁻ΔΔₐₗ. The amount of target and endogenous genes were first calculated from an average of triplicates, and a deltaCt (ΔCt) value was then obtained by subtracting the average Ct of the endogenous control from the averaged Ct value of the target gene. Thereafter, a ΔΔCt value was calculated by subtracting a chosen reference sample (as calibrator) from the ΔCt value of each sample. The relative quantification was then calculated from using the equation RQ=2⁻ΔΔCt, where 2 represents doubling of the amount of the PCR product after each cycle of amplification.
3.5 Protein analysis

3.5.1 ELISA for human immunoglobulin

Total IgA, IgG and IgM were detected using the Human IgA/IgG/IgM ELISA quantitation set (Bethyl laboratories) according to manufacturer’s instructions, with minor modifications.

ELISA is a sensitive and specific method for quantification of antibodies or antigens present in cell culture supernatants, serum, plasma etc. The ELISA used for the determination of human Igs is based on an indirect detection method. Briefly, a 96-well flat bottom polystyrene plate was coated with 100µl of individual capture antibody specific for the relevant antigen of interest (IgA or IgG or IgM) diluted 1:100 in coating buffer and incubated overnight at 4°C. On the next day, the coating buffer was removed by decanting and coated plates were washed three times with TBS, 0.05%Tween-20 (TBS-T), pH 7.6. At the last wash, residual solution was removed by inverting the plate and blotted it against a clean paper towel. Afterwards, each well was blocked with 100µl of 1% BSA in TBS for 30 min at room temperature. The blocking step not only reduces the background noise by inhibiting non-specific binding to uncoated regions of the plate and “sticky” sections of the absorbed protein, but also stabilises the antigenic and functional regions of the adsorbed protein. After incubation, the blocking solution was removed and the plate was washed five times with TBS-T. After washing 100µl of each supernatant at 1:4 dilutions and of serial dilutions of the appropriate standards for the calibration curves were added to the appropriate wells and incubated at room temperature for 1h. Standards and test samples were diluted in 1%BSA in TBS, 0.05%Tween-20. Both standards and samples were done in duplicate.

After incubation and 5 washes, 100µl of the relevant HRP-conjugated detection secondary antibody was applied to each wells and incubated for 1h at room temperature in the dark. The HRP conjugate antibody was used at the following concentrations: 1:20,000 for IgA; 1:50,000 for IgG; 1:20,000 for IgM. After five washes, 100µl of chromogenic substrate TMB (mixture of equal volume of two substrate-reagents) was
added to each well and incubated at room temperature in dark. Plates were allowed to develop at room temperature (with developing times varying among different Ig isotypes) and the enzymatic reaction stopped upon the addition of 100µl/well of 2M H₂SO₄. The OD of each well was determined by measuring the absorbance wavelength of 450nm using an ELISA plate reader (Genio).

Standard curves were generated using reference serum containing a known concentration of antibodies. Each standard was diluted to concentrations of 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml 15.63ng/ml, 7.8 ng/ml and 0ng/ml, defining a variable reference curve. A third-order nonlinear polynomial regression curve was fitted to characterise the relationship between OD and standard dilution (Graphpad Prism) and only curves with \( r^2 \) values of \( \geq 0.99 \) were used in subsequent analyses. Ig concentrations were determined by reading sample dilutions that fell within the standard curve. The resulting value was multiplied by the dilution factor of the sample to correct for the final concentration.
3.5.2 ELISA for BAFF, APRIL and SLPI

In study I (Chapter 4), the cell-free supernatant was collected from each well of 24 wells plate for the analysis of soluble BAFF and APRIL protein concentrations which were measured using the Quantikine human BAFF/BLyS/TNFSF13B (R&D Systems) and the human APRIL (eBioscience) ELISA kits, respectively.

In study II (Chapter 5), the cell-free supernatant were collected from each well of 6-wells and soluble SLPI protein concentration was analyzed using the human SLPI Quantikine ELISA Kit (R&D Systems). All ELISAs were performed following the manufacturers’ instructions.

Known standards curves were included for each determination, and concentrations of BAFF, APRIL and SLPI in culture supernatants were estimated by third-order nonlinear polynomial regression analysis. The resulting value was multiplied by the dilution factor of each sample (according to kit instructions) to correct for the final concentration.

3.5.3 Flow cytometry

3.5.3.1 FACS analysis for membrane-bound BAFF in RASFs

RASFs were seeded into 24-wells plate and treated with 10µg/ml of PIC or left untreated (4 wells per each treatment) when cells reached 90% confluency. After 24h incubation, supernatants were removed from adherent culture cells and the cells were washed twice with magnesium-calcium-free ice cold PBS and then detached by exposure to cold Versene (EDTA solution with PBS, 200µl per well) for 10min at 37°C. Cells from each of the 4 wells with identical treatment were pooled together for subsequent FACS profiling. Cells were regularly vortexed in order to keep them in single cell suspension. Cells were then washed twice and pelleted by centrifugation (5min at 1,500 rpm, 4°C) in cold FACS buffer containing PBS + 2%FCS. Subsequently, cells were blocked with 1% goat serum in FACS buffer on ice for 20min. Following two washes, the cells were resuspended in cold FACS buffer, equally divided (100µl each) and transferred into a 5ml
polystyrene round-bottle tube. The tube was centrifuged at 15,000 rpm for 5 min. Cell pellets were resuspended in cold FACS buffer and incubated with primary antibody (100 μl, 2 μg/ml) on ice for 1 h: rabbit anti-human BAFF polyclonal antibody (Buffy-2, Abcam), or irrelevant rabbit antibody control (Santa Cruz). Cells were then washed twice and incubated with RPE-labelled goat anti-rabbit Ig (100 μl, 1 μg/ml) for 30 min on ice in the dark. Mouse anti-human ICAM-1 conjugated with PE (eBioscience) was used as a positive control for membrane-bound staining. Additional controls (irrelevant RPE-labelled goat anti-rabbit Ig) were run in addition to the unstained control (to correct for cell autofluorescence) in order to evaluate the non-specific binding of the secondary polyclonal antibody to dead or sticky cells. After washing, samples were fixed with 2% PFA and stored in the dark until samples were run using the FACSCalibur flow cytometer (BD Biosciences) and analysed with the FlowJo Cytometry Analysis Software. An overflow of the staining scheme is shown in Table 3.4.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Antibody condition</th>
<th>Step 1 incubation</th>
<th>Antibody</th>
<th>Step 2 incubation</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unstained</td>
<td></td>
<td>Unstained</td>
<td>1 μg/ml, 30 min on ice</td>
<td>Autofluorescence</td>
</tr>
<tr>
<td>2</td>
<td>Rab anti human BAFF (primary antibody)</td>
<td>1 μg/ml, 1 h on ice Wash twice</td>
<td>Goat anti rab-RPE (second antibody)</td>
<td>Wash twice 2% PFA</td>
<td>Membrane BAFF</td>
</tr>
<tr>
<td>3</td>
<td>Rab antibody control</td>
<td></td>
<td>Goat anti rab-RPE</td>
<td></td>
<td>Specific binding of primary antibody control</td>
</tr>
<tr>
<td>4</td>
<td>Unstained</td>
<td></td>
<td>ICAM-PE</td>
<td></td>
<td>Positive control</td>
</tr>
<tr>
<td>5</td>
<td>Unstained</td>
<td></td>
<td>Goat anti rab-RPE</td>
<td></td>
<td>Non-specific binding of secondary antibody control</td>
</tr>
</tbody>
</table>

Table 3.4. An overflow of staining scheme for membrane BAFF and ICAM-1.
3.5.4 Western blot analysis for BAFF and APRIL protein expression

Western blot is a technique which allows detection of specific proteins in complex samples according to the size of the protein. It uses gel-electrophoresis from tissue/cell protein extracts to separate native or denatured proteins followed by transfer into membranes for immunodetection of relevant target proteins using specific antibodies.

3.5.4.1 Total cell protein extraction

RASFs and RADFs were seeded into 6-well plates and half of each plate (total of 3 wells) was either treated with 10µg/ml of PIC or left untreated. After treatment, culture supernatants were collected at indicated time points and maintained at -20°C for subsequent ELISA (BAFF, APRIL and SLPI, see relevant sections) while adherent cells were used for protein extraction. All procedures were performed at 4°C using pre-cooled reagents. Briefly, cells were rinsed twice with ice-cold PBS and whole cells lysates were prepared on ice by adding 200µl fresh disruption buffer/sample [50mM Tris (pH8), 150mM NaCl, 1% Nonidet P-40 (NP-40), 5mM sodium fluoride (NaF), 2mM EDTA, 2mM phosphatase inhibitors (Phosphatase inhibitor cocktail set II, Calbiochem), 1X protease inhibitors (Complete Mini protease inhibitor cocktail, Roche)]. Each sample (one single sample was considered by pouring 3-wells of cell lysate from each treatment) was disrupted with a soft cell scraper, transferred into 1.5ml centrifuge tube and lysed on ice for 1h with frequent vortexing and pipetting. Subsequently, the cell lysate was spun at 4°C for 15min at 15,000 rpm. The recovered supernatant was aliquoted, labelled as cytosol fraction (total whole cell lysate) and stored at -80°C. The remaining pellet mainly containing nuclear fractions was discarded.

Total tonsil cells were used as positive control. The cells were lysed similarly as above with the exception that cells were lysed with 40µl of ice cold RIPA buffer every 2 x 10^6 cells.
<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris pH8</td>
<td>Buffering agent as it is an innocuous substance to most protein and thus preventing protein denaturation</td>
</tr>
<tr>
<td>NaCl</td>
<td>Buffering agent prevents protein denaturation</td>
</tr>
<tr>
<td>NP-40</td>
<td>Non-ionic detergent which are good solubilizers in that they break lipid-lipid and lipid-protein interactions</td>
</tr>
<tr>
<td>NaF (Phosphatase inhibitors)</td>
<td>Phosphorylation/dephosphorylation of proteins influences hydrostatic relationships. Proteins undergo covalent attachment of a phosphoryl group (phosphorylation) at serine, threonine, or tyrosine residues. NaF acts on Serine/Threonine phosphatases</td>
</tr>
<tr>
<td>EDTA</td>
<td>Chelates Ca and Mg ions preventing phosphorylation in the lysate</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Prevents protein degradation</td>
</tr>
</tbody>
</table>

Table 3.5. List of components of the lysis buffer used for protein extraction and their functions.
3.5.4.2 Protein quantification (BCA protein assay)
Protein concentration was determined using the Peirce BCA (bicinchoninic acid) Protein assay (Thermo Fisher Scientific) according to manufacturer’s instruction. Briefly, in order to calibrate the standard curve, bovine serum albumin (BSA, 2 mg/ml) standards were prepared at serial concentrations of 0, 0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2mg/ml with the same disruption buffer as in the cell lysate. The cell lysate samples were prepared at three different dilutions (ratios of 1, 1:3 and 1:9) with disruption buffer and added at 10μl/well to a 96-well plate. Next, 200μl of BCA working reagent was added to each well and the plate was covered and incubated at 37°C for 30 min. Absorbance reading at 560nm was measured using a spectrophotometer (Tecan Genio). A standard curve of absorbance versus micrograms of protein was then used to quantify the amount of protein in each lysate.

3.5.4.3 Immunoblotting

3.4.4.3.1 Electrophoresis
Equal amounts of proteins (max 40 μg/lane) were diluted with 5x Lammeli loading buffer [10% SDS, 7.7% dithiothreiotol, 10% glycerol, 0.44M Tris (pH 6.8), 0.04% bromophenol blue] and boiled for 5min at 95°C for protein denaturation. Lysates were then mixed by brief vortexing and microcentrifugation. Samples were subsequently resolved by Polyacrylamide (13% acrylamide) gel electrophoresis (SDS-PAGE), composed of a stacking gel, comprising of 5% acrylamide mix, 0.125M Tris pH6.8, 0.1% SDS, 0.1% ammonium persulfate (APS), 0.1% TEMED, and a resolving gel, comprising of 13% acrylamide mix, 0.375M Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED, in which proteins are separated according to their molecular weight (the length of polypeptide). The polyacrylamide gels are formed by polymerization of monomeric acrylamide into polymeric polyacrylamide chains with crosslinking of the chains by N-N’ methylenebisacrylamide. The upper (low percentage 5%) stacking gel consists of large pore where the protein were quickly concentrated onto the bottom of the wells and the lower (high percentage 13%) resolving gel, with smaller pore size was where the protein separated. The acrylamide concentration utilised was selected according to the molecular
weight of the protein being tested. Generally, a low percentage gel is selected for resolving high molecular weight proteins. The above 13% resolving acrylamide was optimised for human BAFF (32 and 17 kDa) and APRIL (30 and 17 kDa).

As proteins have different electrical charges that affect their mobility, the SDS in the samples and buffers confers a negative charge to all protein which ensure that all the protein migrate through gel pores toward the positively charged anode when placed under an electrical field. SDS was also used to break up any remaining aggregates and non-covalently bound multimers. For determination of the size of separated proteins, a protein ladder (Precision plus protein all blue standards, Bio-Rad) was loaded in the first well of each gel (8μl/well). Each gel contained appropriate positive controls consisting of tonsil total cell lysates and 2ng human BAFF (Peprotech). The Bio-Rad electrophoresis chamber was then immersed in Tris-glycine electrophoresis buffer (see appendix for details), covered and electrophoresis was accomplished at 100V for 2hr at room temperature.
3.4.4.3.2 Transfer of protein to PVDF membrane

After separation, protein were transferred onto polyvinylidenfluoride (PVDF) microporous membrane (Immobilon-P, Millipore) by “wet transfer” in transfer buffer (see appendix for details) by utilizing a Bio-Rad-Tank. Methanol in the buffer system is necessary to achieve efficient binding to the PVDF membranes, possibly by decreasing the SDS affinity to immobilised proteins. The Immobilon-P membrane required a pre-treatment step by soaking in 100% methanol for 1-2 min, followed by equilibration of the membrane for at least 5 min in the transfer buffer before the transfer of proteins. Briefly, the gel was carefully removed from its running cassette, thereafter, the gel and methanol soaked PVDF membrane were assembled together ensuring that no air bubbles were found between the gel and the membrane. Next, the gel/membrane was sandwiched between filter paper and blotting pads and assembled within the blot module that was tightly locked in the cell. The inner buffer chamber was fully filled up with transfer buffer and the outer chamber was half-filled with transfer buffer. Finally, the transfer of protein was carried out at 150 V for 2 hr in a cold room at 4°C. The correct transfer was verified by the colorisation of the membrane with Ponceau red solution (0.2% Ponceau S in 3% trichloroacetic acid) for 1-2 min and followed by several rinses with deionised water to reveal transferred proteins as multiple bands. Due to its property as a negatively charged dye, Ponceau S binds to the positively charged amino groups of the proteins, resulting in a clear background and red protein bands. This staining technique is reversible allowing downstream immunological detection by immunoblotting. This technique has a sensibility of 250 ng of protein after separation by electrophoresis in polyacrylamide gels and transfer to PVDF membrane. Before immunoblotting, each membrane was blocked using 2.5% milk powder in wash TBS-T buffer comprising 0.05% tween-20 in TBS (20 mM Tris base, 150 mM NaCl in deionised water, pH 7.5) for at least 30 min at room temperature, placed on a rocking platform, and subsequently stored overnight at 4°C in blocking buffer to minimise non-specific bindings.

3.5.4.3.3 Immunostaining for BAFF and APRIL protein detection

For primary antibody incubation, membranes were inserted into 50 ml falcon tubes containing 10 ml of TBS-T, 2.5% milk power, and incubated on a roller for 2 hrs with the
following primary antibodies: 0.5μg/ml rabbit anti-human BAFF polyclonal antibodies (Abcam), 0.05μg/ml mouse anti-human APRIL polyclonal antibodies (Aprily-5, Enzo Life Science). Afterwards, membranes were extensively washed 6 times for a total of 1h in TBS-T and subsequently incubated with secondary antibodies conjugated with HRP, diluted in TBS-T/2.5% milk power solution for 1h at room temperature. The following secondary antibodies were used at a concentration of 1:1000: goat anti rabbit polyclonal antibodies (for BAFF) and goat anti mouse polyclonal antibodies (for APRIL, HAF008 and P0447, respectively, both from R&D system). After further 6 washes, the protein bands were finally detected using enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare) according to the manufacturer’s instructions. The signals were exposed and detected, under red light, by the exposition of autoradiography film, Hyperfilm ECL (GE Healthcare) using Fuji C-ray processor for appropriate exposure times. The film was then serially washed in Developer and Fixer buffers. If proteins of interest were present, a black band would appear on the film. The stained membrane was then stored in humid chambers with TBST at 4°C protecting from light for subsequent stripping and re-probing. Alternatively, the blot could also be placed inside an air-tight plastic bag at room temperature in the dark as an alternative method for long term storage.

3.4.4.3.4 Stripping of western blot

The process of stripping was used to remove the primary and secondary antibodies from a western blot membrane. Therefore, the blot could be reused by stripping and re-probing with an anti-human beta-actin antibody. Stored membranes were firstly soaked in TBS-T and then washed 3 times under agitation for at least 30min at room temperature. Afterwards, the membrane was re-blocked with 2.5% milk power for 15min under agitation at room temperature. The membrane was then submerged in a pre-warmed (50°C) stripping buffer (100mM 2-meracaptoethanol, 2% SDS, 62.5mM Tris HCl, pH6.7), and incubated for 30 minutes at 50°C in a water-bath, with occasional shaking. The blot was washed extensively in TBS-T 3 times (15mins each) and was then probed with mouse anti-human beta-actin monoclonal primary antibody (Sigma, A2228) diluted 1:5000 in TBST. After 6 washes for a total of 1h at room temperature, the membrane was incubated with 1:1000 goat anti-mouse polyclonal antibodies conjugated with HRP (P0447, R&D system),
diluted in TBS-T/2.5% milk power solution for 1h at room temperature. Subsequently, the blot was detected as mentioned above.

