Ectopic lymphoid structures support Epstein-Barr virus persistence and autoreactive plasma cell infection in Rheumatoid Arthritis synovium and Sjogren’s Syndrome salivary glands

By

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DECLARATION

The work presented in this thesis is less than 100,000 words and was planned, performed and analysed by me, with the following exceptions:

Dr. Paola Migliorini performed the ELISA for citrullinated-EBV antigens in Hu/RA SCID mice serum. Dr. Martina Severa and Miss Fabiana Rizzo performed the real-time RT-PCR after selective pre-amplification target genes in RA synovia cDNA. Dr. Michele Bombardieri generated the citrullinated biotinylated fibrinogen. Dr. Barbara Serafini thought me and performed some of the IHC, IF and ISH experiments on the RA synovia.

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Dr. Michele Bombardieri had overall supervision of the work, and took part in the revision of the papers derived from this thesis.

The Candidate First Supervisor Second Supervisor

Cristina Croia Prof. Costantino Pitzalis Dr. Michele Bombardieri
ABSTRACT

The ubiquitous \( \gamma \)-herpesvirus Epstein-Barr virus (EBV) infects B cells and modifies their differentiation programme leading to B cell activation and immortalization. Although different evidences support a link between EBV infection and rheumatoid arthritis (RA) and Sjogren’s syndrome (SS), the exact role of EBV in RA and SS pathogenesis remain elusive.

Recently ectopic lymphoid structures (ELS) have been identified as preferential niches for EBV persistence and reactivation in patients with multiple sclerosis and myasthenia gravis. Independent studies demonstrated that around 50% of RA synovia and 30% of SS salivary glands are characterised by the development of functional ELS, capable to promote local differentiation of autoreactive plasma cells.

In this PhD project I explored the potential role of EBV in RA and SS pathogenesis by analysing EBV infection in the RA synovium and SS salivary glands and its relationship with ELS, in situ autoreactive plasma cell differentiation, pathogenic autoantibodies production and cytotoxic immune response.

In this work I demonstrated that: i) markers of EBV latent and lytic infection are consistently associated with the presence of ELS in the RA synovium and SS salivary glands; ii) latent EBV proteins are preferentially expressed by B cells, while viral reactivation occurs in plasma cells; iii) a large subset of autoreactive plasma cells is EBV lytically infected in the RA synovia and SS salivary glands; iv) antibodies specific for unmodified and citrullinated EBV peptides, known to cross-recognize ACPA, are produced within ectopic lymphoid structures as
demonstrated in vivo in human RA/SCID chimeras; v) SS salivary gland grafts transplanted into SCID mice release human IgG against EBV antigens, whose production correlates with the level of SS-associated auto-antibodies and vi) analysis of CD8+ and CD4+ T-cell localization and granzyme B expression indicated that EBV persistence in ELS-containing RA synovia and SS salivary glands may be favoured by exclusion of CD8+ T cells from B-cell follicles and impaired CD8-mediated cytotoxicity.

Overall, these results redefine a novel and pathogenically relevant role for EBV in B-cell dysregulation and chronic inflammation in RA synovium and SS salivary glands.
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</tr>
<tr>
<td>ELS</td>
<td>ectopic lymphoid structure</td>
</tr>
<tr>
<td>EULAR</td>
<td>European league against rheumatism</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cells network</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>germinal centre</td>
</tr>
<tr>
<td>gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>GPI</td>
<td>glucose-6 phosphate isomerase</td>
</tr>
<tr>
<td>GrB</td>
<td>granzyme B</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>immunohistochemistry</td>
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<tr>
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<td>interleukin</td>
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<td>IM</td>
<td>infection mononucleosis</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
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<tr>
<td>ISH</td>
<td>in situ hybridization</td>
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<tr>
<td>LCL</td>
<td>lymphoblastoid cell lines</td>
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<tr>
<td>LMP</td>
<td>latent membrane protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>MG</td>
<td>Myasthenia Gravis</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MS</td>
<td>Multiple Sclerosis</td>
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<td>NK</td>
<td>natural killer</td>
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<tr>
<td>NSCS</td>
<td>non-specific chronic sialodenitis</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PD1</td>
<td>programmed death 1</td>
</tr>
<tr>
<td>PNAd</td>
<td>peripheral lymph node addressin</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>RF</td>
<td>rheumatoid factor</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RQ</td>
<td>relative quantification</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SHM</td>
<td>somatic hyper mutation</td>
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<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematous</td>
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<td>secondary lymphoid organs</td>
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<td>Sjogren’s syndrome</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetic acid EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffer saline</td>
</tr>
<tr>
<td>Tfh</td>
<td>T-follicular helper</td>
</tr>
<tr>
<td>TLS</td>
<td>tertiary lymphoid structure</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine isothiocyanate</td>
</tr>
<tr>
<td>VCA</td>
<td>viral capsid antigen</td>
</tr>
</tbody>
</table>
VCP    viral citrullinated protein
TBE    tris borate EDTA buffer
CHAPTER 1

INTRODUCTION
1.1 Epstein - Barr virus

1.1.1 Discovery of EBV

Epstein-Barr virus (EBV) was discovered in 1964 by electron microscopy of cells cultured from Burkitt’s lymphoma tissue by Epstein, Achong and Barr (1). Four years later EBV was proved to be the etiological agent of infection mononucleosis (IM). Although primary infection is usually asymptomatic in childhood, it causes IM in about one third of the cases if it occurs during adolescence or adulthood (2). EBV DNA was detected in 1970 in tissues from nasopharyngeal carcinoma (3) and in 1982 it was found to be associated with non-Hodgkin’s lymphoma (4). Since then, EBV has been linked to several cancers and other diseases (i.e. autoimmune diseases). Today we know that EBV is one of the most successful viruses, infecting more than 90% of the world population with lifetime persistence (2). In most cases, EBV establishes an asymptomatic latent infection although the clinical outcome of the infection depends on the fragile interplay between the virus and the host immune response.

1.1.2 Classification of EBV

Epstein-Barr virus or human Herpes virus 4 (HHV4) is one of the 8 known human herpesviruses. This family, characterized by the capacity to persist within their host indefinitely, is divided into three subfamilies on the basis of
the virus biological properties and genome homologies; Alphaherpesvirinae, Bethaherpesvirinae and Gammaherpesvirinae. Alphaherpesvirinae includes herpes simplex virus type 1 and 2 and Varicella-Zoster virus. Bethaherpesvirinae includes cytomegalovirus and human herpesvirus 6. Gammaherpesvirinae, distinguished for their ability to establish persistence in lymphoid cells, includes type 2 (i.e. Kaposi’s sarcoma-associated herpesvirus) and type 1 or lymphocryptoviridae (LCV) (i.e. EBV) viruses. EBV is the only LCV with human tropism, whereas the other components of the family only infect primates. There are 2 subtypes of EBV, which differ from each other in the sequence of some latent antigen genes (i.e. EBNA2, 3A, 3B and 3C) (5). Type 1 is dominant in the Western hemisphere and Southeast Asia, whereas type 1 and 2 are equally prevalent in Africa (6). There are no proved differences that one strain is more “tumorigenic” than the other.

1.1.3 EBV genome and structure

EBV is 120-180 nm in diameter and consists of an inner core of protein and DNA surrounded by a protein capsid. A protein tegument lies between the capsid and the outer envelope that is embedded with glycoproteins important for cell tropism, host range and receptor recognition (6). The EBV genome consists of 184-Kb linear, double-stranded DNA, encoding for approximately 100 genes. The genomic structure of EBV is characterized by short and long unique sequence domains (US and UL, respectively) that contain almost all the genome coding capacity, as well as internal (IR) and terminal (TR) tandem and reiterated direct repeats (2). After EBV infection, the genome becomes circular.
by linking the two TR sequences (7). The precise number of TRs is determined during viral replication and is maintained identical from the parental to progeny cells; consequently, heterogeneity in the number of joined TR distinguishes clonal infection events.

1.1.4 EBV tropism and cell-entry

EBV preferentially infects B lymphocytes, leading to their immortalization and formation of lymphoblastoid cell lines (LCL) in vitro, but it can also infect epithelial cells as part of its normal cycle of persistence in the human host. Differently from B cells, infection of epithelial cells by EBV in vitro results in active replication, with production of new virus and lysis of the cell (8). More rarely the virus infects T cells, natural killer (NK) cells, monocytes and smooth muscle cells (6, 9, 10). The major envelope glycoprotein gp 350/220 is mainly responsible for the high affinity binding of the virus to CD21 (complement receptor type 2/CR2) on the B cell. However, in order to entry into a B cell, EBV also uses a co-receptor, the glycoprotein gp42, that binds the human leucocyte antigen (HLA) class II molecule on the B cell. The entry of EBV in the epithelial cell is less well understood but there are evidences that it can be due either to the interaction between the viral antigen BMRF2 and integrins on the epithelial cell (11) or via two viral glycoproteins gH and gL that bind gHgL on epithelial cells (12). Fusion of the EBV envelope with either B cells or epithelial cells require three glycoproteins, gH, gL and gB (13). Once the virus nucleocapsid is dissolved, the genome is transported in the nucleus where it is replicated by host DNA polymerases and persists as an episome. Viral DNA polymerase
accomplishes linear viral replication, which occurs during the lytic phase of the viral cycle.

1.1.5 EBV Primary infection and persistence

EBV is well-known for its unique ability to transform resting B cells *in vitro* into permanent, latently infected lymphoblastoid cell lines in which all the 9 viral latent genes are expressed. In contrast to *in vitro* studies, the understanding of the biology of EBV infection *in vivo* is still rudimentary. Primary infection normally takes place in the mucosal tissue of the oropharynx, but the initial target cell for the infection is still not clear, being either B cells or epithelial cells. One model suggests that EBV first infects oropharyngeal epithelial cells, which then undergo lytic cycle to generate virions which can in turn infect B cells. Many observations sustain this theory; i.e. the presence of EBV in the desquamate oropharyngeal epithelial cells of IM patients (14) and the presence of EBV DNA, mRNA and proteins in a small subset of tonsillar epithelial cells from healthy EBV+ donors (15), whilst the observed EBV replication in normal tongue epithelium suggests that the tongue may be the source of EBV secretion into saliva (16). An alternative model suggests that B cells may be directly infected by EBV; in fact, the crypt structures in the tonsil, formed by invaginations of the tonsillar lymphoepithelium, might allow direct access of EBV-containing saliva to the B cells in the underlying lymphoid tissue (17).

Once infection is established, there are 4 different gene programs that EBV-infected B cells can express which depend on the localization and differentiation state on the infected B cells. One of these programs is used to
produce infectious virus (lytic program). The other three are all associated with the latency state, in which no infectious virus is produced; these are known as:

1- growth program or latency III which primarily affect tonsillar naïve B cells, and in which all 9 known latent genes are expressed; i.e. EBNA1-6, LMP1,2A,2B.

2- default program or latency II, where just EBNA1, LMP1 and LMP2A are expressed, and is present in germinal centre B cells and

3- latent program or latency I (none or just EBNA1 is expressed) which is typical of memory B cells.

In addition to latent proteins, two small non-translated RNA, EBER1 and 2 are expressed during all the phases of EBV latency.

To explain how this growth-promoting virus achieves its quiescent state in memory B cells, it has been suggested that once EBV has infected naïve B cells, it uses the strategy of transforming latently infected B cells into proliferating blasts since only in this way it can convert these cells into long-lived memory B cells, that enter the peripheral circulation and allow EBV to persist indefinitely in protected niches (“The germinal centre model”) (18-20).

According to this model, EBV needs such a complex scheme of latent gene programs because only by switching from one to the other it can allow the naïve B cells to differentiate into memory B cells through the process of the germinal-centre (GC) reaction (Fig 1.1). In details, when EBV infects a naïve B cell in the lymphoid tissue of the Waldeyer’s ring it uses the growth program to activate the cells to become proliferating blasts. This process mimics what happen when an antigen contacts a naïve B cell via its surface immunoglobulin or B cell receptor (BCR). Usually the naïve B cells will receive survival stimuli from both the antigen and antigen-specific helper T cells. EBV, by then switching to the default program delivers survival signalsto the germinal centre
B cell, independently from the presence of the proper antigen and T cell help. At this stage, EBV switches down almost all the genes, so that the new memory B cells can enter the circulation invisible to the immune response. In response to unknown signals a memory B cell can then differentiate into an antibody secreting plasma cell where the virus can reactivate and replicate, although this also allows detection by cytotoxic T cells which control the degree of viral replication.

The above model is supported by numerous obervations such as the finding that lymphoblasts produced during the growth program resemble antigen-activated B cells both in their cell surface phenotype and morphologic features (21, 22) and the evidence that only naïve B cells express the growth programme in the Waldeyer’s ring in healthy carriers(23). However, alternative interpretations for the persistence of EBV in the B cell compartments have been suggested, such as the model whereby EBV could directly infect memory B cells in the oropharinx. This theory is based on the observation that EBV-infected cells in extrafollicular areas are dominated by clones with stable memory IgG genotypes but no ongoing hypermutation (24). It has also been suggested that EBV-driven proliferation occurs in extrafollicular areas of the tonsil, rather than in germinal centres. This idea comes from the observation that transgenic expression of LMP1 under a constitutively active promoter in mice resulted in lymphoma and arrested GC development (25).
Figure 1.1

Normal B-cell differentiation

Antigen

T-cell help

Naive B cell

B-cell blast

GC B cell

Post-GC B cell

Memory B cell

T-cell help + antigen

Survival signals

Cell division

EBV infection

Growth program

Default program

Latency program

EBNA-1 only
Figure 1.1 The germinal centre model: EBV infects naïve B cells inducing their differentiation into memory B cells through the process of the germinal-centre (GC) reaction

In the lymphoid tissue of Waldeyer’s ring EBV infects resting naive B cells and using its growth program activate them inducing their proliferation. This process parallels the activation of a naive B cell triggered by the cognate antigen. The antigen-activated B-cell blast is rescued through entry into the pool of memory B cells when it receives signals from antigen and antigen-specific T helper cells. EBV switching from the growth to the default program delivers these rescue signals to the latently infected blast. Then the memory cells exit the cell cycle and enter the peripheral circulation. EBV-infected cells enter the peripheral circulation when the virus enters the latent I program, where all protein-encoding genes are shutted-down. Memory B cells occasionally divide, as part of the homeostatic mechanism for maintaining stable numbers of cells. When an infected cell undergoes division, it expresses EBNA-1 alone to allow the viral genome to divide along with the cell. Memory B cells can response to activatory signals differentiating into plasma cells and therefore secreting antibodies. If such a cell contains the latent virus, it will reactivate viral replication producing new infectious virions.

1.1.6 Function of EBV latent antigens

There are 9 different antigens associated with the latent state of EBV, 6 with nuclear localization (EBNA-1, -2, -LP, -3A, -3B and -3C) and 3 on the cell membrane (LMP-1, -2A and -2B). Additionally 2 short not-polyadenylated and therefore not-translated RNAs are expressed during EBV latency, EBER-1 and -2.

EBNA1 mainly ensures the maintenance and replication of the episomal EBV genome through sequence-specific binding to the plasmid origin of viral replication OriP (26). EBNA2 is the first viral protein expressed after B cell infection in vitro and is essential for the transformation process. Moreover, interacting with the transcription factor RBP-Jk, it transcriptionally activates cellular genes, such as CD23 and viral genes such as LMP1 and LMP2A (6). EBNALP interacts with EBNA2 and is required for the efficient outgrowth of virus-transformed B cells in vitro (27). EBNA3A, 3B and 3C modulates the transcriptional activation of EBNA2 mediated by EBNALP (28).

LMP1 is a classical viral oncogene with a critical role in B cell transformation and a strong inhibitor of apoptosis via the up-regulated expression of anti-apoptotic proteins, i.e. Bcl-2 and A20 (29). Moreover, LMP1 functionally resemble CD40 and can provide both growth and survival signals to the B cells (25). In details, the cytoplasmic portion of LMP1 contains carboxy terminal activator regions (CTARs) that are functionally homologous to the cytoplasmic tail of the human CD40 as they are able to interact with the adaptor proteins tumor necrosis factors (TRAFF) and TNF receptor-1 associated death domain (TRADD) (30). Interactions between TRAFFs/TRADD and CTARs lead to the activation of the transcription factor NF-kB. The net result of both LMP1 and CD40 signaling consists in rescuing B cells from apoptosis and driving their
proliferation. The homology between LMP1 and CD40 further supports the hypothesis that EBV, by mimicking the CD40-CD40L interaction, makes a GC-B cell independent from T-cell help thus promoting B cell survival (25).

LMP2A and 2B proteins share a similar structure, consisting of a transmembrane domain made of 12 membrane-spanning loops, with a C-terminal cytoplasmic tail. Additionally, LMP2A has a N-terminal cytoplasmic portion that comprises 8 tyrosine residues, two of which form an immunoreceptor tyrosine-based activation motif (ITAM) (31). The same ITAM sequences are found in the α and β chains of the B cell receptor (BCR). Both LMP2A and BCR are in turn associated with Lyn, a protein kinase. The phosphorylation of the ITAM by Lyn leads in both cases to the recruitment of the Syk tyrosine kinase and the activation of the canonical BCR downstream pathway. The main result of this cascade is the delivery of survival signals to the B cells.

In the context of B cell colonization during primary infection in vivo, LMP2A, by mimicking the BCR signal, acts together with LMP1 surrogate T cell help in order to drive infected cells through the GC reaction. Therefore, LMP1 and LMP2A have the potential to provide the necessary signals, independent of antigen binding and T cell help, to allow latently infected cells to survive and eventually differentiate into memory cells.

Finally, EBER1 and EBER2 encode short non-polyadenylated RNAs expressed in all forms of latency. EBERs can bind directly to the ds-RNA–activated protein Kinase, PKR, which contributes to the antiviral effects of interferons, suggesting that EBER-mediated inhibition of this molecule could promote viral resistance (32). In addition, EBERs also interact with the autoantigen La (33) and the ribosomal protein L22 (34-36), and have been shown to induce
expression of IL-10 in Burkitt’s Lymphoma (37), IL-9 in EBV-infected T cells (38) and insulin-like growth factor in epithelial cells (39, 40).

Table 1.1 summarizes the function, localization and phase cycle expression of the most relevant EBV latent proteins.

### 1.1.7 EBV reactivation: role of lytic proteins

EBV lytic infection occurs in vivo during primary infection and sporadically during persistent infection; probably when memory B cells are induced to differentiate into plasma cells (41). Conversely, in vitro approximately 5% of cells in LCL cultures undergo spontaneous lytic replication. In both cases, lytic replication involves a sequential expression of lytic viral antigens. The process is initiated by immediate early genes BZLF1 (ZEBRA) and BRLF1 (Rta), which transactivate expression of early genes such as BFRF1, BALF2, BHRF1 and BMRF1, which products are involved in viral DNA synthesis. Late lytic genes mainly encode viral structural proteins. Specifically, the late gene BLLF1 encodes the envelope glycoproteins gp350 and gp220, BCRF1 encodes a viral protein that closely resembles IL-10, and BALF4 encodes the glycoprotein gp110 (6). Therefore, production of new virions starts with the synthesis of viral DNA and packaging into capsid in the host cell nucleus followed by budding through the nuclear membrane resulting in cytoplasmic vesicles containing enveloped virions. These vesicles then fuse with the host plasma membrane to release the new virus particles by endocytosis (6). This process also leads to the death of the host cell. Table 1.1 summarizes the function, localization and phase cycle expression of the most relevant EBV lytic proteins.
<table>
<thead>
<tr>
<th>EBV PROTEIN</th>
<th>LOCATION</th>
<th>EBV PHASE CYCLE</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA1</td>
<td>nucleus</td>
<td>latency I, II, III</td>
<td>Gene regulation, extra-chromosomal replication, and maintenance of the EBV episomal genome</td>
</tr>
<tr>
<td>EBNA2</td>
<td>nucleus</td>
<td>latency III</td>
<td>Transcriptional regulator and activator of several cellular and viral genes</td>
</tr>
<tr>
<td>EBNA3</td>
<td>nucleus</td>
<td>latency III</td>
<td>Transcriptional regulator that influences cellular and viral genes</td>
</tr>
<tr>
<td>LMP1</td>
<td>membrane</td>
<td>latency II, III</td>
<td>Anti-apoptotic protein; mimic CD40-induced signalling pathway</td>
</tr>
<tr>
<td>LMP2A</td>
<td>membrane</td>
<td>latency II, III</td>
<td>Mimic BCR and replace survival signal to the B cells</td>
</tr>
<tr>
<td>EBERs</td>
<td>nucleus</td>
<td>latency I, II, III</td>
<td>Induce IL-10 production which can suppress cytotoxic T cells and stimulate B-cell growth; inhibit the antiviral effect of PKR</td>
</tr>
<tr>
<td>BZLF1</td>
<td>nucleus</td>
<td>immediate-early lytic</td>
<td>Mediate the switch between the latent and the lytic forms of EBV infection</td>
</tr>
<tr>
<td>BFRF1</td>
<td>cytoplasm/perinuclear</td>
<td>early lytic</td>
<td>Promote fusion of perinuclear virion envelope with the outer nuclear membrane</td>
</tr>
<tr>
<td>BMRF1</td>
<td>cytoplasm/perinuclear</td>
<td>early lytic</td>
<td>Processivity factor for the viral DNA polymerase</td>
</tr>
<tr>
<td>Gp350/220</td>
<td>cytoplasm/membrane</td>
<td>late lytic</td>
<td>Major EBV glycoprotein that mediates binding of EBV to its B-cell receptor, CD21</td>
</tr>
<tr>
<td>p160</td>
<td>cytoplasm/membrane</td>
<td>Late lytic</td>
<td>EBV capsid protein</td>
</tr>
</tbody>
</table>
1.1.8 Control of EBV infection by the immune system

The innate immune response is an important first line of defense against viral infection; viruses early after infection elicit a strong type I INF response by NK cells. In fact, NK deficiencies are associated with increasing susceptibility to several viral infections, including EBV (42). The adaptive immune response to EBV infection consists both of a humoral and cell-mediated component. Antibodies against EBV antigens limit the spread of infection and are critical in diagnosing infectious mononucleosis (43), whereas the cytotoxic T cells response, eliminating the infected cells that express EBV antigens, are critical in controlling viral replication but also contribute to the severe symptoms of IM (44). Both CD4 and CD8 cells make a robust response to EBV antigens but CD8+ cells tend to dominate the response to the lytic reactivation. However, the immune system is unable to eliminate completely the virus, and as a consequence, viral shedding and virus-infected cells persist at a low level, approximately 1 in 10,000 to 100,000 memory B cells (45).

1.1.9 Viral infections and autoimmune diseases

As a general rule, autoimmune diseases are complex and multifactorial, being genetic, epigenetic and environmental factors involved in their pathogenesis. Among the latter, viral infections are believed to contribute to the maturation of the immune system from the innate to the adaptive phase and therefore may also take part in the induction of autoimmune diseases(46). To simplify the complex viral-host interactions possibly leading to breach of self tolerance and
the onset of autoimmunity, two main sets of events have been strongly associated with viral infection and autoimmunity in susceptible individuals. The first set includes antigen specific mechanisms, such as molecular mimicry, expression of modified or cryptic determinants and superantigens; the other set involves non-antigen specific mechanisms such as enhanced processing and presentation of self-antigens, immune cell activation, cytokines release and apoptosis (47). Among the antigen-specific mechanisms, molecular mimicry may develop when infecting agent incorporate a viral epitope that has a sequence homology with a self-antigen so that the immune response could turn towards the self-antigen due to cross reactivity, leading to the activation of auto-reactive T and B cells specific for the corresponding self-molecule (48). Alternative antigen-specific mechanisms through which a virus can activate the immune response are due to tissue injury, cell death, oxidative stress and reparative changes that happen as a result of the viral infection and that can cause changes in self-proteins, such as altered expression, post-translational modifications, denaturation, misfolding and mutations. These changes could lead proteins usually recognized as self to be targeted by the immune system as not-self and induce an autoimmune response. Among the non-specific immunological mechanisms caused by a virus, the so-called “epitope spreading” is believed to play a pivotal role and consists in the development of autoimmune responses against endogenous epitopes, secondary to the progressive release of self-antigens during a chronic infection, which can, in time, induce the expansion of T and B cell mediated immune responses towards different self-antigens. Conversely, superantigens are proteins produced by virally-infected cells which can bind the T cell receptor, without the necessity of antigenic specificity, resulting in a massive
activation of T lymphocytes of different specificities eventually leading to an autoimmune reaction. Finally, viral infection could also activate the immune response through the induction of cell death; in fact, if dying cells are not removed for different reasons, i.e. deficient scavenging, the apoptotic process could lead to accumulation of nuclear material that can in turn cause autoimmunity(49).

1.1.10 EBV infections in the pathogenesis of autoimmune diseases

EBV fulfills many general properties which indirectly suggest its involvement in the pathogenesis of autoimmune diseases; i) it is ubiquitous and autoimmune diseases have a worldwide prevalence; ii) it has life-long persistence in the human host with occasional reactivation and autoimmune diseases have a chronic evolution with periods of flares; iii) it can alter the host immune response leading to various consequences such as the production of (auto)antibodies that are specifically associated with certain autoimmune diseases (50); iv) EBV has the peculiar ability to inhibit apoptosis, limit anergy and tolerance and induce the production of different pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (51); all these processes are potentially related to the pathogenesis of several autoimmune diseases. In the literature there is a large body of evidence linking EBV with different autoimmune diseases, i.e. multiple sclerosis (MS), rheumatoid arthritis (RA), Sjogren’s syndrome (SS), systemic lupus erythematosus (SLE) and myasthenia gravis (MG). The evidence in support of its association with RA and SS will be reviewed more in details in Sections 1.4 and 1.5 below. However, in general, previous studies revealed a higher EBV viral load in the peripheral blood
together with exaggerated humoral immune response against EBV in autoimmune patients compared to healthy individuals (52-57). Furthermore, there is strong evidence that patients with different autoimmune diseases display an impaired cytotoxic anti-EBV response (58-61), indicating a lack of efficient control of EBV infection. Moreover, there are several examples of molecular resemblance between common self-antigens in autoimmune diseases and EBV proteins (62-67). Furthermore, there is also direct evidence that EBV can infect the target organs in patients with organ-specific autoimmune disease (68-74). Finally, meta-analysis of existing epidemiological data highlighted a strong connection between EBV infection and MS (75-78). However, despite all the above evidence linking EBV with autoimmune diseases, the mechanisms by which EBV might contribute to the development of such diseases remain elusive.

Generally the effects of EBV infection in autoimmunity have been based on the molecular mimicry, suggesting that autoimmunity may be initiated by EBV antigens that cross-react with human proteins (79-81).

A fascinating hypothesis is also that EBV, due to its unique capacity to infect, activate and latently persist in B lymphocytes, would allow survival of autoreactive clones which would be normally depleted in the periphery by physiological mechanisms of B cell homeostasis such as anergy (82). In details, Pender proposed that autoreactive B cells, infected by EBV during primary infection, would differentiate through the GC process in latently infected autoreactive memory B cells that express some virus-encoded anti-apoptotic proteins and become resistant to apoptosis. According to this model, autoreactive memory B cells can, in genetically predisposed individuals, lodge in organs where their target antigen is expressed and produce in situ pathogenic autoantibodies. Subsequently, autoreactive T cells activated in
peripheral lymphoid organs would migrate in the target organs where they receive co-stimulatory survival signal from the infected B cells. The autoreactive T cells would then proliferate and produce cytokines, which in turn will recruit other inflammatory cells leading to inflammation and destruction of the target organ.

Additionally, the same author also proposed (83) that CD8+ T cells dysfunction, that characterizes many autoimmune diseases, contributes to the development of chronic autoimmunity by impairing CD8+ T cells control of EBV infection, with the consequent accumulation of EBV infected autoreactive cells in the target organs where they produce pathogenic autoantibodies and provide costimulatory signal to the autoreactive T cells.