### 3.5.5 Immunocytochemistry

Immunocytochemistry is a technique which allows direct visualization of specific antigens in the cultured cells by the use of antibodies conjugated with enzymes or fluorescent dyes.

#### 3.5.5.1 Detection of BAFF, APRIL and SLPI expression in fibroblasts using immunocytochemistry

Fibroblasts were seeded at 5\times10^4 cells/200µl onto cover slides. They were placed onto petri-dish covered with lid to prevent evaporation and allowed to grow to 90% confluence before treatment. Cells were stimulated with 10µg/ml PIC or left untreated. After 24h (for BAFF and APRIL detection) or 72h (for SLPI), adherent cells were washed in PBS and fixed and permeabilised with ice-cold 1:1 acetone/methanol for 15mins if staining was performed on the same day, or stored in coplin jar containing the above fixative at -20°C until used. Staining scheme is outlined in Figure 3.7.

![Figure 3.7. Diagram showed a staining design on a coverslide.](image)

Adherent fibroblasts were treated with 10µg/ml PIC or medium only. Immunochemical staining for BAFF, APRIL or SLPI on PIC-treated fibroblasts was done in triplicates. Untreated fibroblasts served as control. As negative control, respective isotype antibodies were employed.
To ensure immobilization of antigens on the target cells while retaining authentic cellular and subcellular architecture, the cells undergo adequate fixation. A mixture of two organic solvents was chosen, acetone and methanol, which are both recognised as dehydrating agent and thus they compete with water for protein hydrogen bond. This preserves cellular architecture by fixing proteins via precipitation/coagulation. In an attempt to gain advantages of both reagents, acetone, which is a gentler fixative and methanol, which is a better fixative, were mixed 1:1 (vol: vol) and used at -20°C. Both fixatives can also dissolve lipids from cell membrane making them permeable to antibodies allowing free access of antibodies for binding to their specific antigens. On the day of staining, slides were removed from the coplin jar and excess fixative was flicked off before placing promptly into a humidified chamber, thus preventing cells from drying out. Afterwards, each well on the coverslide was encircled with a PAP pen hydrophobic marker before soaking the slide in TBS containing coplin jar for 5mins under agitation. Labelled StrepAvidin-biotin immunohistochemical (LSBC) method was applied for amplifying staining signals based on high affinity binding effect of biotin (a low molecular weight vitamin H) for avidin (a large glycoprotein). This technique involves a three steps staining procedure: the first layer is an unlabeled primary antibody, the second layer consists of a biotinylated secondary antibody and the third layer is a complex of StrepAvidin-biotin conjugated with a fluorescent dye. Signals are amplified using this system because several biotins are conjugated to antibodies and each biotinylated residue can bind to more than one molecule of streptavidin, as illustrated in Figure 3.8.
Figure 3.8. Diagram showed the use of biotinylated antibodies to amplify immunocytochemical reactions by Labelled StrepAvidin-biotin immunohistochemical (LSBC) method.

(1) The first step of the procedure is to incubate the section with primary antibodies raised against the antigen of interest; (2) Next, biotin-labelled secondary antibodies are added; (3) In the last step of the procedure, the antigen is localised by incubation with streptavidin conjugated with a fluorescent dye.
Because cells may contain endogenous biotin resulting in autofluorescence, a blockage step is essential and can be performed by pre-treating the cells with unconjugated avidin followed by saturating the remaining avidin binding site with free biotin (avidin-biotin block). For this purpose, before primary antibody incubation, fixed cells were depleted of endogenous biotin using a commercial avidin-biotin blocking kit (Vector laboratory). Briefly, slides were incubated for 10mins with unconjugated avidin to block endogenous biotin; after washing, slides were incubated for 10mins with unconjugated biotin moieties to block the remaining biotin-binding activity of the avidin molecule. Slides were then washed three times with TBS under gentle agitation after each blockage step for 5mins each. To inhibit non-specific binding, slides were further incubated for 10mins with DAKO serum-free protein block. This solution contains casein, a hydrophilic protein which is more effective than the classic normal serum block. Any excess protein block was flicked off and wiped away with tissue paper. Without any washing step, cells were then incubated with rat anti-human BAFF (Buffy-2, Enzo Life Sciences) or mouse anti-human APRIL (Aprily-2, Enzo Life Sciences) or their respective isotype controls at 10μg/ml for 1h at RT. Goat anti-human SLPI (R&D) or its respective isotype control were applied at 4μg/ml for 1h at RT. After the primary antibody reaction, the slides were washed and appropriate biotinylated antibodies (donkey anti-rat, Serotec, for BAFF, rabbit anti-mouse, Dako, for APRIL, both at 3μg/ml or rabbit anti-goat, Dako, for SLPI at 2μg/ml) were added for 1h at RT. Primary and secondary antibodies were all diluted in DAKO Antibody Diluent (S3022, DAKO). Afterwards, slides were washed and incubated for 30mins in the dark with Streptavidin ALEXA 555 at 1:100 dilution, (detected on the TRITC channel, for BAFF and SLPI) or Streptavidin ALEXA 488 at 1:300 dilution (detected on the FITC channel for APRIL) followed by three washing steps. For nuclear counterstaining, cells was incubated in the dark with DAPI (1:1000 dilutions) for 10mins and rinsed with TBS for 10mins under gentle agitation. The cells were mounted in an aqueous-based anti-fading mounting medium Mowiol 4-88 reagent (Calbiochem, cat no: 475904) with a coverslip for preservation of the fluorescence tags. Slides were then examined under an Olympus BX60 microscope.
3.5.5.2 Detection of membrane-bound BAFF and ICAM-1 expression in fibroblasts using confocal microscopy

Fibroblasts were seeded at $5 \times 10^4$ cells/200µl onto cover slides and grown to 90% confluence. Cells were then stimulated with 10µg/ml PIC or left untreated. After 24h, cells were washed in PBS and fixed using 4% paraformaldehyde (PFA) at RT for 10mins. PFA is a non-permeabilizing cross-linking reagent which forms intermolecular bridges via free amino acid residues, thus creating a network of linked proteins. After three washing in TBS, unreacted aldehyde can be quenched by incubating slides with 0.1M of amino-containing reagent, glycine, for 10mins at room temperature in order to reduce free aldehyde groups of PFA. This can effectively lower the fluorescent signal to noise in the staining. After further washing, slides were blocked with avidin-biotin blocking kit (Vector laboratory) intercalated by washes with 0.1% BSA in TBS, for 10mins each at RT. Slides were then incubated with rat anti-human BAFF (Buffy-2, Enzo Life Sciences) followed by a rabbit anti-rat biotinylated antibody (DAKO, 1:300) and streptavidin Alexa 488 (Invitrogen). In selected stainings, in order to colocalise membrane-bound BAFF, a mouse anti-human ICAM-1 antibody conjugated with PE (eBioscience) was added at 1:100 dilutions after BAFF staining. Slides were analysed with a Leica DM5500Q confocal microscope (Leica, Wetzlar, Germany). Matching isotype controls were used at the same concentration to exclude non-specific staining. As above, all antibodies were diluted in DAKO Antibody Diluent and all incubation steps were performed at RT in a humidified chamber.
3.6 Statistical Analysis

Differences in quantitative variables were analysed by the Mann Whitney U test when comparing 2 groups and by Kruskal-Wallis with Dunn’s post test when comparing multiple groups. Wilcoxon’s signed-rank test was used to analyse repeated measurements at different time-points. All analyses were performed using GraphPad Prism version 5.01, GraphPad Software, USA. A p value <0.05 was considered statistically significant.
CHAPTER 4

Results

A BAFF/APRIL-dependent TLR3-stimulated pathway enhances the capacity of rheumatoid synovial fibroblasts to induce AID expression and Ig class-switching in B cells

In this part of my PhD project, I demonstrated that signaling between TLR3 and B cell survival factors (BAFF and APRIL) enables autoreactive B cell activation by RASFs: i) BAFF and APRIL were produced by RASFs at mRNA and protein level; ii) BAFF and APRIL were induced by TLRs, particularly TLR3; iii) these cytokines displayed dynamic gene expression (early vs. late gene induction) upon TLR stimulation; iv) RASFs-derived BAFF and APRIL were required for inducing functional B cell activation (AID, IgG/A/M production), v) interfering with signaling through both BAFFR and BCMA on the B cell surface downregulated these processes in the activation of functional B cells.

4.1 TLR3-stimulated induction of BAFF and APRIL mRNA in RASFs, OASFs and RADFs

I first examined whether stimulation of synovial fibroblasts from OA and RA patients (to dissect disease specificity) as well as paired RA dermal fibroblasts (to investigate site specificity) with TLR ligands was capable of inducing mRNA expression of BAFF and APRIL. Since others and I have shown that TLR2, TLR3 and TLR4 are strongly expressed on RASFs and OASFs [360], I focused on specific ligands for these receptors using optimal concentrations as previously established [110]. Fibroblasts were treated with TLR agonists: bLP (TLR2 ligand), PIC (TLR3 ligand) and LPS (TLR4 ligand) and harvested at 4, 8, 24, 48 and 72 h.
BAFF mRNA expression in RASFs and OASFs was markedly induced by PIC (Figure 4.1a, e), whilst there was a more modest induction with LPS and no upregulation with bLP. The exposure of RASFs to PIC triggered a rapid induction of BAFF transcripts at 4h, which peaked at 8h and gradually decreased afterwards. Similar to RASFs, a comparable increase in BAFF mRNA was induced by PIC in RADFs (Figure 4.1c), suggesting that BAFF mRNA upregulation in response to PIC is not disease specific or dependent on the anatomical localisation of the fibroblasts. However, in my experiments I noticed that at baseline RASFs and OASFs displayed increased expression of BAFF mRNA; between 4 and 8-fold higher than RADFs (Figure 4.2a), as previously reported [428]. Conversely, I showed that RADFs were capable of producing significantly higher levels of BAFF mRNA in response to TLR4 stimulation as compared to RASFs or OASFs, and similar to that obtained with PIC (Figure 4.1).

In comparison with BAFF, APRIL was weakly modulated by TLR3 stimulation in RASFs (Figure 4.1b) and OASFs (2.5-fold increase, Figure 4.1f) with no apparent modulation in RADFs (Figure 4.1d). Interestingly, although modestly increased, APRIL displayed a different kinetic of expression compared to BAFF, with a later peak of induction at 24h (Figure 4.1b). Differently from BAFF, no difference in baseline mRNA expression of APRIL was observed in RASFs compared to OASFs and RADFs (data not shown). I finally investigated whether there was a differential expression of TLR3 mRNA in RASFs compared to OASFs and RADFs and I found no difference among these cells in basal TLR3 expression (Figure 4.2b).
Figure 4.1. Induction of BAFF and APRIL mRNA in RASFs, RADFs and OASFs in response to TLR ligands.

Quantitative Taqman real-time PCR analysis of BAFF and APRIL mRNA expression in response to TLR ligands. RASFs (a and b), RADFs (c and d) and OASFs (e and f) were stimulated with 10μg/ml PIC (TLR3), 100ng/ml lipopolysaccharide (TLR4), 300ng/ml bLP (TLR2) or medium alone for 4, 8, 24, 48 and 72 h. Results are expressed as mean±SEM of the mRNA fold increase calculated at each time point relative to the baseline unstimulated expression. Data were normalised using mammalian 18S as an endogenous control gene (18S). *p<0.05, **p<0.01 compared with unstimulated.

OASF, synovial fibroblast from osteoarthritis patient; PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
Figure 4.2. Comparative expression of BAFF and TLR3 mRNA in RASFs and RADFs.

Box and whisker graph showing quantitative Taqman real-time analysis for basal (a) BAFF and (b) TLR-3 mRNA expression in RASFs (n=5) vs. RADFs (n=5). Data are expressed as the difference in threshold cycle (dCt) following normalisation for the endogenous controls. The lower and upper margins of the box represent the 25th and 75th percentile, respectively, while the extended arms represent the 10th and 90th percentiles. The mean is indicated as a horizontal line within the box.

RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
4.2 RASFs but not RADFs release high levels of BAFF in response to TLR3 stimulation

I next examined whether the over-expression of BAFF mRNA in response to TLR3 was mirrored by increased protein expression and release of soluble BAFF. As shown in Figure 4.3a, upon PIC stimulation, high amounts of soluble BAFF was released in the supernatant of RASFs in a time-dependent manner reaching 600pg/ml at 72h. Similar levels were observed in the supernatant of OASFs (Figure 4.3b). Strikingly, despite strong up-regulation of BAFF mRNA, RADFs produced only modest amounts of soluble BAFF in response to PIC, ~10-fold lower compared to RASFs (Figure 4.3a). Similar differences were observed analysing the basal (i.e. unstimulated) levels of BAFF released in the supernatant of RASFs vs. RADFs. While unstimulated RASFs constitutively and time-dependently released BAFF, reaching 200pg/ml at day 3, negligible BAFF production was seen in RADFs (Figure 4.3a). Overall, these data indicate that increase production of BAFF, either at basal level or upon TLR3 stimulation, is characteristic of synovial fibroblasts and dependent on anatomical location.

To further confirm that RASFs and RADFs differ in their capacity to produce BAFF at protein level, I next investigated the intracellular localisation of BAFF upon TLR3 stimulation by immunofluorescence. As shown in Figure 4.3c, weak and granular perinuclear BAFF immunoreactivity was detected in untreated permeabilised RASFs. Upon incubation with PIC for 24h a strong increase in cytoplasmic BAFF as well as membrane-bound BAFF was observed. The strong increase in BAFF staining intensity was observed with the relocation to the periphery of the cells accompanied by transformation of RASFs from a spindle shape into an aggressive stellate phenotype [429]. Similar data were observed in OASFs (Figure 4.3d, result was performed by my collaborator, Dr Brentano). In contrast, following exposure to PIC, RADFs failed to increase the intensity or modify the pattern of BAFF cytoplasmic staining which remained weak and barely detectable. Overall, our histological data corroborate the findings of ELISA data showing that increased production of BAFF, at basal level or upon TLR3 stimulation, is characteristic of SFs but not DFs.
While the ELISA data reflected a processed soluble form of BAFF, I illustrated the presence of cell surface transmembrane BAFF by using flow cytometry and immunofluorescence staining. As revealed by flow cytometry, RASFs constitutively expressed transmembrane BAFF, which was modestly upregulated upon PIC stimulation (Figure 4.4a). The confocal microscopy images showed that the localisation of BAFF along the periphery of the RASFs, as illustrated by the overlapping staining with cell surface adhesion molecule, ICAM-1 (Figure 4.4b). I further used a Western blot approach to discriminate between the transmembrane and processed forms of BAFF. As controls, I ran soluble recombinant BAFF (Peprotech) or tonsillar cells, which identified as a source of mononuclear cell. As shown in Figure 4.5, the antibody used for flow cytometry also worked in Western blot analyses, which was capable of recognising both the full-length as well as the soluble forms of BAFF (corresponding to amino acids 83-285 of human BAFF). Analysis of lysates obtained from RASFs and RADFs revealed a 32kDa band, suggesting the expression of endogenous full-length BAFF (Figure 4.5, lane 1 and 3). The presence of the 32kDa band in RASFs, but not RADFs, became more prominent after 48h of PIC stimulation (Figure 4.5, lane 2 and 4). This result further demonstrating that the increased production of BAFF at basal level or upon TLR3 stimulation was a characteristic of SFs but not DFs. Interestingly, an albeit but modest full-length BAFF in RASFs and RADFs remained low and did not up-regulated upon 24h and 72h incubation with PIC (data not shown), suggesting the mechanism of membrane externalisation appears in a selective kinetic process. In addition to 32kDa species, the anti-BAFF antibody can also recognise a lower molecular weight form of 17kDa, representing the processed form of BAFF. However, my Western blot experiments could not detect the soluble form of BAFF in all the tested lysates. A plausible explanation might be the fact the cleavage of membrane BAFF was dependent on the amount of surface expression and activity of the pro-protein convertase furin, which cleaves and in turn releases surface-bound BAFF to the extracellular medium as soluble form of BAFF at a specific time point. Therefore, the time-point of cell harvesting as well as tissue variability and cell concentration may influence the detection of the 17kDa lower molecular weight form of BAFF in my system.
Figure 4.3. TLR3 stimulation induces intracellular and soluble BAFF in RASFs and OASFs but not RADFs.

(a) Column-bar graph showing the amount of soluble BAFF released in the cell culture supernatant from RASFs and RADFs conditioned for 24, 48 and 72h in the presence or absence of 10μg/ml PIC. RASFs produce significantly higher amounts of BAFF, both in resting and stimulated conditions, than RADFs. Results are represented as mean±SEM of minimum three experiments.

(b) Column-bar graph showing the amount of soluble BAFF released in the cell culture supernatant from RASFs and OASFs conditioned for 24h in the presence or absence of 10μg/ml PIC. RASFs produce similar amounts of BAFF as compared with OASFs, both in resting and stimulated conditions. Results are represented as mean±SEM of minimum three experiments.

(c) Representative microphotographs of BAFF immunostaining on RASFs and RADFs before and after stimulation with PIC. RASFs/RADFs were grown on coverslips and processed for immunofluorescence as described in the Methods. Nuclei are counterstained in blue (DAPI) and BAFF in red (Alexa-555). Primary RASFs/RADFs were left untreated (medium only) or stimulated for 24 h with PIC (10μg/ml). Appropriate isotype control was used to confirm specificity. Images were captured at 20x magnification and with the same exposure time for both conditions.

(d) Representative microphotographs of BAFF immunostaining on OASFs before and after stimulation with PIC. OASFs were grown on coverslips and BAFF (brown) was detected based on the activity of colorimetric horseradish peroxidase (HRP). Primary OASFs were left untreated (medium only) or stimulated for 24h with PIC (10μg/ml). Appropriate isotype control was used to confirm specificity. Images were captured at 200x magnification and with the same exposure time for both conditions. Result was performed by my collaborator, Dr Brentano.