This hypothesis would also partially explain the recent observation that ectopic B cell follicles in the brain of MS and in the thymus of MG patients are an active site of EBV persistence and reactivation (72, 73). Therefore, in this scenario, EBV infection in autoreactive B cells would sustain the development of ectopic lymphoid follicles in the target organs, a frequent finding in organ-specific autoimmune diseases and a site for functional autoreactive B cell activation and differentiation (84, 85).
1.2 Rheumatoid arthritis

1.2.1 Definition and classification criteria

RA is a chronic, systemic inflammatory disease, primarily affecting the diarthrodial joints leading to joint destruction through inflammatory involvement of the synovial membrane, cartilage, and subchondral bone. RA is an autoimmune disease since it is characterized by the presence of circulating autoantibodies, such as rheumatoid factor (RF) (86) and anti-citrullinated protein/peptide antibodies (ACPA) (87-90).

In 1958 the American Rheumatism Association (ARA) proposed the first attempt of diagnostic criteria for RA (91). In 1987 the American College of Rheumatology (ACR) published a revised set of seven classification criteria (92), that are listed in table 1.2.

It should be noted that classification criteria are devised to ensure the standardization of the diagnosis in patients taking part in clinical studies, and to facilitate the analysis of results, but they have significant limitation in diagnosis in daily clinical practice. In particular they are less useful in early arthritis patients. In fact, joint damage is rarely apparent in the very early stages of disease, but rather accumulates consistently over time. Nevertheless an early diagnosis would be ideal since an early therapeutic intervention would improve the clinical outcome preventing or reducing joint damage and the consequent disability. The difficulties to recognize RA patients at early stages necessarily hamper the development of new clinical trials for RA treatments. Recently, in order to progress a new approach for the RA classification, a joint
working group of the ACR and the European League Against Rheumatism (EULAR) was created. The main aim of this group was to develop new classification criteria for RA that were specifically designed in order to capture patients with early RA. In 2010 was therefore developed the ACR/EULAR (93) classification criteria for RA, which are listed in table 1.3.
Table 1.21987 revised American College of Rheumatology criteria for diagnosis of rheumatoid arthritis

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Morning stiffness in and around joints lasting for at least an hour before maximal improvement</td>
</tr>
<tr>
<td>2</td>
<td>Soft tissue swelling (arthritis) of 3 or more joints areas observed by physician</td>
</tr>
<tr>
<td>3</td>
<td>Swelling (arthritis) of the proximal interpharingeal, maticarpophalangeal, or wrist joints</td>
</tr>
<tr>
<td>4</td>
<td>Symmetrical swellings (arthritis)</td>
</tr>
<tr>
<td>5</td>
<td>Rheumatoid nodules</td>
</tr>
<tr>
<td>6</td>
<td>Presence of rheumatoid factor</td>
</tr>
<tr>
<td>7</td>
<td>Radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints</td>
</tr>
</tbody>
</table>

Criteria 1 through 4 must have been present for at least 6 weeks. Rheumatoid arthritis is defined by the presence of 4 or more criteria.

Source adapted from the ACR, 2007, www.rheumatology.org (93)
### Classification Criteria for RA

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

#### A. Joint Involvement

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 large joint*</td>
</tr>
<tr>
<td>1</td>
<td>2-10 large joints</td>
</tr>
<tr>
<td>2</td>
<td>1-3 small joints #</td>
</tr>
<tr>
<td>3</td>
<td>4-10 small joints</td>
</tr>
<tr>
<td>5</td>
<td>≥ 10 joints (at least 1 small joint)</td>
</tr>
</tbody>
</table>

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

#### B. Serology (at least 1 test result is needed for classification)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative RF and negative ACPA</td>
</tr>
<tr>
<td>2</td>
<td>Low-positive RF or low-positive ACPA</td>
</tr>
<tr>
<td>3</td>
<td>High-positive RF or high positive ACPA</td>
</tr>
</tbody>
</table>

#### C. Acute-phase reactant

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal CRP and normal ESR</td>
</tr>
<tr>
<td>1</td>
<td>Abnormal CRP or abnormal ESR</td>
</tr>
</tbody>
</table>

#### D. Duration of symptoms

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 6 weeks</td>
</tr>
<tr>
<td>1</td>
<td>≥ 6 weeks</td>
</tr>
</tbody>
</table>

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 Aletaha et al. Arthritis Rheum. 2010*
1.2.2 Clinical aspects

The most common clinical presentation of RA patients consists of symmetrical arthritis of the wrists and the metacarpophalangeal and proximal interphalangeal joints of the hands. Arthritis of the metatarsophalangeal joints of the feet is also common and occasionally, large joints can be affected in isolation. The number of swollen joints at baseline is used as an indicator of progressive disease and future radiographic progression (94). Early morning stiffness, which can last for over 1 hour, is considered a cardinal feature of RA. Systemic features including flu-like symptoms, fatigue, malaise and weight loss are also common in RA. A more detailed list of extra-articular symptoms is listed in Table 1.4. The presence of rheumatoid nodules, especially over the extensor aspect of the elbows, are a specific feature but occur late in the disease and are only seen in a minority of patients.

Occasionally RA patients present atypical patterns of disease. For example, the so-called ‘polymyalgic’ onset of RA, a pattern sporadically observed in patients over 65 years old, which present heavy stiffness and limb girdle pain rather than peripheral arthritis. Another atypical manifestation is the ‘palindromic rheumatism’, characterised by episodic involvement of one or several joints lasting hours to days and occurring at intervals of days to months.

Notably, there is no single clinical feature or serologic or radiologic exam sufficient to diagnose RA.
Table 1.4 Extra articular manifestations of RA

<table>
<thead>
<tr>
<th>ORGAN</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Vasculitis, nodules</td>
</tr>
<tr>
<td>Cardiac</td>
<td>Pericarditis, Myocardial fibrosis/granulomatous disease</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>Interstitial pneumonitis, Rheumatoid Nodules, Serositis</td>
</tr>
<tr>
<td>Neurological</td>
<td>Mononeuritis multiplex, peripheral neuropathy</td>
</tr>
<tr>
<td>Haematological</td>
<td>Anemia, Felty’s syndrome</td>
</tr>
<tr>
<td>Exocrine</td>
<td>Secondary Sjogren’s syndrome</td>
</tr>
<tr>
<td>Others</td>
<td>Amyloidosis, systemic vasculitis, osteoporosis</td>
</tr>
</tbody>
</table>
1.2.3 Epidemiology

RA is the most common form of polyarthritis and affects between 1-2% of the adult population worldwide. Its prevalence varies between different countries; i.e. there is a lower incidences in parts of China than in Western Europe and the USA (95), and lower prevalence rates in Southern than Northern European countries (96, 97). Furthermore, prevalence rates of up to 5.3% in Native American-Indian population have been reported (98).

RA is virtually undetectable in parts of Nigeria and rural Africa (99), whereas prevalence rates rise to nearly 1% in black populations in urban areas, further suggesting an environmental etiology in disease pathogenesis.

The incidence of RA is typically two to three times higher in women than men, a ratio that falls with increasing age. The onset of RA, in both women and men, is highest among those in their forty, although the disease can affect people at any age.

1.2.4 Etiology

Rheumatoid arthritis involves a complex interplay between genetic susceptibility and environmental triggers.

1.2.4.1 Genetic factors
The genetic contribution to the risk of developing RA has a substantial impact on conferring susceptibility to RA (100), as estimated through studies of monozygotic and dizygotic twin pairs. From studies in Finland and the United Kingdom it was found that concordance rates for RA of 12.3% and 15.4% exist in monozygotic twins, compared to 3.5% and 3.6% in dizygotic twins. On the basis of those observations it was estimated that the heritability of RA is approximately 65% (101).

Multiple loci have been associated with the genetic risk of RA. The HLA locus is the most important of these and accounts for 30% to 50% of overall genetic susceptibility to RA (100, 101). Within the HLA locus, the strongest association is with alleles of HLA-DRB1, encoding a conserved sequence of amino acids in the third hypervariable region of the β-chain of the HLA-DR molecule which is collectively referred to as the “shared epitope” (102). HLA-DR molecules are heterodimers that present antigenic peptides to T lymphocytes. Structural analysis demonstrated that the shared epitope can influence peptide binding as well as contact between HLA-DR and the T cell receptor (103, 104). Therefore, the products of shared epitope HLA-DRB1 alleles may predispose to RA as a consequence of an impaired antigen presenting to T cells. Proposed models include failure to generate appropriate Treg cells, the presentation of arthritogenic peptides to class II-restricted peripheral-effector T cells and the selection of pathogenic T cells during thymic selection (105). Interestingly the HLA-DRB1 shared epitope appears to confer genetic susceptibility for the development of the subgroup of RA that is characterized by ACPA production (106).

Beside HLA-DRB1 shared epitope, other single nucleotide polymorphisms (SNPs) in candidate genes have been linked to RA; i.e. loci involved in T-APC interactions (i.e. CTLA-4), in the regulation of the T cell activation threshold
(i.e. PTPN22), signal transducer and activator of transcription (i.e. STAT4) or the homeostatic chemokine CCL21 (107). Several novel SNPs have been identified using genome-wide association studies (108, 109); however, despite this increasing list of gene associated to RA, the overall susceptibility to RA conferred by the non-HLA genes identified so far is estimated to be below 5%.

1.2.4.2 Environmental factors

Among environmental factors, infectious agents, most notably intracellular pathogens and viruses, have long been suspected to promote the development of RA. Different studies proposed a number of candidate pathogens as a trigger of RA, such as mycoplasma (110), mycobacterium tuberculosis (111), human retrovirus 5 (112), alpha virus (113) EBV (114), cytomegalovirus (115), rubella virus (116) and parvovirus B19 (117). Among those, EBV has been one of the most studied and has been linked to RA on the basis of different observations, as discussed in section 1.4. At present there appears to be no consistent data suggesting that a single infectious agent is responsible for RA development, although many avenues of research are investigating such a possibility.

Others (i.e. non-infectious) environmental factors that have been proposed to contribute to RA are smoking (118), obesity and diet (119, 120). Smoking is extremely interesting as it has been shown that cigarette smoking is an independent risk factor for RA among person with the shared epitope HLA-DRB1 (121), a link possibly explained by the induction of protein citrullination in the lung upon activation of airway macrophages and which might trigger breach of self-tolerance towards citrullinated antigens (122).
Based on the above observations, it is evident that multiple environmental factors may interact in various ways and in different genetically determined individuals in order to trigger the development of RA.

1.2.5 Immunopathogenesis of RA synovitis

Together with activation of the resident stromal cells (i.e. fibroblast-like synoviocytes), one of the features that histologically characterize the inflamed RA synovium is the infiltration of different immune cell subsets, including T cells, B cells, plasma cells, macrophages and DCs. Infiltrating immune cells can follow three main patterns of infiltration; i) diffuse synovitis, which is present in ~50% of the cases and is characterized by predominant macrophage activation and diffuse T cell infiltration; ii) follicular synovitis, whereby T and B cells form compact lymphoid aggregates without a clear GC reaction (~25% of the cases) and iii) in the resting ~25% of the patients synovial tissue presents organized ectopic GC-like structures whereby B/T cell follicles are further characterized by the differentiation of CD21+ follicular dendritic cells networks (FDCs)(123, 124). The latter two patterns can be defined as ectopic lymphoid neogenesis or tertiary lymphoid structure (TLS) formation as they display features typical of secondary lymphoid organs (SLOs) such as T/B cell segregation, differentiation of HEVs and expression of lymphoid chemokine CXCL13 and CCL21, which respectively segregate in the B and T cell areas of the aggregates. This phenomenon has been described in diverse chronic inflammatory conditions of autoimmune, infectious or cancer-related origin (listed in table 1.5) and, in the case of autoimmunity, it has been associated
with the development of autoantibodies and in some cases a more severe
disease outcome.

The possibility that during chronic inflammation the same pathways implicated
in the physiological development of lymphoid organs could be aberrantly re-
activated and lead to the formation of TLS gave rise to a growing interest in the
dissection of the pathways and regulatory systems normally involved in
physiological lymphoid organogenesis.
Table 1.5 Human chronic inflammatory diseases with lymphoid neogenesis

<table>
<thead>
<tr>
<th>Disease</th>
<th>Target tissue</th>
<th>Percentage of patients with ectopic tissues</th>
<th>T-cell aggregates with CCL19”CCL21”stromal cells, DCs and HEV-like vessels</th>
<th>Antigen recognized by antibodies generated in ectopic GCs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Autoimmune diseases</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Diarthrodial joints</td>
<td>10–15%</td>
<td>Present; PNAD CCL21- blood vessels and PNAD CCL21 HEVs</td>
<td>Rheumatoid factor (I)</td>
<td>9, 14, 17, 18, 41, 45, 71, 80</td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis (hyperthyroidism)</td>
<td>Thyroid gland</td>
<td>100%</td>
<td>Present; HECA-452:CCL21 HEVs</td>
<td>Thyroglobulin, thyreoperoxidase</td>
<td>1, 4, 15, 43, 44</td>
</tr>
<tr>
<td>Graves’ disease (hypothyroidism)</td>
<td>Thyroid gland</td>
<td>54–63%</td>
<td>Present; HECA-452:CCL21 HEVs</td>
<td>Thyroglobulin, thyreoperoxidase</td>
<td>1, 4, 15, 43, 44</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>Thymus</td>
<td>Mainly patients with early-onset myasthenia gravis</td>
<td>Present; CCL21 HEVs</td>
<td>Nicotinic acetylcholine receptor</td>
<td>2, 28, 30</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
<td>Salivary glands</td>
<td>17%</td>
<td>Present; PNAD CCL21 HEVs</td>
<td>SSA/Ro, SSa/La</td>
<td>16, 35, 58, 81</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Central nervous system</td>
<td>30–40% of patients with secondary progressive multiple sclerosis</td>
<td>Absent; CCL21 HEVs</td>
<td>Not determined</td>
<td>3, 46, R. Magliozzi, personal communication</td>
</tr>
<tr>
<td>Cryptogenic fibrosing adenitis</td>
<td>Lungs</td>
<td>83–90%</td>
<td>Not determined</td>
<td>Not determined</td>
<td>83, 84</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis and primary biliary cirrhosis</td>
<td>Liver</td>
<td>None</td>
<td>Present; CCL21/MADCAM1 HEVs</td>
<td>Not determined</td>
<td>42, 56</td>
</tr>
<tr>
<td><em>Other chronic inflammatory diseases</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulcerative colitis*</td>
<td>Gut</td>
<td>27%</td>
<td>Present; CCL21/PNAD blood vessels</td>
<td>Not determined</td>
<td>53, 71, 123, 124</td>
</tr>
<tr>
<td>Crohn’s disease*</td>
<td>Gut</td>
<td>Not determined</td>
<td>Present; CCL21/PNAD blood vessels</td>
<td>Not determined</td>
<td>53, 71, 123, 124</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Arteries</td>
<td>32%</td>
<td>Present; HECA-452 HEVs</td>
<td>Not determined</td>
<td>106, 107</td>
</tr>
<tr>
<td><em>Infectious diseases</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic hepatitisC</td>
<td>Liver</td>
<td>33–65%</td>
<td>Not determined</td>
<td>Not determined</td>
<td>98, 99</td>
</tr>
<tr>
<td>Helicobacter pylori (or Campylobacter pylori)-induced gastritis</td>
<td>Stomach</td>
<td>27–100%</td>
<td>Present; PNAD HEVs</td>
<td>Bacterial antigens</td>
<td>40, 53, 60, 63, 97</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>Joints</td>
<td>17%</td>
<td>Present; CCL21 HEVs</td>
<td>Not determined</td>
<td>102, 103</td>
</tr>
<tr>
<td>Tumours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal breast carcinoma</td>
<td>Breasts</td>
<td>33–100%</td>
<td>Not determined</td>
<td>Tumour-associated and normal breast tissue antigens</td>
<td>105, 109</td>
</tr>
</tbody>
</table>

*Inflammatory bowel diseases, it is more difficult to distinguish between lymphoid neogenesis and hyperplasia of meso-in-associated lymphoid tissues: CCL21, CCL - chemokine ligand 2; DC, dendritic cell; FDC, follicular dendritic cell; GC, germinal centre; HECA-452, high endothelial cell antigen-452; HEV, high endothelial venule; MADCAM, mucosal addressin cell-adhesion molecule; PNAD, peripheral node addressin; SSA/Ro, Sjogren’s syndrome antigen A (ribonucleoprotein autoantigen); SSa/La, Sjogren’s syndrome antigen B (autoantigen La).

1.2.5.1 Microarchitecture of TLS

Secondary lymphoid organs (SLOs) are characterized by the presence of B cell (follicle) and T cell (paracortex) areas, and specialized vascular and canalicular systems. Their complex structure is essential for regulating leukocyte traffic and compartmentalization. Specifically SLOs provide the proper environment that allows the encounter between the antigen (via antigen presenting cells migrating from the periphery through afferent lymphatics) and naïve lymphocytes (entering from the peripheral blood), which in turn cause the activation and differentiation of antigen-responsive T and B cells (Fig 1.2 A). The process of lymphoid neogenesis is highly dynamic since it allows sparse lymphocytes to aggregate and eventually organize in GCs surrounded by a T-cell area where also DCs and HEVs are located (Fig 1.2 B)(125-127). Ectopic GCs are made by proliferating B cells and a network of CD21+ FDCs, which allow the B cell maturation by presenting the cognate antigen in the form of immunological complexes, stimulate proliferation and prevent apoptosis of GC B cells (128, 129). Ectopic GC are characterized by the presence of naïve B cells centroblasts, centrocytes, memory B cells and plasma cells, indicating that the complete process of B cell maturation occurs in those structures. Also T-cells are found in ectopic GC, most probably acting as cognate T-cell help and therefore contributing to the development of the GC B cell response (130).

The basic cellular constituent of B and T cell areas in TLS are similar to the one found in SLOs, but the general structure of the TLS markedly differs from that of conventional SLOs (Fig 1.2). In fact, differently from SLOs, TLS lack the surrounding capsule, which implies that they are directly exposed to external signals such as the stimulated antigens or cytokines produced in the inflamed environment. Moreover, TLS also lack of the afferent lymph vessels that
normally represent the way through which antigens and DC enter the SLOs. This incomplete development of TLS can therefore cause an irregular access of DCs, lymphocytes and macromolecules in TLS, causing abnormal B and T cell activation.

Various members of the tumor necrosis factor (TNF) family play a crucial role in regulating the development of ectopic lymphoid structures in chronic inflammatory conditions. One of the most important is the heterotrimeric lymphotoxin α1β2 (LT-β), which is mainly produced by B cells during chronic inflammation. This molecule plays a pivotal role in the cellular organization of ectopic lymphoid structures by inducing PNAd+ high endothelial venules (HEV) (131), the ectopic expression of CXCL13, CCL21 and CCL19, which are responsible for the segregation of B and T cells into different areas (132) and follicular dendritic cell network differentiation (133). Notably, ectopic expression of CXCL13 and CCL21 within the inflammatory aggregates in RA synovia is observed both in the context of highly organized GC-like structures, where there are distinct B/T cell areas and the presence of FDC networks, as well as in minor organizational stages, devoid of FDC networks (126).
Figure 1.2 Basic structure of secondary and tertiary lymphoid organs

A. Lymph node (secondary lymphoid organ, SLO) architecture. An external capsule mainly made of collagen surrounds and protects the whole lymph node. The inner cortex is composed by the B cell area, which can form primary follicles or, after encounter the antigen, secondary follicles with an ongoing GC-reaction, and the paracortex where T-cells but also DCs and HEVs reside. B and T lymphocytes enter the lymph node via HEVs, whereas DCs and antigens are carried there via afferent vessels. The medulla, formed by numerous lymph-draining sinuses separated by medullary cords, mainly contains macrophages, plasma cells and memory T cell.

B. Tertiary lymphoid organs (TLOs) architecture. Similarly to SLO, TLOs present a segregated B and T cell area. B cells can form primary follicles or well-structured secondary follicles. In the last case there is the formation of a polarized GC containing a dark and light zone respectively composed by proliferating centroblasts or smaller centrocytes. HEVs, DCs and sparse plasma cells reside in the mantle zone, which is the T-cell area. Differently from SLOs, TLOs lack the surrounding capsule and afferent vessels.

Source: Aloisi at al. Nature Review Imm. 2006
1.2.5.2 Functionality of TLS

GC within lymphoid tissues are the main sites where antigen-driven antibody response takes place, leading to B cells affinity maturation and differentiation into memory B and plasma cells via the processes of somatic hypermutation (SHM) and class-switch recombination (CSR) of the IgG genes (134, 135). It is now clear that AID is the enzyme responsible to initiate and control both SHM and CSR (136). Although the exact mechanisms are still not entirely understood, SHM is initiated by AID by introducing single point mutations in WRC (W = A/T, R = A/G) motifs which are mutational “hot spots” within the Ig variable genes, which encode for the antigen-binding region of the antibody (137, 138). This process results in affinity maturation occurring through cycles of antigen-dependent selection within the GC whereby a B cell acquire one new mutation at each proliferation cycle. In addition, AID regulates CSR via its carboxy-terminal region (139) by introducing double-strand breaks which allows recombination of Ig switch regions of DNA followed by excision of switch circles within the constant heavy-chain region. Ig class switch from IgM and IgD to IgG, IgE or IgA is an important mechanism that has a strong impact on the biological functions of antibodies enhancing their effector capacity (140).

In the mid-90s, several groups, by performing analysis of the V-gene repertoire from total RA synovial RNA extracts or from follicular structures microdissected from the RA synovium, clearly demonstrated that a significant fraction of B lymphocytes accumulating within the synovial membrane of RA patients have an oligoclonal repertoire with highly mutated V regions, compatible with a local antigen-driven GC reaction (141, 142). These data provided strong indirect evidence that RA synovium can support in situ diversification and expansion of
B-cell clones. The direct demonstration was recently provided by our group, reporting that in the RA synovium lymphoid aggregates characterized by FDC network formation invariably express AID(85). Importantly, AID can also be expressed in the absence of full compartmentalization of the GC into dark and light zones, a morphological feature that distinguish RA synovium TLS from secondary lymphoid organ GC (141). Humby et al also demonstrated that AID expression is almost exclusively observed within FDC network, which are detectable in around half of the RA samples with Grade 3 lymphocyte cluster, i.e. where radial number of lymphocytes aggregates is >10 (126, 143). Moreover, it was also demonstrated that a further 20% of patients with RA synovitis histologically characterized by T/B cell segregation but without expression of FDC networks, expressed AID mRNA in the presence of detectable mRNA encoding CD21 long isoform (85), a specific isoform of the complement receptor 2, which is selectively expressed by FDCs but not B lymphocytes. Thus, the majority of RA synovia with T/B-cell lymphoid aggregates appears to present GC-related immunological activity, even in absence of histologically detectable FDC networks.

In SLO, both naïve B cells, which accumulate in the mantle zone surrounding GCs, and memory B cells can enter or re-enter the GC and undergo de novo or new rounds, respectively, of selection and affinity maturation (135). According the presence of both antigen-inexperienced IgD+CD27- B cell as well as IgD+CD27+ unswitched and IgD-CD27+ switched memory B cells have been detected in the RA synovia. Thus both naïve and memory B-cell subsets could possibly undergo affinity maturation in ectopic lymphoid structures. Berek et al (141, 144) by analyzing the Ig V gene repertoire of B-cell clusters microdissected from RA synovia, have demonstrated that both scenarios are likely to take place. They observed that in some synovial aggregates B cells
display identical rearrangements and only differ by single nucleotide mutations, indicating that the entire diversification of the Ig V region repertoire of some clones occurs within the synovial membrane. They also suggested that already mutated memory B cells undergo further rounds of SHM within the synovial membrane with the acquisition of a highly mutated and diversified repertoire. Another possibility is that hypermutated memory B cells proliferate without further diversification.

There are many evidences proving that B cells also actively undergo CSR within the RA synovium. Humby et(85) analyzing the expression of circular transcripts, which are specific transcribed by CSR, demonstrated that CSR invariably occurs in the RA synovium characterized by AID+/CD21L+ lymphoid structures, which thus support the production of high affinity IgG (auto)antibodies. In the same study it was demonstrated that locally differentiated plasma cells produce RA associated autoantibodies such as those directed against citrullinated proteins. These findings strongly suggest that ectopic lymphoid structures within RA synovium not only sustain in situ Ig V repertoire diversification and class switching but also allow the in situ differentiation of autoreactive plasma cells.

1.2.6 Autoantibodies in RA

RF was the first autoantibody demonstrated to be linked to the RA pathogenesis in 1940 by Emil Waaler (145). After that, many other autoantibodies have been found associated with RA pathogenesis. The pool of autoantibodies found in RA patients is extremely heterogeneous; in fact, they can target collagen, cartilage proteins, heat shock proteins, enzymes, nuclear proteins and, most importantly, citrullinated proteins such as fibrin or
vimentin. Notably, anti-citrullinated protein antibodies (ACPA) have a high degree of specificity for RA, while all other antibodies including RF may also occur in other diseases and even in healthy individuals. Although it lacks specificity, RF is still the most widely used serological marker of RA and represents one of the seven classification criteria of the American College of Rheumatology for the disease.

ACPA and RF are strongly associated with a more severe and erosive disease, suggesting that auto-antibodies, possibly also including those not routinely used for diagnostic purposes, may contribute to the pathological processes characteristic of RA such as chronic synovitis and erosiveness; however, a formal demonstration of the pathogenicity of autoantibodies is still missing.(146).

1.2.6.1 Rheumatoid factors

RFs are autoantibodies directed against the Fc portion of IgG. IgM is the main isotopic form of RF found in serum and synovial fluid of RA patients. It should be noted that transiently increased levels of IgM-RF are also found during normal immune response and during infections, probably in reaction to immune complexes containing microbial antigens (147, 148). Thus, low-affinity IgM-RF can be found in a small percentage (<15%) of healthy individuals (149, 150). In contrast, RA is characterised by chronic persistence of high-titre IgM-RF (as well as the presence of IgG and IgA subtypes). Around 60-80% of RA patients with established disease are positive for IgM-RF, whereas the prevalence of the same antibody in patients with early RA is significantly lower. High titres of IgM-RF can also be detected in patients with primary Sjögren's
syndrome or mixed cryoglobulinaemia, whereas low titres are found in other rheumatic diseases (151, 152). Both IgG, IgA and IgM-RF can be detected in the earliest stages of the disease and can even precede the onset of RA by several years (153).

1.2.6.2 Anti-citrullinated protein antibodies (ACPA)

Anti-citrullinated protein antibodies (ACPA) are autoantibodies directed against epitopes containing the unusual amino acid citrulline that is generated by post-translational deimination of arginyl residues by the enzyme peptidyl arginine deiminase. ACPA are the only autoantibodies specifically found in serum of RA patients. Although citrullination of proteins occurs in normal epidermis and elsewhere (154), the physiological role of citrullination remain uncertain.

Filaggrin was the first protein whose citrullinated epitopes were demonstrated to be specific in RA. Filaggrin is a protein that binds keratin fibres in epithelial cells and is exclusively expressed in squamous epithelial cells. Notably, filaggrin is also the target of anti-keratin antibodies that had been reported to be highly specific for RA (155). Considering that filaggrin is not expressed in the joint, it is reasonable to think that it is not the primary target structure of ACPA but most probably represents a cross-reacting antigen. Beside citrullinated filaggrin, other established antigens that bind ACPAs are citrullinated type II collagen (CII), α-enolase, fibrinogen and vimentin (156, 157).

All the different citrullinated antigens can be measured by ELISA, although in the clinical setting, ACPA are the most used and are mainly detected using the anti-cyclic citrullinated peptide (anti-CCP) test. In facts, ACPA not only
represent the most specific serological marker antibodies for RA but also, similarly to RF, can be detected in RA serum even before the clinical onset (156, 158). Moreover, presence of ACPA in RA patients has been associated with radiographic disease progression (i.e. bone damage) and with the presence of RA associated HLA-DR alleles ("the shared epitope"). Thus, ACPA are currently the most used and reliable diagnostic marker for RA, whose determination is particularly useful in patients with early arthritis.

1.3 Sjögren Syndrome
1.3.1 Definition and classification criteria

Sjogren’s syndrome (SS) is a chronic autoimmune disease characterized by immune cell infiltration of the exocrine glands leading to glandular dysfunction, aberrant autoantibody production and extra glandular systemic features, including arthritis, fatigue and diverse organ involvement (159). This syndrome may present as a primary disease (pSS) or in association with other autoimmune rheumatic disease such as rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis defining as secondary Sjogren’s syndrome (sSS) (160).