PIC, polyinosinic:polycytidylic acid; OASF, osteoarthritis synovial fibroblast RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
Figure 4.4. Expression of membrane-bound BAFF in RASFs.

(a) FACS analysis demonstrating membrane-bound expression of BAFF and ICAM-1 (as positive control) in RASFs in resting conditions (UnS) and upon TLR3 stimulation (PIC). Voltage settings for the fluorescence photomultiplier tubes were set so that unstained cells lie close to the origin of an FL1/FL2. FL2 refers to recorded fluorescence after light emitted from the sample has passed through a PE/RPE filter (i.e., orange fluorescence). Stained (and accordingly more fluorescent) cells appear further along the axes than do unstained cells. **Top row right:** Histogram with the number of particles (*counts*) against the relative fluorescence intensity of RPE. Membrane-bound BAFF in unstimulated (light grey line) and TLR3 stimulated (dark grey line) RASFs. An irrelevant antibody control was used to exclude non-specific binding (black line). **Top row left:** Quantitative Dot-plot analysis of irrelevant antibody staining. **Middle and bottom row:** Quantitative Dot-plot analysis of BAFF (left) and ICAM-1 (right) membrane expression in the presence or absence of TLR3 stimulation (PIC). The percentage of positive cells for each quadrant is indicated within each plot.

(b) Confocal microscopy images of RASFs immunostained for BAFF and ICAM-1. Representative images of RASFs stained with Alexa-488-conjugated antibodies (green) to detect BAFF immunoreactivity (**left panel**). Identification of surface antigen on RASFs by PE-conjugated mouse-anti-human ICAM-1 antibody (red, **middle panel**). Merged double staining for BAFF (green) and ICAM-1 (red) highlighted their appearance on the membrane of RASFs by yellow colour (**right panel**, asterisks). Original magnification 200x.

PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
Figure 4.5. Expression of full-length BAFF in RASFs and RADFs.

Assessment of RASFs and RADFs on the expression of transmembrane and soluble form of BAFF. RASFs and RADFs conditioned for 48 h in the presence (+) or absence (-) of 10μg/ml PIC. Protein was separated in 13% SDS-PAGE and the cell lysate were analysed by western blot using anti-BAFF Buffy-2 polyclonal antibodies (higher molecular weight of 32kDa represented full-length BAFF; lower molecular weight of 17kDa represented the processed soluble form of BAFF). Beta actin was used as endogenous control to confirm equal loading (lower membrane). Tonsil cells and recombinant human BAFF were used as positive controls.

PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
4.3 RASFs but not RADFs constitutively produce large amounts of APRIL

I next investigated the ability of RASFs and RADFs to release APRIL in resting and stimulated conditions. Unlike RADFs, RASFs produced abundant basal levels of soluble APRIL, reaching ~900pg/ml on day 3 (Figure 4.6a). In keeping with the previous gene expression analysis showing that APRIL transcripts were poorly modulated by TLR3, only a marginal increase in soluble APRIL was observed upon PIC stimulation (Figure 4.6a). However, APRIL was released at a higher rate within the first 24h in PIC-stimulated RASFs. In contrast to RASFs, APRIL levels in RADFs were extremely low in basal conditions and remained low following TLR3 activation. Finally, in contrast with previous data [314], I did not detect expression of BCMA mRNA in RASFs (or RADFs, data not shown).

Due to the higher basal production of APRIL protein in RASFs compared to RADFs, I then wanted to confirm differential expression of APRIL by immunocytochemistry. As shown in Figure 4.6b, immunofluorescence staining for APRIL demonstrates that while resting RASFs express intense cytoplasmic immunoreactivity with bright perinuclear localisation, possibly corresponding to vesicle-sequestered or Golgi-associated APRIL [317], whilst RADF were characterised by a significantly weaker staining. Overall, our findings indicated that even after several passages, RASFs (but not RADFs) retain the capacity to release constitutively high levels of APRIL, which can only be modestly increased upon TLR3 stimulation.

To further confirm the above argument, I used a western blot approach to determine the expression of APRIL in RASFs and RADFs. As controls, I ran tonsillar cells, which are a source of mononuclear cells. As shown in Figure 4.7, the polyclonal antibody recognised the 30kDa unprocessed form of APRIL, which is prominently expressed in resting RASFs early at 24h. This finding mirrored the ELISA data above that RASFs constitutively express high levels of APRIL. In contrast, RADFs expressed a much less APRIL, both at the basal level and upon PIC stimulation. Similar observations were obtained throughout the time-course for both RASFs and RADFs, where the level of...
expression was dampened at later time points of 48h and 72h. In addition to the 30kDa species, the anti-APRIL antibody recognised a lower molecular weight 17kDa form of APRIL (processed form of APRIL), which was only evident after conditioning RASFs for 72h (data not shown). Undetectable levels of the soluble form of APRIL in my cell lysates from these experiments might be due to the fact that APRIL is cleaved in the golgi apparatus by a furin convertase before secreted as a soluble ligand.
Figure 4.6 Intracellular and soluble APRIL expression in RASFs vs. RADFs.

(A) Column-bar graph showing the amount of soluble APRIL released in the cell culture supernatant from RASFs and RADFs conditioned for 24, 48 and 72h in the presence or absence of 10μg/ml PIC. Data are presented as mean±SEM of at least three experiments.

(B) Representative microphotographs of APRIL immunostaining on RASFs and RADFs in resting conditions. RASFs/RADFs were grown on coverslips and processed for immunofluorescence as described in the Methods. Nuclei are counterstained in blue (DAPI) and APRIL is stained in green (Alexa-488). Appropriate isotype control was used to confirm specificity. Images were captured at 20x magnification and with the same exposure time for both conditions.

PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblasts; RASF, rheumatoid arthritis synovial fibroblasts.
Figure 4.7. Expression of full-length and processed form of APRIL in RASFs and RADFs.

Assessment of RASFs and RADFs on the expression of transmembrane and soluble forms of APRIL. RASFs and RADFs conditioned for 24h in the presence (+) or absence (-) of 10μg/ml PIC. Protein was separated in 13% SDS-PAGE and the cell lysates were analyzed by western blot using anti-APRIL polyclonal antibodies (higher molecular weight of 30kDa represented full-length APRIL; lower molecular weight of 17kDa represented the processed soluble form of APRIL). Beta actin was used as endogenous control to confirm equal loading (lower membrane). Tonsil cells were used as a positive control.

PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblasts; RASF, rheumatoid arthritis synovial fibroblasts.
4.4 Purification of $\geq 97\%$ IgD$^{hi}$ naïve unswitched human B cells from tonsil by positive selection using magnetic-activated cell-sorting (MACS)

Given the capacity of PIC to induce high levels of BAFF in RASFs, I next investigated whether TLR3 stimulation was capable of enhancing the well established capacity of RASFs to promote Ig production in B lymphocytes [363]. In addition, because TLR3 promotes the release of significant levels of soluble BAFF, with RASFs constitutively producing high amount of soluble APRIL, I investigated whether the capacity of RASFs to activate B cells was dependent on direct cell-cell interactions or could also be mediated by soluble mediators. To this aim, I first isolated IgD$^+$ unswitched B cells isolated from human tonsils (and peripheral blood). By using well established MACS separation protocols I achieved a preparation of freshly isolated B cells with $\geq 97\%$ purity for surface IgD (Figure 4.8).

Flowcytometric analysis of B cells distinguishes different subpopulations using a combination of surface markers: IgD, CD20 and CD27. In pre-sort population: (1) CD20 expression was used to distinguish between three B cell populations, where CD20$^{lo}$ defined the resting B cell populations where CD20 intermediate the GC founder cells and CD20$^{hi}$ the GC cells (second panel left row); (2) IgD surface expression distinguished two populations, IgD$^{lo}$, representing centroblasts/centrocytes where IgD$^{hi}$ indicated mature resting B cells (first panel left row); (3) Double staining with IgD/CD27 demonstrated four populations: class switched memory B cells (CD27$^+$, IgD$^-$), pre-switched memory B cells (CD27$^{lo}$, IgD$^{hi}$), non-class switched mature resting B cells (CD27$, IgD^{hi}$), and cells negative for both CD27 and IgD (CD27$^-$, IgD$^-$) (last panel left row).

After enrichment, $\geq 97\%$ of these B cells showed strong surface expression for IgD$^{hi}$ (Figure 4.8, first panel in central row), and around 94% surface expression for CD20 (Figure 4.8, last panel in central row), indicating almost all cells in the after-sort fractions are of B cells origin. $<2\%$ were CD27-positive cells demonstrating a lack of memory B
cells in the purified fraction (Figure 4.8, third panel in central row). Moreover, although
the majority of the cells in the purified fraction expressed CD20 at intermediate level, a
feature of GC founder cells, this population also expressed IgD in high amounts,
suggesting our selected population (IgD<sup>hi</sup> CD20<sup>+</sup> CD27<sup>neg/low</sup> B cells) represented
unswitched resting mature B cells and was selected for subsequent culture experiments.
Figure 4.8. FACS characterisation of tonsillar B cell subpopulations before and after isolation of IgD\(^+\) cells by magnetic-activated cell-sorting (MACS).

A forward scatter/side scatter (FSC/SSC) plot showing the “live” gate used to exclude cellular debris and dead cells; this gate is then applied to all subsequent plots (Left panel). Voltage settings for the fluorescence photomultiplier tubes are set so that unstained cells lie close to the origin of an FL1/FL2. FL1 refers to recorded fluorescence after light emitted from the sample has passed through a FITC filter (i.e., green fluorescence) and FL2 refers to fluorescence recorded after the light has been passed through a PE/RPE filter (i.e., orange fluorescence). Stained (and accordingly more fluorescent) cells appear further along the axes than do unstained cells. Tonsillar mononuclear suspensions before (pre-sort, second panel) and after (after-sorted, third panel and flow-through, fourth panel) IgD\(^+\) MACS isolation were single or double stained with FITC-conjugated anti-IgD (horizontal axis), RPE-conjugated anti-CD20 or PE-conjugated anti-CD27 Ab (vertical axis). Fluorescence analysis was performed on the total lymphocyte gate based on forward and 90° scatter profile. The percentage of positive cells in each quadrant is indicated beside each plot. A purity of $\geq97\%$ IgD\(^+\) B cells was obtained after sorting with the vast majority of IgD-CD27\(^+\) switched memory B cells in the flow-through.
4.5 BAFF and IL-4 induced Ig production, AID expression and Iγ-Cµ circular transcripts in tonsillar IgD⁺ B cells.

To examine the possible role/involvement of TLR3 engagement on fibroblasts in B cell survival, proliferation and activation, I first constructed a system to clarify the basis of regulation on activated human unswitched IgD⁺ B cells. I evaluated the ability of well-described activating cytokines, BAFF and IL-4, to induce the expression of AID, a gene that is exclusively expressed in active B cells, in the selected B cell populations.

In my initial study, increasing cells densities of 1 x 10⁶ and 2 x 10⁶ IgD⁺hi CD20⁺ CD27neg/low unswitched resting B cells were cultured in the presence or absence of 1μg/ml BAFF and 100ng/ml IL-4 for 24h and 72h and cells were collected for determination of AID at transcriptional levels. AID transcripts were detectable within 24h of BAFF and IL-4 stimulation and the expression were presented at least up to 72h, where a weak expression was observed in unstimulated B cells (Figure 4.9). Compared with untreated cells, increased cell density was associated with increased AID expression, where the application of BAFF and IL-4 induced an almost 200-fold increase of AID mRNA with 1 x 10⁶ IgD⁺ B cells and 300-fold increase with 2 x 10⁶ IgD⁺ B cells following 72h of incubation. These results suggested that BAFF synergised with IL-4 induced AID gene expression in human B cells. 1 x 10⁶ IgD⁺ B cells has been chosen for all subsequent culture experiments, allowing meaningful comparisons.
Figure 4.9. Effect of different B cell densities on AID induction in purified IgD$^+$ naïve human tonsillar B cells.

Purified B cells (1 x 10$^6$ and 2 x 10$^6$/well) from tonsil were cultured with 1μg/ml BAFF and 100ng/ml IL-4 (+) for 72h or left untreated (-). Cells were then harvested, RNA extracts and QT-PCR performed to evaluate AID transcripts. AID mRNA expression, normalised to 18S, was affected by both stimuli and population density. Application of BAFF/IL-4 displays a clear comparable AID mRNA expression.
My results revealed that BAFF and IL-4 induced AID expression in IgD+ tonsillar B cells, and as AID is required for class-switching, I further determined whether its expression was associated with the ability to induce class-switching. 1 x 10^6 of IgD+ B cells were stimulated in culture in the presence or absence of BAFF and IL-4. As shown in Figure 4.10a, cultures treated with BAFF and IL-4 strongly enhanced IgG synthesis. The release of IgG was regulated time-dependently and reached the maximal level after 72h of stimulation. Treatment of BAFF and IL-4 resulted in a 4-fold increase of IgG levels (~80 ng/ml) compared to untreated control (~20ng/ml). Conversely, IgA production was unaffected by the treatment. Constitutive production of polyreactive IgM was observed, and treatment with BAFF/IL-4 provided an enhancing effect on IgM secretion, suggesting the maintenance of B cell survival/proliferation in my culture system. Figure 4.10b demonstrated that AID expression was induced by BAFF and IL-4 in a time-dependent manner. AID transcript was found to be induced by ~20-fold at 24h and ~300-fold after 72h of incubation. The specificity of the AID and endogenous control PCR products were confirmed by agarose gel electrophoresis (Figure 4.10c). In order to confirm that AID was functional, I determined the presence of Iγ 1/2 -Cμ CTs, specific by-products of ongoing CSR from IgM to IgG1/2 in B cells, which are reported to disappear 48h after CSR [263]. In my system, a weak basal expression of Iγ 1/2 -Cμ CTs was detected throughout the incubation period, and the addition of BAFF and IL-4 markedly upregulated CT expression in B cells (Figure 4.10d). Iγ 1/2 -Cμ CTs were strongly expressed at 24h, and expression was dampened but maintained up to 72h of incubation.
Figure 4.10. BAFF/IL-4 induce Ig production, AID expression and Ig- Cµ circular transcripts in tonsillar IgD+B cells.

(a) BAFF and IL-4 increased the production of IgG and IgM in tonsillar IgD+B cells in a time-dependent manner. IgA synthesis was not modulated by the treatment. For comparison, cells were left in medium only (-).

(b) QT-PCR performed to evaluate AID mRNA expression, normalised to 18S, before and after BAFF/IL-4 stimulation. Baseline expression was set as 1.

(c) 1.8% agarose gel showing AID, 18S and BAFFR PCR products to confirm specificity of the PCR. Bands of the corrected size were observed for all the genes analysed AID (99 bp), BAFFR (150 bp), 18S (187 bp). Expression of AID, but not BAFFR, was induced by BAFF/IL-4.

(d) CT Ig-Cµ expression was determined by running PCR amplified product on 1% agarose gel (inverted colour, amplicon 502 bp, upper gel). 18S was used as endogenous control to confirm equal loading (lower gel). Tonsil cells were used as a positive control. Results are represented as mean ± SEM. * p<0.05, **p<0.01 compared to unstimulated.
4.6 TLR3 stimulation of RASFs, but not RADFs, enhances class-switched immunoglobulin secretion from IgD+ human B lymphocytes

Tonsil IgD+ unswitched B cells at ≥97% purity (Figure 4.8) were co-cultured either in cell-cell contact or in a transwell system (see method for details) with RASFs, OASFs or RADFs. As shown in Figure 4.11a, tonsillar IgD+ B cells cultured alone produced low amounts of IgG and IgA, which were not significantly modified after incubation with PIC, suggesting that TLR3 stimulation is not sufficient per se to trigger Ig production. Conversely, when the same numbers of B cells were culture together on a confluent monolayer of RASFs, spontaneous IgG was enhanced (two-to-threefold increase in IgG production already at 72h) and IgG levels were further increased upon TLR3 stimulation (Figure 4.11a), indicating that RASFs following PIC stimulation releases factors capable of triggering class-switched Ig production in B cells. Similar, albeit more modest induction of IgA was also evident. Paired RADFs were not capable of significantly enhancing IgG production either in resting or stimulated conditions (Figure 4.11b), while OASF displayed a similar behaviour compared to RASFs (Figure 4.12, result was performed and obtained from my collaborator, Dr Brentano).

I next determined if IgG class-switching induced RASFs in B cells required direct cell-cell interactions. As shown in Figure 4.11, a cell-cell contact was not necessary, since CSR to IgG/IgA still occurred (albeit with slightly lower efficiency) when tonsillar IgD+/CD27- B cells (but also from IgD+/CD27- peripheral blood mature B cells, Figure 4.13, result was performed and obtained from my collaborator, Dr Brentano) were cultured in transwells system without physical contact with RASFs in the culture system, indicating that soluble factors released by RASFs are sufficient to promote CSR. Overall, the treatment of RASFs with PIC, either in the cell-cell or transwell system, selectively induced the production of IgG, and to a much lesser extent IgA, but did not augment the secretion of polyreactive IgM. The phenomenon that the treatment of RASFs with PIC minimally modulated IgM production suggests that RASFs do not simply enhanced the survival/proliferation of B cells, but rather induced functional activation as indicated by the increased IgG/IgM ratio after 8 days compared to 24h and 72h (Figure 4.14d).
Figure 4.11. TLR3 stimulation of RASFs but not RADFs enhances their capacity to promote the production of class-switched antibodies in IgD⁺ B cells (purified from tonsil)

Purified tonsillar IgD⁺ B cells were cultured with fibroblasts for 72h. B cells were cultured alone (open bars) or in co-culture fibroblasts in direct physical contact (grey bars) or a transwell system (black bar). All cultures were incubated with (+) or without (−) 10μg/ml PIC. IgG, IgA and IgM levels in supernatants were measured by ELISA. Results are presented as mean±SEM of at least three experiments. *p<0.05, **p<0.01 compared with unstimulated.