The main targets of the inflammatory process in SS are the exocrine glands (minor and major salivary glands, lacrimal glands etc), determining the hallmark clinical feature: the sicca syndrome (159). Moreover, common feature of SS is the presence of a large number of autoantibodies suggested to be involved in the pathogenesis of the disease. The strict correlation between the presence of some of these antibodies (anti 52-kDa SSA/Ro, 60-kD SSA/Ro, and SS-B/La) and the development of the disease has prompted their inclusion as a critical criterion for the diagnosis of SS. The most accepted and better validated criteria for the classification of the disease are listed in the 2002 revised version of the 1993 European criteria proposed by the American-European Consensus Group (160)(table 1.6). As discussed above for RA, classification criteria are not strictly designed for use in diagnostic in daily clinical practice. In fact, only when sensitivity and specificity of classification criteria are both close to 100% they can be used as diagnostic criteria. The reported specificity and sensitivity of the revised criteria for SS, are respectively 97% and 90% (160).
Table 1.6 Classification criteria for Sjögren’s syndrome

I. Ocular symptoms
II. Oral symptoms

III. Ocular signs: positive result for at least one of the following two tests:
1. Schirmer’s I test
2. Rose Bengal score or other ocular dye score

IV. Histopathology: In minor salivary glands focal lymphocytic sialoadenitis with a focus score >1 defined as a number of lymphocytic foci (more than 50 lymphocytes) per 4 mm² of glandular tissue

V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:
1. Unstimulated whole salivary flow (<1.5 ml in 15 minutes)
2. Parotid sialography
3. Salivary scintigraphy

VI. Autoantibodies in the serum to Ro(SSA) or La(SSB) antigens, or both

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:

a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive
b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey

For secondary SS
In patients with a potentially associated disease (for instance, another well defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS

Exclusion criteria:
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft versus host disease
Use of anticholinergic drugs


1.3.2 Epidemiology
SS is considered one of the commonest autoimmune diseases, affecting between 0.1% to 4.8% of the population. The great variability in the prevalence of the disease is found not only in different area of the world but also in the same country (161). This discrepancy can be due to methodological differences such as in the definition and application of SS diagnostic criteria or different reporting systems (i.e., community vs. hospital-based reports). On the other hand, genetic and environmental factors, which play a crucial role in the pathogenesis of autoimmune diseases, may clarify some of these geographic and ethnic differences.

The highest prevalence of SS has been documented in northern Europe, while the lowest rates are observed in some parts of Asia and comparable rates have been reported in North America and mainland Europe. Anyway, it should be noted that there is little epidemiological data for SS in region outside Europe and North America(162).

Sjogren's syndrome occurs in patients of all ages but it generally manifests during the fourth and fifth decades of life with a female to male ratio of 9:1 (163, 164).

Recently, Lin DF et al. (165) documented different clinical manifestations of SS in their cohort of 573 Chinese patients. In this area of the world, the age of disease onset was much younger compared with western cohorts and up to 30% of patients were diagnosed before the age of 30 years. Furthermore, their reported different symptoms, and fever, pericardial effusion, pulmonary and renal manifestations were relatively common.

1.3.3 Clinicalaspects
SS exocrine glands are typically characterized by a chronic autoimmune process leading to severe tissue damage and glandular dysfunction. The ductal epithelium represent the main target of this process and therefore SS is currently defined as “autoimmune epithelitis” (166).

Dry mouth and dry eyes (sicca syndrome) together with problems in swallowing, taste alterations and a broad range of ocular problems represent the main symptoms of this disease. Hallmark of the ocular involvement in SS is the development of a keratoconjunctivitis sicca, considered the main consequence of qualitative and quantitative alterations in the ocular tear flow. As a consequences of the lack of saliva, SS patients often report symptoms like dental caries, mucositis and oral candidiasis (167). However, it is still debated whether the loss in the secretory component of the glands represents the only cause of the glandular dysfunction. It is strongly believed that serum autoantibodies might contribute to this process by interfering with neurological stimuli that regulate glandular secretion (167).

According to its definition of autoimmune disease, SS might affect diverse organs: joints, gut, respiratory tract, skin, neurological system can all be involved during the disease course, diversely influencing disease morbidity and prognosis.

From 42% to 83% of SS patients report muscoloskeletal symptoms, more often in the female population. The spectrum of articular manifestations is large and in one third of the patients articular manifestations develop before the onset of the sicca syndrome (167). SS articular involvement is often polyarticular and symmetric with morning stiffness and arthralgia; mono-oligoarticular involvement is described in the early phases of the disease. More rarely, SS patients can develop a frank inflammatory arthritis, although commonly non-erosive (168). Development of myalgia represents another frequent
musculoskeletal manifestation of SS, often associated with fibromyalgic-like symptoms and severe fatigue (167).

Haematological abnormalities are commonly reported in SS patients: anaemia, lymphopenia, polyclonal and monoclonal gammopathies and lymphoproliferative disorders have been described with various prevalence in different populations (169). Often SS patients present skin manifestation like skin xerosis which is found in between 23 to 67% of SS patients and most probably related to an impaired sebaceous and sweet glands glandular secretion (170). Less common cutaneous manifestations include vasculitis, urticaria vasculitis and annular lesions (171, 172). One third of SS patients presents with Raynaud’s phenomenon often in association with articular involvement (173).

Neurological involvement includes the development of peripheral neuropathies and, less frequently, central-nervous-system involvement (167). Between 2 to 75% of SS patients can be affected by a broad range of respiratory involvement (174-176). Nasal cavity involvement with bleeding and hyposmia, xerotrachea and bronchial hyper reactivity has been described (167, 174). Furthermore, interstitial lung disease is not uncommon in SS patients, although frequently this is not symptomatic but easily detectable at CT scans. As a consequence of the diffuse distribution of exocrine glands in the gut, the whole gastrointestinal tract might be involved in SS with the most common manifestation being dysphagia, occasionally associated with oesophageal dysmotility (177).

A significant association between SS and primary biliary cirrhosis has been described being sicca syndrome more frequent in patients with primary biliary cirrhosis, with anti-hepatocytes and anti-mitochondrial antibodies (AMA) not infrequently found in SS patients (178).
Histologically chronic active hepatitis with prevalent periductal involvement and portal fibrosis has been detected in the liver of SS patients (178). Moreover, the development of sicca syndrome has been associated with liver involvement during Hepatitis C virus (HCV) infection (159). Interestingly, in SS patients the prevalence of anti HCV antibodies is higher compared to the healthy population (4 to 19% of the patients) (179). However, given the tropism of HCV for salivary glands, HCV infection is currently an exclusion criterium in the classification of SS.

SS patients might also suffer of acute pancreatitis, malabsorption and calcification, since the exocrine components of the pancreas are seldom a target of the disease (180).

Renal involvement has been described in SS patients with renal tubular acidosis being the most common manifestation. Glomerulonephritis is rare while a mild proteinuria is present in 44% of the patients. Defects in urine concentration have been described in 22 to 44% of SS patients. Rarely renal failure has been described. Renal involvement in SS has been related to the aberrant production of anti-tubular antibodies or to cell-mediated tissue damage by antigen specific lymphocytes (167).

Interstitial cystitis is commonly found in SS patients. Also autonomic bladder dysfunction has been reported in association with anti-muscarinic receptors antibody production.

Gynaecological symptoms are often referred and in particular dryness and development of gynaecological infection related to impaired gland secretion (167).

Among other autoimmune disease, Hashimoto thyroiditis and coeliac disease have been reported to be frequently associated to SS (181).
Finally, the most serious complication of SS is the evolution towards haematological malignancies, in particular lymphoma development which involves approximately 5% of primary SS patients. Lymphomas in SS are most commonly non-Hodgkin B cell lymphomas which usually arise within the major salivary glands, most often the parotids, with the histopathologic pattern of a low-grade marginal zone lymphoma of the mucosa-associated lymphoid tissue (MALT-L). The development of lymphoma is the main factor influencing morbidity and mortality in SS patients (182).

1.3.4 Etiology

The etiology of SS is still unknown. However it is considered a multifactorial disease where a combination of genetic background, gender, immunological abnormalities, environmental factors and infectious antigens play a role in development of the disease (159).

1.3.4.1 Genetic factors

Patients affected by primary SS compared with normal subjects display a higher frequency of selected histocompatibility antigens, in particular HLA B8, DR3 and DRw52 (183). In particular, the HLA DRB1*03-DQB1*02 haplotype has been strongly associated with the production of anti-Ro and anti-La antibodies in patients affected by SS and by SLE, suggesting an overlap in the genetic background of these two diseases (184). Moreover, several cytokine and pro-
inflammatory gene polymorphisms have been suggested to play a role in the SS pathogenesis (185, 186).

1.3.4.2 Role of pathogens

The role of infectious agents in the mosaic of autoimmunity is probably the best-established one, although no strict correlation has been found between SS and any particular virus. Cytomegalovirus, Hepatitis viruses C (HCV), Human Immunodeficiency virus (HIV) and Human T Cell Leukemia Virus type 1 (HTLV-1) have been often associated with the development of sialoadenitis and the occurrence of a SS-like syndrome in humans and in mouse models. Similarly, Epstein-Barr virus has been demonstrated to elicit, in humans, latent infection of salivary glands and, upon reactivation, to generate a T cell-dependent B-cell activation together with cytokine production typical of SS (IL-12 and IL-18)(159). HTLV-1 has also been associated with SS; evidences came from both in vivo studies using HTLV-1 transgenic mice that develop a SS-like syndrome, and from evidence of a higher prevalence of SS in areas endemic for HTLV-1 infection (187).

Upon HIV infection between 2 and 5% of patients develops a salivary gland infiltrates similar to primary SS, although no autoantibody production has been detected in these patients compatible with SS.

HCV infection in mice and humans can mimic several SS clinical aspects and despite HCV infected patients are excluded from the diagnosis of SS, some authors suggest HCV as etiological factor for SS (188).
Recent evidence demonstrated a cross reaction between a linear B cell epitope of Ro60-kd autoantigen and a protein of the Coxsackie virus 2B, strongly suggesting a role for Coxakie virus infection in SS pathogenesis (189) although this was not confirmed in subsequent studies (190). Diverse mechanisms can explain the relationship between viral infection and SS development. It is strongly believed that molecular mimicry between viral antigens and self-epitopes in SS can play an important role in the pathogenesis of the disease (191). In addition it has long been suggested that cryptic antigens displayed on the cellular surface of the glandular epithelium upon virus-mediated infection in association with an aberrant class II MHC expression by the same epithelial cells play a key role favouring the activation of self-reacting lymphocytes and maintaining the inflammatory process (192).

1.3.5 Immunopathogenesis of salivary gland involvement in Sjogren’s syndrome

1.3.5.1 The inflammatory infiltrates

The majority of the data describing the formation of the typical inflammatory aggregates (inflammatory foci) in SS glands derives from studies on minor and major salivary glands. This is due not only to the fact that salivary glands and in particular minor salivary glands are commonly involved in the disease course, but also because they can be easily taken with minor surgical procedures (i.e. labial salivary gland biopsies).
Salivary glands are divided in major (parotid, submandibular and sublingual) and minor glands (labial, buccal and palatine). The acini and the ducts represent the gland secretory component; a stromal component is also present with nerves, blood and lymphatic vessels. The ducts are classified in intercalated (surrounded by myoepithelial cells), striated and excretory. Typically SS lymphomonocytoid inflammatory infiltrate is organized around the excretory ducts. Histologically the diagnosis of SS is based on the Chisholm and Mason score and refers to the presence of at least 1 focus score (a focus is defined by the presence of 50 periductal lymphocytes) in 4mm$^2$ of tissue area (187). The immune cells that constitute these follicular structures, which share similar features with lymphoid follicles in SLOs as described in the previous chapter, are predominantly lymphomonocytic cells. In particular, CD4+/CD45RO+ memory T cells represent the major cellular component of these aggregates. Cytotoxic CD8+ T cells are less abundant and usually localise around acinar structures where they could contribute to acinar loss via production of perforin/granzymes (193). B lymphocytes are the second main lymphocytyc subset, constituting 20-30% of the infiltrating cellular component, particularly at later stages of salivary gland involvement (194). Typically, the number of recruited B cells increases during the expansion and organization of the periductal inflammatory focus.

In a subset of SS patients, B and T lymphocytes can become organized in discrete areas, with T/B cell compartmentalization, and in around 30-40% of SS minor salivary glands it is possible to observe the presence of ectopic GCs characterized by CD21+ follicular dendritic cells networks (194). Therefore, also Sjogren’s syndrome, as previously discussed for Rheumatoid Arthritis, is a chronic inflammatory condition characterized by ectopic lymphoid structures formation.
Different evidences have demonstrated that ectopic expression of lymphotoxins (LT) and homeostatic chemokines play a crucial role in regulating the development of ecopic lymphoid structures in SS salivary glands. Similarly to the mechanism of SLO organogenesis, CXCL13, CCL21 and CCL19 are up-regulated in the SS salivary glands and their expression correlate with the gradual acquisition of lymphoid features and FDC networks formation (195-197). Lymphoid chemokines are differentially expressed by different cell types in discrete anatomical structures within ELS in SS salivary glands, allowing the formation of separate T and B cell zone. In particular, CXCL13 is capable of selectively recruit leukocytes expressing the specific receptors CXCR5, while CCL21 selectively recruit CXCR7+ cells. Consequently CXCL13 is found in the B cell-rich areas of the aggregates and CCL21 localise in close contact with HEVs in the surrounding T cell area (195, 198, 199). Therefore both CXCL13 and CCL21 play a fundamental role in regulating the progressive organization and maintenance of periductal foci in SS salivary glands.

Ectopic lymphoid structures have been suggested to be functional in SS salivary glands, promoting Ag-driven B cell activation, selection and proliferation with the in situ production of autoantibodies (123). In support of this hypothesis, B cells with highly organized foci in SS salivary glands express somatically hypermutated Ig genes (200) and can produce autoantibodies such as anti-Ro/SSA and anti-LA/SSB (201, 202). Recently, it has been demonstrated that ectopic GCs in SS salivary glands are functional, since they invariably express AID in periductal inflammatory foci characterized by features of secondary lymphoid organs such as T/B cell segregation and FDC networks (203). Since AID expression has been demonstrated to be sufficient for, and exclusively expressed in, B cells undergoing CSR and/or SHM (204), its detection in SS salivary glands indicate that B cells activate their molecular machinery
responsible for hypermutation of Ig genes in ectopic lymphoid tissue. Together with the finding that autoreactive B cells are detected in situ in SS salivary glands and correlates with serum detection of the same autoantibodies (202), this discovery reinforce the notion the ectopic lymphoid neogenesis in SS is accompanied by local selection and proliferation of autoreactive B cells and that circulating autoantibodies may, at least in part, reflect local production in the salivary glands.

### 1.3.5.2 The ductal epithelium

The ductal epithelium plays an important role in the SS pathogenesis since it is involved in the immune cell recruitment within the glands, in the process of antigen presentation and in the direct stimulation of the immune cells (205). Epithelial cells derived from the SS salivary glands are capable to secrete molecules involved in immune cells recruitment and organization (196). This suggests that, upon antigen stimulation the ductal epithelium provides the first signals for leukocyte migration. SS ductal epithelial cells have also been suggested to actively participate to the organization of the immune response by presenting the antigen/s and providing the costimulatory molecules capable to activate the infiltrating lymphocytes. In fact has been demonstrated that primary cultures of SS ductal epithelial cells retain the expression of costimulatory molecules such as HLA-DR/DP/DQ (206), CD80/CD86 (207) and CD40 (208) as well as adhesion molecules such as VCAM-1 and ELAM-1, suggesting an active role of the ducts in the antigen presentation process in vivo. Ductal epithelial cells can also release proinflammatory cytokines (IL-1, IL-6, and TNF-a) and
metalloproteinase (209), that can in turn stimulate the infiltrating immune cells. Finally the ductal epithelium show an increased apoptosis rate in SS salivary glands, as demonstrated by the aberrant expression of Fas and Fas ligand (210), suggesting that it can play a role in the break of tolerance towards autoantigens and in the perpetuation of the autoimmune process by releasing apoptotic blebs containing autoantigens. However, it is still not clear whether the activation of the ductal epithelium is a constitutive feature of SS salivary glands or merely reflects a secondary event due to pathogen infection. In the most severe cases of SS, the ductal epithelium can be infiltrated by the immune cells forming areas of lymphoepithelial proliferation or lymphoepithelial sialoadenitis (LESA). The presence of this hystopathological lesion has been specifically associated with the development of the mucosa associated lymphoid tissue (MALT) type B-cell lymphomas.

1.3.5.3 Apoptotic mechanisms involved in tissue damage in SS salivary glands

The major mechanisms believed to play a pivotal role in determining abnormal apoptotic rate in SS salivary glands are discussed beloved. It is important to note that the increased apoptosis of epithelial cells in SS salivary glands may not only explain, at least in part, the exocrine dysfunction observed in SS, but also contribute to clarify the mechanism underling the breach of tolerance and generation of autoantibodies. In fact, some autoantigens in SS are typically associated with apoptotic process. In particular Ro/SSA and Ro/SSB are expressed within apoptotic “blebs”(211, 212) while the 120Kd antigenic form
of α-fodrin is generated during apoptosis upon cleavage by caspases and calpains (213).
Together with the Fas/Fas-L pathways, cytotoxic lymphocytes, the key players in cell-mediated immunity, may induce apoptosis through exocytosis of cytolytic granules. This mechanism depends on the synergy of a calcium-dependent pore-forming protein (perforin) and a battery of proteases (granzymes), and results in penetration of the target cell by effector molecules. This machinery is believed to be partly responsible for epithelial cell apoptosis. Of particular interest, CD8+ T lymphocytes producing perforin and granzyme B are localized around acinar structures in SS salivary glands. In addition it has been observed that acinar cells surrounded by CD8+ cytotoxic T cells displayed an increase apoptotic ratio (193), supporting the hypothesis that direct cytotoxic mechanism also mediate epithelial cell injury via aberrant apoptosis.

1.3.6 Autoantibodies in Sjogren’s syndrome

Autoantibody production in SS seems to reflect a breakage in the immune tolerance towards autoantigens, often cryptic. In this regard, the development and maturation of autoreactive B cells encoding for these autoantibodies has been largely investigated. It is believed that at glandular level the aberrant presentation of antigens/autoantigens can favour the development of autoreactive B cells, whose survival and spreading is supported by the excess of antigens presented by local APC and by the aberrant expression of B cell survival factors within the glands.
The main self-proteins implicated in SS pathogenesis are Ro/La ribonucleoproteins and fodrin whilst the main target for potentially pathogenic antibodies has been suggested to be the acetylcholine muscarinic receptor M3.

1.3.6.1 Anti Ro/SSA and anti La/SSB

Autoantibodies to the ribonucleoprotein particles Ro/SSA and La/SSB are commonly found in sera of patients with SS but are also generally detected in sera of patients with SLE. Their presence in SS patients is associated with longer disease duration, increased frequency of non-exocrine manifestations and the presence of lymphocytic infiltrates in the minor salivary glands(214, 215).

The Ro/La ribonucleoprotein complex is composed of the Ro 60 kDa, Ro 52 kDa and La 48 kDa proteins that are associated with one small cytoplasmic RNA (Y-RNA).

The Ro/SSA antigen is a ribonucleoprotein particle composed of hY-RNAs and two protein components (60 kDa and 52 kDa) conforming a ribonucleoprotein complex.

The La/SSB antigen is also a ribonucleoprotein particle associated with RNA polymerase III transcripts, including the hY-RNAs.

It is not clear what trigger tolerance breakdown and autoantibody response to Ro/SSA and La/SSB in SS. It has been suggested that stresses, such as ultraviolet radiation, viral infections and apoptosis can lead to undesirable cell surface exposure of autoantigens to the immune system (216).

Although there is not much genetic information explaining SS pathogenesis, it is known that a single nucleotide polymorphism in intron 3 of the Ro52 gene is
strongly associated with the presence of anti-Ro52 autoantibodies in primary SS (217).

1.3.6.2 Anti-α-fodrin

Fodrin is the main component of the cortical cytoskeleton of most eukaryotic cells, composed of α and β subunits. Many studies have associated fodrin with apoptotic mechanisms. It was reported an abnormal location of α-fodrin on the surface of apoptotic-induced cells (218). Moreover, it was demonstrated that α-fodrin is specifically cleaved by granzyme B during cytotoxic lymphocyte granule-induced cell death (219). Also, it was found that Epstein–Barr virus (EBV) reactivation induces an increase in apoptotic protease activities leading to progression of α-fodrin proteolysis (220). More recently, Maruyama et al. (221) described a 150 kDa cleaved product of α-fodrin that is exposed as a neoepitope to the immune system. These studies indicate that an abnormal proteolysis of α-fodrin may lead to an altered location of this autoantigen in the external surface of apoptotic epithelial cells, triggering the autoimmune process in the salivary glands.

Although several authors have further investigated the prevalence and clinical significance of anti-α-fodrin antibodies in SS describing prevalence of antibodies in 55–64% of adult patients with primary SS and in 40–86% of those with secondary SS (depending on the Ig isotype study) (222) and in child/juvenile SS patients (223, 224), there is evidence that these antibodies do not have significant additional diagnostic value (225).
1.4 Link between EBV and Rheumatoid arthritis: evidences and controversies

The evidences linking EBV to RA include serological data, a higher EBV viral load in RA patients, cross-reactivity between EBV proteins and RA self-antigens, the expression of EBV DNA/RNA/antigens in the RA synovial membrane and a cell-mediated response against EBV systemically and within the RA joints (68). Among all the above aspects, both positive and negative findings have been reported. Here, I shall review the main relevant observations.

1.4.1 Humoral response to EBV antigens in rheumatoid arthritis

EBV was first implicated in the pathogenesis of RA by Alspaugh and Tan (226), who reported that sera from patients with RA were reactive against a nuclear antigen in EBV-transformed lymphocytes. This “RA nuclear antigen” (RANA) was identified as a glycin/alanine-rich repeat in EBNA1. Since then, there has been a general consensus in several reports to conclude that RA patients, compared to healthy controls, have higher titers of antibodies specific for both latent and lytic EBV antigens, not only in the serum but also in the synovial fluid (56, 227-231). However, EBV titres in RA serum were found usually equal to, or greater than, those in the synovial fluid, an observation which led to the controversial suggestion that the synovial fluid titres may merely reflect the serum anti-EBV antibodies levels and not local production (228, 232, 233).
1.4.2 Cell-mediated immune response to EBV in rheumatoid arthritis

Several groups reported that cytotoxic CD8+ T cells directed against EBV antigens are altered in frequency (61, 231, 234) and are functionally impaired in RA patients (61, 235). In a recent study CD8+ T cells, with a decreased ability to produce INF-γ, were demonstrated to be directed predominantly against lytic EBV peptides in RA patients (61).

In keeping with these reports, there is strong evidence to suggest that the impairment of T cells from RA patients does not allow an adequate control of EBV-induced B cell proliferation (235, 236). Accordingly, B cells from RA patients undergo lymphoblastoid transformation in vitro more often compared to controls and the time needed to transform lines is shorter (237). Interestingly, the deficiency in the CD8+ T-cell function seems not to be present in the early stages of RA and has been therefore related to an acquired phenotype possibly due to chronic inflammation rather than to genetic factors. In any case, the net result of functional defects in the EBV-specific CD8+ T cell compartment is a reduced control of EBV infection.

1.4.3 Molecular mimicry between EBV antigens and self-proteins in RA

In the molecular mimicry hypothesis, cross reactivity is assumed to occur between viral and human proteins. Different findings suggest a role for molecular mimicry between EBV and self-antigens in RA patients. Significant homology has been described among several sequences of both EBNA-6 and HLA DQB1*0302 (238).
Glycine/alanine repeats identical to those found in EBNA-1 are also present in cytoskeleton proteins including cytokeratin and type 2 collagen (63) which are putative autoantigens in RA. Interestingly, an antibody to these glycine/alanine repeats strongly reacted with a 62-kDa protein expressed by synovial lining cells in RA patients (239) while reduced reactivity with synovium from patients who had other joint diseases (osteoarthritis, psoriatic arthritis, lupus, and villonodular synovitis) was noted. Although the target protein has not been fully characterized, it seems to be located on lining macrophage synovial cells.

The RA susceptibility sequence QKRAA located in HLA-DRB1*0401 shares homology with a sequence in the gp110 EBV glycoprotein (240), which is a lytic cycle antigen expressed by the endoplasmic reticulum and on the surface of EBV-transformed lymphoblastoid cells (241). The gp110 protein is among the herpes virus gB proteins, which are targets of the immune response to infection (242). Both B cells and T cells cross react to the QKRAA sequences in gp110 and HLA-DRB1*0401 (240). Therefore, EBV infection may lead to an immune response against HLA-DR molecules containing the shared epitope. The humoral response to purified EBV gp110 and EBNA-1 varied with the expression of the shared epitope, being strongest in patients carrying RA susceptibility alleles (243).

1.4.4 Cell-mediated response to EBV antigens within the joint

CD8+ T-cell clones directed against BZLF1 and BMLF1 proteins were identified in the joint fluid from RA patients. Both BZLF1 and BMLF1 are transactivators produced during the EBV lytic cycle (244). This T-cell response was stronger in the joint than in peripheral blood, suggesting accumulation of EBV-specific T
cells within joints. However, a similar T-cell response was also found in the synovium from patients with other joint diseases (psoriatic arthritis, spondyloarthropathy, and reactive arthritis), as well as against cytomegalovirus proteins (244).

More recently, another study demonstrated the presence of T cells directed against gp110 in the synovium of both RA patients and patients with other inflammatory joint diseases (spondyloarthropathies, connective tissue diseases, and chondrocalcinosis) (234). Remarkably the strength of the response, as assessed using the limit dilution method, was greater in the RA patients compared to controls. Further, the response in the RA patients was stronger in joint fluid than in peripheral blood (234).

These data strongly suggest that within the RA joints there is an ongoing cell-mediated response to lytic-cycle EBV antigens. The stronger response in the synovial fluid compared to the peripheral blood is probably due to the preferential migration of EBV-reactive cytotoxic T cells from the bloodstream to the joints.

1.4.5 EBV load in RA patients

The percentage of EBV-infected B cells is higher in RA than in healthy subjects. The EBV-burden in RA patients was investigated in different studies showing an EBV load 10-time higher in RA patients than in controls (56, 245). In particular, Balandraud et al observed that the higher EBV burden remained stable over 1-year follow-up period (56). More recently, it was observed that the increased EBV load is not associated with any particular HLA-DR alleles (114).
1.4.6 EBV and anti-citrullinated proteins antibodies in RA

EBV antigens can undergo post-translation citrullination, thus becoming targets for anti-CCP, highly specific in RA. Antibodies towards a citrullinated EBV peptide derived from EBNA1 (VCP1) have been identified in 50% of sera from RA patients compared to only 5% of controls (66). The VCP1 antibody titres correlated with anti-CCP titres and were more common among patients with rheumatoid factors. The same group demonstrated that anti-VCP1 antibodies from RA sera cross-reacted with citrullinated EBNA1 and citrullinated peptides that are autoantigens in RA, such as fibrinogen and filaggrin (66).

Recently, another viral citrullinated peptide, VCP2, derived from EBNA2 was suggested to be a substrate for ACPA (246). Accordingly, pre-incubation of RA sera (positive for CCP and VCP2) with VCP2 inhibited the binding to anti-CCP by as much as 60%. VCP2 has been demonstrated to be a good and sensitive tool in the diagnosis of RA, being detected almost exclusively in RA patients with respect to control subject and patients with other autoimmune diseases (247). Moreover, it was demonstrated that anti-VCP2 antibodies have a similar sensitivity and sensibility of anti-CCP in predicting RA (248).

1.4.7 Presence of EBV nucleic acids and proteins in the RA synovia

The investigation of EBV within the synovial tissues of RA patients has proved by far the most controversial aspect of EBV involvement in RA. Although EBV presence in the RA synovial membrane has been investigated using different techniques, such as immunohistochemistry (IHC), in situ
hybridization (ISH) and PCR techniques, these have never been performed in parallel on the same cohort of patients.