PIC, polyinosinic:polycytidylic acid; RADFs, rheumatoid arthritis dermal fibroblasts; RASFs, rheumatoid arthritis synovial fibroblasts; TLR, toll-like receptor.
Figure 4.12. TLR3 stimulation of OASFs enhances their capacity to promote the production of class-switched antibodies in IgD⁺ B cells (purified from tonsil)

Purified tonsillar IgD⁺ B cells were cultured with fibroblasts for 72h. B cells were cultured alone (open bars) in co-culture fibroblasts in direct physical contact (grey bars) or a transwell system (black bar). All cultures were incubated with or without 10μg/ml PIC. IgG, IgA and IgM levels in supernatants were measured by ELISA. Results are presented as mean±SEM of at least three experiments. *p<0.05, **p<0.01 compared with unstimulated.

Result was performed and obtained from my collaborator, Dr Brentano.

PIC, polyinosinic:polycytidylic acid; OASF, osteoarthritis synovial fibroblast.
Figure 4.13. TLR3 stimulation of RASFs enhances their capacity to promote the production of class-switched antibodies in IgD⁺ B cells (purified from peripheral blood).

Purified IgD⁺ B cells from peripheral blood were cultured with fibroblasts for 72h. B cells (black bars) or RASFs (grey bars) were cultured alone or RASF were co-culture with B cells in direct physical contact (open bars). All cultures were incubated with or without 10μg/ml PIC. IgG, IgA and IgM levels in supernatants were measured by ELISA. Result was performed and obtained from my collaborator, Dr Brentano. Results are presented as mean±SEM of at least three experiments. *p<0.05 compared with unstimulated.

PIC, polyinosinic:polycytidylic acid; RASF, rheumatoid arthritis synovial fibroblast.
4.7 TLR3-stimulated RASFs induce up-regulation of AID and circular transcripts in B cells

Although the above data strongly pointed towards the ability of RASFs to actively induce class-switching in B cells, a formal demonstration of the capacity of RASFs to induce class-switching requires evidence of AID expression, which is the enzyme required to initiate CSR, as well as the appearance of circular transcripts (CTs).

RASFs strongly induced AID mRNA after 24h and 72h of co-culture in cell-cell and transwell systems, a capacity further enhanced upon TLR3 stimulation (Figure 4.14a, b). Importantly, IgD⁺ B cells alone (Figure 4.14a, b) or cultured with RADFs (data not shown) displayed no or barely detectable levels of AID mRNA. The specificity and the correct size of the AID and endogenous control PCR products were confirmed by agarose gel electrophoresis (Figure 4.10c). Interestingly, there was a trend towards increased expression of AID when RASFs were physiologically isolated from B cells in a transwell. The mechanism by which AID mRNA is increased in the co-cultures remains to be defined. One possible explanation could be due to some unidentified cell surface molecules, which may influence the production of AID. Indeed, it has been demonstrated that RASFs expressed cell surface death receptors, such as Fas/CD95, and that crosslinking of these receptors by antibodies can induce apoptosis [104]. Therefore, I hypothesised that the cell surface expression of death receptors may positively correlate with the stimulation of PIC. Accordingly, the transwell co-culture system, in which B cells are cultured in the absence of direct cell-cell contact with RASFs, may potently impair apoptosis induced by the death receptors expressed on RASFs. Nevertheless, my current data demonstrates that, in the presence of PIC and RASFs, IgD⁺ B cells up-regulate the expression of AID more than IgD⁺ B cells cultured with un-treated fibroblasts.

Next, in order to confirm that AID was functional, I determined the presence of Ig₁/₂-Cµ CTs. I investigated their expression 72h after co-culturing B cells with RASFs. As shown in Figure 4.14c, no Ig₁/₂-Cµ CTs were detected in IgD⁺ B cells alone at 72h, while co-
culture with unstimulated RASFs was sufficient to induce their expression. Importantly, TLR3 triggering strongly enhanced CSR from IgM to IgG (Figure 4.14c, top gel, lane 4), a result in keeping with evidence of increased IgG production upon PIC stimulation. RASFs-dependent induction of Iγ1/2-Cµ CTs and IgG production was similar to that observed using recombinant BAFF and IL-4 (Figure 4.10d) where I confirmed that sustained expression of CT Iγ1/2-Cµ up to 72 h was dependent on the presence of the agonist, indicating the importance of an activated state of IgD⁺ B cells for active CSR at 72h in my co-culture system. While no Iγ1/2-Cµ CTs were detected in IgD⁺ B cells alone, co-culture with unstimulated RASFs was sufficient to induce CTs expression up to 72h, which was clearly enhanced upon TLR3 stimulation (Figure 4.14c, top gel, lane 4).

Overall, these data provide conclusive evidence that RASFs release soluble factors able to directly activate and sustain the expression of AID and induce the molecular machinery required for ongoing CSR in unswitched B cells and that TLR3 stimulation enhances this effect.
Figure 4.14. TLR3 stimulation of RASFs enhances activation-induced cytidine deaminase (AID) expression and ongoing class-switching in co-cultured IgD+ tonsillar B cells.

(a and b) Purified tonsillar IgD+ B cells were cultured with RASFs for 24h (a) and 72h (b) with 10μg/ml PIC or left untreated in the presence (transwell) or absence (cell–cell contact) of a cell insert. AID mRNA expression was determined by quantitative real-time PCR normalised to an endogenous control gene (18S) and the fold difference relative to baseline expression was calculated by setting AID expression in B cell alone as 1. *p<0.05, **p<0.01 compared with unstimulated.

(c) Representative 1% agarose gel (inverted colour) of Iγ-Cμ circular transcripts (CTs; amplicon 502 bp, upper gel), specific by-products of ongoing class-switching from IgM to IgG. The lower gel represents the endogenous control, mammalian 18S (amplicon 187 bp). CTs were amplified from B cell cDNA using specific primers (Methods). B cells were cultured alone or in transwell with RASFs for 72h in the presence or absence of 10μg/ml PIC. Tonsil was used as a positive control.

(d) Progressive production of IgG over IgM in B cells co-cultured in transwell with TLR3-stimulated RASFs after 24h, 72h and 8 days, demonstrating. Data are presented as mean IgG to IgM ratio ±SEM of at least three experiments per time point. *p<0.05, **p<0.01 compared with unstimulated.

PIC, polyinosinic:polycytidylic acid; cel:cel, cell to cell contact; trans, transwell; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
4.8 Dual blockade of BAFFR and BCMA abrogates TLR3-induced AID expression and class-switching

I finally wondered whether the activating capacity of RASFs to induce AID expression, CSR and IgG production in IgD+ B cells in response to TLR3 was dependent on the release of BAFF and/or APRIL. In order to test this possibility, I inhibited BAFF/APRIL-dependent signal transmission by using neutralising antibodies. By using neutralising antibodies against BAFFR (specific for BAFF) and BCMA (binding both BAFF and APRIL) alone I showed that in the transwell system individual receptor blocking displayed significant but partial inhibition of TLR3-dependent IgG and IgA production (with BCMA interference appearing more efficient than BAFFR blockade at the same dose of neutralising antibody), while the combination of anti-BAFFR/anti-BCMA completely suppressed TLR3-induced IgG and IgA production (Figure 4.15a).

In addition, blockade of BAFFR and/or BCMA downregulated TLR3-induced RASFs-dependent AID mRNA expression as well as the production of Iγ1/2-Cµ CTs (Figure 4.15 b and c, respectively). Experiments in cell-cell contact displayed similar results, although the inhibitory effect was slightly smaller (data not shown). Overall, these findings suggest that the molecular machinery for CSR induced in IgD+ B cells by RASFs via TLR3 stimulation is mostly dependent on the production of BAFF and APRIL and can be blocked by interference with their specific receptors.
Figure 4.15. Blockade of BAFFR and/or BCMA reverses TLR3 induced RASF-dependent IgD\(^+\) B cell activation.

(a) IgG, IgA and IgM production by tonsillar IgD\(^+\) B cells cultured in transwell with RASFs for 72 h in the presence of 10\(\mu\)g/ml of either anti-human BCMA or anti-human BAFF-R blocking antibodies or the two combined. Suppression of IgG and IgA production is expressed as the percentage of reduction of the effect induced by TLR3 stimulation. *p<0.05, **p<0.01 compared with PIC stimulated.

(b) Modulation of activation-induced cytidine deaminase mRNA expression in B cells co-cultured in transwell with RASFs for 72h after blockade of BAFFR and/or BCMA signaling. Results are expressed as mean±SEM of the mRNA fold increase calculated by setting the baseline expression as 1. *p<0.05, **p<0.01 compared with PIC stimulated.

(c) Representative 1\% agarose gel (inverted colour) of Ig-\(\gamma\)C\(\mu\) circular transcripts (CTs, amplicon 502 bp, upper gel) expression in B cells co-cultured in transwell with RASFs for 72 h after blockade of BAFFR and/or BCMA signaling. Endogenous control, mammalian 18S (amplicon 187 bp) is shown in the lower gel.

PIC, polyinosinic:polycytidylic acid; cel:cel, cell to cell contact; trans, transwell; aBCMA, anti-human BCMA blocking antibody; aBAFFR, anti-human BAFF-R blocking antibodies; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
CHAPTER 5

Results

Dual role of SLPI as a negative regulator of TLR3-induced BAFF expression in rheumatoid synovial fibroblasts and of B cell functional activation

SLPI has been previously described as an anti-inflammatory protein that is constitutively expressed in multiple cell types where it functions to counteract localised tissue destruction by its anti-inflammatory, antimicrobial and anti-protease properties. Recently, SLPI has been demonstrated to restrain BAFF production released by the activated ECs [286]. In this part of my PhD project, I demonstrated that TLR induced expression of SLPI in RASFs where it functions to downregulate functional B cell activation: i) SLPI was abundantly produced by RASFs at mRNA and protein level and its basal expression level was around 60-fold higher than RADFs; ii) SLPI expression was induced by TLRs particularly TLR3; iii) SLPI displayed a different dynamic gene expression as compared to BAFF (SLPI transcripts level peaked at 72h vs. 8h for BAFF) ; iv) RASFs-derived SLPI downregulated BAFF mRNA/protein; v) RASFs exerted a direct and indirect refractory effect on functional B cell activation via (AID, IgG/A/M production).

5.1 TLR3-stimulated induction of SLPI mRNA in RASFs, OASFs and RADFs

I first examined whether TLR stimulation of RASFs and paired RADFs (to investigate site specificity) induced SLPI mRNA. SLPI mRNA was significantly and markedly modulated by TLR3 stimulation in RASFs (Figure 5.1a), while there was a modest induction with LPS and bLP stimulation. Interestingly, in comparison with BAFF mRNA, which was rapidly induced by PIC and peaked at 8h post-stimulation (see Chapter 4, Figure 4.1), TLR3 stimulation provoked a much delayed and gradually increased induction of SLPI transcripts, which peaked 72h after stimulation. Importantly, although
a comparable increase in SLPI mRNA from basal expression could be induced by PIC in RADF (Figure 5.1b), RASFs displayed a significantly higher basal expression (over 60-fold) of SLPI mRNA as compared to RADFs (Figure 5.1c). Thus, these data demonstrated i) that RASFs are able to produce this homeostatic regulator at transcript level with a basal expression significantly higher compared to matching RADFs, and that ii) SLPI induction can be further induced after sensing the TLR3 ligand PIC, a process common to both synovial and dermal fibroblasts.
Figure 5.1. Induction of SLPI mRNA in RASFs, RADFs and OASFs in response to TLR ligands.

(a, b) Quantitative Taqman real-time PCR analysis of SLPI mRNA expression in response to TLR ligands. RASFs (a), RADFs (b) were stimulated with 10μg/ml PIC (TLR3), 100ng/ml lipopolysaccharide (TLR4), 300ng/ml bLP (TLR2) or medium alone for 4, 8, 24 and 72h. Results are expressed as mean±SEM of the mRNA fold increase calculated at each time point by setting the baseline unstimulated expression as 1. Data were normalised using mammalian 18S as an endogenous control gene (18S). *p<0.05, **p<0.01 compared with unstimulated. PIC, polyinosinic:polycytidylic acid; RADFs, rheumatoid arthritis dermal fibroblasts; RASFs, rheumatoid arthritis synovial fibroblasts; TLR, toll-like receptor.

(c) Comparative expression of SLPI mRNA in RASFs and RADFs. Box and whiskers graph showing quantitative Taqman real-time analysis for basal SLPI mRNA expression in RASFs (n=5) vs. RADFs (n=5). Data are expressed as the difference in threshold cycle (dCt) following normalisation for the endogenous controls. The lower and upper margins of the box represent the 25th and 75th percentile, respectively, while the extended arms represent the 10th and 90th percentiles. The median is indicated as a horizontal line within the box. ***p<0.001 compared with RADFs.
5.2 RASFs, but not RADFs, produce high levels of SLPI constitutively and in response to TLR3 stimulation

I next investigated the ability of RASFs and RADFs to release SLPI in resting and stimulated conditions. Unlike RADFs, RASFs time-dependently produced abundant basal levels of soluble SLPI in the supernatant of RASFs reaching up to ~500 pg/ml on day 3 and on average ~10-times higher than RADFs (Figure 5.2a). Upon TLR3 stimulation further increase in SLPI production by RASFs was observed, resulting on average in a 2-fold increase in SLPI concentration (Figure 5.2a) and up to ~2ng/ml on day 3. Consistent with the low basal expression of SLPI in RADFs, TLR3-induced SLPI production by these cells was 10-times lower compared to RASFs.

In order to confirm SLPI expression in RASFs and to investigate its intracellular localisation in basal conditions and upon TLR3 stimulation, I performed immunofluorescence on RASFs and RADFs cell monolayers. As shown in Figure 5.2b, resting permeabilised RASFs (see Chapter 3 for methods) expressed an intense perinuclear and cytoplasmic SLPI immunoreactivity which was strongly increased upon incubation with PIC. In contrast, RADFs were characterised by a significantly weaker SLPI staining, and, following exposure to PIC, its expression remained barely detectable in RADFs. Overall, ELISA and IF data indicate that RASFs, but not RADFs, are capable of releasing high level of SLPI in basal and TLR3 stimulated conditions.
Figure 5.2. TLR3 stimulation induces intracellular and soluble SLPI in RASFs but not RADFs.

(a) Column-bar graph showing the amount of soluble SLPI released in the cell culture supernatant from RASFs and RADFs conditioned for 24, 48 and 72 h in the presence or absence of 10μg/ml PIC. RASFs produce significantly higher amount of SLPI both in resting and stimulated conditions than RADFs. Results are represented as mean±SEM of minimum three experiments.

(b) Representative microphotographs of SLPI immunostaining on RASFs and RADFs before and after stimulation with PIC. RASFs/RADFs were grown on coverslips, permeabilised and processed for immunofluorescence as described in the Methods. Nuclei are counterstained in blue (DAPI) and SLPI in red (Alexa-555). Primary RASFs/RADFs were left untreated (medium only) or stimulated for 72h with PIC (10μg/ml). Appropriate isotype control was used to confirm specificity. Images were captured at 20x magnification and with the same exposure time for both conditions.

PIC, polyinosinic:polycytidylic acid; RADF, rheumatoid arthritis dermal fibroblast; RASF, rheumatoid arthritis synovial fibroblast; TLR, toll-like receptor.
5.3 SLPI attenuates B cell activation via direct inhibition of Ig production and AID expression

I next examined whether SLPI could directly attenuate the capacity of B lymphocytes to produce class-switched Ig and down-regulate AID expression. As mentioned in Chapter 4, I showed that stimulation with a combination of BAFF and IL-4 significantly and markedly induced the expression of AID, and the release of class-switched IgG production in tonsillar IgD+ B cells (Figure 4.10). Thus, in this part of my project I used the same experimental conditions in order to dissect the possible role of SLPI in modulating B cell activation. IgD+ tonsillar B cells were treated with or without graded concentrations of recombinant SLPI at 10, 50 and 500ng/ml in the presence or absence of BAFF/IL-4 stimulation. As shown in Figure 5.3a, I found that SLPI markedly down-regulated the production of IgG production induced by BAFF/IL-4, in a dose-dependent manner, with a reduction from ~500ng/ml down to ~300ng/ml at the highest dosage of SLPI used (500ng/ml). Importantly, the basal (i.e. unstimulated) production of IgG by IgD+ tonsillar B cells was also partially suppressed after treatment with SLPI (Figure 5.3a). Similarly, albeit a more modest significant reduction in basal IgA production was also evident following SLPI treatment, while 500ng/ml SLPI completely abrogated the effect of BAFF/IL-4 on IgA production. Conversely, treatment of B cells with SLPI only minimally modulated IgM production, suggesting that SLPI promotes functional suppression of B cell activation rather than simply affecting the survival/proliferation of B cells.

In keeping with notion that SLPI has a direct suppressive effect on the molecular machinery involved in Ig class-switching, I next demonstrated significant downregulation of AID mRNA expression in stimulated B cells following incubation with SLPI (Figure 5.3b). All doses of SLPI were able to downregulated AID expression by 40-50%; however, in contrast to Ig production AID expression was not modulated in a dose-dependent manner and no further decrease in AID could be observed at increasing doses of SLPI. Thus, it is possible that SLPI may exert additional mechanisms that affect Ig secretion over and above inhibition of CSR. Nevertheless, these in vitro findings clearly
demonstrate that CSR activity induced in IgD⁺ B cells by a combination of BAFF/IL-4 can be directly attenuated by SLPI, confirming the immunoregulatory capacity of this factor.
Figure 5.3. SLPI attenuated BAFF/IL-4-induced AID expression and class-switching.