In some PCR and RT-PCR studies, RA patients were significantly more likely to have EBV DNA or RNA in synovial fluid or membrane compared to controls (245, 249). In addition, EBV DNA was identified in the synovial membrane also by Southern blot analysis (69).

Other studies detected EBV in the RA synovium mainly by ISH and IHC. Takey et al (70) demonstrated the expression of EBER and LMP1 in synovial lining cells from about 25% of the RA cases analysed, but not in OA controls. They also found that the incidence of EBV-positive synovial lining cells was higher in RA synovial samples with high lymphocyte infiltration than in the moderately infiltrated ones. Takeda et al (69) also found evidence of EBV DNA, EBER and EBV-lytic antigens in a high proportion (about 50%) of synovial tissues from RA patients but no evidence of EBV infection in synovial tissues from OA patients.

Despite these evidences, there have been also conflicting reports, especially in the analysis made by ISH and IHC. For example, Alspaugh et al (71) studying the pannus extracted from RA patients, investigated the presence of RA-associated nuclear antigen (RANA), anti-RANA, EBV specific antigens, and EBV genomes. Although they detected rarely some RANA and commonly anti-RANA, they did not detect any EBV-specific antigens or genes. They therefore concluded that EBV is not the primary event causing RA through direct infection. Brousset et al (250) did not detect any transcripts associated with the latent and early-lytic phase of EBV in RA synovial membranes using ISH technique. Niedobitek et al (251) failed to detect LMP1 or BZLF1 positive cells in RA synovial tissue, although they found EBER-positive lymphocytes.

In summary, even though converging evidence indicates that EBV infection and both humoral and cellular immunity toward the virus are dysregulated in
patients with RA, evidence that EBV infection is altered in the RA synovium remains controversial and the exact role played by the virus in the pathogenesis of RA is yet to be clarified.
1.5 Link between EBV and Sjogren’s syndrome: evidences and controversies

EBV is an excellent putative candidate to be involved in the pathogenesis of SS. In fact, EBV replicates in the oropharynx during primary infection and it has its normal site of latency in salivary and lacrimal glands. Since EBV can induce a strong T-cell and B-cell response, the immune recognition of the virus at its site of latency or activation might contribute to exocrine gland damage. Several observations support a link between EBV and SS both in the peripheral and exocrine gland compartment.

1.5.1 Peripheral compartment

Firstly, in comparison with healthy controls, SS patients show an increased humoral response towards the virus. Independent investigations revealed higher titres of anti-EBV antibodies (especially anti-EBNA1, -2 and -3) in serum of SS patients compared to controls (252, 253). Secondly, there are different examples of molecular resemblance between self-antigens in SS and EBV proteins. For instance anti-La/SSB antibodies from SS patients precipitate a cytoplasmic protein, which is complexed with EBER-1 and EBER-2 (33). Recently Navone et al (254), trying to identify novel autoantigens in SS, screened a random peptide library with pooled IgG derived from SS patients. They found a single peptide recognized by the majority of patients’ sera, but not by the control sera. This peptide shows homology with an EBV-derived protein, with tear lipocalin (a protein highly expressed in tears and saliva) and
with alpha-fodrin (a cytoskeleton protein considered an important autoantigen in SS). Anti-peptide antibodies affinity purified from SS sera recognized both the viral protein and tear lipocalin and alpha-fodrin. Moreover, Inoue et al (220) demonstrated that EBV reactivation can increase the apoptotic protease activity sustaining alpha-fodrin proteolysis and therefore causing the production of a cleavage product of alpha-fodrin that represent a key organ-specific autoantigen in the SS pathogenesis.

Furthermore, in vivo evidences of a link between EBV and SS come from a work from Miysaka et al (255), where they demonstrated that transferring B cells from SS patients to SCID mice induces a monoclonal lymphoproliferative disorder that resembles those arising in SS.

### 1.5.2 Exocrine compartment

As in the RA synovium, it is still highly controversial whether an increased expression of EBV nucleic acids and proteins are still expressed in SS salivary and lacrimal glands.

It was recently demonstrated that the saliva of patients with SS can activate EBV (256). In this study the influence of saliva was examined on an EBV-negative cell line transfected with BZLF1, a trans-activating EBV gene promoter. The results showed that saliva of SS patients exerts a significant effect on the EBV gene promoter.

Using ISH, IHC and dot blot hybridization Wen et al observed that two different forms of EBV infection may exist in SS salivary glands(74). One is EBER1-negative latency in epithelial cells and the other is EBER1-positive latency in lymphocytes. They did not detect any EBV lytic protein in the samples
analysed. Conversely Fox et al (257) using IHC observed that more than 50% of the SS salivary glands analysed were positive for the EBV early lytic antigen (EA) although only in epithelial cells. They did not detect any EBV+ lymphocytes. Differently, Pfugfelder et al (258), using IHC, PCR and ISH, found that lacrimal glands from SS patients express high level of EBV DNA and latent antigens both in epithelial cells and in lymphocytes. Early and late lytic antigens were the only EBV antigens expressed exclusively in epithelial cells of SS lacrimal glands. They also detected a significantly reduced or no EBV DNA/proteins expression in the control lacrimal glands, where only epithelial cells were infected. Few other works confirmed a higher expression of EBV DNA in SS salivary glands compared to the control, only in epithelial cells (259). Conversely, there are other studies which failed to observe an increased EBV expression in SS compared to control salivary glands. Venables et al (260) found no differences between SS patients and normal controls analysing the presence of EBV in the salivary glands either by ISH or IHC. Using IHC Schuumrman et al (261) did not detect any EBV antigen expression neither in SS or control salivary glands. Using ISH for EBV DNA the same group detected reactivity in around 50% of the SS salivary glands analysed, only in epithelial cells. Merne et al (262) using IHC, found instead similar level of expression of EBV latent antigen in SS and control salivary glands.

Thus, although there is a general consensus that dysregulated EBV infection and aberrant T/B cell EBV-reactivity are present in the RA and SS patients, significant controversies exist regarding the expression and the pathogenic role of this virus in the target tissues of both disease, namely the RA synovium and the SS salivary glands.
Common limitations of previous studies have been i) the use of a limited number of techniques to detect the virus; ii) generally small cohorts of patients analysed and, most importantly, iii) the absence of immunophenotypical characterization of the inflammatory infiltrates, in particular regarding the presence of ectopic B cell follicles and GC-like structures. These are fundamental limitations as the virus may express different programs of gene expression at different stages and in different cell types which can only be assessed via a comprehensive approach with the parallel use of diverse techniques among the same tissues.

Furthermore, the evidence that EBV has preferential tropism for B cells and the recent observation that ectopic B cell follicles are preferential niches for EBV latency and reactivation, highlight a fundamental deficiency of previous studies, mainly the characterization of tissue for the presence/absence of ectopic lymphoid structures.

With these limitations in mind, I set up the goal of overcoming previous pitfalls in the investigation of EBV in the RA synovium and in SS salivary glands as described in the next sections of this PhD thesis.
CHAPTER 2

RATIONALE OF THE THESIS AND AIMS
2.1 Rationale of the thesis and aims

Epstein-Barr virus (EBV) infects B cells and modifies their differentiation programme leading to B cell activation and immortalization. Increasing evidences support a link between EBV and common B cell-related autoimmune diseases, such as multiple sclerosis (MS) and myasthenia gravis (MG) where ectopic lymphoid structures (ELS), in the brain and the thymus respectively, are preferential sites of EBV persistence and reactivation. Since a subset of RA and SS patients are also characterised by such ELS formation in target organs, in the first instance I investigated whether EBV infection is deregulated and preferentially associated to ELS also in the RA synovia and SS salivary glands. Furthermore, in this PhD thesis I investigated whether a deregulated EBV infection in RA synovia and SS salivary glands play a pathological role in these conditions either via contribution to local autoimmunity through the in situ differentiation of autoreactive plasma cells and/or the activation of a local cytotoxic immune response.

In details the main aims of my PhD were:

1) To set the long outstanding issue of whether EBV proteins/RNA are expressed in the RA synovia and SS salivary glands and if their expression is related to the presence of ELS. In order to clarify this aspect, I used a comprehensive array of techniques (i.e. IHC, IF, ISH, RT-PCR) on a large number of RA synovial and SS salivary gland biopsies fully characterised for the level of inflammation and the presence of GC-like structures. Different experimental approaches to investigate EBV expression were employed since previous
discrepancies in EBV RNA/protein detection could have been due not only to tissue heterogeneity but also variability of the specific sensibility of different techniques.

2) To investigate whether there is a relation between the cytotoxic T cells infiltration and function and EBV reactivation in SS salivary glands and RA synovia. This aim will test whether EBV reactivation contributes to elicit a T cell response in the target organ promoting local inflammation and tissue destruction.

3) To clarify whether anti-EBV antibodies are directly manufactured in situ in the RA synovium and SS salivary glands. For this purpose I first detected anti-EBV antibodies (anti-EBNA1, anti-VCA and anti-EA) in matching RA sera vs synovial fluids, and then investigated local production in the synovial and salivary gland compartments using the RA/SCID and SS/SCID chimeric models. For this purpose, mice were engrafted with ELS+/EBV+ RA synovia or SS salivary glands and the production of human IgG antibodies directed against EBV antigens assessed in the mouse circulation.

4) To assess whether antibodies against citrullinated EBV antigens are actively manufactured by GC-like structures in the RA synovium, possibly contributing to the development and persistence of local autoimmunity in the RA joints.

5) To test the hypothesis that EBV infection favours B cell survival and proliferation, and their differentiation into autoantibody-producing plasma cells in RA synovia or SS salivary glands. In order to address this question, I aimed to investigate whether anti-citrullinated fibrinogen antibody-producing
plasma cells in RA synovia and anti-Ro52/SSA and anti-La/SSB antibody-producing plasma cells in SS salivary glands were affected by EBV infection/reactivation.
CHAPTER 3
MATERIALS AND METHODS
3.1 Samples used in this study

3.1.1 Synovial tissue from RA and OA patients

Synovial tissue was collected from 43 RA patients (30 females, 13 males, mean age (range) 64.2 years (28-82), mean disease duration 4.5 years (1-16), mean CRP 38.7mg/l (5-132), mean ESR 34 (5-71), 72% RF+, 58.1% ACPA+) fulfilling the revised 1987 ACR criteria for RA (92). All patients attended the Rheumatology Department at Barts & The London School of Medicine and Dentistry, Queen Mary University of London and were treated with conventional DMARDs in monotherapy or in combination. Synovial tissue was collected after informed consent (LREC07/Q0605/29 granted by the East London &The City Research Ethics Committee 3) either by ultrasound-guided joint biopsy or total joint replacement or following synoviectomy.

As negative control population, 11 cases with OA (8 females and 3 males; mean age 72.8, range 68-82) undergoing total joint replacement were included in the study.

Freshly collected synovial tissue was part embedded in paraffin or frozen in OCT (optimal cutting temperature) for immunohistochemical studies and part dissected in multiple pieces and stored in a 10:1 v/v of RNA Later (Ambion) at -80°C for RT-PCR analysis.

A summary of of histological, immunohistochemical, ISH and RT-PCR findings in RA synovial samples is reported in Table 3.1.
Table 3.1 Summary of histological, immunohistochemical, ISH and RT-PCR findings in RA synovial samples

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g2: n.p. +/-

96
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**Diffuse synovitis**

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n.p. = not performed

+ = present

- = absent

+/- = present only in some areas
3.1.2 Minor Salivary glands from SS and NSCS patients

Minor salivary gland samples were obtained from patients attending the Rheumatology/Oral Medicine Combined Sjogren’s Clinic at Barts and The London NHS Trust. Samples were stored at the EMR Sjogren’s tissue bank. Biopsy samples were obtained from 29 patients (25 women and 4 men) with a mean age of 48.3 years (range 27-65 years) and mean disease duration of 5.9 years (range 1-14). All patients fulfilled the American–European Consensus Group. Sixteen of 29 (55%) were positive for anti-Ro/SSA and/or anti-La/SSB.

As negative control population, 7 patients with non-specific chronic sialodenitis (NSCS) (6 women and 1 man) were chosen as a control group. Their mean age was 48.7 years (range 16-73 years), and their mean disease duration was 3.14 years (range 1-10 years). Salivary gland biopsies were performed for routine diagnostic purposes after obtaining the patient’s consent (LREC 05/Q0702/1).

Freshly collected salivary glands were part embedded in paraffin for immunohistochemical studies and part dissected in multiple pieces and stored in a 10:1 v/v of RNA Later (Ambion) at -80°C for RT-PCR analysis.

A summary of histological, immunohistochemical and ISH finding in SS salivary glands as well as and clinical data of those SS patients is summarized in table 3.2.
Table 3.2 Summary of histological, immunohistochemical and ISH findings in SS salivary glands and clinical data of the relatedSS patients

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e.c. = epithelial cells; + = present; - = absent; F= female; M= male

100
3.1.3 Collection of human tonsils

Normal human tonsils were obtained from patients undergoing tonsillectomy. Freshly collected tonsils were embedded in paraffin for immunohistochemical studies or stored in a 10:1 v/v of RNA Later (Ambion) at -80°C for RT-PCR analysis.

3.1.4 Tonsil from an IM patient

Paraffin-embedded sections from a patient with IM were kindly provided by Dr. Francesca Aloisi at ISS, Rome, Italy.

3.1.5 RA and OA serum and synovial fluid

Paired sera and synovial fluid were obtained from 7 RA patients (6 females and 1 male with mean age 54, range 42-67; mean disease duration 10.5 years, range 5-16). All patients attended the Rheumatology Department at Barts & The London School of Medicine and Dentistry, Queen Mary University of London and were treated with conventional DMARDs in monotherapy or in combination.

As negative control population, paired sera and synovial fluid from 10 OA patients (5 females and 5 males with mean age 64.2, range 61-77), were kindly provided by Dr. Cristiano Alessandri (Policlinico Umberto I, Rome, Italy).
Sera from blood and synovial fluid from each patient were collected at -20°C until tested.

3.1.6 Serum from SCID mice transplanted with RA synovia

In this study I used the serum collected from 30 Beige SCID mice double transplanted with a total of 60 samples of human synovial tissue from 4 RA patients undergoing arthroplasty (2 with diffuse and 2 with CD21+ aggregate synovitis) (263). Each mouse was double-transplanted with tissue obtained from adjacent pieces of synovium from the same patient, thus minimizing variability of transplants within each mouse. Sixteen mice were transplanted with diffuse RA synovia EBV negative and 14 with ELS+/CD21+/EBV+RA synovia.

In this model full engraftment of human tissue is reached after 7 days, and significant levels of circulating human ACPA antibodies are detectable from week 2 onwards (264). Four weeks post-transplantation animals were sacrificed and underwent a terminal bleed; serum was collected and stored at -20°C for subsequent analysis of human ACPA and anti-EBV antibodies. All procedures were performed according to the Home Office regulations (PPL 70/7001).

3.1.7 Serum from SCID mice transplanted with SS salivary glands
In this study I used the serum collected from 8 Beige SCID mice double transplanted with a total of 4 salivary glands from 2 different SS patients anti-SSA/Ro and anti-SSB/La positive. Each mouse was double-transplanted with tissue obtained from adjacent pieces of salivary gland from the same patient. Serum from mice were collected at week 1, 2, 3 and 4 and stored at -20°C until used. All procedures were performed according to the Home Office regulations (PPL 70/7001).
3.2 Immunohistochemistry

3.2.1 Sample fixation and embedding

Fixatives preserve cells and tissues, make the antigen insoluble and therefore detectable and protect the tissue against the detrimental effects of subsequent processing and staining procedures.

Paraffin samples were fixed with formalin or paraformaldehyde (aldehyde-based fixatives) for 28-48 hours prior the embedding.

Frozen samples were fixed before the staining with cold acetone (-20°C for 10 minutes).

Acetone fixes the tissue by coagulating the proteins and inducing their denaturation. Paraformaldehyde and formalin determine the formation of cross links between the proteins, making them insoluble.

3.2.1.1 Paraffin embedding

Formalin fixed followed by paraffin embedding allow to fix and store material with good preservation of the tissue architecture and cell morphology.

Fresh tissue was trimmed and immediately fixed in 4% paraformaldehyde (Appendix 1) for 24 hours at room temperature. The tissue was then fitted into the embedding cassettes and processed as follows:

1) 70% Ethanol, two changes, 1 hour each

2) 80% Ethanol, two changes, 1 hour each
3) 95% Ethanol, two changes, 1 hour each
4) 100% Ethanol, two changes, 1 hour each
5) Xylen, three changes, 1 hour each
6) Paraffin wax (56-58 °C), two changes, 1 hour and half each.

Finally the tissue sample was embedded into paraffin blocks and placed in a cold plate to set for at least 2 hours.

Paraffin embedded samples were stored in boxes at room temperature until used.

3.2.1.2 Frozen sample embedding

OCT was placed on the top of a labelled small piece of cork, and the sample, previously trimmed, was arranged on the OCT. More OCT was placed on the sample to cover completely the tissue. The cork with the embedded sample was then immersed within ice-cold isopentane, previously bought at its freezing point (-160°C), until completely frozen. The sample was then wrapped in aluminium foil and transferred within a falcon tube in the liquid nitrogen for transportation. Embedded samples were stored in -80°C freezer until used.

3.2.2 Samples cutting

3.2.2.1 Paraffin-embedded samples
Paraffin samples were placed in ice filled boxes to cold down 20 min before cutting. Three µm thick sequential sections were cut in a Leica Microtome (knowlhill, UK). The sections were then floated on a warm bath (45 °C), before being picked up onto microscope Superfrost Plus slides (previously labelled with sequential numbers) and allowed to drain on a hot plate set at 60°C. After 30 min the slides were removed from the hot plate and stored in boxes at room temperature until used.

3.2.2.2 Frozen samples

Six microns sequential sections were obtained by cutting the specimens in a cryostat (Leyka), which is essentially a microtome inside a freezer. Each section was then mounted on a glass slide (superFrost Plus). Slides were allowed to dry overnight at room temperature and then individually wrapped in aluminium foil, placed in boxes and stored at -80°C until use. Each slide was allowed to adjust at room temperature before being unwrapped and used for immunohistochemistry/immunofluorescence.

3.2.3 Slides deparaffinization and rehydration

Before proceeding with the staining protocol, the slides must be first deparaffinized and then rehydratated in order to permeabilize the tissue before the staining. Notably incomplete paraffin removal can cause poor staining of the section.
Before each immunohistochemical procedure (including Haematoxylin and Eosin staining) the slides were treated according to the following protocol. Slides with paraffin sections were immerse in xylene (2 changes, 5 minutes each), followed by passages in 100% absolute ethanol (2 changes, 5 minutes each) and 70% ethanol (2 changes, 5 minutes each). Slides were then rinsed with distilled water for 5 minutes and placed in a jar containing Tris buffer saline (TBS) (Appendix 1) until used. Frozen slides do not need to be rehydrated before use.

3.2.4 Haematoxylin and Eosin (H&E) staining

Haematoxylin and Eosin (H&E) staining was performed in order to visualize cells and tissue components of the samples. The H&E staining, by using two different dyes, allows the visualization and differentiation of the cellular nuclei and cytoplasm under light microscopy. Haematoxylin stains the chromatin within the nucleus, with a deep blue colour, while Eosin stains the cytoplasmic material, connective tissue and collagen with a pink colour. Frozen or paraffin sections were immersed in Mayer’s Haematoxylin for 5 minutes. Slides were then rinsed in tap water and placed few seconds in 1% Acid Alcohol (Appendix 1) to remove the excess of Haematoxylin from the cytoplasm, allowing Haematoxylin to stain only the nuclei. The slides were then washed in running tap water and immersed in Eosin for 5 minutes. Finally, the slides were washed in tap water, dehydrated and mounted in organic solvent soluble mounting medium (DePeX).
3.2.5 Antigen retrieving

Formalin-fixed tissues requires an antigen retrieval step in order to remove methylene bridges (inter- and intra- molecular bridges), which cross link proteins and therefore mask antigenic sites.

Generally two different methods of antigen retrieval are used; the proteolytic-induced antigen retrieval and the heat-induced antigen retrieval. Some antigens prefer enzymatic to heat mediated antigen retrieval and vice versa.

3.2.5.1 Proteolytic-induced Antigen retrieval

Different enzymes can be used to break the protein cross-links formed during the fixation of formalin-fixed paraffin embedded samples, such as Trypsin, Proteinase K, Pepsin and Pronase. However, the use of enzyme digestion method may destroy some epitopes and tissue morphology. Therefore the optimal enzyme concentration and incubation time need to be tested.

In this study I used proteinase K. Proteinase K was warmed up at 37°C (in order to achieve the optimal enzymatic activity) and placed on the slides for 5 minutes. After incubation, slides were rinsed in TBS and placed in TBS until staining.

3.2.5.2 Heat induced Antigen retrieval
Heat treatment at temperatures of 95°C in appropriate buffer solutions (citrate buffer pH 6 or EDTA buffer pH 9) can reconstitute the antigenicity of many proteins that have been rendered non-reactive during the fixation and paraffin embedding process. Although citrate buffers at pH 6 is historically been the most widely used retrieval solutions, high pH buffers have started being implemented for many cases showing improved end results. In this study I used both solutions at pH6 and pH9 (Target Retrieval Solution, DAKO, UK). Plastic jars with sufficient quantity of Target Retrieval Solution were placed in heat water bath at 95°C. After deparaffinization and rehydration, the slides were immersed into preheated Target Retrieval Solutions and placed either in the water bath at 95°C for 45 minutes or subjected to 3 cycles of 3 minutes at 750 Watt in a microwave oven. After incubation, jars with slides were removed from water bath/microwave and allowed to cool down for 20 minutes at room temperature. After retrieving slides were rinsed in TBS and left in TBS until stained.

3.2.6 Endogenous peroxidase and protein block

Some cells or tissues contain endogenous peroxidase. Using HRP conjugated antibody may result in high, non-specific background staining. This non-specific background can be significantly reduced by pre-treatment of tissues with hydrogen peroxide prior to incubation with HRP conjugated antibody. In every staining procedure that required the use of 3, 3’-diaminobenzidine (DAB) as substrate for colour development, a peroxidase block was performed by incubating each slide for 20 minutes with 0.3% of hydrogen peroxidase. After
the blocking, slides were rinsed in TBS and left in TBS until used in following steps.

Another common cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking those sites with normal serum or specific solution. In this study I performed the protein block either incubating the slide with 20% normal serum in TBS for 30 minutes or 10 minutes with a ready to use Protein Block Solution (DAKO, UK).

3.2.7 Immunohistochemical detection of the antigens

Immunohistochemistry allows the localization of specific antigen/s on tissue sections. This system is based on the recognition of a specific antigen/s by a conjugated (direct method) or unconjugated (indirect method) primary antibody. The direct method is a one-step staining method and involves a labelled antibody (i.e. a fluorescence dye or an enzyme such as horseradish peroxidase HRP or alkaline phosphatase AP) reacting directly with the antigen in tissue sections. This technique, using just one antibody is simple and rapid, but the sensitivity is low due to little signal amplification. The indirect method involves an unlabelled primary antibody (first layer) that binds to the target antigen in the tissue and a labelled secondary antibody (second layer) that reacts with the primary antibody. The secondary antibody can be linked to an enzyme or a fluorescent dye. Further amplification can be achieved if the secondary antibody is conjugated to several biotin molecules, which can recruit enzyme-linked Avidin complexes.
While a fluorescent dye can be detected directly using a specific fluorescence microscopy, the use of an enzyme-linked-antibody needs a substrate solution and a chromogen, that reacting with the used enzymes allows the precipitation of the colour (colorimetric methods) on the site of antibody-antigen binding.

3.2.7.1 Colorimetric methods: horseradish peroxidase and alkaline phosphatase

Colorimetric reactions most often use antibodies chemically conjugated to the enzymes horseradish peroxidase (HRP) or alkaline phosphatase (AP). The HRP reacts with diverse chromogen substrate, i.e. DAB and AEC. The DAB results in a brown precipitate insoluble in alcohol and other organic solvents, while AEC develops in an intense red product that is soluble in alcohol and therefore must be used with an aqueous counterstain and mounting media.

The AP catalyses the hydrolysis of phosphate groups from a substrate molecule resulting in a pink or blue product. AP has optimal enzymatic activity at a basic pH (pH 8 - 10). Since endogenous alkaline phosphatase is diffusely expressed in human tissue, in order to avoid unspecific staining in the section, it is necessary inhibit the endogenous enzyme. In this study Vector Red (Vector Lab) was used as chromagen and Levamisole was used to inhibit the endogenous activity of the alkaline-phosphatase.

3.2.7.2 Staining procedures: the Avidin-Biotin complex (ABC) Methods
The avidin-Biotin (ABC) method is a widely used technique for immunohistochemical staining and is based on the strong affinity that Biotin (a small molecular weight vitamin) displays for Avidin (a large glycoprotein of 68 Kd). Avidin has four binding sites for biotin providing the backbone of a macromolecular amplificatory complex. Avidin can be labelled with enzymes (HRP or AP) or fluorochromes, allowing diverse methods of detection to be used. A DAKO Avidin-Biotin Complex (ABC) (Appendix I) linked to HRP or AP was used in this study. The technique involves three layers: the first layer is unlabelled primary antibody, the second layer is the biotinylated secondary antibody and the third layer is the enzymatic HRP or AP-Avidin conjugated. The used enzyme is then visualised by the use of the appropriate chromogen and substrate.

Since endogenous biotin is found in many tissues, it is necessary to pre-treat tissues with unconjugated avidin saturated with biotin, in order to inhibit the endogenous enzyme. For this purpose a commercially available Biotin Blocking System (DAKO, UK) was used. After 10 minutes of incubation with the avidin solution, slides were rinsed in TBS and incubated with the biotin solution for 10 minutes. Then slides were washed and left in TBS.

3.2.7.3 Staining procedures: Dako EnVision System Polymeric Method

This system uses a secondary antibody directly linked to a dextran polymer binding a large number of HRP molecules. The advantages of this method are many including higher sensitivity as compared with the ABC method, and reduced number of staining layers. In details, after the first layer of unconjugated primary antibody, the EnVision secondary is applied on the slides
and after washing and application of the substrate chromogen, the colour is developed.

3.2.7.4 Immunofluorescence

Immunofluorescence is an antigen-antibody reaction where the antibodies are labelled with a fluorescent dye and the antigen-antibody complex is visualized using ultra-violet (fluorescent) microscope. Fluorochromes are dyes that absorb ultra-violet rays and emit visible light. This process is called fluorescence. Commonly used fluorochromes are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). When those molecules with luminescent properties absorb light, they emit light of a different wavelength. Fluorescent materials give off light because of their atomic structure. Electrons are arranged in discrete energy levels surrounding the atom’s nucleus with each level having a predetermined amount of energy. When an electron absorbs energy from a photon of light it becomes “excited” and jumps to a higher, less stable energy level. The excited state does not last long. The half-life of the excited state is generally less than 10 seconds. The electron loses a small amount of energy as heat and the remainder of the extra energy is given off in the form of a photon. The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light. A range of wavelengths of light can excite the electrons of a fluorochrome. For example, fluorescein will fluoresce when hit by light with any wavelength between 450 nm and 520 nm. However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly,
the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 515 nm (visualised in green). In this study I used secondary antibodies conjugated with red-emitting TRITC, green-emitting FITC, green-emitting alexa 488 and red-emitting alexa 555. In some experiment a double staining was performed, using a mixture of primary antibodies raised in different species followed by secondary antibodies conjugated with fluorescent dye with a well separated emission spectra (i.e. TRITC/FITC or alexa 488/alexa 555). The use of two different dyes permits unequivocal demonstration of colocalization or not of two antigens.

In all immunofluorescence experiment nuclei were stained using DAPI (4',6 diamidino-2-phenylindole), a fluorescent dye that strongly bind to A-T rich regions in DNA. When bound to double-stranded DNA DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). Therefore the nuclei of tissue incubated with DAPI are visualised in blue.

All the incubation steps were performed in a humid chamber at room temperature. All the washes were performed in TBS. All primary antibodies were unlabelled, diluted in the antibody diluent and incubated for 1h or 1h and half at room temperature or overnight at 4°C. All secondary antibodies, either biotinylated or fluorochrome-conjugated, were diluted in the DAKO antibody diluent (DAKO, UK) and incubated for 1 hour at room temperature. Single stainings were performed using either the ABC method or the Envision System or fluorescence dye. Double staining were performed using two different fluorescent dyes.
The protein block was performed before each immunofluorescence staining and additional peroxidase or biotin block was performed when using the Envision or ABC methods, respectively. Once slides had been stained, they were either mounted with aqueous mounting medium (when using AEC or fluorescence) or dehydrated through passages in alcohol and xylene and mounted in xylene soluble mounting medium (DePex, VWR, UK, when using peroxidase).

### 3.2.7.5 Antibodies and titration experiments

For primary and secondary antibodies, pilot titrations were run to determine the optimal working dilution and duration of incubation on positive controls. The optimal conditions were then used in subsequent experiments. A complete list of primary antibodies and secondary antibodies used in this study are listed in Table 3.3.
Table 3.3 Primary and secondary antibodies used for immunohistochemistry and indirect immunofluorescence

-Primary antibodies-

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>SPECIFICITY</th>
<th>CLONE</th>
<th>HOST</th>
<th>SOURCE</th>
<th>DILUTION</th>
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<td>T lymphocytes</td>
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<td>Cytotoxic/suppressor T cell subset</td>
<td>C8/144B</td>
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<td>Dako</td>
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</tr>
<tr>
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<td>Macrophages</td>
<td>KP1</td>
<td>Mouse</td>
<td>Dako</td>
<td>1:50</td>
</tr>
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<td>Dako</td>
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<td>GB7</td>
<td>Mouse</td>
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<td>Biolegend</td>
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<td>Mouse</td>
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<td>CS. 1-4</td>
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<td>Dako</td>
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## Secondary antibodies

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<th>NAME</th>
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<td>Invitrogen</td>
</tr>
<tr>
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<td>Invitrogen</td>
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<td>Goat anti-mouse IgG2a ALEXA 488</td>
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<td>Invitrogen</td>
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<td>Goat anti-mouse IgG2a ALEXA 555</td>
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<td>Dako</td>
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</table>
3.2.7.6 Image capture

Images from IHC and IF were captured and visualised using CellIP imaging software on an Olympus BX61 motorised microscope with ColorView II and F View II digital cameras (Olympus Microscopy, UK). Fluorescence signals were viewed using the U Plan APO objectives (4x/0.16na, 10x/0.40na, 20x/0.70na, 40x/0.85na) and FITC (HQ480/40 - HQ535/50m - 510-560nm), TRITC (HQ535/50 - HQ610/75m - 572.5-647.5nm) and DAPI (EXD360/40 - EMD460/50m - 435-485nm) filter sets. Captured images were exported as TIFF files and adjusted using Photoshop software (Version 7.0) (Adobe Systems Incorporated, USA).

3.2.8 Evaluation of the Synovitis Score and presence of lymphocytic aggregates in RA synovia

Each RA synovia was evaluated for the synovitis score and for the presence of lymphocytic aggregates (table 3.1).

To evaluate the synovitis score I followed the method described by Krenn et al (265). Each paraffin-embedded 3 µm thin RA synovia section was stained with haematoxylin and eosin and then semi-quantitatively evaluated according to the synovial lining cell layer, stroma cell density and inflammatory infiltrate. Each parameter was evaluated on a semiquantitative scale from 0 to 3 and the sum was interpreted as follow: 0-1 (no synovitis); 2-4 (low grade synovitis); 5-9 (high grade synovitis).

To grade lymphocytic aggregates I referred to the method devised in our group and previously described by Manzo et al (126). In details, the number of radial...
cells was counted in lymphocytic aggregates: between 2 and 5 cells (G1); between 6 and 10 cells (G2); more than 10 cells (G3).

3.2.9 Characterization of the inflammatory infiltrates in RA synovia

The histological grading of RA synovial tissues and the level of inflammation were assessed by immunohistochemical staining on 3 μm thick paraffin-embedded, serial sections. Each sample was single stained for CD20 (B cells), CD138 (plasma cells), CD3 (T cells), CD68 (macrophages) and CD21 (FDC network). In details, each section was dewaxed in xylene, rehydrated through graded alcohol solutions and washed in TBS. For CD20, CD3, CD138 and CD68 staining, the antigen retrieval was performed incubating each slides in Citrate Buffer (pH 6) for 30 minutes at 95°C and the primary antibody was incubated at the appropriate dilution for 1 hour at RT. For CD21 proteolytic digestion with Proteinase K (DAKO, UK) was used as antigen retrieval method followed by overnight incubation with the primary antibody at appropriate dilution. After exposure to the primary antibody, sections were incubated for 30 minutes at RT with the Dako HRP-conjugated polymer (Dako, UK). After 3 washes with TBS, colour reaction was developed using DAB substrate-chromogen and slides were counterstained with Meyer’s haematoxylin (Sigma, UK) and dehydrated through graded ethanol solutions and xylene and mounted in DePex (BHD, UK). Negative controls included either the omission of the primary antibodies or isotype controls. A tonsil was routinely used as positive control.

The presence of CD20, CD3, CD138 and CD68 was scored from 0 (representing no infiltration) to 4 (representing intense infiltration) as described by Tak et al.
CD68 was scored both for the number of positive cells in the synovial lining layer and in the sub-lining space. CD21 was analysed in each sample in order to identify the presence/absence of FDC networks. Samples were divided in 3 different groups according to the synovial pathotype (table 3.1): 1) diffuse synovitis, mainly characterised by macrophage infiltration and by none or just few and spread B/T and plasma cells, 2) aggregate synovitis without FDC networks, characterised by inflammatory aggregates of B/T cells, variable number of plasma cells but without clear FDC networks and 3) aggregate synovitis with FDC networks identifying GC-like structures. From 9 RA patients, snap frozen synovial tissue was also available for immunohistochemical studies. Six-µm acetone-fixed sections were immunostained for CD20, CD3 and CD138 as described above without antigen retrieval. Samples were classified as ELS+ or ELS-/diffuse on the basis of the presence/absence of B cell aggregates.

3.2.10 Characterization of the inflammatory infiltrates in SS and NSCS salivary glands

The histological grading of the tissue and the evaluation of level of inflammation was assessed by immunohistochemical staining on sequentially cut sections from paraffin-embedded SS and NSCS salivary glands. Each sample was stained for CD20, CD3, CD138 and every antigen was scored from 0 to 4 by adapting the synovial score described above. On the basis of these stainings samples were classified as ELS+ or ELS- (table 3.2). All ELS+ samples were further stained for CD21 in order to classify samples as ELS+/FDC+ or ELS+/FDC- (table 3.2).
CD20, CD3, CD138 and CD21 staining were performed as described in the previous paragraph.

Negative controls included the omission of the primary antibodies or the use of Ig isotype controls.

### 3.2.11 Analysis of EBV antigens expression

Analysis of latent and lytic EBV proteins expression was performed on 3 µm-thick paraffin-embedded biopsies (RA and OA synovia, SS and NSCS salivary glands). In details after deparaffinised, sections were placed in citrate buffer (pH 6.0) or EDTA buffer pH 9 and subjected to 3 cycles of 3 minutes in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by incubating tissues for 20 minutes in 0.3% H₂O₂ in TBS. To block non-specific binding, sections were incubated with 10% normal serum matched to the host species of the secondary antibodies. Samples were then immunostained with antibodies against EBV latent (LMP1, LMP2A, EBNA1), early lytic (BFRF1 and BMRF1) and late lytic (p160 and gp350/220) proteins. Primary antibodies were incubated overnight at +4°C (BFRF1, BMRF1 and EBNA1), for 1 hour (LMP1, p160 and gp350/220) or 90 min (LMP2A) at room temperature. For EBNA1 immunostaining, sections were permeabilized with 0.1% Triton X100 for 10 min and 0.05% Triton X100 was added to the primary antibody-containing medium.

Sections were then incubated with biotinylated secondary antibodies and avidin-biotin peroxidase complex (Elite Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The final reaction was visualized with diaminobenzidine and sections were counterstained with Mayer’s haematoxylin. Negative controls
include omission of primary antibody and the use of IgG isotypes. Tonsil from a patient with mononucleosis was used as positive control. From 9 RA patients, snap frozen synovial tissue was also available for immunohistochemical studies. Three-µm acetone-fixed sections were immunostained for LMP1, LMP2A and BFRF1 as described above without antigen retrieval.

3.2.12 Identification of EBV+ cells

Three µm-thick, deparaffinised tissue sections (from RA and OA synovia, SS and NSCS salivary glands) underwent antigen retrieval with buffer citrate and protein block with 10% normal serum matched to the host species of the secondary antibodies. Sections were then incubated with the unconjugated primary antibodies overnight + 4°C (anti-EBV lytic antigens BMRF1 and BFRF1) or 1 h at room temperature (anti-B cells CD20 and CD79a; anti-plasma cells CD138 and anti-EBV latent antigens LMP2A and LMP1), alone or in different combinations for double staining (i.e. CD20/LMP2A, CD79a/LMP1, CD138/BFRF1, CD138/BMRF1). Slides were then incubated with the corresponding fluorochrome-conjugated secondary antibodies for 1 hour and sealed in DAPI-containing medium (Invitrogen). Negative controls include omission of the primary antibodies and the use of IgG isotype control. Tonsil from a patient with IM was used as positive control for EBV stainings. From 9 RA patients, snap frozen synovial tissue was also available for immunofluorescence studies. Three-µm acetone-fixed sections were double stained for CD138/BFRF1 and CD20/LMP2A as described above without antigen retrieval.
3.2.13 Cytotoxic T cells localization and relationship with EBV+ cells

The relationship between cytotoxic T cells, B cells and EBV infected cells was investigated by immunofluorescence using 3 µm-thick paraffin-embedded biopsies (from RA synovia, SS and NSCS salivary glands). Deparaffinised tissue sections underwent antigen retrieval with buffer citrate incubated for 30 minutes at 95°C and protein block with Dako Protein Block Solution. Double stainings CD20/CD8 were performed to analyse the localization of CD8+ T cells respect to B cell follicles. Double stainings CD8/Granzyme B (the last a serine protease whose expression is restricted to cytotoxic lymphocytes), CD56/GranzymeB or CD4/Granzyme B were performed to identify the subset of cytotoxic T cells. Double staining CD8/BFRF1 or Granzyme B/BFRF1 were performed to identify the localization of cytotoxic T cells respect to the EBV lytically infected cells. Each unconjugated primary antibody was incubated 1 hour RT, a part of BFRF1 that was incubated overnight +4°C. Slides were then incubated with the corresponding fluorochrome-conjugated secondary antibodies for 1 hour and sealed in DAPI-containing medium (Invitrogen). Negative controls include omission of the primary antibodies and the use of IgG isotype control. Tonsil from healthy donor was used as positive control.

3.2.14 Quantitative cell counts

Cell counts were performed in five 40X microscopic fields per section (RA synovia and SS and NSCS salivary glands).
The percentages of CD20+ B cells expressing LMP2A and of CD138+ plasma cells expressing BFRF1 were assessed by counting the number of double positive CD20+/LMP2A+ cells versus total CD20+ cells and the number of double positive CD138+/BFRF1+ versus total CD138+ cells.

The absolute numbers of CD8+, CD4+ and Granzyme B+ cells were assessed by counting the medium number of positive cells in 5 random 40X microscopic fields per section. The percentages of CD8+, CD56+ and CD4+ cells co-expressing Granzyme B were assessed by counting the number of double positive CD8+/Granzyme B+, CD56+/Granzyme B+ and CD4+/Granzyme B+ versus the total CD8+, CD56+ or CD4+ cells respectively. The percentage of Granzyme B+ cells co-expressing CD8, CD56 or CD4 was assessed respectively by counting the number of double positive CD8+/Granzyme B+, CD56+/Granzyme B+ and CD4+/Granzyme B+ versus the total Granzyme B+ cells.
3.3 EBER in situ hybridization (ISH) on RA, OA, NSCS and SS tissues

3.3.1 EBER ISH

In situ hybridization was performed on paraffin-embedded synovial tissue sections from RA and OA patients and salivary glands from SS and NSCS patients using the Epstein-Barr virus (EBER) PNA Probe/Fluorescein and PNA ISH detection Kit (DAKO), as described below. Sequential cut sections were stained for CD20, CD138 and CD3 in order to analyse in which lymphocytic area EBER+ cells localise. IHC stainings were performed as described below.

RNA ISH is used to measure and localize transcripts within tissue sections, using a labelled RNA strand complementary to the target region (i.e. probe). Probes that hybridize to the target sequence are generally labelled with either radio-, fluorescent- or antigen-labelled bases (i.e. biotin, alkaline phosphatase) and are respectively localized and quantified in the tissue using either autoradiography, fluorescence microscopy or immunohistochemistry.

To localised EBER in tissue sections of RA and SS, the PNA ISH Detection Kit (Dako, UK) was used. All the experimental steps were performed using RNAse-free conditions in order to avoid RNA degradation and all the reagents used were contained in the kit. Briefly, 3µm thick paraffin-embedded sections were dewaxed in xylene, rehydrated through graded alcohol solutions and washed in TBS. Slides were then placed in a humid chamber and incubated for 20 minutes with 1:10 diluted in Baxter-treated Water Proteinase K. Slides were then washed in RNAse-free water. In order to eliminate water, slides were immersed in 96% ethanol for 5 sec and then were allowed to dry at room
temperature for 5 min. The hybridization was performed adding the fluorescein-conjugated PNA probe and incubation was performed at 55°C for 1½ hour. For each experiment an irrelevant probe and a probe for GAPDH (both from Dako) were used as internal negative and positive controls, respectively. After hybridization slides were immersed in a preheated stringent wash solution and incubated for 25 minutes at 55°C with shaking. The low salt concentration of the stringent solution and high temperature incubation facilitate to remove imperfectly matched sequences and therefore increase the sensibility of the technique. Slides were then placed at room temperature for 10 min allowing the cool down and washed in TBS. Slides were then incubated with an anti-FITC AP-conjugated antibody for 30 min at RT in a humid chamber. Slides were then washed in TBS, rinsed in pure water and then incubated with a substrate solution (5-bromo-4chloro-3indolyphosphate (BCIP)) and nitroblue tetrazolium (NBT) for 30 minutes or until the colour was properly developed (max 1 hour). The reaction develop a blue colour, in fact BCIP is oxidized by NBT, forming an insoluble dark blue diformazan precipitate after reduction. Slides were then washed in tap water for 5 min, and sealed with an aqueous mounting medium (Dako).

3.3.2 EBER ISH combined to CD20 or CD138 immunohistochemistry

In order to identify which cells were EBER+, paraffin-embedded samples, after EBER ISH, underwent the CD20 or CD138 IHC. In brief, all the ISH procedure described above was first performed on the tissue slides until blocking of the substrate with tap water. Afterwards slides were washed in TBS for 5 min and
incubated with TBS/0.3% H$_2$O$_2$ to block the endogenous peroxidase. After the protein block (Dako), sections were incubated with the primary antibody (CD20 or CD138) for 1 hour at RT, then washed in TBS and incubated with a secondary biotinylated antibody. Sections were then incubated with the avidin-biotin peroxidase complex (Elite Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and the final reaction was visualized with AEC (Dako). After washing in dH2O slides were sealed with the aqueous mounting medium.
3.4 Quantitative Taqman real-time PCR

3.4.1 Extraction of total RNA from RA and OA synovia and SS and NSCS salivary glands

RNA was extracted using the Quiagen RNAesy mini kit according to manufacturer’s instructions. Tissues stored at -80°C in RNA Later were defrosted on ice, weighted and then cut in order to obtain approximately 20 mg of starting material (30 mg of tissue is the maximum amount suggested in the protocol to avoid reduction in RNA yield and purity). Tissues were placed in a sterile, RNAase-free tube and 600 µl of buffer RLT (containing denaturing guanidine thiocyanate and with the addition of 10 µl of fresh β-Mercaptoethanol/ml of RLT buffer) were added. Tissues were homogenised using a rotor-stator homogenizer until the sample was uniformly homogeneous (usually 20-40 sec). Tissue lysate were centrifuged for 3 minutes at maximum speed (13000 rpm) in a microcentrifuge tube. An equal volume of 70% ethanol was added to the lysate and immediately mixed by pipetting in order to precipitate RNA, which remains in the aqueous phase. An aliquot (700 µl) of the sample were added to the RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to allow the RNA to bind to the silica column. The flow-through was discarded and the remaining sample (approximately 500 µl) added to the RNeasy mini column and centrifuged as above. To avoid any possible DNA contamination, a DNase step was included according to manufacturer’s instruction. 350 µl of Buffer RW1 was added to
the column, centrifuged for 15 s at ≥8000 x g and the flow-through discarded.

10 µl of DNAse I stock solution (previously prepared by dissolving solid DNase I (1500 Kunitz units) in 550 µl of RNAase-free water) were added to 70 µl of Buffer RDD, gently mixed and added to the RNeasy mini column silica-gel membrane. Following 15 min incubation at RT, 350 µl of buffer RW1 were added to the column, centrifuged for 15 s at ≥8000 x g and the flow-through discarded.

The RNeasy columns were transferred into a new 2 ml RNAs free collection tube. In order to wash away contaminants (residual DNA and proteins) in the organic phase, 500 µl of Buffer RPE were pipetted onto the RNeasy column and centrifuged for 15 s at ≥8000 x g to wash the column. The flow-through was then discarded and another 500 µl Buffer RPE carryover the tube was centrifuged again for 1 min at full speed (13.000 rpm).

For elution the RNeasy column was transferred to a new RNAase free 1.5 ml collection tube, and 30 µl of RNAase-free water was pipetted directly onto the RNeasy silica-gel membrane and the tube centrifuged for 1 min at ≥8000 x g. The elution was immediately frozen at -80°C until required.

3.4.2 Quantification of total RNA

Nucleic acids are traditionally quantified using UV absorption using a spectrophotometer. In its simplest form the absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. An absorbance of 1 unit at 260 nm is equivalent to about 40 µg/ml of RNA. RNA has its absorption maximum at 260 nm and the ratio of the
absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 1.8-2.0.

In this study the concentration of total RNA isolated with RNeasy Kit was determined by using the Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). This instrument allows an exact quantification with the use of 1 µl of eluted RNA.

### 3.4.3 Determination of RNA quality and integrity

RNA integrity was assessed by resolving total RNA on a 0.8% agarose gel. Agarose powder, in 1x Tris Acid EDTA (TAE) buffer (Appendix II), was boiled in a microwave for 2 minutes, then the ethidium bromide (fluorescent dye that binds to nucleic acids) was added and the solution poured into a minigel tray where a well-comb was inserted. After polymerization, the comb was removed and the gel was placed in the minitank and covered with 1x TAE running buffer. Samples were mixed with blue loading buffer dye to monitor migration, loaded into the gel wells and run at 80 V (voltage) for 30 minutes. RNAs were visualised by fluorescence of the incorporated ethidium bromide using a UV light transilluminator and examined for RNA degradation and DNA contamination.

RNA should demonstrate 2 bands on electrophoresis (corresponding to the 28S and 18S of eukaryotic ribosomal RNA), with the intensity of the 28S band being approximately double that of the 18S band. If the 18S band appears more intense than the 28S band, or a smear is present, RNA degradation is likely. Extra bands at high molecular weight normally indicate genomic DNA carry-over.
3.4.4 Reverse Transcription PCR

In Reverse-Transcription PCR (RT-PCR), mRNA is converted to complementary DNA (cDNA) using a reverse transcriptase before being amplified. RNA was reverse transcribed to cDNA using the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). One µg of extracted total RNA from each sample was mixed with 1 µl of Oligo(dT)20 Primers (50 µM) or Random Hexamers (50 ng/mL) and 2 µl of 10 mM dNTP mix and brought to a 12 µl volume reaction with DEPC-treated water in a 0.5 ml PCR tube. After brief spinning down in a microcentrifuge, samples were incubated for 5 min at 65°C to denature RNA tertiary structure and samples were immediately cooled on ice to allow RNA and oligos to anneal. For the final reaction, 8 µl of a master mix containing 1 µl of Thermoscript™ RT (15 U/µl), 1 µl of 0.1 M DTT, 1 µl of RnAsiOUT™ Ribonuclease Inhibitor (40 U/µl), 1 µl of DEPC-treated water and 4 µl of 5X cDNA synthesis buffer were added. After mixing and a brief spin down of the tubes, the reverse transcription to cDNA was run in a PCR machine (Applied Biosystems 9700) for 1 hour at 50°C and the reverse transcriptase was inactivated at 85°C for 5 min. In order to remove the original RNA that could interfere with the quantitative real-time PCR analysis, RNA digestion was performed using 1 µl of E. coli RNasi H (2 units/µl) at 37 °C for 20 min. Finally, the completed cDNA strand was diluted to a final concentration of 10 ng/µl with DEPC-treated water and stored until used.

3.4.5 Quantitative Taqman real-time PCR
PCR involves amplification of a specific DNA sequence, which spans between two sequences of primers. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each cycle (in real time) as opposed to the endpoint detection by conventional quantitative PCR methods: it is based on the detection and quantitation of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in reaction.

For quantitative Taqman real-time evaluation of mRNA expression levels of genes, sequence-specific primers and probes from Applied Biosystems were used. Each gene expression assay contains, together with the forward and reverse primers, a Taqman MGB probe with a FAM reporter dye at the 5’ end. Within the probe, the dye is linked to a non-fluorescent quencher; during the polymerase reaction, the probe is detached from the cDNA and the quencher is released allowing fluorescence emission from the reporter dye.

The real-time PCR were run in triplicate on 384-well PCR plates (Applied Biosystem) with an equal loading of 10 ng of cDNA/well and detected using the ABI PRISM 7900HT Instrument. The thermal cycling condition used comprised a 2 min UNG activation step at 50°C, a 95°C Taq polymerase enzyme activation step for 10 min and cycles of 95°C denaturation for 15 sec and 60°C annealing/extension for 1 min. Results were then analysed after 40 cycles of amplification using the ABI PRISM 7900HT Sequence Detection System Version 2.3 (SDS 2.3). cDNA from normal human lymph nodes was used as calibrator. Relative quantification was measured using the comparative Ct (Threshold Cycle) method. As endogenous control, 18S was used to normalise for the cDNA of each sample. The ΔCt for each of the triplicates were calculated. When a single value within each triplicate differed substantially from the other two values the skewed value was excluded from the analysis. To calculate
ΔΔCt, the ΔCt of each sample was subtracted to the chosen reference sample (usually a synovia from an OA patient or a salivary gland from a NSCS patient). The relative quantity was then calculated following the equation 

$$RQ = 2^{\Delta\Delta C_{t}}$$

where 2 represents doubling of the amount of the product of amplification after each PCR cycle. In order to assess the efficiency of the real-time PCR for each gene, a standard curve was prepared by serial 2^5 dilutions (i.e. 1:1, 1:32 and 1:1024) of the cDNA from a positive control tissue (tonsil). Optimal PCR efficiency must have a difference of 5 Ct between each dilution.

### 3.4.6 Quantitative Taqman real-time evaluation of gene expression in SS salivary glands and RA synovia

Total RNA was extracted from RA and OA synovia and SS and NSCS salivary glands using the Quiagen RNeasy Mini Kit (Quiagen) and reverse transcribed to cDNA using Thermoscript Kit (Invitrogen) as described above. The following target genes were evaluated: CXCL13, AID and Blimp-1 in the salivary glands and CXCR13 and AID in the synovial tissue. CXCL13 was chosen as previous work from my group demonstrated that the expression of this lymphoid chemokine in the target tissue closely segregates with the presence of ELS. Similarly, AID was shown to be a sensitive and specific marker of functional ectopic GC-like structures. Finally, Blimp-1 is a transcription factor specifically expressed in plasma cells and was thus used as a marker of plasma cell infiltration within the tissues.

All primers and probes were obtained from Applied Biosystem and the complete list is reported in table 3.4. Samples were run in triplicate at 10 ng of cDNA/well, detected using the ABI PRISM 7900HT Instrument and results
analysed using the ABI PRISM 7900HT Sequence Detection System Version 2.1. Relative quantification was assessed using the Comparative Ct Method calculated as described above. Mammalian 18S or human β-actin were used as endogenous control to normalize for the cDNA of each sample. cDNA from a tonsil was used as a positive control.

3.4.7 Pre-amplification of specific genes and quantitative PCR

Analysis of EBV genes (EBER, LMP2A, and EBNA1) was performed by quantitative real-time RT-PCR after the selective pre-amplification of target genes. In addition to viral genes, GAPDH as housekeeping gene and CD19 as control for the presence of infiltrating B cells were also analysed after selective pre-amplification.

The pre-amplification step increase the quantity of the desired cDNA targets for gene expression, helping to avoid false negative results while analysing low expressed genes. Since EBV-infected B cells likely represent a small fraction of the total RNA extracted from the resident and inflammatory cell populations within synovia/salivary glands, it should be suspected that detectability of viral nucleic acids in whole synovia/salivary glands tissue might be well below the sensitivity of conventional real-time PCR techniques. Thus, cDNAs were pre-amplified as previously described (268) using the PreAMP Master Mix Kit (Applied Biosystems) according to manufacturers’ instructions. Each preamplification reaction was prepared in a 0.2 ml tube mixing 25 µl of TaqMan PreAmp MasterMix (2 X), a mix of 90 nM forward and reverse primers and 120 ng cDNA in a final volume of 50 µl.
After mixing, samples were run in a PCR machine (Applied Biosystem 9700) for 10 cycles by using the following thermal cycling conditions: 10 min at 95°C for 15 sec and 60°C for 4 minutes. Preamplification products were diluted 1:5 in DNase-free H2O and analysed in duplicates by quantitative PCR assays using the ABI PRISM 7900HT machine (Applied Biosystems). Each PCR reaction was prepared in a final volume of 10 µl containing 2 µl of pre-amplified cDNA in the presence of 5µM primers’ mix, hot-start DNA polymerase (Quiagen), 0.1X SYBR green (Sigma-Aldrich) and 0.2X ROX dye (Invitrogen). The thermal cycling conditions used were: 2 minutes at 50°C to activate the UDG enzyme, 15 minutes at 96°C, 40 cycles at 96°C for 15 sec and 60°C for 1 minute.

3.4.8 Quantitative real-time evaluation of gene expression after selective preamplification in RA synovia and SS salivary glands

RNA extracted from RA and OA synovia was pre-amplified and then analysed by quantitative RT-PCR assays as described above. The following target genes were evaluated: CD19, LMP2A, EBNA1, EBER, and GAPDH. The primers used were designed and previously tested in the laboratory of Dr. Eliana Coccia (ISS, Rome, Italy) and primers sequences are listed in table 3.4. PCR products derived from cDNA of EBV+ P3HR-1 cells and human primary B cells were included in each run as positive controls for EBV and CD19 gene expression respectively, together with a negative control (positive control in the absence of RT enzyme). Values were normalized to GAPDH and calculated using the 2-ΔCt formula. The experiments in RA and OA tissues were performed during my stay in the laboratory of Dr. Eliana Coccia, ISS, Rome, Italy.
Conversely, detection of the same target genes in the salivary glands of SS and NSCS were performed in the EMR lab using the same technique and primers. RNA extracted from SS and NSCS salivary glands was pre-amplified and then analysed by quantitative RT-PCR assays as described above. The following target genes were evaluated: CD19, EBER1 and GAPDH. cDNA from HRCS57 and Ramos cells line (a kind gift of Dr. Emanuela Carlotti, Institute of cancer, QMUL) was used as a positive and negative control for EBV, respectively. cDNA from tonsil was used as positive control for CD19 expression.