(a) SLPI decreased the production of IgG induced by BAFF/IL-4 in tonsillar IgD⁺ B cells in a dose-dependent manner. IgA synthesis was weakly modulated by the treatment. For comparison, cells were left in medium only (-). Suppression of IgG is expressed as the percentage of reduction of the effect induced by BAFF/IL-4 stimulation. Results are represented as mean ± SEM. * p<0.05, **p<0.01, ***p≤0.001 compared with unstimulated and BAFF/IL-4 stimulated.

(b) QT-PCR performed to evaluate AID mRNA expression. Data were normalised using mammalian beta actin as an endogenous control gene, before and after SLPI treatment. Baseline expression was set as 1.
5.4 SLPI directly inhibits TLR3-stimulated induction of BAFF in RASFs

Previously in this thesis I reported that RASFs can produce B cell survival factors, at both mRNA and protein level, upon TLR stimulation (Chapter 4, Figure 4.1, 4.3-4.7). Therefore, in this part of my PhD project I sought to determine whether SLPI can restrain B cell survival factors expression upon TLR3 stimulation in RASFs. Here I specifically focused on TLR3 since I showed that BAFF expression in RASFs is mainly modulated by the TLR3 ligand PIC. I investigated two aspects of SLPI in regulating the expression of BAFF. Firstly, RASFs were treated with the TLR3 agonist, PIC, with/without pre-incubation with recombinant SLPI at 50 and 500ng/ml and subsequently harvested at 4, 8, 24, 48 and 72h. As shown in Figure 5.4a, treatment with recombinant SLPI rapidly elicited an inhibitory effect on the expression of BAFF mRNA as early as 4h in a dose-dependent manner. The inhibitory effect was somewhat decreased at 8h post-stimulation and was lost afterwards (data not shown). Conversely, SLPI did not induce any significant modulation of APRIL mRNA (data not shown), suggesting a distinct function of SLPI on BAFF vs. APRIL expression in RASFs.

I next aimed to confirm whether SLPI-induced downregulation of BAFF mRNA was mirrored by decreased protein expression. As shown in Figure 5.4b, treatment with SLPI significantly reduced the amount of soluble BAFF release at 72h in a dose-dependent manner. Conversely, only marginal suppression was observed at 24 and 48h. Secondly, in preliminary experiments, RASFs were treated with PIC with/without pre-incubation with an anti-human SLPI blocking antibody. As shown in Figure 5.4c, blocking of endogenous RASFs-released SLPI resulted in increased expression of BAFF mRNA both in unstimulated conditions and after TLR3 stimulation at 4h. This effect was somewhat decreased at 8h post-stimulation and was lost afterwards (data not shown). Overall, transcript and protein analysis consistently demonstrated that RASFs-released SLPI can directly control the production of BAFF induced by TLR3 stimulation.
**Figure 5.4. SLPI attenuated TLR3-induce BAFF expression in RASFs.**

**(a)** RASFs were stimulated with 10μg/ ml PIC (TLR3) in the presence of 50ng/ml or 500ng/ml SLPI or medium alone for 4h. Results are expressed as mean±SEM of the mRNA fold increase calculated at 4h by setting the baseline unstimulated expression as 1. Data were normalised using mammalian 18S as an endogenous control gene (18S). *p<0.05 compared with PIC stimulated.

**(b)** Column-bar graph showing the amount of soluble BAFF released in the cell culture supernatant from RASFs conditioned for 24, 48 and 72h in the presence or absence of 10μg/ml PIC and with or without 50ng/ml or 500ng/ml SLPI. SLPI suppressed the level of BAFF release from RASFs in a dose-dependent manner at 72h of incubation. Results are represented as mean±SEM of minimum two experiments.

**(c)** RASFs were stimulated with 10μg/ml PIC (TLR3) in the presence of 10μg/ml anti-human SLPI/relevant isotype control or medium alone for 4h. Results are expressed as mRNA fold increase calculated at 4h by setting the baseline unstimulated expression as 1. Data were normalised using mammalian 18S as an endogenous control gene (18S).

PIC, polyinosinic:polycytidylic acid; RASF, rheumatoid arthritis synovial fibroblast; SLPI, secretory leukocyte protease inhibitor.
5.5 SLPI suppressed AID expression and class switched immunoglobulin secretion from IgD+ human B lymphocytes in culture with TLR3-stimulated RASFs

The evidence obtained from the experimental work in sections 5.3. and 5.4 led me to hypothesise that SLPI may exert a dual regulatory role on RASF/B cell interactions whereby it may act at two different cell levels; the first in RASFs where it modulates the release of BAFF and the second at the B cell level where it can antagonise AID expression and IgG/A production. Thus, in this final part of my PhD project I investigated whether the capacity of RASFs to induce AID expression and class-switched Ig production in IgD+ B cells in response to TLR3 was regulated by SLPI.

By using graded concentrations of recombinant SLPI, I showed that exogenous addition of SLPI completely suppressed TLR3-induced IgG and IgA production (Figure 5.5a). Of note, the levels of class-switched Ig were suppressed by SLPI in a dose-independent manner, both in the absence and presence of TLR3 stimulation. In contrast to anti-BAFFR and/or anti-BCMA, a minimal reduction effect was also observed in IgM levels, suggesting that, in this context, SLPI influences not only the class-switching machinery (see below) but may also alter the mechanisms of Ig secretion and/or the survival of B cells.

I next investigated whether the ability of SLPI to restrain the production of class-switched Ig in B cells was associated with the concurrent downregulation of AID expression. As shown in Figure 5.5b, I found that SLPI was able to downregulate the expression of AID.

Importantly, the concentration of SLPI in the synovial fluid of RA patients is ~80ng/ml (Figure 5.6), which falls within the selected dosage range of recombinant SLPI I used to treat RASFs (10, 50, and 500 ng/ml). Therefore, my in vitro system resembles the physiological condition and is biological relevant.
Figure 5.5. SLPI attenuated activation-induced cytidine deaminase (AID) expression and class-switching in co-cultured TLR3-stimulated RASFs and IgD⁺ tonsillar B cells.

(a) IgG, IgA and IgM production by tonsillar IgD⁺ B cells cultured in cell-cell contact with RASFs for 72h in the presence of SLPI. An increasing concentration gradient of drug (50ng/ml or 500ng/ml) was represented by the grey triangle. Suppression of IgG of is expressed as the percentage of reduction of the effect induced by TLR3 stimulation. *p<0.05, **p<0.01 compared with PIC stimulated.

(b) Modulation of activation-induced cytidine deaminase mRNA expression in B cells co-cultured in cell-cell contact with RASFs for 72h after treatment with SLPI. Results are expressed as mean±SEM of the mRNA fold increase calculated by setting the baseline expression as 1. *p<0.05 compared with PIC stimulated.

PIC, polyinosinic:polycytidylic acid; RASF, rheumatoid arthritis synovial fibroblast; SLPI, secretory leukocyte protease inhibitor.
Figure 5.6. SLPI concentrations in the synovial fluid from RA and OA patients.

SLPI concentrations in the synovial fluid (syn fluid) of RA patients is significantly higher (***p<0.001) compared with OA patients. Results are represented as mean±SEM from 18 of RA patients and 18 of OA patients.
CHAPTER 6
Discussion

_In situ_ autoreactive B cell activation and autoantibody production within the RA synovium is a typical feature of a subset (~50%) of RA patients in which synovitis is characterised by the formation of ectopic lymphoid structures (ELS) [152]. It has long been shown that within RA lymphoid aggregates infiltrating B lymphocytes undergo antigen-driven affinity maturation, clonal expansion, Ig isotype switching and PC differentiation [153, 430, 431]. Our group recently showed that these processes: (i) require the expression of AID, the enzyme responsible for the initiation of SHM and CSR of the Ig genes, (ii) are self-sustained for several weeks in the SCID/RA chimeric model in the absence of recirculating immune cells and (iii) develop in the context of prolonged expression of B cell survival and proliferating factors, BAFF and APRIL [152]. In addition, the functionality of synovial B cell follicles is dependent on the interaction with networks of stromal cells, which express CD21 [141, 146] and closely resemble FDC in secondary lymphoid organs (SLO) [432]. Accordingly, the diffuse form of synovitis, characterised by the absence of B cell aggregates, lacks AID expression and ongoing Ig class-switching [152]. Despite strong evidence that ELS support the production of autoantibodies within the RA synovium is correlate with, our group and others demonstrated that circulating autoantibodies in RA do not associate with the presence of synovial ELS [148, 152, 433], suggesting that SLO are also important sites for autoreactive B cell activation.

Although it is currently unclear whether synovial FDC differentiate from resident stromal cells [96], fibroblast-like synoviocytes isolated from the RA synovium (RASFs) have been shown to display features of FDC, such as the capacity to promote the survival of B cells by rescuing them from apoptosis [364, 365, 434, 435] and their differentiation into Ab-producing cells [363, 365, 434, 436]. It is tempting to suppose that when the
requirement for FDC increases in the GC during immune response, SFs may migrate to lymphoid follicles in response to certain chemokines and differentiate to FDCs as a result of interaction with GC B cells. Lindhout et al have demonstrated that SFs could be induced to express the FDC phenotype by treatment with IL-1β and TNF-α. Because fibroblasts are ubiquitous cells, it remains to be elucidated whether all kinds of fibroblasts have the ability to differentiate into FDCs or whether this property is limited to certain types of fibroblasts.

Despite previous works clearly highlighting a critical and direct role for RASFs in B cell activation and differentiation, the specific factors regulating these interactions have not been fully investigated. Cell-dependent mechanisms involving adhesion molecules (i.e. VCAM-1) and chemokines (i.e. SDF-1) only partially account for the observed B cell activation [365], suggesting a critical contribution of additional factors. In addition, because previous works invariably used total B cells for co-culture experiments, in which they did not differentiate between naïve, switched and unswitched memory B cell subset, therefore the actual capacity of RASFs to induce the molecular machinery required for Ig class-switching in naïve B cells was questionable.

In Chapter 4, I provided a series of novel observations which contribute to the understanding of key mechanisms in regulating the crosstalk between RASFs and B cells within the RA synovium. First, I demonstrated that RASFs constitutively produce high levels of B cell survival, activating and proliferating factors BAFF and APRIL, both at mRNA and protein level, confirming and expanding previous evidence of over-expression of these factors at mRNA level in RASFs [292, 314, 428]. Furthermore, I showed that TLR3 stimulation was able to induce strong (between 200- and 600-fold) and rapid up-regulation of BAFF mRNA. Of particular interest is the dynamic expression of BAFF mRNA in RASFs in response to TLR3, in which an increased expression was already observed at the earliest time-point of 4h, with peak expression seen at 8h and progressively returning to the basal level at 72h. In contrast, APRIL was more modestly modulated by TLR3 and displayed a delayed kinetic of expression, with high transcripts at 24-48h post-stimulation.
In contrast to our observation that BAFF mRNA peaked at 8h and progressively decreased up to 72h (Figure 4.1a), previous reports demonstrate that after the stimulation of RASFs with TNF-α or IFN-γ, BAFF mRNA peaked at 24h and maintained this level for at least 2 days [428]. The differential expression of BAFF transcripts in response to TLR3 as compared to TNF-α ans IFN-γ agonists could be due to: (1) the requirement for a rapid response to recognise the acute challenge by dsRNA activation, (2) the existence of a rapid homeostatic function of TLR3 in cells turnover (3) a unique feature of how TLR3 recognises and binds its ligands (i.e. different association and dissociative rate of agonist-receptor interaction). Moreover, the mechanisms by which TLR3 recognises extracellular viral dsRNA is currently unknown. While TLR2 and TLR4 have been proposed to exert their role as cell surface receptors, TLR3 expression and localisation are regulated in a cell-type specific manner [383, 437]. For instance, human TLR3 has been demonstrated to be localised to the endosomal compartments in myeloid DCs, while it localised to both the cell surface and endosome of human fibroblasts cell line MRC5 [438], ECs [439]and macrophages [440].

Although the localisation of TLR3 on human SF is not well clarified, a recent study from Brentano et al showed that the expression of IFN-β mRNA and the production of pro-inflammatory cytokines and chemokines (IL-6, CXCL10 and CCL5) in RASFs were inhibited when pretreating the cells with chloroquine [110]. This phenomenon suggesting that TLR3 may localise in the interior of the fibroblast from arthritic joints, and that the TLR3 signaling arising in an intracellular compartment, requires endosomal maturation. However, the mechanism by which dsRNA is delivered from the extracellular fluid to the intracellular dsRNA sensors remains unresolved.

TLR3 is specific for dsRNA and the synthetic analog PIC. The dsRNA ligands may be derived from genomes of viruses that are detected only by endosomal TLRs, and the endosomal TLRs are particularly crucial to their containment. Host cells internalise microbes into different endocytic pathways: (1) phagocytosis, including the mannose receptor, Fc receptor and scavenger receptors; (2) fluid phase endocytosis/macropinocytosis, allowing uptake of suspended or cell-adherent particles;
(3) receptor-mediated endocytosis, mediated by caveolae (taken place at membrane rich in cholesterol and sphingolipid) or clathrin which absorbs a variety of particles and molecules; (4) fusion with the plasma membrane and later being swept into the endosomes either before or after the process of replication. Ultimately, the vesicles form at the plasma membrane and will quickly uncoat and allow fusion with endosomes [441].

Of interest, it has been demonstrated that IFN-β production was inhibited by pre-treating monocyte-derived immature DCs with cytochalasin D (a phagocytosis inhibitor) and chlorpromazine (a clathrin-mediated endocytosis inhibitor) but not with methyl-β-cyclodextrin (a caveolae-mediated endocytosis inhibitor), suggesting that the clathrin-dependent endocytic pathway may participate in the dsRNA-mediated TLR3 activation [442]. The same group further demonstrated that a major cytoplasmic lipid raft protein, Raftlin, is translocated from the cytoplasm to the plasma membrane where it colocalises with PIC, and thereafter associates with clathrin to participate in cargo sorting and mediate cell entry of PIC to the TLR3-positive endosome [443].

On the other hand, it remains to be clarified whether TLR3 could shuttle between the cellular surface and other intracellular compartments and where its exact location is when TLR3 accumulates intracellularly. It has also been illustrated that TLR3 localises to unidentified intracellular vesicles and that it initiates intracellular signaling in conjunction with endosomal maturation [444]. Furthermore, the engagement of cell surface or intracellular TLR3 with its respective ligands could activate different signaling pathways [445]. Therefore, a distinct localisation and downstream signaling pathway of TLR3 might possibly explain why the incubation of RASFs with PIC, but not bLP or LPS, could lead to a high induction of BAFF mRNA. Finally, it remains to be determined whether there is an alternative sensor for dsRNA, because PIC can still induce IFN responses in the absence of TLR3 or TRIF. The newly discovered cytosolic dsRNA receptor called retinoid-inducible gene (RIG-1) like receptor might be one of the possible candidates for this, as it has been reported to recognise both virus-derived RNA and PIC [446]. Interestingly, it has been demonstrated that TLR3 activation was associated with
elevated levels of RIG-1, suggesting the possibility of a crosstalk between two models of dsRNA detection [447].

Despite RASFs over-expressing TLR4 [360], LPS treatment (as well as TLR2 stimulation) induced only limited levels of BAFF mRNA (Figure 4.1a and 4.1c), consistent with recent data on airway fibroblasts [448]. These results are in keeping with previous evidence that TLR4 can even down-regulate BAFF mRNA expression in RASF in the presence of IFN-γ [293]. Because TLR2 and TLR4 mainly signal through a MyD88-dependent pathway, these data suggest that BAFF (and APRIL) transcription is under the control of TRIF (Toll/IL-1R domain-containing protein inducing IFNB) rather than MyD88-dependent pathways.

Although the mechanisms behind the increased expression of BAFF by TLR3 stimulation have not been fully elucidated, it is likely that the preferential activation of the TRIF-mediated pathway downstream of TLR3 activation is required to switch on the proinflammatory pathway (ultimately via NF-kB) for BAFF/APRIL production in addition to the unique induction of type I IFN and IFN-responsive gene expression (via IRF). In this regard, increased expression of IFN-β, known to be upregulated in the RA synovium [117], might also contribute to the pathogenesis of RA by exacerbating Th17, as recently suggested [449].

Interestingly, the modest but reproducible induction of BAFF in TLR4-activated RASFs and OASF may be explained by the evidence that TLR4 can also signal via a MyD88-independent pathway involving TRAM as a bridging adaptor for TRIF (Figure 6.1). Based on these findings, I can therefore hypothesise that TRIF-mediated signaling is the main pathway involved in the marked induction of BAFF in TLR3-activated RASFs and OASF. Further plans for the investigation of this signaling pathway in RASFs will be discussed in detail in Chapter 7 (Future plans).
Figure 6.1 Participation of TIR-domain containing adaptor molecules in TLR2, TLR3 and TLR4 signaling pathway.

Importantly, I showed that the capacity of synoviocytes to respond to TLR3 and release high levels of BAFF was not restricted to RASFs, as OASFs displayed similar expression of BAFF/APRIL in resting and stimulated conditions. Conversely, RADFs produced negligible amounts of BAFF/APRIL constitutively and failed to significantly increase their protein expression despite similar mRNA induction in response to TLR3. Overall, this suggests that the over-expression of BAFF and APRIL in SFs is dependent on epigenetic abnormalities related to the anatomical localisation rather than disease specificity [426]. In addition, the importance of TLR3 is relevant to RA pathogenesis as RASFs can be activated in the synovial microenvironment by RNA released from necrotic synovial cells [110].