Relative quantification was assessed using the Comparative Ct Method calculated as described above.
### Table 3.4 Primers used in real-time RT-PCR

**-From Applied Biosystem-**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>RefSeq</th>
<th>Gene Expression Assay ID</th>
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<tr>
<td>AID</td>
<td>NM_020661</td>
<td>Hs00221068_m1</td>
</tr>
<tr>
<td>CXCR13</td>
<td>NM_006410.1</td>
<td>Hs00757930_m1</td>
</tr>
<tr>
<td>BLIMP</td>
<td>NM_001198.3</td>
<td>Hs00153357_m1</td>
</tr>
<tr>
<td>Human 18S</td>
<td>XO3205.1</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>NM_001101</td>
<td>Hs99999903_m1</td>
</tr>
</tbody>
</table>

**-Designed primers-**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP1</td>
<td>5'-CCC TTT GTA TAC TCC TAC TGA TGA TCA C-3'</td>
<td>5'-ACC GAA GAT GAA CAG CAC AAT-3'</td>
</tr>
<tr>
<td>LMP2A</td>
<td>5'-TTC TGG CTC TTC TGG GAA CA-3'</td>
<td>5'-ATT GCC CCA ATC TGA GTC CT-3'</td>
</tr>
<tr>
<td>EBNA1</td>
<td>5'-GGA GCC TGA CCT GTG ATC GT-3'</td>
<td>5'- TAG GCC ATT TCC AGG TCC TGT A-3'</td>
</tr>
<tr>
<td>CD19</td>
<td>5'-AGA ACC AGT ACG GGA ACG TG-3'</td>
<td>5'-CTG CTC GGG TTT CCA TAA GA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ACA GTC CAT GCC ATC ACT GCC-3'</td>
<td>5'-GCC TGC TTC ACC ACC TTC TTG-3'</td>
</tr>
</tbody>
</table>
3.5 Identification of autoreactive plasma cells in RA synovia and SS salivary glands

Autoreactive plasmacells can be identified on a tissue-section using a biotinylated self-antigen against which the plasma cells produce antibodies. The binding of the biotynilated antigen can be visualised either by IHC (using the Elite Vectastain ABC kit or the Dako EnVision System Polymeric Method) or by IF (using a fluorochrome-conjugated Streptavidin). In order to identify the autoreactive plasma cells in RA and SS tissues, double IF using a biotinylated antigen known to be associated with RA or SS was performed. After incubation of the biotinylated antigen, a fluorochrome-conjugated streptavidin was added and followed by incubation with an anti-CD138 antibody as marker of plasma cells. In this study biotinylated citrullinated fibrinogen or biotinylated Ro 52-Kd and La 48-Kd were used to identify the autoreactive plasma cells in RA synovia and SS salivary glands respectively.

3.5.1 Identification of anti-citrullinated protein/peptide antibodies (ACPA)-producing cells in RA synovia

In order to identify the autoreactive plasma cells producing anti-citrullinated protein antibodies (ACPA) in the RA synovia we used a citrullinated biotinylated fibrinogen (CFb) using an in vitro method previously optimised by Dr M. Bombardieri at EMR (85). Plasminogen-depleted human Fb (Calbiochem: EMD BioSciences) was incubated at 0.86 mg/ml with 10 U/ml of rabbit skeletal
muscle peptidyl arginine deiminase (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 7.4) (Appendix I), 10 mM CaCl₂, and 5 mM DTT for 2 h at 50 °C. The enzyme was inactivated by adding 2% SDS and heating at 100 °C for 3 min and removed by serial spinning and washing with 0.01 M sterile bicarbonate buffer in a 100 kDa Amicon filter device (Millipore). CFb was then biotinylated using NHS-LC biotin (Pierce Biotechnology). In addition, an aliquot of unmodified Fb of the same concentration as the CFb was biotinylated to be used as a negative control.

To detect and localize ACPA-producing cells and study their relationship with EBV infection I used high-inflamed and EBV+ paraffin-embedded RA synovial tissue from ACPA+ RA patients. Three µm-thick, deparaffinised tissue sections underwent antigen retrieval with buffer citrate and protein block with the Dako Protein Block Solution. Sections were then incubated with the unconjugated primary antibodies (CD20, CD138 or BFRF1) followed by incubation with the specific secondary antibodies conjugated with ALEXA-488 or -555. Biotinylated CFb was then added at 1:20 dilution, incubated for 1h and followed by incubation with streptavidin ALEXA-488 or -555. Sections were counterstained with DAPI. Biotinylated, unmodified fibrinogen was used as negative control for CFb.

3.5.2 Analysis of anti-Ro 52-Kd and anti-La 48-Kd antibodies-producing cells in SS salivary glands by IHC

Paraffin-embedded ELS+ and EBV+ SS salivary glands from Ro52/SSA+ and La/SSB+ SS patients were stained using the biotinylated Ro 52-Kd and La 48-Kd (a kind gift of Prof. Roland Jonsson at University of Bergen, Norway).
Three µm-thick, deparaffinised tissue sections underwent antigen retrieval with buffer citrate and endogenous peroxidase, avidin, and biotin activity was blocked as described above. After the protein block with the Dako Protein Block Solution, sections were incubated with the biotinylated Ro 52-Kd or La 48-Kd (both diluted 1:20) for 1 hour RT, followed by incubation of biotinylated secondary antibodies and avidin-biotin peroxidase complex (Elite Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The final reaction was visualized with diaminobenzidine and sections were counterstained with Mayer’s haematoxylin.

For the identification of anti-Ro52 and/or anti La 48-Kd producing cells, 3 µm-thick, deparaffinised tissue sections underwent antigen retrieval with buffer citrate and protein block with the Dako Protein Block Solution. Sections were then incubated with the unconjugated primary antibodies (CD20, CD138 or BFRF1) followed by incubation with the specific secondary antibodies conjugated with ALEXA-488 or -555. Biotinylated Ro 52-Kd or La 48-Kd were then added at 1:20 dilution, incubated for 1h and followed by incubation with streptavidin ALEXA-488 or -555. Sections were counterstained with DAPI.
3.6 Serological studies

3.6.1 Detection of ACPA (anti-CCP) antibodies

ACPA were detected in both RA and OA sera and synovial fluid (diluted 1:100) and in sera of SCID mice transplanted with RA synovium (diluted 1:10) using a commercially available anti-cyclic-citrullinated antibody (anti-CCP2) ELISA kit (Axis Shield), following the manufacturer's instructions. Results are expressed as optical density (OD).

3.6.2 Anti-unmodified EBV antibodies

Human IgG antibodies against recombinant unmodified VCA, EBNA1 and EA were detected using commercially available ELISA kits (Serion, Würzburg, Germany), following the manufacturer’s instructions. All 3 antibodies were screened in RA sera and synovial fluid (diluted 1:100) and in mouse sera (diluted 1:10). OA sera and synovial fluid (diluted 1:100) were screened only for anti-EBNA1 antibodies. All results are expressed as optical density (OD). The cut-off was calculated as described in the kits.

3.6.3 Anti-citrullinated EBV antigens antibodies
All SCID mice sera found anti-CCP+ were further screened for the presence of anti-viral citrullinated peptide antibodies VCP1 or VCP2 by ELISA. This set of experiments was performed in the laboratory of Dr. Paola Migliorini (University of Pisa, Italy). Briefly, 10 µg/ml VCP1 or VCP2 was added to polystyrene plates (Nunc MaxiSorp F96; Nunc, Roskilde, Denmark) in PBS and incubated overnight at 4°C. Saturation was carried out with PBS containing 3% bovine serum albumin (BSA) for 45 min at room temperature. Sera diluted 1:50 in PBS containing 1% BSA and 0.05% Tween 20 were then incubated in duplicate for 3h at RT. After washing with PBS 1% Tween and PBS, alkaline phosphatase conjugated anti-human IgG (Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 2 h at RT. Alkaline phosphatase activity was revealed with p-nitrophenyl phosphate in 50 mm Na2CO3 (pH 9.6). All results are expressed as optical density (OD).

3.6.4 Anti-SSA/Ro and anti-SSB/La antibodies

Anti-SSA/Ro and anti-SSB/La antibodies of IgG isotype were measured in mice serum (diluted 1:10) by commercial ELISA Kit (Euro Diagnostica). Cut-off was calculated as described in the Kits. Results are expressed as U/mL.
3.7 Statistical analysis

Differences in quantitative variables were analysed by Mann Whitney U-test or Kruskal-Wallis with Dunn’s post-test when comparing two or multiple groups, respectively. Fisher’s exact test was used to evaluate associations of qualitative variables. All statistical analyses were performed using GraphPad Prism version 3.03 (GraphPad Software, San Diego Ca, USA). A p value <0.05 was considered statistically significant.
CHAPTER 4  RESULTS

Detection of EBV in RA synovia
In my PhD project, I first aimed to clarify the long-standing issue whether EBV protein and nucleic acids are expressed in the RA synovia, performing a comprehensive analysis of EBV antigens/RNA expression using different techniques (i.e. RT-PCR, ISH, IHC and IF) in a large cohort of RA patients. Importantly, each RA synovia was also analysed for the level of inflammation and the presence of ELS by IHC and/or RT-PCR. This is extremely relevant as RA synovitis is a highly heterogeneous inflammatory process in terms of degree of inflammation as well as lymphoid organization, and novel as no one so far attempted to correlate EBV presence with either the level of immune cell infiltration or the presence of GC-like structures. The latter is of pivotal important as it was recently suggested that ELS are preferential niches of EBV persistence and reactivation in chronic autoimmune diseases (72, 73).

4.1 Histological characterization of RA synovial samples

Fifty paraffin-embedded synovial tissue samples from 43 RA patients were first analysed for the global inflammation and synovitis scores determined as described in materials and methods. On the basis of the synovitis score, 2 RA synovia were classified with no synovitis (score 0-3), 18 with low-grade synovitis (4-6) and 30 with high-grade synovitis (7-9). Each sample was first analysed for the presence and size of lymphocytic aggregates (G1, G2, G3) using H&E staining as described in materials and methods. Furthermore, in order to better characterise the degree of cellularity, the predominant infiltrating cell subsets and the presence of ELS, each sample was stained in
sequential sections, for CD20, CD3, CD138, CD68, and CD21 and the presence of each antigen was scored as described in materials and methods and as shown in Fig 4.1. On this basis RA samples were further classified as follow; 12 displayed perivascular synovitis with large B-cell aggregates containing CD21+ FDC networks (ectopic GC) and the typical segregation of B and T cell areas, 18 showed B-cell enriched lymphocytic aggregates without FDC networks (these 2 groups were collectively classified as ELS+), and 20 were characterised by diffuse synovitis with sparse or no B cells and dominant macrophage infiltration (Fig 4.2).

Additionally, CD138+ plasma cells were significantly more abundant in ELS+ synovia compared to diffuse synovitis. Of relevance, while plasma cells mostly localized at the periphery of ectopic B-cell follicles suggesting local differentiation in ELS+ samples, they displayed prevalent perivascular accumulation in samples with diffuse synovitis, rather suggesting recent migration from the peripheral compartment.

In order to enrich the variety of material used in this study (i.e. some EBV antigens cannot be detected on paraffin samples), we also characterised the level of inflammation in 9 RA synovial snap-frozen samples staining for CD20, CD3 and CD138. On the basis of the stainings 4 RA synovia were characterised as ELS+ and 5 as samples with diffuse inflammation devoid of B-cell aggregates.
Figure 4.1

CD20

CD138

CD68 lining

CD68 sub-lining
Figure 4.1 RA synovitis score

The atlas shows representative stainings of each antigen for each score. Each antigen was scored from 0 (no positive cells) to 4 according to the number of stained cells. CD68 scoring was analysed separately for the lining and sub-lining compartments. Bars: 50 µm in all panels.
Figure 4.2

RA synovia with diffuse inflammation (ELS-)

RA synovia with aggregates (ELS+) but without FDC networks

RA synovia with aggregates (ELS+) and FDC networks
Figure 4.2 Classification of RA synovial samples according to their synovial pathotype

RA synovial sections were immunostained for CD20 (B cells), CD3 (T cells), CD138 (plasma cells), CD68 (macrophages) and CD21 (follicular dendritic cells) and classified as: i) ELS- with diffuse inflammation, mainly characterised by macrophage infiltration, with sparse or no B and plasma cells and negative for CD21. ii) ELS+ without FDC, characterised by variable number and dimension of B/T cell aggregates with surrounding plasma cells, variable macrophage infiltration but negative staining for CD21. iii) ELS+ with FDC networks characterised by numerous and large B/T cell aggregates with surrounding plasma cells, variable macrophages infiltration and positive for CD21. An example of each pathotype is shown. Bars: 50 µm in all panels.
4.2 EBV latent antigens are expressed by B cells and are strictly associated with ELS formation in the RA synovia

Among the paraffin-embedded RA synovial samples characterised for the level of inflammation and presence of GC-like structures, 38 were selected for the IHC screening of EBV latent antigens (LMP1 and LMP2A) in single staining and in double immunofluorescence with B-cell specific marker (CD20 and CD79a) in order to identify whether infiltrating B cells express EBV latent antigens in the RA synovia. 11 OA were selected as disease control. The specificity of all anti-EBV antibodies used in this study was previously tested in EBV+ and EBV- tissues and cell lines (72, 73). However, positive and negative control tissues were included in all cases.

LMP2A expression was at first analysed by single IHC or IF in combination with single staining for CD20, CD3, CD138 and CD21 in sequential sections (Fig 4.3 A-G). LMP2A expression mostly localised in the B cell area of B cell-follicles. By double IF for CD20/LMP2A (Fig 4.3 H) I could confirm that LMP2A was mainly expressed by B cells inside B cell aggregates. Rarely did I observe few LMP2A+/CD20- cells, which were identified as plasma cells on the basis of their morphology (Fig 4.3 I). Quantitative analysis demonstrated that LMP2A was expressed inside B-cell aggregates in all RA synovial samples with ectopic GC (n = 10) and 7 out of 14 samples containing B-cell aggregates without FDC (Fig 4.3 table). The percentage of CD20+ B cells expressing LMP2A varied among different B-cell aggregates, ranging from 8.2% to 54.7% (mean±SD, 19.3±12.8%). Outside B-cell aggregates, a very small proportion of CD20+ B cells (3±6%) co-expressed LMP2A. All 14 RA samples containing diffuse immune infiltrates and sparse B cells were negative for LMP2A (Fig 4.3 J and
table). All OA sections stained as a negative control tissue also did not express LMP2A (Fig 4.3. K).

In selected RA synovial samples with ectopic GC (n=4) I also performed immunostaining for LMP1, another viral membrane protein expressed during the growth and default programs of EBV latency (2). Similarly to LMP2A, LMP1 immunoreactivity was mainly present inside B-cell aggregates. Double stainings for LMP1 and the B-cell marker CD79a confirmed that LMP1 was expressed in B cells (Fig. 4.3 L).

I also analysed 9 snap-frozen samples for LMP2A and LMP1 expression by immunofluorescence. LMP2A was further analysed in double IF with CD20. As expected and as observed with paraffin-embedded RA synovia, the latent antigens of EBV were mostly expressed by B cells within B cell follicles of ELS+ RA synovia and virtually absent in RA synovia characterised by diffuse/no B cell inflammation.
Figure 4.3

<table>
<thead>
<tr>
<th>RA synovia</th>
<th>N</th>
<th>LMP2A+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELS+ FDC+</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>ELS+ FDC-</td>
<td>14</td>
<td>50%</td>
</tr>
<tr>
<td>diffuse</td>
<td>14</td>
<td>0%</td>
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Figure 4.3 Detection of the EBV latency proteins LMP2A and LMP1 in RA synovia

Almost sequential RA synovial sections, stained in bright field for LMP2A and CD20, show LMP2A+ cells co-localising in the B cell area of B cell aggregates (A-B). Sequential cut RA synovia sections stained for LMP2A in IF and for CD20, CD3, CD138 and CD21 in IF/IHC show that LMP2A expression co-localised in the B cell area of a GC-like structure characterised by B/T cell segregation, surrounding plasma cells and an FDC networks (C-G). Double IF CD20/LMP2A demonstrate that LMP2A is expressed by B cell of a B-cell follicle (H) and the inset in H highlight the typical membrane localization of LMP2A. RA synovia showing few LMP2A+/CD20- cells, identified as plasma blast on the basis of their morphology (I). RA synovia characterised by diffuse B cell infiltration and LMP2A negative (J). OA synovia LMP2A negative (K). Double IF CD20/LMP1 show that LMP1 is expressed by B cells within a B cell follicle (L) and in the inset is highlighted the typical membrane localization of LMP1.

The table shows the percentage of ELS+/FDC+, ELS+/FDC- and diffuse RA synovia LMP2A+. Bars: 50 μm in A,B, E, G, K; 10 μm in C, D, F, H, I, J,L.
4.3 EBER is mostly expressed by B cells in RA synovia and is positively associated with ELS formation

Next I sought to visualize EBV infected cells using in situ hybridization for EBER, the gold standard technique for EBV detection in pathological tissues (269). Paraffin synovial samples from 19 RA patients (15 with aggregate and 4 with diffuse synovitis) and 8 OA cases were analysed. To check for RNA integrity, GAPDH ISH was performed. Fifteen of 19 RA synovial samples and all OA synovial samples displayed a suitable GAPDH signal (Fig 4.4 A). In sections of a tonsil from a case with infectious mononucleosis, which was used as positive control tissue, EBER signals were present at a very high frequency and were localized mainly outside B-cell follicles (Fig. 4.4 B). None of the 5 GAPDH- RA samples was EBER+ while 10 of the 15 GAPDH+ RA samples displayed EBER signals. Among the latter, the highest frequency of EBER+ cells was detected in those containing ELS with ectopic GC and FDC networks (6 EBER+ samples out of 7 analysed) (Fig 4.4 table). Analysis of RA ELS+ samples immunostained for CD20 in combination with EBER ISH revealed that EBER was expressed by B cells inside B cell-follicles as well as CD20- cells localising at the border of the follicles (Fig 4.4 C). It should be noted that the double staining CD20/EBER did not give a clear EBER signal, probably because the strong CD20 signal in dark red cover the blue EBER staining, especially within B cell follicles where many positive CD20 cells accumulate. I therefore preferred to analyse EBER localization in sequential sections. Immunostaining of adjacent sections for CD20, CD138, CD3 and EBER showed that EBER+ cells were located both inside and at the border of intrasynovial B-cell follicles (Fig. 4.4 D-G), around T-cell areas (Fig. 4.4 H, I) and in B-cell-rich perivascular aggregates (Fig. 4.4 J, K).
In RA samples with less organised B-cell aggregates (without FDC), fewer and mainly scattered EBER+ cells were detected in 4 out of 5 biopsies (Fig 4.4 L, M). Conversely, no EBER+ cells were detected in samples with diffuse synovitis (N=3), which generally lacked a substantial B-cell infiltrate (Fig 4.4 N). EBER signals had the expected nuclear localization in the large majority of EBV infected cells (Fig. 4.4 inset in M). No signal was detected in RA synovia using a non-specific probe (Fig. 4.4 O). EBER signals were not detected in any of the 8 OA specimens analysed despite presence of infiltrating B cells and plasma cells in some samples (Fig. 4.4 P).

In summary, the above experiments demonstrated the presence of EBV latency transcripts and virally infected cells in RA synovial samples characterized by well-preserved RNA and aggregated B-cell infiltrates, the highest frequency of EBER+ cells being associated with ectopic GC.
Figure 4.4

<table>
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<tr>
<th>RA synovia</th>
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</tr>
</thead>
<tbody>
<tr>
<td>ELS+ FDC+</td>
<td>7</td>
<td>86%</td>
</tr>
<tr>
<td>ELS+ FDC-</td>
<td>5</td>
<td>80%</td>
</tr>
<tr>
<td>diffuse</td>
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<td>0%</td>
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</table>
Figure 4.4 Detection of EBER+ cells by ISH in the RA synovia

GAPDH ISH on an ELS+ RA synovia shows a good RNA preservation (A). EBER ISH in a tonsil from a patient with infectious mononucleosis (B). RA synovial sample ELS+ immunostained for CD20 in combination with EBER ISH show several EBER+ cells inside and at the border of a B-cell aggregate. The EBER signal is mostly covered by the strong CD20 staining (C). CD20 immunostaining (D) and EBER ISH (E) in serial sections of a RA synovia with ectopic B-cell follicles show a high frequency of EBER+ cells both inside and at the border of B-cell follicles. EBER ISH (F) and CD138 immunostaining (G) performed in serial sections reveal a high frequency of EBER+ cells in an area enriched in plasma cells. CD3 immunostaining (H) and EBER ISH (I) performed in serial sections of a RA synovium with aggregate synovitis reveal that EBER+ cells accumulate around a T-cell aggregate (marked with an asterisk) which is EBER negative. Perivascular accumulation of CD20+ (J) and EBER+ (K) cells in serial sections of a RA synovia with lymphocytic aggregates. Presence of scattered CD20+ cells (L) and EBER+ cells (M) in an area of diffuse immune infiltration in a RA synovium with B-cell infiltration but without FDC. The typical nuclear localization of EBER+ cells (inset in M). Absence of EBER+ cells in a RA synovia characterised by diffuse infiltration (N). Negative control performed by ISH using a no sense probe on a RA synovia ELS+ (O). OA synovial samples immunostained for CD20 in combination with EBER ISH show some CD20+ cells EBER negative (P).

The table shows the percentage of ELS+/FDC+, ELS+/FDC- and diffuse RA synovia EBER+. Bars: 10 µm in A, C, E-G, I-M, P; 50 µm in B, D, H, N, O.
4.4 The presence of EBV latent transcripts is associated with the level of AID, CD19 and CXCL13 gene expression in RA synovia

Search for EBV transcripts in RNA later-preserved RA synovial samples was performed using a highly sensitive and specific quantitative real time RT-PCR including a cDNA pre-amplification step that selectively enriches for the genes of interest (268). Thirteen RA synovial samples with optimal RNA quality were selected for this set of experiments. Of these, 9 samples were classified as ELS+ aggregate synovitis based on histological and immunohistochemical characterization, presence of AID RNA and higher frequency of CXCL13 and CD19 transcripts, and 4 samples were classified as diffuse synovitis, being devoid of lymphocytic aggregates, a detectable AID signal and substantial CXCL13 and CD19 gene expression (Fig. 4.5 A, B). Four synovial tissues from patients with established osteoarthritis (OA) were used as control.

Three genes expressed during EBV latent infection (EBNA1, LMP2A, and EBER1) were analysed. The frequency of CXCL13, CD19, EBER1 and LMP2A transcripts was found to be significantly higher in RA samples with ELS than in those with diffuse synovitis (Fig. 4.5 A-D). EBNA1 transcripts were not detected. The 4 OA samples were negative for CXCL13 and all EBV transcripts and CD19 was detected only in the most inflamed OA case (Fig. 1 A-D). These findings further confirm at mRNA level the association between the degree of B-cell infiltration, lymphoid organization and EBV infection in the RA synovium.
Figure 4.5 Detection of EBV transcripts in the RA synovia

Quantitative real-time RT-PCR reveals a higher expression of the CXCL13 gene in ELS+/AID+ RA synovia compared to diffuse/AID- RA synovia or OA synovia (A). Pre-amplification of selected genes followed by real time RT-PCR shows that CD19 (B), EBER (C) and LMP2A (D) gene expression is significantly higher in ELS+AID+ than in diffuse/AID- RA samples. A low level of CD19 mRNA, but no latent viral transcripts, can be detected in OA synovia.

*p<0.05, ** p<0.01
4.5 EBV reactivation occurs in plasma cells and is associated with ELS formation in the RA synovia

To investigate whether EBV also reactivate in the RA synovia, I first stained 34 RA synovial samples for BFRF1, an antigen associated with the early phase of the viral lytic cycle (270). In details, I performed both IHC staining on sequential sections for CD20, CD138 and BFRF1 and double IF for CD138/BFRF1 on RA paraffin-embedded synovial tissues. All 7 RA synovia with ectopic GC contained cells were immunopositive for BFRF1 (Fig. 4.6 table). Differently from LMP2A+ and LMP1+ cells, BFRF1+ cells mainly localized at the periphery of B-cell follicles and in the interfollicular areas and were identified as CD138+ plasma cells (Fig 4.6 A-E). CD138-/BFRF1+ cells were rarely observed. The percentage of CD138+ plasma cells expressing BFRF1 in ELS+ RA cases with ectopic GC ranged between 3 and 23% in individual microscopic fields (mean 10.1±7.3%, mean ± SD). Only a few BFRF1+ plasma cells either isolated or in small clusters, were detected in 5 out of 14 RA samples with B-cell aggregates without FDC (Fig. 4.6 F and table). No BFRF1 immunoreactivity was detected in 13 RA synovia with diffuse inflammation (Fig 4.6 G and table). As expected, all OA synovia analysed were BFRF1 negative (Fig 4.6 H).

In selected RA synovia with ectopic GC (n=6) I also analysed the expression of BMRF1, another viral lytic antigen. Performing double stainings CD138/BMRF1 I found that BMRF1, similarly to BFRF1, was mainly expressed by peri-follicular plasma cells (Fig 4.6 I and inset).

I also analysed 9 snap-frozen samples for BFRF1 expression performing double IF for BFRF1/CD138. As expected and as observed with paraffin-embedded RA synovia, BFRF1 was mostly expressed by plasma cells outside B cell follicles of 3
out of 4 ELS+ RA synovia and absent in all 5 RA synovia characterised by diffuse/no B cell inflammation. These observations strongly suggest that EBV reactivation in the RA synovium is associated with highly organised B-cell follicles and an ongoing GC reaction which allows the in situ differentiation of plasma cells.

To understand whether EBV reactivation in the RA synovium could lead to production of mature viral particles, expression of the EBV structural proteins gp350/220 and p160 was analysed in 6 BFRF1+ RA samples with ectopic GC. Only two small clusters of gp350/220+ cells and p160+ cells were detected in 2 different samples (Fig. 4.6 J, K). These findings suggest abortive EBV reactivation or rapid elimination of productively infected cells.
Figure 4.6

<table>
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<th>RA synovia</th>
<th>N</th>
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</tr>
</thead>
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</tr>
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<td>ELS+ FDC-</td>
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<td>35%</td>
</tr>
<tr>
<td>diffuse</td>
<td>13</td>
<td>0%</td>
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</table>

A. CD20
B. CD138
C. BFRF1
D. CD138
E. CD138
F. CD138
G. CD138
H. CD138
I. CD138
J. CD138
K. Gp250/320
L. p160
Figure 4.6 Detection of EBV lytic proteins BFRF1 and BMRF1 in RA synovia

The table on top shows the percentage of BFRF1+ samples among ELS+/FDC+, ELS+/FDC- and diffuse RA synovia. Representative immunostainings for CD20 (A), CD138 (B) and BFRF1 (C) performed in serial sections of a RA synovial sample with ectopic B-cell follicles reveals that both plasma cells and BFRF1+ cells localize at the B-cell follicle border. The inset in C highlights the typical cytoplasmic localization of BFRF1. Sequential sections show numerous plasma cells accumulating around a large B cell follicle (D) and many of them displaying reactivity against BFRF1 (E). A few CD138+/BFRF1+ cells are detected among scattered CD138+ plasma cells in a RA synovial sample with aggregates devoid of FDC (F). A RA synovia characterised by diffuse inflammation show no reactivity for BFRF1 (G). An OA samples BFRF1 negative is also shown (H). Double IF for CD138/BMRF1 performed on a RA synovia ELS+/FDC+ show several plasma cells displaying reactivity against BMRF1+ (I); those plasma cells localise just outside a B cell follicle, identified by sequential staining for CD20 (inset in I). Immunohistochemistry for the EBV late lytic proteins p160 (J) and gp350/220 (K) reveals two groups of productively infected cells in 2 different RA synovial samples with ectopic GC. The inset in K shows few gp350/220+ cell at high power magnification. Bars: 50 µm in A-D, H, K; 10 µm in E-G, I, J.
4.6 Ongoing cytotoxic activity at sites where EBV lytically infected cells accumulate in the RA synovia

Since CD8+ T cells play a key role in the control of EBV by eliminating infected cells and respond vigorously to viral lytic antigens (271), I investigated the relationship between CD8+ T cells, B cells and EBV infected cells in 13 RA synovial samples (3 ELS+/FDC+, 2 ELS+/FDC- and 8 diffuse). As reported previously (272), CD8+ T cell infiltration was much more abundant in ELS+ RA synovia than in those lacking ELS (Fig 4.7 graph a). However, double immunofluorescence stainings for CD8 and CD20 revealed that in ELS+ RA samples CD8+ T cells were mostly excluded from B-cell follicles and infiltrated the surrounding plasma cell-rich area (Fig. 4.7 A, B). Conversely, in RA synovia with smaller B-cell aggregates or diffuse synovitis CD8+ T cells were intermingled with B cells (Fig. 4.7 C, D). To visualize possible interactions between intrasynovial CD8+ T cells and EBV lytically infected cells, I performed double immunofluorescence for CD8 and BFRF1. Only occasional contacts between CD8+ and BFRF1+ cells were observed (Fig. 4.7 E).