Autoimmune or autoinflammatory diseases develop when self DNA or RNA in extracellular milieu bind to host nucleic acid sensors, therefore, a unique system should exist in order to ensure that the damaged cells and their endogenous TLR ligands are silently eliminated. Under normal circumstances, host DNA is sequestered in nuclei/mitochondria, and the release of free nucleic acid are hydrolysed by extracellular and endosomal DNases and RNases in order to prevent improper activation of the innate immune system. Accordingly, nuclease-resistant phosphorothioate-stabilised or 3’-polyguanosine oligonucleotides, which are resistant to nuclease degradation, have been reported to be strong activators for TLR9 [451, 452]. Several studies have reported an interplay between host nucleic acid and autoimmunity: (1) dysfunction of Flap endonuclease 1 has been shown to result in residual un-digested self DNA within apoptotic bodies leading to chronic inflammation and autoimmunity [453]; (2) deficiencies in extracellular DNase I results in systemic lupus erythematosus (SLE)-like disease in mice [454]; (3) a low DNase I activity with minor mutation within the DNase 1 gene have also been detected in sera from human SLE patients [455]; (4) dysfunction of lysosomal DNase II also results in insufficient degradation of DNA, leading to a variety of autoimmune syndromes including arthritis [456]. Recently, it has been reported that (B Zimmermann Ann Rheum Dis 2012;71:Suppl 1 Article 89) extracellular RNA (exRNA) is present in the synovial lining layer and that the RNase activity in the synovial fluid seems to be reduced in RA patients as compared to OA. Accordingly, the reduced RNase
activity may explain the accumulation of dsRNA released from the necrotic synovial fluid cells, which subsequently activates TLR3 signaling within the arthritic joint.

Next, I confirmed that RASFs express BAFF at protein level, both at surface-bound and soluble form. This is of critical importance as BAFF can elicit its biological functions both in a transmembrane and a soluble form [457]. Interestingly, the soluble BAFF concentrations observed in resting and TLR3 activated RASFs were comparable or higher to those released by other activated cell types, such as DC and airway EC, which express biologically functional amounts of BAFF and efficiently respond to TLR3 stimulation [279, 458]. Of interest, the limited increase in the amount of transmembrane BAFF expression on RASFs after PIC stimulation was not mirrored with the respective high release of soluble BAFF, suggesting that BAFF is continuously cleaved from the cell membrane. An alternative explanation could be that processing of BAFF in RASFs occurs intracellularly before being released as soluble protein, as previously observed in neutrophils [279]. Despite strong mRNA upregulation, it is tempting to suppose that the failure of RADFs in releasing BAFF protein may be due to an inactive/desensitised state of the furin protease, and that the regulation of such cleavage may be critical to the ultimate levels of soluble form of BAFF. Another interesting observation is that despite the huge upregulation of BAFF mRNA (~1000-fold) (Figure 4.1) in TLR-stimulated RASFs, there is only 4-5 fold increase in soluble BAFF (Figure 4.3), suggesting that there is a non-linear relationship between mRNA and protein induction of BAFF which likely undergoes profound post-transcriptional/post-translational modifications in fibroblasts. Indeed, recent analyses indicate that mRNA levels only partly correlate with the corresponding protein concentration and it has been postulated that the differences in protein concentrations are only 20%–40% attributable to variable mRNA levels, underlining the significance of post-transcriptional regulation [459]. Additionally, I provided the first demonstration that RASFs constitutively release high levels of soluble APRIL (approximately 10-folds higher for APRIL as compared to unstimulated RADF at 72h). In keeping with the mRNA data, although TLR3 activation did not induce a large induction of APRIL in RASFs, APRIL was released at a higher rate within the first 24h of stimulation, reflecting a possible role for TLR3 in the early mobilisation of
intracellular APRIL. Interestingly, immunostaining of resting RASFs demonstrated that APRIL was not homogeneously expressed among RASFs, demonstrating some heterogeneity within the RASF population.

One important aspect that I carefully considered in my experimental work was to exclude the presence of contaminating myelomonocytic cells as a possible alternative source of BAFF/APRIL, both constitutively and in response to TLR stimulation in RASFs. Indeed, I only used RASFs between passages 4-8 in which the culture is devoid of contaminating lymphocytes and macrophages, and SFs maintain a fully activated and stable phenotype. In order to further confirm that the culture synoviocytes are devoid of contaminating lymphomononuclear cells, I investigated the expression levels of IL-10 mRNA, which was undetectable in all the RASFs cultured that I examined. This is of particular relevance as synovial macrophages, rather than SFs, are known to produce considerable amount of IL-10, and therefore, IL-10 production can be taken as a contamination measurement for the presence of synovial macrophages [427]. In keeping with this evidence, Ritchlin and colleagues demonstrated that IL-10 could still be detected in RASFs up to passage 3 [460].

As described throughout in this thesis, BAFF and APRIL are critically involved in promoting functional B cell activation, AID expression and Ig class-switching [244, 259, 461]. Thus, in my PhD project I next investigated whether: (1) RASFs are capable of supporting AID expression and class-switching in co-culturing with tonsillar unswitched IgD+ B cells; and (2) this effect is dependent on the release of BAFF/APRIL by RASFs. It should be pointed out that the use of unswitched B cells allowed me to dissect the actual capacity of RASFs to enable the molecular machinery for CSR rather than just simply enhance the survival/proliferation of class-switched B cells as previously reported. By these means, I showed that RASFs and OASFs, but not RADFs, in co-culture with IgD+ mature human tonsillar B cells (but also from IgD+/CD27+ peripheral blood mature B cells, Figure 4.13), are sufficient in inducing class-switched antibodies, primarily IgG and to a lesser extent IgA. Further stimulation of RASFs with TLR3 ligands strongly
enhanced the production of IgG and IgA in co-cultured B cells, an effect directly dependent on RASFs as IgD+ B cells alone did not respond to PIC.

The production of isotype class-switching requires AID and occurs via excision of switch circles following the introduction of double strand breaks in the Ig switch regions of DNA, with the subsequent substitution of IgM and IgD with IgG, IgA, or IgE [253]. Accordingly, I showed that RASFs, but not RADFs, induced a rapid and sustained de novo expression of AID in co-cultured IgD+ B cells, an effect significantly potentiated by TLR3 simulation. Subsequently, I demonstrated that AID expression induced Iγ-Cμ, being transiently produced for up to 48h after [263]. Therefore, the increased detection of Iγ-Cμ CTs after over 72h of co-culture demonstrated that class-switching from IgM to IgG was actively ongoing and that TLR3 stimulation of RASF potentiated CSR in IgD+ B cells. Accordingly, the IgG/IgM ratio was over 10-fold higher 8 days after co-culture in TLR3-stimulated conditions than after 24h. Of relevance, TLR3-induced RASFs-dependent B cell activation was maintained when B cells and RASFs were physically separated via a cell insert, suggesting that soluble factors are sufficient to induce B cell activation. This evidence is of particular importance as BAFF can elicit its biological functions both in a membrane-bound and in a soluble form [457]. Of importance, Figures 4.3-4.5 demonstrated constitutive expression of membrane-bound BAFF in resting RASFs, confirming previous results by Rochas et al [292], and my data further showed increased membrane expression upon TLR3 stimulation. However, I would like to point out that in my transwell system, AID expression, CT production and Ig class-switching could still be detectable in the absence of direct cell-cell contact between RASFs and B cells, similar to what has been shown in epithelial/B cell co-cultures [244, 286]. Furthermore, I also showed that this effect is dependent on soluble BAFF/APRIL, as shown in Figures 4.11-4.13. This confirms the original work by Schneider et al [457], who showed that both membrane and soluble forms of BAFF are biologically functional. Moreover, my work is the first to show increased expression of the processed form of APRIL in resting RASFs compared to RADFs, as shown by ELISA, which is important (together with soluble BAFF) to explain B cell activation in the transwell system.
Importantly, although previous reports [259, 457] pointed out that BCR triggering (such as incubation with anti-IgM and/or treatment with soluble CD40 ligand (CD40L) or CD40 mAb) is essential, together with BAFF and/or APRIL stimulation in order to induce class-switching in B cells, later work has clearly shown that BAFF/APRIL can induce Ig class-switching independently from BCR triggering in the presence of other factors (i.e. cytokines such as IL-4, IL-6, IL-10). It was previously shown that RASFs can contribute to B cell activation via IL-6 [292] and potentially IL-10 [363] and IL-6 is most likely also contributing to B cell activation in my co-culture system. In agreement with the independency from the BCR trigger, stimulation with BAFF/IL-4 is sufficient to induce AID, CT and Ig class-switching (Figure 4.10). This conclusion is also supported by previous work demonstrating that in the absence of BCR, TLR3 activation of oral and intestinal ECs induces Ig class-switching in B cells via soluble BAFF and/or APRIL [244, 286].

Given the high amount of soluble BAFF and APRIL released by RASFs, I finally questioned whether these factors are critically involved in promoting functional B cell activation, AID expression and Ig class-switching. Using a set of comprehensive experiments, I showed that TLR3-dependent IgG and IgA production was completely inhibited after interfering with signaling through both BAFFR (specific for BAFF) and BCMA (binding both BAFF and, with greater affinity, APRIL) on the B cell surface. Of relevance, only minimal effects on IgM production were observed, suggesting that reduced B cell activation rather than survival was responsible for the down-regulation of IgG/IgA. Accordingly, BAFFR/BCMA blockade can also down-regulated the expression of AID and the production of Iγ-Çμ CTs. These in vitro data support in vivo observations in the RA/SCID model, in which functional niches of autoreactive B cells required BAFF and APRIL for Ig production, AID expression, ongoing class-switching and production of ACPA antibodies [152].

Importantly, the freshly isolated tonsillar B cells rapidly die when cultured in the absence of proliferative and/or survival stimuli. The viability of B cells is controlled by activation of an internally coded suicidal program called programmed cell death/apoptosis, an active
characterised by specific morphologic and biochemical changes leading to \textit{in vitro} endonucleolytic degradation of the DNA at nucleosomal intervals. Therefore, in cultured B cells, the culture media is supplemented by fetal bovine serum.

My established co-culture system indicate that the presence of stromal cells (OASFs, RASFs, and RADFs) can promote the production of secreted Ig from B cells, suggesting that soluble factors or cytokines provided by the stromal cells is able to support/induce B cells survival \textit{in vitro}. In addition, when B cells cultured without the presence of stromal cells, an albeit but modest level of IgM remains detectable, indicating that the isolated B cells become able to survive alone \textit{in vitro}. It could be argued that the enhanced survival seen in the B cell cultures reflects an increased number of viable cells due to more cell division rather than reflecting protection from death. Nevertheless, in the future, an aliquot of the collected B cell samples can be assayed for viability (intact plasma membrane) and cell death (damaged plasma membrane) by dye exclusion method. Cells with disturbed plasma membrane permeability are stained, while undamaged (viable) cells are not stained with dyes that do not penetrate the plasma membrane (“exclusion dyes”). The most frequently used dye for exclusion tests is trypan blue. In addition, a fluorescent dye, propidium iodide (PI), is membrane impermeant and therefore does not enter undamaged cells with intact membranes. When PI does gain access to nucleic acid, it become highly fluorescence and is therefore used to identify dead cells. The stained and unstained cells are counted with a light microscope (trypan blue), or flow cytometer (PI).

In an effort to understand the specific roles of BAFF/APRIL during later stages of B cell differentiation, it is also necessary to understand the expression of BAFF/APRIL receptors in B-lineage cells at various stages of maturation. Expression of BAFF-R has been found on most B-lineage cells, with low levels of expression on GC B cells and absence in long-lived PC. In contrast, the precise developmental stage(s) at which B cells express TACI and BCMA remains controversial. Regarding BCMA, Darce \textit{et al} [462] recently proposed that the balance between the expression of BAFFR/BCMA on the B cell surface may be involved in B cell’s decision to either differentiate into a memory cell.
or PC. They showed that upregulation of BCMA develops in parallel with a loss of BAFF-R during B cell differentiation into rapidly-dividing Ig-secreting cells (plasmablast) and PC. This result reinforces evidence from Zhang et al showing that human tonsillar B cells receive BAFF signal through different receptors as they differentiate into PC, whereby naïve B and GC-B cells utilise BAFF-R, differentiated PC precursors and PCs use BCMA and TACI [329]. In regard to TACI, its expression on human B cells remains controversial. It has been occasionally reported that TACI is expressed on: (1) tonsillar memory B cell alone, [329] (2) both mature naïve and memory B cells [463], or (3) all tonsillar B-lineage cells [464]. Despite its unclear expression on human B cells, it has been recently demonstrated that TACI expression is required for CSR in humans [465].

Unlike BAFF, APRIL does not bind to the BAFF-R and thus it is not involved in the selection or survival of naïve B cells. In turn, APRIL has a unique function in class-switching to IgA via the interaction with TACI, allowing the maintenance of mucosal immunity. APRIL is also essential for the survival of PCs in neonatal bone marrow. Interestingly, Sakurai et al showed that TACI has a dual role depending on the ligand, as it acts as a positive regulator in the case of APRIL-induced IgA production and as a negative regulator of BAFF-induced B cell responses, such as B cell proliferation and production of IgA and IgG in human peripheral blood B cells in vitro [323]. In addition, the same group demonstrated that cell surface HSPG is essential for APRIL-induced B cell responses. HSPG is widely expressed throughout the B cell lineage as a transmembrane protein in association with molecules such as CD44 and syndecan-1. Moreover, syndecan-2 has been reported to be widely expressed in mesenchymal cells including RASFs. Thus, the expression of APRIL by RASFs may not only have a paracrine effect on infiltrating B cells, but might also act as an autocrine stimulus for RASFs themselves, most likely upon interaction with the cell surface HSPG expressed on RASFs. Therefore, it is possible to hypothesise that APRIL might exert different regulatory functions on two cell levels (i.e. B cells and RASFs) in my co-culture system. Finally, Nagatani et al reported that BCMA, but not BAFFR/TACI can be expressed on RASFs; further suggesting that APRIL may act on RASFs in an autocrine manner via
direct interactions with its main receptor BCMA [314]. However, despite extensive investigations, I consistently failed to detect BCMA expression in RASFs after passage 4 (data not shown). Therefore, based on my own data (possibly obtained in “cleaner” experimental conditions compared to previous reports), it is unlikely that BCMA expression may have a major impact in the autocrine signaling of APRIL on RASFs.

Apart from the interaction with B cells, BAFF/APRIL has been shown to exert diverse and profound effects on other leukocytes and thus contribute to the amplification of inflammation at the site of injury. For instance, BAFF-R has been reported to be expressed on some T cells and may thereby modulate T cell activation by promoting the production of IFN-γ and IL-17 production [466, 467]. Moreover, activated monocytes and DC are reported to be primarily express TACI intracellularly, but cell surface TACI expression may be induced upon inflammatory response. BAFF/APRIL inhibitors have been recently been shown to exert clinical efficacy in human trials. The rationale for targeting BAFF/APRIL is not only related to their diverse immunoregulatory functions, but also to the evidence that they are overexpressed in several autoimmune diseases and have been successfully targeted in murine models of autoimmunity [275].

Despite considerable immunologic differences between rodents and humans, the results of the human studies are largely in line with those of the mouse studies. In humans, BAFF blockade preferentially depletes naïve and transitional B cells, while little effect is observed on class switched memory B cells and long-lived PC [468]. Selective BAFF blockade has less effect on serum Ig levels as compared to the blockade of both BAFF and APRIL, and both agents decrease the serum levels of IgM preferentially. The increased serum BAFF levels in RA patients together with the observation that high levels of both BAFF and APRIL, and their respective receptors, are found in the rheumatoid synovium [469], suggesting the need for BAFF inhibitors in RA treatment. However, the clinical use of both belimumab (a monoclonal antibody specific for soluble BAFF) and atacicept (TACI-Ig fusion protein, acts as a dual BAFF/APRIL inhibitor) have shown only modest effect [470], in contrast with the promising data obtained from murine studies where down-modulation of both DC and T cell functions was observed
along with the reduction of local BAFF expression within the affected joints [466]. It is interesting to ask why atacicept, as compared to belimumab, has no discernible effect on clinical benefit even though it eliminates ARPII in addition to BAFF. It is tempting to speculate that: (1) RA is a heterogeneous disease characterised by the presence of ELS in RA synovium and is maintained in the presence of high expression levels of BAFF and/or APRIL. Accordingly, different degrees of cellular organisation (phenotype of diffuse, aggregates and GC) may be associated with different requirement of BAFF/APRIL in different RA patients. Indeed, Humby et al reported that there is no difference in the expression pattern of BAFF when comparing CD21+ and CD21− synovial tissues, but an up-regulation of APRIL mRNA in GC+ synovitis [152]. It is therefore possible that: (1) atacicept is an ideal agent only for targeting those RA patients with GC+ synovitis; (2) atacicept may only neutralise circulating BAFF and APRIL, but not those that are tightly bound to their respective receptors within the synovium. Therefore it may only be an ideal agent to those patients who objectively exhibit B cell hyperactivity at the time of treatment.

A recent phase II clinical trial of a different anti-BAFF antibody, Tabalumab (LY2127399), that targets both transmembrane and processed forms of BAFF has shown efficacy in RA and further trials are now in progress [471] (M Genovese, abstract 1923). Thus, the observations provided in my current study have profound relevance, not only in the understanding of the pathophysiological processes regulating BAFF/APRIL expression/function within the rheumatoid joint, but may also in understanding the role of stromal-derived B cell survival factors in the mechanism of response/resistance to novel biological targeting of BAFF/APRIL.

In addition to BAFF/APRIL, this work highlights a fundamental role for TLR signaling in the induction and maintenance of the activated phenotype by RASFs. In particular, apart from the traditional pathogen-associated ligands, there is increasing evidence showing that the endogenous breakdown products from the extracellular matrix, such as hyaluron or intracellular components, are released when cells rupture, thereby, playing an important pathogenic role in the inflammatory arthritis. Therefore it would be of interest
to further investigate the function of TLRs in sensing tissue damage signals during chronic inflammatory responses. There are some new treatment strategies for targeting tissue regeneration applications, such as CQ-07001, an endogenous human protein that has been shown to be a powerful agonist for TLR3. This drug is currently in preclinical development (Clinquest Group obtains exclusive worldwide rights to clinical development of this drug - Amsterdam, the Netherlands, 06-03-2007), in which it is used for anti-inflammatory and tissue regeneration applications. In terms of treating RA, hydroxychloroquine, which has been reported to be efficacious in combination with methotrexate compared to the treatment with methotrexate alone, is also able to modulate TLR function by preventing intracellular TLR activation [472]. Furthermore, effective targeting of TLRs in RA is supported by a phase II clinical trial with chaperonin 10 that reacts against TLR2 and TLR4 signaling [473]. Based on my current work, it is possible to speculate that novel, yet undeveloped TLR3 antagonists might have significant potential in modulating RASFs function, synovial B cell activation and humoral autoimmunity in RA synovium.

In the second part of my experimental work (Chapter 5), I uncovered a novel counterimmunoregulatory pathway centered on an endogenous expression of SLPI by RASFs upon TLR stimulation. I proposed that SLPI, which is primarily known for its antiprotease and antibacterial activities at mucosal barriers, plays a key role in modulating RASFs functions, B cell survival factor production and downstream B cell activation with the net result of contributing to the control of inflammation within the joint microenvironment (see also Chapter 8). In this thesis I am the first to show that SLPI is expressed and secreted by the resident stromal cells isolated from the RA synovium. I demonstrated that RASFs produced significant levels of SLPI, both at the mRNA and protein level, in resting conditions. Furthermore, I showed that TLR3 stimulation, but not TLR2 or TLR4, was able to induce a further strong up-regulation of SLPI mRNA.

Similar to BAFF, RADF produced negligible amounts of SLPI and failed to increase their protein expression despite similar mRNA in response to TLR3. It is of interest to note
that baseline SLPI transcript levels in resting RASFs are around 60-fold higher compared to RADFs from the same patients, again, suggesting that profound epigenetic abnormalities confer the activated state of synovial stromal cells over fibroblast as different anatomical localisations. Of interest, the dynamic of SLPI mRNA expression in RASFs in response to TLR3 demonstrated that SLPI is induced at a late stage, with a progressive peak of production at 72h in response to TLR3 stimulation. This delayed kinetic of expression is opposed to the one observed for BAFF which is characterised by early expression at 4-8h.

In addition to mRNA expression, I demonstrated that the levels of soluble SLPI secreted from resting RASFs at 48h appeared to be much higher than those described in human mast cells and a lung EC line (A549) after 96h of culture [474]. Moreover, the expression of SLPI mRNA was paralleled by a similar pattern of SLPI protein expression, suggesting that a transient time is required for the induction of SLPI mRNA transcription, translation, and the subsequent intracellular transport, protein secretion as well as protein accumulation into the culture supernatant.

Overall, the above observations raised the question of whether BAFF mRNA expression could be, at least partially, under the transcriptional control of SLPI (BAFF mRNA peaks at 8h whereas SLPI mRNA peaks at 72h). In keeping with this hypothesis, I provided compelling evidence to support such conclusion by demonstrating that, i) the administration of exogenous SLPI to TLR3-stimulated RASFs downregulated the production of BAFF mRNA and protein expression and that, ii) incubation with a anti-SLPI blocking antibody significantly upregulated BAFF mRNA in resting and stimulated conditions. This further suggests that endogenous RASFs-derived SLPI is biologically relevant in modulating RASFs activation. Previous reports showed that SLPI is able to reduce LPS-induced TNF-α production in a macrophage cell line [338, 475], suggesting that SLPI might preferentially interfere with members of the TNF-family. However, I showed that this inhibitory effect was specific for BAFF, as it was not associated with the close homolog of BAFF, APRIL (data not shown).
Although the exact mechanisms by which SLPI could interfere with the production of BAFF in RASFs remains to be determined, it is well established that SLPI can penetrate into several cell types and downregulate critical transcription factors, such as NF-kB. Whether this is also true for RASFs is an interesting area that I am currently pursuing (See Chapter 7 for details).

Another interesting observation worth noting is that despite the high constitutive expression of SLPI mRNA and protein by RASFs in resting conditions, which are further increased upon PIC stimulation, such levels of endogenous SLPI are not sufficient to completely counterbalance the activation state of RASFs, although its selective blockade clearly showed that endogenous SLPI contains some biologically inhibitory functions in the culture system that I used. The exact biological contribution of SLPI in the broader control of synovial inflammation remains under investigation.

An additional mechanism by which SLPI might modulate BAFF release by RASFs is related to its strong and broad antiprotease activity. In this regard, as the soluble form of BAFF is secreted upon the cleavage of the membrane-bound BAFF by a protease termed furin, it is possible to speculate that SLPI might inhibit the production of BAFF in RASFs also by inhibiting proteases that are necessary for the release of BAFF. Whatever the prevalent mechanism, my findings imply that a negative feedback loop exists between SLPI and BAFF, resulting in the fine and selective tuning of BAFF production in RASFs with potential consequences on BAFF-dependent downstream B cell activation.

In addition to its regulatory activity on RASFs, I also demonstrated that exposure of human B cells to exogenous SLPI resulted in the inhibition of class-switching by interfering with the expression of AID and the molecular machinery for CSR. My observation is in line with recent finding by Xu et al. who showed that SLPI from tonsillar ECs restrained class-switching by inhibiting AID in B cells. Interestingly, the same group demonstrated that SLPI can penetrate into the B cell nucleus and cytoplasm. Furthermore, Taggart et al demonstrated that SLPI enters the U937 monocytic cell line or
peripheral blood monocytes and rapidly localises into the cytoplasm and nucleus where it affects NF-κB activation by direct binding to NF-κB sites in a site-specific manner.

I first confirmed in my study that SLPI could inhibit the expression of AID and IgG class-switching in tonsillar B cells induced by BAFF/IL-4. In addition, my data suggest that SLPI may exert its inhibitory activity on Ig class-switching via two different levels of regulation by affecting both Ig transcriptional regulation (AID expression) and the post-transcriptional control of gene expression (Ig production). This is reflected by the fact that SLPI showed a dose-dependent response in terms of progressive inhibition of IgG/IgA production, this was only partly mirrored by the downregulation of as AID expression, which was already maximally reduced at the lowest dosage of SLPI (10ng/ml) and its expression remained the same throughout the different dosage of SLPI treatment.

Having demonstrated that SLPI exerted its inhibitory effects both by (1) dampening the expression of RASFs-derived BAFF in resting conditions and upon TLR3 stimulation and (2) inhibiting B cell activation directly, I finally investigated the next effect by adding exogenous SLPI to my co-culture systems of RASFs and B cells and analysed the downstream effects on AID expression and production of class-switched Ig. By these means, I showed that TLR3-dependent IgG/IgA production was significantly inhibited after altering signaling with exogenous SLPI. Of relevance, a minimal reduction effect on IgM production was observed, in this context, SLPI likely to influence not only the class-switching machinery (see below), but also alter the mechanisms of Ig secretion and/or the survival of B cells. Accordingly, SLPI can also downregulate the expression of AID in co-cultured B cells.

Importantly, it would be of interest to investigate whether blocking the TLR3-stimulated-RASFs derived SLPI in the RASFs/B cell co-culture system with a blocking antibody could upregulate the production of BAFF by RASFs and the subsequent Ig production by B cells. Studies on this point are in progress and are discussed in Chapter 7.
In summary, in this thesis I demonstrated that (Chapter 4) i) RASFs, but not RADFs, constitutively release high levels of BAFF and APRIL; ii) BAFF expression is strongly induced by TLR3 ligands, a very relevant observation for RA synovitis as RASFs have been shown to be continuously activated in the synovial microenvironment by RNA released by necrotic cells [110]; iii) TLR3 stimulation strongly enhance the capacity of RASFs to directly promote AID expression, isotype class-switching and IgG/IgA production in unswitched IgD+B cells; iv) these effects can be efficiently blocked by inhibition of BAFF/APRIL signaling through their receptors on the B cell membrane. Overall, the first part of my PhD thesis contributes to significantly increase my knowledge of the mechanisms regulating the crosstalk between stromal cells and B cells in the synovial microenvironment and highlights the importance of the BAFF/APRIL system in sustaining the functionality of synovial niches of autoreactive B cells, with relevant implications for biological treatments targeting B cell-related pathways in RA.

In Chapter 5, I provide several lines of evidence with carefully controlled experiments to support the conclusion that the capacity of resting and TLR3-activated RASFs to directly contribute to AID expression and Ig class-switching in B cells can be regulated via the endogenous production of SLPI. In particular I showed that i) RASFs, but not RADFs, constitutively release high levels of SLPI mRNA and protein; ii) TLR3 stimulation of RASFs further increases SLPI expression, iii) the ability of class-switching in BAFF/IL-4 stimulated B cells is strongly restrained by SLPI; iv) the induction of BAFF expression via TLR3-stimulated RASFs is restrained by SLPI; v) the ability of TLR3-induced effects on B cells co-cultured with RASFs are partially blocked by SLPI treatment.

The logical conclusion of this set of experiments is that the effect of TLR3 on class-switching is dependent on BAFF/APRIL released by RASFs via a SLPI-regulated signaling programme. Overall, this work contributes to elucidate the mechanisms regulating the crosstalk between stromal cells and B cells in the synovial microenvironment and highlights the importance of the BAFF/APRIL/SLPI system in sustaining the functionality of synovial niches of autoreactive B cells. This
enhances the current understanding of the disease pathogenesis and identifies a novel promising strategy for therapeutic targeting in RA.
CHAPTER 7

Future Plans

In my PhD thesis I highlighted an important role for TLR3 signaling in RASFs in mediating the release of B cell survival/proliferation factors resulting in the enhanced capacity of RASFs to promote AID expression and ongoing class-switching in unswitched IgD⁺ mature naïve B cells. Although these effects could be effectively blocked by inhibition of BAFF/APRIL signaling through their receptors on the B cell membrane, an alternative possibility that I am pursuing is to alter the TLR3 signaling pathway in order to directly regulate BAFF/APRIL expression by RASFs. In particular, one pivotal aspect of my future research efforts will focus on the identification of signaling molecules that participate in TLR3-mediated RASFs activation upstream of BAFF/APRIL production.

Previous reports showed that TRIF-deficient mice defective in both TLR3- and TLR4-mediated expression of IFN-β and activation of IRF3. Importantly, whereas TRIF-deficient mice revealed a complete loss of TLR3-mediated NF-kB activation, TLR4-mediated NF-kB activation was only completely abolished in mice with disrupted genes encoding both MyD88 and TRIF [476]. The finding that both PIC induced NF-κB and IRF3 activation are completely abolished in TRIF-deficient mice, demonstrating that TLR3 mediates its signaling only via TRIF-dependent pathways. In support of this conclusion, Jiang et al reported that mutation of the TRAF6-binding site of TRIF only abolished its ability to activate NF-κB but not IRF3 [477], suggesting that TLR3-mediated the activation of NF-κB and IRF3 might bifurcate at TRIF. I therefore hypothesise that selective disruption of the signaling via the TRAM/TRIF pathway (the sole adaptor used by TLR3), may be crucial to limit the release of B cell survival factors by RASFs in resting conditions and upon TLR3 and TLR4 activation. In turn, I postulate...
that this inhibition will impairs the downstream B cell activation in co-culture with RASFs.

The future experimental plan is to employ a lentiviral delivery system for knocking down the TRIF-dependent pathway by using a construct encoding for a dominant negative TRIF that acts as a negative regulator of TRIF signaling. A preliminary experiment has been performed by Dr. Taher Taher, a post-doctoral fellow who has successfully generated a lentiviral vector encoding a dominant negative TRIF. The next immediate steps that I plan to perform include the stable transfection of RASFs with the TRIF lentivirus in order to investigate three specific aims:

1) To dissect in vitro the modulation of basal/post-TLR3/4-induced BAFF mRNA/protein (and other TLR3-activated cytokines) in TRIF-lentivirus-transfected RASFs;
2) To investigate in vitro whether the TRIF-lentivirus transfected-RASFs have reduced ability to modulate class-switching and activation of AID in IgD⁺ unswitched B cells in the co-culture system;
3) To examine in vivo in the human RA/SCID chimeric model, whereby SCID mice are transplanted with rheumatoid synovium, what the effect of TRIF down-modulation on the release of B cell survival factors and downstream B cell activation/autoantibody production in the in the presence or absence of TLR3 stimulants.

Such combination of in vitro and in vivo experiments should be able to provide further information on the mechanisms regulating the fine tuning of BAFF/APRIL release from RASFs, and provide proof of concept in support of the rationale for blocking the TRIF signaling pathways (specifically within the rheumatoid synovial tissue) as a tool to control critical pro-inflammatory pathways that are aberrantly activated in the RA joints.

In parallel with this work, I plan to capitalise on the results described in Chapter 5, in which I highlighted a novel endogenous pro-resolving route mediated by an SLPI-
dependent pathway in RASFs. In my work so far I demonstrated that the addition of recombinant SLPI reduced the production of BAFF at both the mRNA and protein level in TLR3-stimulated RASFs and that blockade of endogenous SLPI with antagonistic anti-SLPI antibodies exerts opposite effects, suggesting that SLPI is an important regulator of BAFF expression in RASFs. Moreover, I showed that SLPI could directly attenuate the expression of AID and inhibit the machinery responsible for Ig class-switching in human B cells either alone or in co-culture with RASFs.

Thus, critical questions that remain to be elucidated include:

i) Which are the mechanisms through which SLPI exerts its immunoregulatory properties in RASFs/B cell interactions and,

ii) Whether modulation of SLPI in gain/loss of function experiments results in profound alteration of critical pro-inflammatory pathways, which are active in RA synovitis.

Regarding the first aspect, I will primarily focus on the capacity of SLPI to impair NF-kB activation in resting and activated RASFs and B cells, alone and in co-culture experiments; I will employ the electrophoretic mobility-shift assay and supershift assay (EMSA) in order to detect NF-kB nuclear translocation in response to exogenous SLPI and anti-SLPI antibodies. The prediction is that exogenous SLPI will result in attenuated nuclear translocation of NF-kB in response to inflammatory stimuli, such as TLR ligands. In addition, I will investigate whether the capacity of SLPI to inhibit the release of soluble BAFF is also related to its anti-protease properties and in particular whether SLPI can affect the enzymatic activity of the furin convertase resulting in accumulation of membrane bound BAFF.

Regarding the second aspect, I plan to dissect the functional role of SLPI in modulating RA synovitis and its potential as a therapeutic adjuvant in controlling inflammatory arthritis. As previously mentioned, Song et al demonstrated that SLPI ameliorates arthritis in the streptococcal-cell wall induced rat model by inhibiting proteolytic tissue destruction as well as in suppressing the production of the inflammatory mediator TNF-α.
Therefore, here I plan to use two in vivo models in order to dissect the effect of SLPI gain/loss of function experiments and the possible therapeutic role of SLPI in RA disease. In the first in vivo model, of which I present preliminary data in Chapter 8 (Supplementary Results), where I demonstrate that exogenous SLPI is able to modulate collagen-induced arthritis (CIA) in DBA/1 mice by delaying the onset of arthritis and reducing clinical severity of synovitis. Based on such promising data, in the near future I plan to better characterise the effect of SLPI in the CIA model by:

1) Performing histological analysis for inflammatory cell infiltration, cartilage destruction and osteoclast activation/bone erosion;
2) Investigating the downstream effect on the production of inflammatory cytokines within the inflamed synovium and;
3) Assessing the effect of SLPI in modulating autoantibody production in the CIA model, in particular the production of anti-type II collagen antibodies.

In the second in vivo model, I plan to translate these observations in the human SCID-RA chimeric model. Here the plan is to engraft SCID mice with RA synovium with/without ectopic B cell follicles and investigate whether recombinant SLPI (loss of function) or anti-SLPI antibodies (gain of function) are able to: i) modulate basal or TLR3 stimulated production of proinflammatory cytokines and B cell survival factors; ii) affect B cell survival/proliferation and modulate AID activation and ongoing CSR; iii) regulate the production of ACPA antibodies within the synovial tissue.

Overall, these data should provide novel and relevant observations on the functional capacity of SLPI to influence the microstructural organisation of RA synovial tissues, which modulate autoreactive B cell activation in the ectopic microenvironment and affect in situ autoantibody production. If successful, this set of experiments could be highly relevant in paving the way to exploit the anti-inflammatory, pro-resolving and immunomodulatory properties of SLPI for therapeutic purposes in RA synovitis.
CHAPTER 8

Supplementary Data: An anti-inflammatory role for SLPI in collagen-induced arthritis

In this final part of my PhD thesis, I will briefly present and discuss preliminary data obtained in vivo in the collagen-induced arthritis (CIA) model focusing on the therapeutic potential of SLPI in ameliorating inflammatory arthritis. I will briefly introduce the CIA model and the experimental setting used for this in vivo work.

8.1 Background: Animal models of RA

RA is a complex multifactorial inflammatory disease. Experimental models of arthritis therefore serve as valuable tools for investigating the underlying mechanisms of disease pathogenesis and provide the possibility to investigate novel potential treatments of arthritis with the possibility of translation into humans. Nowadays, no particular model of arthritis mirrors all the pathogenic and clinical aspects of the human disease, but various animal models have been used to delineate several different pathways, all leading to arthritis onset. Overall, this approach has allowed a rapid and greater understanding of the cellular and immunological aspects underlying the development of synovitis and breach of tolerance, which would not have been possible to evaluate in humans due to obvious ethical reasons.