In order to establish whether intrasynovial CD8+ T cells displayed cytotoxic activity, I analysed the expression of granzyme B, a serine protease whose expression is restricted to cytotoxic lymphocytes and correlates with functional cytolytic activity (273). As expected, a significantly higher number of granzyme B+ cells were found in ELS+ compared to ELS- RA samples (Fig 4.7 graph b). Although granzyme B+ cells were also present in perifollicular areas populated by BFRF1+ cells, physical contacts between EBV lytically infected cells and granzyme B+ cells were rarely observed (Fig. 4.7 F).

By performing double immunofluorescence stainings for CD8 and granzyme B I observed that the lytic enzyme was expressed in both CD8+ and CD8- cells (Fig.
However, the percentage of CD8+ cells co-expressing granzyme B was more than 2-fold higher in EBV- diffuse synovitis than in EBV+/ELS+ RA samples (Fig 4.7 graph c). These findings indicate that the majority of CD8+ T cells infiltrating ELS+ synovia do not display cytotoxic activity, as previously suggested (274). Accordingly, the percentage of CD8+/granzyme B+ cells relatively to the total granzyme B+ cell population was significantly higher in ELS-/EBV- RA samples compared to ELS+/EBV+ RA samples (Fig. 4.7 G, H and graph d). Since granzyme B is expressed in the cytoplasmic granules of cytotoxic T cells and NK cells, I next investigated if the numerous CD8-/GranzymeB+ cells in ELS+ RA synovia were NK CD56+ cells. Therefore I performed double staining for CD56/Granzyme B on selected (n=4) ELS+ RA synovia characterised by a high number of CD8-/GranzymeB+ cells. Surprisingly, only 3% of the total granzyme B+ cells were CD56+ too (Fig 4.7 I). Since previous studies (275-278) observed that in chronic viral infections, including EBV and in some chronic inflammatory diseases, including RA, the CD4+ T cells can acquire lytic activity, I next analysed the level of inflammation and the lytic state of CD4+ T cells in ELS+ versus ELS- RA samples. I found that CD4+ T cell infiltration was more abundant in ELS+ RA synovia compared to those lacking ELS (Fig 4.7 graph e). Of relevance, the percentage of GrB+/CD4+ T cells among GrB+ cells was significantly higher in ELS+EBV+ RA samples compared to EBV- samples and samples with diffuse synovitis (p<0.001) (Fig. 4.7 J, K and graph f). These findings suggest that in EBV+/ELS+ RA synovia, but not in diffuse synovitis, a large subset of CD4+ T cells acquire cytolytic activity.
Figure 4.7
Figure 4.7 Distribution and frequency of cytotoxic cells relatively to EBV infected cells in the RA synovia

CD8+ cells in ELS+ synovia do not enter large B-cell follicles and localize mainly in plasma cell areas (A,B). The distribution of CD138+ cells is shown in the inset of panel A. Conversely, in ELS- RA synovia CD8+ cells are found intermingled with B cells (C, D). BFRF1+ and CD8+ cells at the border of a B-cell follicle in ELS+ RA synovium. Only rarely, CD8+ cells were observed in direct contact with BFRF1+ cells (E and inset). Granzyme B+ cells in a perifollicular area where EBV lytically infected cells are also present (F). The inset in F shows cell-cell contact between a granzyme B+ cell and a BFRF1+ cell. CD8+ cells are mainly granzyme B negative in ELS+ RA synovium (G). Instead, in ELS- RA synovium a high percentage of CD8+ cells express granzyme B (H). In ELS+ RA synovia Granzyme B+ cells do not express CD56 (I). In ELS+ RA synovium many granzyme B+ cells are CD4+ (J and inset) whereas in ELS- RA synovium the majority of CD4+ cells are granzyme B-negative (K). Bars: 50 µm in A-C; 10 µm in D-K.

The absolute number of CD8+ cells is significantly higher in ELS+ compared to ELS- RA synovium (graph a). The absolute number of Granzyme B+ cells is significantly higher in ELS+ than ELS- RA synovium (graph b). The percentage of CD8+/Granzyme B+ cells on the total CD8+ cells is significantly higher in ELS- than ELS+ RA synovium (graph c). The percentage of CD8+/Granzyme B+ cells on the total Granzyme B+ cells is significantly higher in ELS- than ELS+ RA synovium (graph d). The absolute number of CD4+ cells is significantly higher in ELS+ than ELS- RA synovium (graph e). The percentage of CD4+/Granzyme B+ cells on the total Granzyme B+ cells is significantly higher in ELS+ than ELS- RA synovium (graph f). *p<0.05, **p<0.01, ***p<0.001
CHAPTER 5   RESULTS

Detection of EBV in SS salivary glands
EBV has been previously associated with ELS in MS and MG (72, 73) and I confirmed a strong link between EBV and ELS in RA (Chapter 4). Thus, the concept emerging from these data is that EBV may not be associated to a specific autoimmune disease, but that a dysregulated EBV infection is a common feature of different autoimmune diseases characterised by B cell-dysregulation and formation of ELS.

To better validate this hypothesis I next extended the analysis of EBV infection to another chronic autoimmune disease characterised by ELS formation in the target organs, the salivary glands of Sjogren’s syndrome. For this purpose, similarly to RA, I performed a deep analysis of the level of inflammation/presence of ELS in each SS salivary glands analysed, both by IHC and RT-PCR and analysed the status of EBV latent and lytic infection by different techniques (i.e. IHC, IF, ISH and real-time RT-PCR), focusing on the relationship between EBV expression/reactivation and ELS formation.

5.1 Histological characterization of SS salivary glands

Salivary glands from 13 SS and 5 NSCS patients were stained for the presence of CD20, CD3, CD138 and CD21 and each antigen was scored as described in materials and methods. CD68 staining was not performed on SS salivary glands since it was previously observed in the RA synovia that macrophages infiltration was mostly unrelated with the status of EBV infection.

Among the 13 SS salivary glands analysed, 6 (46.1%) were characterised by the presence of large B-cell enriched lymphocytic aggregates which displayed FDC networks, while 5 (38.4%) showed smaller lymphocytic aggregates without FDC
and 2 (15%) were characterised by diffuse inflammation with sparse or no B cells (Fig 5.1).

Among 5 NSCS salivary glands analysed 2 showed small lymphocytic aggregates of less than 50 cells and 3 showed diffuse inflammation with no/sparse B cells. Notably none of the SS NSCS salivary glands analysed was FDC+.

I observed a typical B/T cell segregation with surrounding plasma cells in most of the aggregates with FDC networks and in some of the FDC- ones.
Figure 5.1

**SS salivary glands with diffuse inflammation (ELS-)**

**SS salivary glands with aggregates (ELS+) without FDC+ networks**

**SS salivary glands with aggregates (ELS+) and FDC+ networks**
Figure 5.1 Immunohistochemical characterization of the inflammatory infiltrates in SS salivary glands

Sequential sections of SS salivary glands were stained for CD20, CD3, CD138 and CD21 and samples were classified as: i) with diffuse inflammation with no or sparse B cells; ii) ELS+ FDC- (characterised by B/T cell aggregates, surrounding plasma cells without FDC network; iii) ELS+FDC+ (characterised by B/T cell aggregates, surrounding plasma cells and FDC network). An example of each pathotype is shown. Bars: 50 µm in all panels.
5.2 EBV latent antigens are mainly expressed by B cells in SS salivary glands and are strictly associated with ELS formation

I next analysed the status of EBV latent infection in SS salivary glands by immunohistochemistry for LMP2A expression. Since EBV is mainly expressed by B cells and/or plasma cells, we first selected 4 SS salivary glands ELS+/FDC+ and stained serial sections for LMP2A and CD20. LMP2A+ cells were detected in all ELS+ samples and localised in the B cell-rich areas (Fig 5.2 A, B).

Next I performed double staining for CD20/LMP2A in all 13 SS samples and confirmed that LMP2A was mainly expressed by B cells (Fig 5.2 C and D). LMP2A positive B cells were found in 100% of ELS+/FDC+ (Fig 5.2 C and table) and ELS+/FDC- (Fig 5.2 D and table) SS salivary glands, mostly in B cell-aggregates. Conversely samples with sparse or no B cells did not display CD20+/LMP2A+ cells (Fig 5.2 E and table). Similarly, I did not detect CD20+/LMP2A+ cells in any NSCS salivary glands (Fig 5.2 F).

A second pattern of LMP2A+ expression outside CD20+ B cell areas was also observed in both SS and NSCS salivary glands. In this context, LMP2A seemed to be mainly expressed by ductal/acinar epithelial cells; thus I performed double IF staining for a cytokeratin marker and LMP2A in all SS and NSCS samples. By these means I could observe LMP2A expression in all SS samples analysed and mostly in ductal epithelial cells, although with a rather low prevalence (<10 cells/sample) (Fig 5.2 G, H and table). Also 4 out of 5 NSCS samples displayed few LMP2A+/cytokeratin+ cells.

In selected SS salivary glands with ectopic GC (n=4) I also performed immunostaining for LMP1, and as observed for LMP2A, this EBV latent antigen was mostly expressed by B cells inside B-cell aggregates but also by few ducts/acin cells. Double staining for LMP1 and the B-cell marker CD79a
confirmed that LMP1 was expressed by B cells mostly inside B-cell follicles (Fig 5.2 I).
Interestingly, the presence of either LMP1+ and/or LMP2A+ ductal epithelial cells was not associated with the formation of periductal inflammatory infiltrates.
SS salivary glands  | N  | B cells LMP2A+ | Epithelial cells LMP2A+
---|---|---|---
ELS+ FDC+  | 6  | 100% | 100%
ELS+ FDC-  | 5  | 100% | 100%
diffuse    | 2  | 0%   | 100%
**Figure 5.2 Detection of LMP2A+ and LMP1+ cells in SS salivary glands**

Sequential sections from ELS+ SS salivary glands stained for CD20 and LMP2A show LMP2A+ cells co-localising in the B cell area of a B–cell follicle (A, B). A representative B cell follicle, with CD21+/FDC networks as demonstrated by sequential staining (inset in C) is extensively LMP2A+ (C). Some B cells LMP2A+ in a B cell aggregate FDC- (D). In the inset in D a high power magnification of a CD20+/LMP2A+ cell is shown. A SS salivary gland characterised by diffuse inflammation is LMP2A negative (E). A salivary gland from a NSCS patient is LMP2A negative (F). Few LMP2A+ epithelial cells in a SS (G) and a NSCS (H) salivary gland. Double IF for CD79a/LMP1 in an ELS+FDC+ SS salivary gland shows LMP1+ B cells in a small aggregate (I).

The table shows the percentage of LMP2A+ SS salivary glands among samples with ELS+FDC+, ELS+FDC- and diffuse inflammation. Bars: 10 µm in all panels.
5.3 EBER is mainly expressed by plasma cells and B cells and is associated with ELS formation in SS salivary glands

In order to confirm the presence of EBV latency in SS salivary gland, I next performed ISH for EBER transcripts. I first performed ISH for GAPDH on 9 SS (5 ELS+/FDC+, 3 ELS+/FDC- and 1 with diffuse inflammation) and 5 NSCS salivary glands (2 with small aggregates and 3 with diffuse inflammation) in order to confirm good RNA preservation. All samples analysed showed a good GAPDH signal (Fig 5.3 A) and were therefore analysed for EBER expression by ISH. EBER was expressed in 8 out of 9 and in 1 out of 5 of the SS and NSCS salivary glands respectively (Fig 5.3 table). Of relevance, the only SS sample which was found EBER- was the one characterised by diffuse inflammation (Fig 5.3 B and table). Conversely, the only NSCS sample found EBER+ also displayed small B cell follicles.

Staining sequential sections of ELS+ SS salivary glands for EBER/CD20/CD138/CD3 I observed that EBER+ cells mostly accumulate in the B/T/plasma cell-rich areas, with maximal enrichment around ectopic B cell follicles (Fig 5.3 C-F). Less frequently and less numerous EBER+ cells were observed in area of diffuse B/plasma cell infiltration in ELS+ samples (Fig 5.3 G-I). In order to identify the cells expressing EBER I analysed 2 ELS+ SS samples, combining EBER in situ hybridization with immunohistochemistry for CD20 or CD138. Using this approach, I could observe that both CD138+ plasma cells (Fig 5.3 J) and CD20+ B cells (Fig. 5.3 K and inset) expressed EBER. Interestingly, I could not detect any epithelial cells EBER+, despite LMP2A/LMP1 positivity (Fig 5.3 L). This suggests that epithelial cells in the salivary glands express a different latent EBV programme compared to infiltrating B cells.
Finally, no EBER signal was detected in SS or NSCS salivary glands using a non-specific probe (Fig. 5.3 M).
Figure 5.3

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Figure 5.3 Detection of EBER+ cells in SS salivary glands

GAPDH ISH on a representative SS salivary gland showing good RNA preservation and uniform signal (A). A representative SS salivary gland characterized by diffuse inflammation is EBER negative (B). Serial sections of ELS+FDC+ SS salivary gland stained for CD20 (C), CD21 (inset in C), CD3 (D), CD138 (E) and EBER (F) show an accumulation of EBER+ cells in an area with a B-cell follicle and FDC networks surrounded by numerous T cells and plasma cells. Serial sections of an ELS+ FDC- SS salivary gland stained for CD20 (G), CD138 (H) and EBER (I) show EBER+ cells accumulating in the same area where plasma cells accumulate. A double staining for CD138/EBER on ELS+ SS salivary gland confirms that most EBER+ cells are plasma cells (J) while a minority display double EBER/CD20 staining demonstrating that a subset of B cells also expresses EBER (K). In the inset in K a large magnification of a CD20+/EBER+ cell is shown. Double staining for CD20/EBER show some EBER+ cells accumulating around a duct which is EBER negative (L). No signal was detected in SS salivary glands using a non-specific probe (M).

The table shows the percentage of EBER+ salivary glands among SS samples with ELS+FDC+, ELS+FDC- and diffuse inflammation. Bars: 50 µm in all panels.
5.4 The expression of EBV latent transcripts correlates with the level of CD19, AID, and BLIMP mRNA in SS salivary glands

I next aimed to confirm the presence of EBV latency in SS salivary glands at transcript level and its correlation with the B cell and plasma cell content. For this purpose I analysed the mRNA expression of EBV latent genes EBER1/EBER2, a gene marking B cell and plasma cells (CD19) as well as markers of functional B cell activation (AID), plasma blasts/cells differentiation (Blimp) and a master regulator of ELS formation (CXCL13). EBV latent genes and CD19 expression were detected using a highly sensitive and specific quantitative real time RT-PCR which includes a cDNA pre-amplification step that selectively enriches for the selected genes. Conversely, conventional TaqMan RT-PCR with no pre-amplification step was used for AID, CXCL13 and Blimp quantitative mRNA assessment.

Twenty-nine SS salivary glands with optimal RNA quality were selected for this analysis. RNA from 4 NSCS salivary glands was used as disease control. Furthermore, RNA from HRC57 and Ramos cell lines were used as positive and negative controls for EBER genes, respectively. Finally, RNA from a human tonsil was used as positive control for AID, CXCL13 and BLIMP expression.

Thirty-one out of 33 samples (27 SS and 4 NSCS) displayed a good GAPDH signal and were selected for further analysis. Variable level of CD19 expression was detected among the SS samples analysed (Fig 5.4 A). A significantly lower average level of CD19 expression was detected among the 4 NSCS samples analysed (Fig 5.4 A). As expected, I observed a significant positive correlation between the level of CD19 expression and AID positivity (Fig 5.4 B). Detectable levels of EBER were observed in 21 (77%) of the SS samples analysed respectively. EBER expression significantly correlates with the level of AID and
CD19 mRNA (Fig 5.4 C-D). EBER signal showed also a significant correlation with Blimp mRNA expression (Fig 5.4 E), a plasma cell marker, confirming the ISH data indicating predominant EBER expression within infiltrating perifollicular plasma cells. Finally the expression of EBER was positively associated with the level of CXCL13 expression (Fig 5.4 F), confirming that EBER expression is closely associated with the formation of ELS in SS salivary glands. Interestingly, only 1 out of 4 NSCS samples displayed barely detectable EBER positivity in the context of a low level of CD19 expression (Fig 5.4 A).

A 1% agarose gel was used to confirm specificity of the EBER, CD19 and GAPDH amplificons (Fig 5.4 G). As expected, specific PCR products of 167 bp (EBER), 115 bp (CD19) and 266 bp (GAPDH) were observed.

Overall, these data confirmed that EBV latency in SS salivary glands is strictly associated with ELS formation, B cell infiltration and plasma cell differentiation.
Figure 5.4
Figure 5.4 Detection of EBER transcripts in SS salivary glands by quantitative TaqMan RT-PCR

Significantly higher levels of CD19 mRNA are expressed in SS respect to NSCS salivary glands (A). CD19 (B) and EBER (C) gene expression positively correlate with the level of AID expression. EBER gene expression also significantly correlates with the level of CD19 (D) and BLIMP-1 (E). EBER expression was positively associated with the level of CXCL13 (F). 1% agarose gel showing EBER, CD19 and GAPDH PCR products confirms specificity of the PCR on ELS+ and ELS- SS salivary glands. Band of the correct size were observed for all the genes analysed. Higher level/frequency of CD19 and EBER but not GAPDH gene expression were detected in ELS+ compared to ELS- SS salivary glands (G).

*** p<0.001; ** p<0.01; *p<0.05
5.5 **EBV reactivation occurs in plasma cells and is associated with ELS formation in SS salivary glands**

I next analysed whether EBV also displays reactivation in the SS and NSCS salivary glands, performing double IF for CD138/BFRF1 on 13 SS and 4 NSCS salivary glands. I observed numerous BFRF1+ cells (>20+ cells/section) in all ELS+FDC+ SS samples (Fig 5.5 A and table) and few BFRF1+ cells (< 5 cell/section) in 4 out of 5 ELS+FDC- SS samples (Fig 5.5 B and table). All SS samples with diffuse inflammation were BFRF1 negative (Fig 5.5 C and table). Among the 4 NSCS samples one displayed reactivity against BFRF1, although only rare positive cells were identified in the whole section (Fig 5.5 D). Notably, the only NSCS sample BFRF1+ was the one displaying some small B cell follicles, albeit this patient did not fulfil Chisholm and Mason criteria for histological diagnosis of SS.

BFRF1+ cells were invariably identified as CD138+ plasma cells (Fig 5.5 A, B, D) and mostly localised at the periphery of B cell aggregates (Fig 5.5 E-F).

In order to confirm the lytic reactivation of EBV in SS salivary glands I next investigated the expression of another early lytic EBV antigen, BMRF1, in 3 selected ELS+FDC+ SS samples performing double IF for CD138/BMRF1. As expected, I observed BMRF1+ cells in all the samples analysed mostly localising at the border of B cell aggregates and co-localizing with CD138 (Fig. 5.5 G).

Overall, these observations strongly suggest that EBV reactivation in the SS salivary glands is dependent of in situ plasma cell differentiation within highly organised B-cell follicles in an ongoing GC reaction.
Figure 5.5

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<td></td>
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Figure 5.5 Detection of BFRF1+ and BMRF1+ cells in SS salivary glands

Numerous CD138+/BFRF1+ cells are observed in ELS+FDC+ SS salivary glands (A) compared to ELS+/FDC- SS salivary glands (B). Rare plasma cells BFRF1 negative are also observed in one SS sample lacking B cell follicles (C). One single sample of NSCS salivary glands displays 2 BFRF1+ plasma cells (D). Serial sections show BFRF1+ cells (E) accumulating around a B cell follicle (F) in an ELS+ SS salivary gland. Serial sections show BMRF1+/CD138+ cells (G) accumulating around a B cell follicle (inset in G) in a ELS+FDC+ SS salivary gland, confirming EBV reactivation.

The table shows the percentage of BFRF1+ SS salivary glands among samples with ELS+FDC+, ELS+FDC- and with diffuse inflammation. Bars: 10 µm in all panels.
5.6 Ongoing cytotoxic activity in SS salivary glands preferentially occurs at sites where EBV lytically infected cells accumulate

I next investigated the localization of cytotoxic CD8+ T cells and their possible association with EBV reactivation in SS salivary glands. Performing double immunofluorescence staining for CD8 and CD20 in 13 SS samples I observed that CD8+ T cells mostly localised outside B cell follicles in ELS+ samples (Fig 5.6 A). CD8+ T cells were significantly more abundant in ELS+ compared to ELS- samples (Fig 5.6 graph a). Double IF for CD8/BFRF1 on ELS+/BFRF1+ SS salivary glands (n=10) demonstrated that CD8+ T cells can accumulate in BFRF1+ areas around B cell follicles with seldom direct cell-cell contact with BFRF1+ cells (Fig 5.5 B and inset).

To assess whether CD8+ T cells in SS salivary glands display cytotoxicity I next performed double IF for CD8/Granzyme B on both ELS+ and ELS- SS salivary glands. Interestingly, I observed numerous Granzyme B+ cells in the same areas where BFRF1+ cells accumulate (Fig 5.6 C), revealing ongoing cytotoxic activity at sites of EBV lytic reactivation. Several cell contacts between BFRF1+ and Granzyme B+ cells were observed, suggesting that cytotoxic cells may be acting against EBV-reactivation (Fig 5.6 C inset). However, only a small proportion of CD8+ T cells also expressed granzyme B, and the percentage of cytotoxic double positive Granzyme B/CD8 T cells was significantly higher in ELS- compared to ELS+ samples (Fig 5.6 D-E and graph b).

Conversely, double IF for CD4/Granzyme B on 3 ELS+ SS salivary glands revealed that around half of the infiltrating Granzyme B+ cells were CD4+ (Fig 5.6 F). In order to investigate whether the Granzyme B+/CD4+/CD8- cells belonged to the recently described subset of activated T helper cells I next
performed on 2 selected SS samples double IF for PD1/Granzyme B and observed that over 90% of CD4+/Granzyme B+ cells were also PD1+ (Fig 5.6 G). On the basis of the above data it is possible to speculate that in ELS+/EBV+ SS salivary glands, a defective cytotoxicity in the CD8+ T cell population is present which seems to be compensated by the acquisition of cytotoxic activity by a subset of activated Th cells.
Figure 5.6
Figure 5.6 Analysis of the link between cytotoxic T cells and EBV reactivation in SS salivary glands

CD8+ cells accumulate out of a B cell follicle in an ELS+ FDC+ SS salivary gland (A). Just outside a B cell follicle, numerous CD8+ T cells accumulate in the same area of some BFRF1+ cells (B). In the inset in B a direct cell-cell contact between a BFRF1+ and CD8+ cell is shown. Many Granzyme B+ cells just outside a B cell follicle, in an area where BFRF1+ cells accumulate (C). In the inset in C a contact between a BFRF1+ and a Granzyme B+ cell is shown. In an ELS+/FDC+ SS salivary gland most of the CD8+ T cells are non-cytotoxic (D), whilst most of the CD8+ cells are Granzyme B positive in SS salivary glands with diffuse inflammation (E). In the inset in E a double positive CD8/Granzyme B cells is shown. Most of the CD4+ T cells express Granzyme B in ELS+/FDC+ SS salivary glands (F) with the inset in F showing a high magnification of a double positive CD4/Granzyme B cell. Some Granzyme B+ cells co-express PD1 in an ELS+ SS salivary gland (G) with the inset in G shown a high magnification of a double positive PD1/Granzyme B cells. Bars: 50 µm in A; 10 µm in B-G.

Graph: The absolute number of CD8+ T cells is significantly higher in ELS+FDC+ and in ELS+FDC- compared to ELS- SS salivary glands (a). The percentage of CD8+/Granzyme B+ cells on the total number of Granzyme B is significantly higher in ELS- compared to ELS+FDC+ SS salivary glands (b).

*p<0.05, ** p<0.01
CHAPTER 6  RESULTS

EBV reactivation, humoral autoimmunity and anti-EBV responses in patients with RA and SS
It has long been postulated that EBV may contribute to the development of autoimmunity via the rescue, survival and activation of autoreactive B cells which would be otherwise depleted in the periphery (82). However, this hypothesis has never been formally tested. Thus, in the final part of my PhD project I investigated the relationship between EBV and autoimmunity in patients with RA and SS. First, I assessed the humoral response against EBV antigens in the serum and synovial fluid compartment of RA patients and analysed its correlation with immunoreactivity towards citrullinated antigens. Second, I took advantage of the human/mouse chimeric SCID model in order to investigate the direct production of native and/or translationally modified (i.e. citrullinated) human anti-EBV antibodies by human RA synovia or SS salivary glands engrafted into SCID mice and their association with either ACPA (RA) or anti-SSA/Ro, anti-SSB/La antibodies (SS). As the only source of human antibodies in this system are those locally produced within the target organ, this experimental approach allowed me to directly investigate in situ production within ELS. Finally, I used RA and SS-specific recombinant autoantigens (citrullinated fibrinogen and Ro52, respectively) in order to investigate whether plasma cells infiltrating the RA synovium and SS salivary glands and showing evidence of EBV reactivation (see Chapters 4 and 5) were autoreactive and producing antibodies against disease-specific autoantigens. In Chapter 6 I present the results of this analysis in RA patients first, followed by a parallel investigation in SS.
6.1 Humoral anti-EBV response, ACPA and synovial EBV reactivation in RA patients

I first analysed the production of anti-EBV antibodies in paired sera and synovial fluids from RA and OA patients. This analysis aimed first of all to clarify whether there is a differential anti-EBV response in RA vs OA patients. Secondly, comparing the titres of anti-EBV antibodies in paired serum and synovial fluid from the same patients, I aimed to investigate whether anti-EBV antibodies are enriched in the synovial fluid as an indirect indication of a local production within the RA synovia. Following this, I tested the hypothesis that anti-EBV antibodies may cross-react with self-antigens, potentially contributing to the local production of autoantibodies. I first investigated whether there was a correlation between the levels of anti-EBV antibodies with those of anti-CCP antibodies in the serum/synovial fluid of RA patients. Second, in order to directly analyse the local production of anti-CCP and anti-EBV antibodies in the RA synovia and its association with ELS formation I investigated the production of anti-EBV, anti-citrullinated EBV and anti-CCP human IgG antibodies in the sera of SCID mice transplanted with ELS+ or ELS- RA synovia. Finally, I tested the hypothesis that EBV contributes to the survival of autoreactive plasma cells in the RA synovia, investigating whether ACPA-producing plasma cells, identified by binding of biotinylated citrullinated fibrinogen (cFb) were infected with EBV.
6.1.1 Analysis of anti-EBNA1, anti-VCA, anti-EA and anti-CCP antibodies in paired serum and synovial fluid from RA patients

Paired serum and synovial fluid from 7 RA patients were screened by commercial ELISA in order to detect antibodies anti-EBNA1 IgG (marker of EBV past infection), anti-VCA IgG (marker of EBV primary and recent infection) and anti-EA IgG (marker of acute infection or recent reactivation).

Detectable level of anti-EBNA1 (Fig 6.1 A) and anti-VCA IgG antibodies (Fig 6.1 B) were found in all RA serum and synovial fluid analysed. Anti-EA IgG were detected in 2 out of 7 RA sera (28%) and in 3 out of 7 RA synovial fluids (42%) (Fig 6.1 C). Anti-VCA IgG antibodies production was the only that significantly correlated comparing serum and synovial fluid from RA patients (Fig 6.1 D). Interestingly, some synovial fluids displayed higher levels of anti-EBV antibodies compared to the serum (higher anti-VCA in 1 RA patient and higher anti-EBNA1 and anti-EA in 2 RA patients), suggesting that antibodies anti-EBV may be locally produced in the synovium in a subset of RA patients.

Anti-CCP production in serum and synovial fluid of the 7 RA patients was significantly correlated (Fig 6.1 E). Remarkably, I demonstrated that the anti-CCP level in the synovial fluid of RA patients significantly correlated with the synovial fluid levels of anti-VCA (Fig 6.1 F) and anti-EBNA1 (Fig 6.1 G) antibodies.