In principle, there are three main basic factors to consider in experimental models of arthritis in rodents: (1) animal strains, (2) antigen used for immunisation, and (3) the use of adjuvants. Adjuvants are immunostimulatory agents that are applied together with an antigen in order to enhance the transportation and presentation of Ags to the local lymph node, primarily via the induction of a local inflammatory reaction at the site of immunisation, leading to the release of chemokines and up-regulation of adhesion
molecules. Subsequently, the local and/or systemic cytokines increase the infiltrating population of immune cells and stimulate the innate response through accessory cell PRRs. In a very schematic and non-comprehensive way, animal models of inflammatory arthritis can be subdivided into: (1) induced models whose development is based on immunisation with antigen that contains peptides or protein in the presence/absence of an adjuvant; (2) induced models whose development is based on chemical agents (non-immunogenic adjuvants) which lacks bacterial cell wall components or any other components that contain peptides; (3) spontaneous models whose development is based on genetic manipulations. While each of these models has advantages and disadvantages, collagen-induced arthritis (CIA) has been the most widely studied model of RA.

8.1.1 Collagen-induced arthritis

CIA was first described in rats, but was subsequently found also to develop also in susceptible strains of mice and non-human primates following immunization with type II collagen (CII), the major constituent protein of articular cartilage, emulsified in either complete form, Freund's Complete Adjuvant (CFA), composes of inactivated and dried mycobacteria (usually *Mycobacterium tuberculosis* [MTB] suspended in mineral oil) or incomplete form (IFA), which lacks the mycobacterial components (hence consist of just the water in oil emulsion). The “gold standard” CIA disease model has been described in genetically susceptible DBA/1 mice that carry the MHC Class II I-Aq haplotype (H-2q) [478]. Following immunisation, this CIA mouse model shares many similarities with RA and has been used in preclinical evaluation of RA drugs currently used in the clinic (e.g. etanercept)

The immune response to CII is characterised by both the stimulation of collagen-specific T cells and the production of antibody specific for both the immunogen (heterologous CII) and the autoantigen (mouse CII) by B cells. Accordingly, immunoglobulin concentrates of sera from CIA mice are able to transfer the disease even to resistant mouse strains, demonstrating an active role of antibodies in mediating the pathology in this model [479]. Similar protocols are now used to induce arthritis and are collectively termed collagen antibody-induced arthritis (CAIA). The chief pathological features of
CIA include synovial hyperplasia, mononuclear cell infiltration, pannus formation, cartilage degradation, erosion of bone, and fibrosis. As in RA, pro-inflammatory cytokines, such as TNF-α and IL-1β are abundantly expressed in the arthritic joints of CIA mice and blockade of these molecules results in a reduction of disease severity [480]. This provides some hope that the results with CIA can be transferred to humans to treat and perhaps cure RA.

8.2 Methods: Induction of CIA

8.2.1 Immunisation

A classic protocol for the development of CIA model was employed. The DBA/1JLacJ mouse strain was obtained from Jackson Labs. The colony was housed in a purpose-built area and maintained strictly according to the international and United Kingdom guidelines for animal care. All mice were maintained under germ-free conditions using specially adapted laminar flow cabinets and filter-top cages. Breeding and procedures were carried out according to Home office Guidelines on the Operation of the Animal (Scientific Procedure) Act 1986 (PPL 70/6109). A total of 26 male DBA/1 mice were used at 12-13 weeks of age and were randomly assigned to four groups as follows: control untreated animals (n=5); control CIA-injected animals, which received PBS on day 17 (n=7); CIA-injected animals treated with recombinant human SLPI (R&D) on day 17 (n=7) at a lower dosage of 30μg (in 100μl); and CIA-injected animals treated with recombinant human SLPI on day 17 (n=7) at a higher dosage of 100μg (in 100μl). PBS and drugs were injected to CIA mice model intraperitoneally (i.p). From day 10 post-collagen injection, all mice were monitored for clinical signs of arthritis. The severity of arthritis was determined by blind scoring of each ankle, digit and wrist joint, based on the degree of swelling/oedema (examined by plethysmometry) and disfigurement (Supplementary Figure 1). The overall clinical score was calculated by adding up the scores from all limbs during the course of the study (generally, clinical scoring was performed every other day from day 10 to day 35).
8.2.2 Type II collagen preparation

Collagen is a fibrous protein and therefore it is insoluble in normal physiological conditions. Consequently, in order to keep CII in solution for the use in the CIA model, it should be solubilised and stored in diluted acetic acid. Briefly, 6mg of bovine native CII (home made by the Bone and Joint Department at the William Harvey Research Institute, QMUL) was reconstituted overnight on a stirrer in 1.5ml of 0.01M acetic acid in the cold room. This process should be carried out at 4°C using pre-chilled reagent and glassware to avoid denaturing CII.

8.2.3 Complete Freund’s adjuvant (CFA) preparation

The best arthritis incidence is obtained if an emulsion is made with proper adjuvant. Freund's adjuvant is a solution of Ag emulsified in mineral oil and utilised as an immunopotentiator (booster). Most mouse strains require immunization with CFA to elicit a high incidence of full-blown arthritis. It has been suggested that the stimulatory capacity of heat-killed Mycobacteria in the adjuvant promotes an inflammatory response that initiates an immune response to CII [481]. In short, CFA was prepared by grinding and mixing 6mg heat-killed MTB (strain H37RA, Difco Laboratories, 231141), in a mortar and pestle with 1.5ml of IFA (Sigma F5506). CII (6mg in 1.5ml of acetic acid) was then added to the CFA, and therefore the CII/CFA emulsion contained collagen and MTB at a final concentration of 2mg/ml. The CII/CFA emulsion was then homogenised on ice with various pulses. A satisfactory emulsification could be observed if the emulsion did not dissipate on water where it floated as a discrete circle.
8.2.4 Induction of collagen-induced arthritis in DBA/1 mice

CIA was induced in DBA/1 mice by intradermal immunization at the base of the tail with 200 µg (100µl) of bovine CII/CFA emulsion using a syringe and a 25-G needle, followed by a boost with bovine CII/IFA on day 21. From day 10 after immunization, mice were examined daily for the onset of disease (Supplementary Figure 1). Paw thickness and clinical score were measured every other day until day 35 post-arthritis. At the end of the 35 day period of monitoring, mice were sacrificed and processed as follows: (1) arthritic paws were collected and placed in 20ml sterile universal tubes containing RNA later for at least 24h at 4°C before transferring to -80°C for long term storage; (2) draining inguinal, axillary and popliteal lymph nodes, were collected and placed in cryotubes containing RNA later as mentioned above; (3) blood was collected and placed into an 1.5ml eppendorf tube followed by gently mixing with pipette tips in order to prevent blood clots and was centrifuged at 1,500 rpm for 10min. The centrifuged blood samples were separated into two distinct layers, with red blood cells (RBCs) pelleted to the bottom of the centrifuge tube and the liquid supernatant (serum) formed at the top portion, which was carefully collected by sterile Pasteur pipettes into a cryotube and stored at -80°C.
Supplementary Figure 8.1. Collagen-induced arthritis (CIA) in DBA/1 mice with maximal arthritis.

Each numeral represents one clinical score.
8.3 Preliminary result

8.3.1 Recombinant SLPI delays the onset and reduces the severity on joint inflammation in the CIA model of RA

As shown in Supplementary Figure 2, the hallmarks of chronic arthritis (joint swelling, erythema, and disfigurement) appeared after the re-boost of type II collagen at day 21. As compared to the control (CIA-mice injected with PBS), a single i.p. administration of SLPI at 100ng/ml was shown to delay the onset and reduce the severity of arthritis at day 26. A subtle suppressive effect was observed with the lower dosage of 30ng/ml SLPI. Finally, SLPI did not appear to completely resolve synovial inflammation as some clinical progression still appeared during the course of the CIA and the clinical inflammatory score the clinical symptoms reappeared during the late chronic disease stage even after a second injection of SLPI. Of particular interest in this context is how exogenously delivered recombinant SLPI exerts its therapeutic effect (reduces disease severity) within the joint. One explanation may be the fact that such low-molecular-mass molecule has advantages of size, diffusibility (negatively charge), and possible anchorage to the synovium, which is where the action is. Moreover, the pharmacodynamic activity of SLPI may be accounted for, in part, by its half-life within the joint. In asthma, where the SLPI has a short half-life value of 6.5h in the epithelial lining fluid [482], little is known within the synovial fluid. However, it should not rule out the fact that the period between the first dosage of SLPI (day 17) and the initiation of arthritis (day 21) may provide sufficient time for tissue distribution to maximise its inhibitory activity. As a result of intracellular compartmentalization of SLPI or distribution to the surface in the synovium, the half-life value determined from synovial fluid may fail to fully quantify SLPI in the synovium.

In our experiments, the two treatment groups, i.e. mice which received recombinant SLPI (100ng/ml) and PBS (control), demonstrated a trend of increased disease severity after day 26, probably due to the limited half-life of SLPI. The lost of ability in inhibiting responses when administered after the initiation of arthritis responses (day 21),
suggesting a potential utility of SLPI as a delay agent rather than a rescue therapy. Moreover, my current data has only demonstrated an overall effect of SLPI against pathophysiologic responses associated with arthritis, additional works are required to fully determine the extent of the ability of SLPI to prevent joint pathology in association with B cells/the stromal cells (SCID model, see Chapter 7). Another important observation is that the concentration of SLPI within RA patients is around 80ng/ml (Supplementary Figure 3), suggesting the selected dosage of 100ng/ml of recombinant SLPI is closely mimics physiological conditions. Although preliminary, my data provides initial preclinical evidence for a promising role for SLPI in delaying the onset and/or slow down the progression of arthritis in CIA mice. In addition, this platform provides the basis for a large series of experiments to further elucidate the role of SLPI in modulating synovial inflammation and B cell autoreactivity (see Chapter 7, future plans), thus providing a stronger rationale for further pursuing SLPI as a critical regulator of synovial inflammation and exploit its anti-inflammatory properties for therapeutic purposes.
Supplementary Figure 8.2. Suppression of experimental arthritis by recombinant SLPI.

Arthritis was initiated by a single intradermally injection of type II collagen on day 0 and the clinical score measured as described above for the course of the study. An intraperitoneal injection of 30ng and 100ng of recombinant human SLPI was administered at day 17 and again at day 30 after re-boost of type II collagen on day 21. Each point represents the mean clinical score ± SEM for each group of animals.
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Appendix

**MEDIA AND MEDIA SOLUTIONS**

**Cell culture medium**
DMEM/F12 500ml
10% FBS (50ml)
50IU/ml Pen/strep (5ml)
10mM Hapes (5ml of 1M Hapes)

**Freezing medium**
10% DMSO
90% FBS
Add 1ml per vial of cell

**BUFFERS**

**20μl Master Mix (PCR x 1 reaction)**
12.3μl Baxter water
0.4 μl 10mM dNTP
2μl 10X buffer
1.2μl 25mM MgCl₂
1μl sense primer
1μl anti-sense primer
0.1μl Taq polymerase
2μl template (cDNA)

**Wash buffer (Western blot)**
4.84g Tris-base
17.52g NaCl
Make up to 2L with deionised water, pH 7.5
Add 1ml Tween-20
5X Tris-glycine eletrophoresis buffer, pH 8.3
25mM Tris (15.1425g of 1M Tris in 1L)
250mM Glycine (93.84g of Glycine in 1L)
0.1% SDS (100ml of 10% SDS in 1L)
Dilute 1:5 with deionized water before use and adjust pH if necessary.

Stripping buffer (80ml, pH6.7)
62.6mM Tris (5ml of 1M Tris)
100mM of beta-mercaptoethanol (559μl of 14.3M beta-mercaptoethanol) 16ml of 10%
20% SDS (16ml of 10% SDS)
Make up the solution with 58.4ml of deionized water*
*Large amount of 10M HCL (around 10ml) was required for adjusting the buffer to pH6.7

2ml Disruption buffer (Western blot cell lysate preparation)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Dilution factor</th>
<th>2ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris (pH8)</td>
<td>1:20</td>
<td>100μl</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>1:33</td>
<td>60μl</td>
</tr>
<tr>
<td>10% NP40</td>
<td>1:10</td>
<td>200μl</td>
</tr>
<tr>
<td>100mM NaF</td>
<td>1:20</td>
<td>100μl</td>
</tr>
<tr>
<td>500mM EDTA</td>
<td>1:250</td>
<td>8μl</td>
</tr>
<tr>
<td>500mM phosphatase inhibitor</td>
<td>1:250</td>
<td>8μl</td>
</tr>
<tr>
<td>1X Protease inhibitor</td>
<td>1:100</td>
<td>20μl</td>
</tr>
</tbody>
</table>

Bring up to 2ml with deionized water

Laemmli 5X lysis buffer
2.5 SDS
1.93g Dithiothreiotol (DTT)
2.5ml Glycerol ***
11ml 1M Tris pH6.8
5ml 0.2% Bromophenol Blue in methanol *
6.5ml deionised water **
* Bromophenol Blue will turn to orange in methanol, once dilute in water, the colour should change to blue
** add just before glycerol
*** last step for the recipe

1.5M Tris pH 8.8
Dissolve 18.17g of Tris-base with 100ml of deionised water
Stored the solution at 4°C

1X Transfer buffer
2.9g glycine
5.8g Tris-base
800ml deionised water
pH should be adjust to around 8.3
Add 200ml methanol
Note: All solution prepared for Western Blot were required to be diluted in deionised water rather than distilled water

POLYACRYLAMIDE AND AGAROSE GEL SOLUTIONS

20ml Tris-glycine SDS polyacrylamide gel (always 13%)
5.9ml deionised water
8.7ml of 30% acrylamide mix
5ml 1.5M Tris (pH8.8)
0.2ml 10% SDS
0.2ml 1% ammonium sulphate*
0.008ml TEMED*
*Lastly add fresh ammonium sulphate and TEMED as these agents cause polymerization
**10ml Stacking gel (always 5%)**

- 6.8ml deionised water
- 1.7ml of 30% acrylamide mix
- 1.25ml of 1M Tris (pH6.8)
- 100μl of 10% SDS
- 100μl of 10% ammonium sulphate*
- 10μl of TEMED*

*Add Last

**Transfer**

Soak 4 pieces of Whatman filter paper, 2 sponge pads and 1 piece of PVDF membrane* (cut to size of gel to ensure current running through the cassette is directed through correctly) in chilled transfer buffer.

Assemble transfer cassette as follows:

<table>
<thead>
<tr>
<th>Sponge pad</th>
<th>Black electrode</th>
<th>Sponge pad</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Sheets Whatman</td>
<td>Nitrocellulose membrane</td>
<td>Polyacrylamide gel</td>
</tr>
<tr>
<td>Black electrode</td>
<td>Sponge pad</td>
<td>(+) POSITIVE ELECTRODE. RED</td>
</tr>
</tbody>
</table>

When assembling cassette sandwich ensure there are no air bubbles between any of the layers otherwise transfer will be impaired. Use a universal to roll over the membrane in order to push air bubbles out.

Proteins run from (-) **negative to (+) positive** (i.e. *Assemble black plate to black electrode*).

* Place the PVDF membrane in methanol for few sec to soak before transferring it to a containers with the chilled transfer buffer
**IMMUNOHISTROCHEMISTRY**

**10X Tris (hydroxymethyl)methylamine-buffered saline (TBS)**
60.6g Triza HCL  
13.9g Triza base  
90g NaCl Mix to dissolve to adjust pH to 7.6 using concentrated HCl. Storage at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

**100mM glycine in PBS**
Add 1.5g glycine to 300ml of PBS  
Adjust pH to 7.2  
Filter to sterilise and store at 4oC

**4% paraformaldehyde (PFA)**
8g of (PFA) into 100ml deionised water (performed under the fume hood). The mixture was allowed to dissolve by heating at 65oC with frequent stirring. 100μl of 10M NaOH was then added to fully dissolve the mixture into solution, which was then neutralised with 1ml of 1M HCl. 20ml of 10X PBS was added with 80ml deionised water added to make up the final solution of 100ml of 4% PFA. The solution was finally filtered and keeps at 4oC.

**Mowiol 488 mounting medium**
2.4g of Mowiol was added into a conical flask containing 6 g of glycerol. 6 ml of distilled water then added and allow to mixture at room temperature for at least 2h with a small magnetic stir bar. Thereafter, 12ml of 0.2M Tris (pH8.5) was added and heated at 50oC for 10min with occasional mixing in order to dissolve the Mowiol. Mowiol was then clarify by centrifugation at 5000g for 15min for the removal any undissolved solids. The supernatant was aliquot and sotre in 1ml eppendorf tubes at -20oC. Mowiol was warm to room temperature before use and opened tubes can be stored at 4oC for 1month.
**ELISA**

**Coating buffer (pH 9.6)**
3.7g sodium Bicarbonate  
0.63g sodium carbonate  
1L distilled water

**Wash solution**
1L 1X TBS (pH 8, see below)  
0.5ml Tween-20

**Blocking solution**
1X TBS  
0.5g BSA (Sigma, A7030)*  
*best blockage effect for the specific albumin from bovine serum

**Sample/Conjugate diluent**
50ml wash buffer (see above)  
0.5g BSA (Sigma, A7030)

**Stop solution**
1.11ml of sulphuric acid (sp. gravity 1.84) in 10ml of deionised water
DYES, STAINS, AND COLORANTS

Loading dye (Bromophenol-blue dye 6X)
300ml of 30% glycerol and 400ml of EDTA 0.5M (pH8) were added to 300ml of deionized water and mixed well. 2mg of bromophenol-blue were added to the solution and dissolved by inversion. Solution was stored at -20°C and diluted to working at the time of use.

10X Ponceau S
2% Ponceau S
30% trichloroacetic acid (TCA)
Diluted to 1X before use (can be re-used many times)

OTHER SOLUTIONS AND REAGENTS

1M Tris pH 7.5
Dissolve 12.14g of Tris-base with 100ml of deionized water

0.5M Ethylenediaminetetra-acetate (EDTA, pH 8.0)
Add 16.81g of EDTA to around 50ml deionized water.
While stirring with a magnetic stirrer, adjust to pH8.0 with pellets of NaOH (as EDTA only dissolved at pH8, large amount ~20g of NaOH might be required)
The solution was stored at room temperature
Publication