As expected, analysis of paired synovial fluids and sera from 10 OA patients demonstrated detectable anti-EBNA1 in all cases (Fig 6.1 H), with no substantial difference between RA and OA anti-EBNA1 titres. Moreover, level of anti-EBNA1 antibodies was not significantly different in serum compared to synovial fluid from all OA patients (Fig 6.1 H), suggesting that anti-EBV antibodies are not locally produced in OA synovia.
Figure 6.1 Analysis of anti-EBV and anti-CCP antibodies in paired sera and synovial fluida from RA and OA patients

All sera and synovial fluid from RA patients were anti-EBNA1 IgG (A) and anti-VCA IgG (B) positive. Two out of 7 RA sera and 3 out of 7 RA synovial fluid displayed detectable level of anti-EA IgG antibodies (C). Anti-VCA IgG levels were significantly correlated in serum and synovial fluid from RA patients (p=0.02; R=0.7) (D). Anti-CCP level were significantly correlated in serum and synovial fluid from RA patients (p=0.001; R=0.9) (E). Level of anti-CCP were significantly correlated with anti-VCA (F) or anti-EBNA1 (G) IgG in the RA synovial fluid (both p=0.02; R=0.6 and 0.7 respectively). Anti-EBNA1 level was not significantly different in sera and synovial fluid from OA patients (H).

The red line in graph A-C and H indicates the cut off for positivity.

sf= synovial fluid
6.1.2 ELS+ RA synovia engrafted into SCID mice produce antibodies against unmodified EBV antigens

I next examined in an *in vivo* model whether production of antibodies against unmodified EBV antigens takes place within isolated RA synovium and correlates with ACPA production. For this purpose, Hu-RA/SCID mice chimeras were generated by implanting fragments of RA synovial tissue (ELS+ or ELS-) into SCID mice (Figure 6.2 A, B). The presence of synovium-derived circulating human antibodies was analysed after 4 weeks. As previously observed (85) levels of human IgG ACPA were significantly higher in sera of mice transplanted with ELS+ compared to ELS- RA synovia (Figure 6.2 C). ELS+ RA synovial grafts also produced significantly higher levels of IgG antibodies directed against the EBV proteins EBNA1 and VCA compared to ELS- (Figure 6.2 D, E), while no production of anti-EA antibodies was detected (Figure 6.2 F). Those findings directly demonstrate for the first time that antibodies against native EBV latent antigens are locally produced in the RA synovia in the presence of ELS and their production is significantly associated with ACPA.
Figure 6.2
Figure 6.2 Unmodified anti-EBV antibodies are locally produced in RA synovia and correlate with ACPA production

Synovial tissue from 4 RA patients was transplanted into 30 SCID mice (arrows depict site of transplants) (A). Transplanted RA synovial tissue in SCID mouse (B) (A-B are reproduced from Humby et al, PLoS Med, 2009). Four weeks after transplantation mouse sera were analysed by ELISA for the presence of human ACPA and anti-EBV IgG antibodies. ELS+ RA synovia engrafted into SCID mice produce significantly higher levels of ACPA (C), anti-EBNA1 (D) and anti-VCA (E) antibodies compared to ELS- synovia, while no production was observed for anti-EA antibodies (F).

*** p<0.001; ns= statistically not significant
6.1.3 ELS+ RA synovia engrafted into SCID mice produce antibodies against citrullinated EBV antigens which closely correlate with local ACPA production

Given the strong association between ACPA and anti-EBV antibody production, I next investigated whether the observed anti-EBV synovial humoral immune response could be directed against citrullinated EBV antigens derived from EBNA1 and EBNA2 (VCP1 and VCP2, respectively), as previously demonstrated in the sera of RA patients (66). For this purpose I analysed ELS+ HuRA/SCID sera, previously found positive for anti-CCP, anti-EBNA1 and anti-VCA antibodies (see Figure 6.2). As shown in Fig. 6.3 A and B ELS+ HuRA/SCID sera reacted with VCP1 and VCP2 and the binding to the citrullinated viral antigens positively correlated with the level of ACPA. These findings support the conclusion that citrullinated EBV proteins are antigenic targets of the humoral immune response generated in ectopic GC in the RA synovium. Moreover, since ACPA cross-react with citrullinated EBV antigens in RA (66, 246), this findings further suggest that local production of VCP1 and VCP2 in RA synovia could play a role in the induction of RA-specific antibodies.
Figure 6.3 Citrullinated anti-EBV antibodies are locally produced in the RA synovia and correlate with ACPA production

ACPA+/anti-EBNA1+/anti-VCA+ sera from Hu/RA SCID mice engrafted with ELS+ RA synovia were analysed for production of antibodies to citrullinated viral antigens VCP1 and VCP2. In vivo production of anti-citrullinated VCP2 EBV antibodies is closely associated with local ACPA production (A, B).

** p<0.01, ns= statistically not significant
6.1.4 ACPA-producing synovial plasma cells are infected with EBV

In order to understand whether EBV might contribute to RA pathology by supporting the survival and differentiation of pathogenic B-cell clones we asked whether synovial autoantibody-producing B cells were infected with EBV. ACPA-producing plasma cells in the RA synovium were identified by the binding of biotinylated citrullinated fibrinogen (CFb). This set of experiments was performed in 8 ACPA+ ELS+ RA synovial samples with ectopic GC and numerous EBV infected cells, and one ACPA+ RA sample with diffuse inflammation and no detectable EBER+ cells. Serial sections of these samples were double stained for CFb and CD138 (to identify ACPA-producing plasma cells), CD138 and BFRF1 (to identify EBV lytically infected plasma cells), and BFRF1 and CFb (to identify EBV lytically infected ACPA-producing cells). As negative control, we used an excess of biotinylated non-citrullinated antigen. In addition, 2 ELS+ salivary glands of patients with Sjogren’s syndrome were used as controls for the specificity toward citrullinated antigens.

In 6 out of 8 ELS+ RA synovia analysed around 30% of the CD138+ plasma cells at the periphery of FDC+ B-cell follicles bound CFb (Fig 6.4 A-C), but not non-citrullinated Fb (Fig. 6.4 D). The same perifollicular areas were populated by lytically infected BFRF1+/CD138+ plasma cells (Fig. 6.4 E, F). When double staining for BFRF1 and CFb was performed, we observed that about 70% of ACPA-producing plasma cells at the periphery of ectopic GC co-expressed the EBV lytic antigen (Fig. 6.4 G-H). Neither ELS- RA synovia (Fig 6.4 I) nor SS salivary glands bind CFb (Fig 6.4 J). These findings clearly indicate that in GC-containing RA synovia a subset of autoreactive plasma cells are infected with EBV.
Figure 6.4 Detection of the EBV early lytic protein BFRF1 in autoreactive plasma cells in ELS+ RA synovia.

Double immunofluorescence staining for CD20 and CD138 shows a B-cell follicle surrounded by numerous plasma cells (A). At the border of the same B-cell follicle (marked with an asterisk in B, D, E) many of the CD138+ plasma cells react with cytrullinated fibrinogen and express BFRF1, indicating that they produce APCA, and are EBV lytically infected. No binding of non-citrullinated Fb is detected in the same perifollicular area (D). Double immunofluorescence staining for CFb and BFRF1 indicates that a fraction of the ACPA-producing cells (CFb+) is positive for BFRF1 (G-H). Double immunostainings were performed on serial sections and the areas shown in G-H correspond to the areas immediately outside the B-cell follicle shown in A-E. No binding of CFb is detected in ELS- RA synovia (I) or in SS salivary gland (J). Bars: 50 µm in A, B, J; 10 µm in C-I.
6.2 Local humoral anti-EBV response, autoimmunity and EBV reactivation in SS salivary glands

Following the observation that EBV reactivation, local production of anti-EBV and of RA-associated antibodies are closely associated in the RA synovium with ELS, I next tested the hypothesis that i) anti-EBV antibodies are locally produced also in ELS+ SS salivary glands characterised by a dysregulated EBV infection; ii) their local production is associated with that of SS-associated autoantibodies and iii) EBV reactivation affects anti-Ro52/SSA and/or anti-La/SSB autoreactive plasma cells in SS salivary glands.

6.2.1 SS salivary glands engrafted into SCID mice produce anti-Ro52/SSA, anti-La/SSB and anti-EBV antibodies

Eight Hu-SS/SCID mice chimeras were generated by implanting fragments of SS salivary glands into SCID mice. The presence of salivary glands-derived circulating human antibodies was analysed by culling 2 mice at each time point at week 1, 2, 3 and 4 post-engraftment. I observed detectable levels of anti-Ro52/SSA and anti-La/SSB antibodies at week 1 and 2 with a peak of production at week 2 (Fig 6.5 A, B). The same sera were next analysed for anti-EBV antibodies production. As shown in Fig 6.5 C, D I found positive levels of anti-EBNA-1 and anti-VCA antibodies at week 1 and 2 with a peak of production at week 2. Conversely, I could not detect anti-EA antibodies at any time point (Fig 6.5 E). Interestingly, a significant positive correlation between the intra-glandular production of SS-associated autoantibodies (anti-La/SSB and anti-Ro/SSA) and anti-EBV antibodies (anti-EBNA1 and anti-VCA) was
observed (Fig 6.5 F-I). Since Ro52 and EBNA1 proteins share homology in the sequence (81), it is possible to speculate from this preliminary data that EBV infection might trigger an immune intra-glandular response via cross-reaction with self-antigens, thus contributing to autoimmunity in SS.
Figure 6.5 Anti-La/SSB, anti/Ro/SSA and anti-EBV antibodies are locally produced in SS salivary glands

Detection of anti-La/SSB (A), anti-Ro/SSA (B), anti-EBNA1 (C), anti-VCA (D) and anti EA (E) antibodies in the serum of the Hu-SS/SCID after 1, 2, 3 and 4 weeks after transplantation. Levels of anti-La were significantly correlated with level of anti-EBNA1 (p=0.02; R=0.6) (F) and anti-VCA (p=0.002; R=0.8) (G) antibodies in the serum of Hu-SS/SCID mice. Level of anti-Ro were significantly correlated with level of anti-EBNA1 (p=0.004; R=0.9) (H) and anti-VCA (p=0.008; R=0.8) (I) antibodies in the serum of Hu-SS/SCID mice.

The red line in graph A-E indicates the cut off.
6.2.2 Anti-Ro52 autoantibody-producing peri-follicular plasma cells in SS salivary glands display evidence of EBV reactivation

In order to investigate whether EBV play a direct role in the survival and differentiation of autoreactive plasma cells in SS salivary glands, in the final part of my PhD project I investigated whether anti-Ro52/SSA and anti-La/SSB producing-plasma cells are EBV-lytically infected. For this purpose I analysed 5 SS salivary glands biopsies; 3 ELS+ EBV+ and 2 ELS- EBV- from anti-Ro/SSA anti-La/SSB positive SS patients.

Autoreactive plasma cells were first identified by analysing immunoreactivity against biotinylated recombinant Ro-52Kd and La-48Kd antigens, generated as previously described (202) (a kind gift of Prof Roland Jonsson, University of Bergen).

I first tested the suitability of Ro-52 and La-48 biotinylated antigens on ELS+ SS salivary glands. While biotinylated Ro-52 gave the predicted staining in large cells surrounding ectopic B cell follicle, La-48 failed to demonstrate a specific immunoreactivity and was not carried forward for further analysis.

Serial sections of ELS+ SS salivary glands were then double stained for Ro-52/CD138 (to identify autoreactive plasma cells), for CD138 and BFRF1 (to identify EBV lytically infected plasma cells), and for BFRF1/Ro-52 (to identify EBV lytically infected anti-Ro52 producing cells). In all ELS+ SS salivary glands analysed, I detected numerous cells localising just outside B cell follicles and binding Ro-52 (Fig 6.6 A). Performing double IF for CD138/Ro-52, CD138/BFRF1 and Ro-52/BFRF1 on sequential sections I were able to identify that a sizeable subset of Ro52-producing autoreactive CD138+plasma cells display EBV reactivation (Fig 6.6 B-D). As expected, I did not detect any immunoreactivity against Ro52 in ELS- EBV- salivary glands (Fig 6.6 E). Finally, staining with
biotinylated Ro-52 on ELS+/EBV+ RA synovia failed to demonstrated any anti-Ro52 immunoreactivity in synovial plasma cells (Fig. 6.6 F). These data strengthen that EBV-infected locally-differentiated plasma cells maintain specific autoreactivity against target autoantigens which are specific for each autoimmune disease, being selectively expressed within the local inflammatory milieu.
Figure 6.6 EBV infects autoreactive plasma cells in SS salivary glands

Ro52+ cells localised just outside a B cell follicle in an ELS+ SS salivary glands (A). Serial sections stained for CD138/BFRF1 (B), CD138-Ro52 (C), and Ro52/BFRF1 (D) reveal that i) all Ro52+ cells are plasma cells. ii) In the same area some EBV lytically infected plasma cells accumulate. iii) A subset of the EBV-lytically infected cells displays reactivity against Ro52. Either ELS- SS salivary gland (E) or RA synovia (F) show reactivity for Ro52.

Bars: 50 µm in A; 10 µm in B-F.
CHAPTER 7

DISCUSSION
7.1 Significance of Epstein-Barr virus infection in the Rheumatoid Arthritis (RA) synovium

Epstein-Barr virus is able to promiscuously and efficiently infect any resting B cells in culture, leading to cellular activation, proliferation, and the outgrowth of transformed lymphoblastoid cell lines (2). Not surprisingly, the virus is pathogenically associated with important human malignancies that include B cell-lymphomas (Burkitt’s, Hodgkin’s and immunoblastic lymphomas in the immunosuppressed) and carcinomas (nasopharyngeal and gastric) (2, 18). EBV has long been suspected also in the pathogenesis of various autoimmune diseases, including RA. Perturbed EBV infection is present in patients with RA (50) as demonstrated by i) serological data (227, 230, 279), showing an increased prevalence of anti-viral antibodies in RA patients; ii) a higher EBV blood viral load (56, 231); iii) molecular mimicry between self-antigens and EBV antigens (63, 66, 238, 239) and iv) clonal expansion of CD8+ T cells reacting against the virus in the joints of RA patients (244). However, EBV dysregulation in the RA synovium has remained a point of contention and whether EBV proteins/RNA are expressed in the inflamed joints of RA patients is still highly controversial. In fact, some works failed to detect EBV proteins or RNA expression in the RA synovia (71, 250, 251), whereas others reported a significant number of EBV+ cells (69, 70).

A likely explanation for these controversies is that none of the previous works has attempted to investigate the presence of EBV in the context of the degree of synovial B cell infiltration and, more importantly, the formation of ELS. This is extremely relevant since the group of Francesca Aloisi at the ISS in Rome recently demonstrated a close link between EBV infection and ELS formation in the meninges of patients with MS (73) and in the thymus of patients with MG.
(72), two autoimmune diseases characterised by ELS formation in their respective target organs.

These high impact publications demonstrated EBV persistence and reactivation in the brain of MS and the thymus of MG patients mainly restricted to ectopic lymphoid follicles where they also observed signs of an ongoing anti-viral immune response. Thus, previous work suggested that a response towards EBV in the context of ELS could contribute to exacerbate the local inflammation. Moreover, the presence of EBV in ectopic B cell follicles, would strongly support the “GC model”, the most robust explanation currently available to elucidate the mechanisms by which EBV gains access to the memory B cell compartment (18). This model assumes that the virus uses the growth latency program to drive newly infected resting B cells into the cell cycle so that they can differentiate into resting memory state (where EBV can persist for lifetime) via a germinal centre reaction. As a result, the potential intersection of EBV and the GC reaction, both of which can drive B-cell growth and differentiation by delivering anti-apoptotic and proliferative signals, may provide a powerful combinatorial risk factor for the development of B-cell related autoimmune diseases (82).

Moreover, the observation that ELS represent preferential niches for EBV persistence and reactivation suggested that EBV-containing ELS might be considered a signature of organ-specific autoimmune diseases where ectopic lymphoid neogenesis develops.

Starting from this overall hypothesis and the previous investigations linking EBV with RA pathogenesis, in the first part of my PhD project I aimed to investigate whether a perturbed EBV infection was also present in the RA synovia characterised by the presence of ELS.
My group and others have previously described ELS in the joints of around 50% of patients with RA; these structures not only recapitulate the most important morphological features of the secondary lymphoid organs (SLO), such as T/B cell segregation, HEV differentiation and expression of lymphoid chemokines CXCL13 and CCL21 (84, 126, 143), but also retain the functionality typical of SLO as demonstrated by the presence of network of follicular dendritic cells and the expression of AID (the enzyme required for SHM and CSR). Furthermore, recent evidences from my group also demonstrated a direct link between ELS and the in situ differentiation of autoreactive plasma cells reactive against citrullinated self-proteins (85, 280).

Thus, my first aim was therefore to settle the longstanding issue of whether EBV proteins/RNA are expressed in the RA synovia. As discussed before, previous studies are highly controversial and the observed discrepancies are likely to a combination of i) technical reasons, i.e. sub-optimal sensibility of the techniques used, lack of a comprehensive approach, questionable preservation of the synovial tissue, and ii) the lack of stratification of RA synovia on the basis of the B cell infiltration and ELS formation. In order to perform a more exhaustive and clear-cut analysis of EBV expression in the RA synovia I used a large variety of techniques (such as IHC, IF, ISH and quantitative real-time PCR) on a large number of RA synovial tissues accurately quality-controlled for a good preservation of the tissue. In addition, I deeply characterised the level of inflammation and the presence of ELS in all RA synovial specimens, staining sequential sections for CD20, CD138, CD3, CD68 and CD21 allowing the classification of the samples in 3 main subgroups, ELS+FDC+, ELS+FDC- and sample with diffuse synovitis. As expected from previous published data on synovial histopathology, around half of the RA synovia showed diffuse inflammation, characterised mainly by macrophages.
infiltration and synovial fibroblast proliferation with diffuse T cells and few/no B cells, with the remaining half presenting ELS, characterised by the presence of B cell follicles with common segregation of B and T cells in separate areas and surrounding CD138+ plasma cells. Among the ELS+ samples, I further characterised them as those displaying clear evidence of CD21+ FDC+ networks (40%) and those lacking FDC (60%). Once the synovial pathotype of the RA samples was assessed and the samples carefully selected, I then moved to the investigation of whether EBV latent antigens (LMP2A and LMP1) were expressed in the RA synovia and were linked to the presence of synovial ELS. By using double IF CD20/LMP2A and staining sequential sections for CD20, CD3, CD138, CD21 and LMP2A as well as staining for LMP1, I clearly demonstrated a strong association between LMP1 and LMP2A expression, the presence of ELS and the differentiation of FDC networks.

According to the GC model of EBV persistence, EBV employs four sequentially expressed, virally-encoded latency transcription programs which drive infected naive B cells to become proliferating blasts and participate in GC reactions (where both LMP1 and LMP2A are expressed) in secondary lymphoid tissues, and finally enter the memory B-cell compartment where the virus establishes persistent infection. LMP1 and LMP2A possess the signalling properties of CD40 and the B-cell receptor, respectively, and have the capacity to promote the development of infected B cells in the absence of cognate antigen by providing pro-survival signals (281). Thus, my data are consistent with the GC model, whereby both LMP1 and LMP2A are expressed in EBV infected B cells transiting ectopic GC in the RA synovium, suggesting that these viral proteins may be involved in the abnormal expansion and survival of EBV infected B cells observed in the pathological tissue sustaining local B-cell activation and
differentiation. Moreover, these results provide a clear explanation for the high variability in the frequency of EBV infected cells in RA synovial tissues reported in previous studies and strengthen the concept that stratification upon synovial B-cell infiltration is essential to reliably assess the presence of EBV infection in the RA joints.

I also confirmed the presence of EBV latency in the RA synovia at mRNA level by using two different techniques, ISH and RT-PCR. Performing ISH for EBERs which are EBV-encoded non-translated small RNAs combined with IHC for CD20 CD3, CD138 on sequential sections, I clearly demonstrated that the highest frequency of EBER+ cells was associated with ectopic GC. EBER+ cells were detected both inside and at the border of intrasynovial B-cell follicles, around T-cell areas and in B-cell-rich perivascular aggregates. Conversely, EBER was virtually absent in RA samples with diffuse inflammation. Overall, these findings confirmed the histological data allowing to conclude that the latent phase of EBV is strongly associated with ELS in the RA synovia and it is mainly expressed by B cells.

The observation of EBER in plasma cells in the absence of LMP2A/LMP1 expression is likely related to a relative higher expression of EBER within the cells, thus allowing greater sensitivity compared to immunostaining of LMP2A and LMP1. Notably, 33% of the RA synovia analysed were excluded due to suboptimal RNA preservation, as assessed by GAPDH ISH. Therefore, a poor tissue preservation may explain the failure of previous works to detect EBER+ cells (282).

The presence of EBV RNA and its association with ELS in RA synovia was further confirmed by TaqMan real time RT-PCR, a very sensitive method which also allows quantitative assessment of gene expression. The analysis was performed only on samples that showed an optimal RNA integrity, assessed by
resolving total RNA on an agarose gel. Moreover, to avoid false negative results due to low target expression, I also performed an additional pre-amplification step of target genes, which amplifies the starting material and increase the sensitivity of the technique. This technique has been optimised and used by our collaborators in previous work (268). EBV-infected B cells represent only a small fraction of the total resident and infiltrating synovial cellular compartment and the sensitivity of conventional real-time PCR techniques might not be sufficient to detect EBV RNA even in highly infiltrated tissue samples.

Consistent with the results obtained by IHC, IF and ISH, EBV latent transcripts (LMP2A, LMP1 and EBER) were strongly associated with the expression of several genes closely related to the formation and function of ELS such as CXCL13, a B cell chemoattractant and AID, the enzyme responsible for IgH V gene somatic hypermutation and Ig class-switching in B cells.

Only one EBV latent-expressed gene was consistently undetectable in the RA synovium irrespectively from the presence of ELS, EBNA-1. This gene product acts as a transcriptional activator and is essential for the replication and persistence of EBV as an episome in infected B cells (2). In the absence of a clear explanation for the lack of synovial EBNA-1 expression, it is possible to speculate that the negative EBNA1 findings could be due to a low level of RNA expression in the RA synovium below the detectability of the method.

After this comprehensive assessment of EBV latency, I next investigated whether EBV merely persists in its latent phase in the RA synovia or also undergo reactivation. For this purpose I analysed the synovial expression of BFRF1 and BMRF1, which are antigens associated with the early lytic cycle of EBV infection (283, 284). By double staining and sequential section analysis, I clearly demonstrated that BFRF1 is expressed almost invariably by plasma cells.
and is strictly associated with ELS and FDC formation being, detected in 100%, 36% and 0% of ELS+/FDC+, ELS+/FDC- and diffuse RA samples respectively. Typical BFRF1+/CD138+ plasma cells were observed at the periphery of B-cell follicles and in the interfollicular areas but always in association with ELS+ samples, strongly suggesting local differentiation within GC-like structures. Thus, in this scenario viral reactivation is initiated as a consequence of the differentiation of EBV infected B cells in ELS whereby the local inflammatory milieu induces the switch from the latent to the lytic EBV gene programme during local plasma cell differentiation. In contrast, EBV structural proteins, like p160 and gp350/220, were rarely detected in synovial ELS suggesting abortive viral replication or very efficient elimination of cells expressing EBV late lytic antigens.

Because EBV has been shown to break tolerance mechanisms and induce autoreactive B-cell activation when LMP2A is constitutively expressed in transgenic mice (285), EBV infection has been discussed as a biologically plausible mechanism allowing potentially pathogenic autoreactive B-cell clones to escape deletion and cause autoimmunity. Thus, a fundamental question that was not addressed previously in RA or in any other autoimmune disease is whether EBV infection in ELS is directly linked to humoral autoimmunity. Since EBV delivers growth and survival signals to the infected B cell, it has been proposed that EBV transformation promotes the survival of autoreactive B cells (82). The RA synovium represents an ideal experimental setting to test this hypothesis since synovial ELS support a GC response leading to in situ differentiation of plasma cells that produce ACPA (85, 280).

Here, I showed for the first time that a remarkable proportion (about 70%) of ACPA-producing plasma cells at the periphery of ectopic GC are infected with EBV and that about 60% of EBV lytically infected cells in the same areas are
ACPA-producing plasma cells. Persistence of ACPA-producing, EBV infected plasma cells in the RA synovium implies that completion of the EBV lytic cycle is somewhat impaired and is consistent with intrasynovial production of potent plasma cell survival signals, such as CXCL12 and APRIL, which normally support long-lived plasma cells in the bone marrow (286, 287). Therefore, the evidence that EBV infects a subset of autoreactive plasma cells in the RA synovia, support the hypothesis that EBV, with its unique ability to immortalise B cells, play a role in the survival of auto-antibody producing plasma cells, that directly contribute to RA pathogenesis. These findings raise the possibility that EBV infection favours the survival of pathogenic autoreactive B cells that participate in the formation of ectopic GC in the RA synovium. These structures and the local availability of self-antigens likely provide the appropriate environment that allows EBV infected autoreactive B cells to selectively expand, undergo affinity maturation and differentiate into autoantibody-producing plasma cells. The recent finding that EBV persists in self-reactive memory B cells (288) lends support to the idea that EBV may contribute to the survival of self-reactive B cells that home to the RA synovium. As previously discussed the induction of anti-viral humoral immune responses cross-reactive with self-antigens is another possible mechanism through which EBV may contribute to autoimmunity (289, 290). In a normal adult, latent EBV infection is associated with moderate, stable and highly correlated levels of IgG antibodies against VCA, EBNA-1, and EA-R, with very low or undetectable levels of antibodies against EBNA-2 and EA-D(291-293). In situations of decreased cellular immunity, however, EBV reactivation, or the transition from latent to lytic infection, can occur. Anti-VCA IgG antibodies, anti-EBNA-2 antibodies, and anti-EA antibodies are often elevated in these situations, which is consistent with EBV reactivation. Latent EBV can replicate and spread despite the
presence of antibodies, and antibody titers correlate with viral activity rather than with the degree of protection afforded (293). In many diseases strongly associated with EBV, such as nasopharyngeal carcinoma and Burkitt's lymphoma, anti-EBV serology is abnormal many years before the onset of disease.

Different studies revealed that anti-EBV titres are higher in patients with RA than in controls (228-230, 279) but very few attempts have been made to compare immune response to EBV in RA sera vs synovial fluids yielding contrasting results results (228, 233). In particular, although anti-EBV antibodies can be detected in the synovial fluid of RA patients, it is still not clear if those antibodies are locally produced in the RA synovia or merely reflect the systemic production. I therefore aimed to evaluate if a perturbed EBV infection in RA patients can cause a systemic and/or local synovial humoral anti-EBV immune response, eventually capable to interfere with self-antigens and exacerbate the pathological immune response. For that purpose I first analysed paired sera and synovial fluids from RA patients and found a significant correlation between the levels of anti-EBV antibodies in the two compartments with few cases in which anti-EBV antibodies were higher in synovial fluid compared to the serum, suggesting that a humoral anti-EBV response can be triggered directly into the joint of a subset of RA patients. Anti-EBNA1 antibodies have been shown to cross-react with different autoantigens in RA, including denatured collagen and keratin (294), leading to the hypothesis that anti-EBNA1 immunoreactivity may fuel breach of self tolerance in the RA synovia, via molecular mimicry. This would result in local production of anti-EBV antibodies, possibly cross-reacting with self-antigen and therefore contributing to the RA pathogenesis via autoantibody production.
In support of this hypothesis, I first observed a significant positive correlation between the synovial fluid production of anti-EBNA1 and anti-CCP antibodies, a highly specific marker of RA. Anti-CCP production also significantly correlated with the level of anti-VCA antibodies in the RA synovial fluid. Following this in vitro evidence, I moved to in vivo studies in the Hu-RA SCID mice chimera, which is generated by engrafting fragments of RA synovial tissues in immunodeficient recipient mice. This model allows detection of circulating human antibodies into the mice circulation which are by definition locally produced in the RA synovia, as this is the only possible source of human antibodies in this model. Using this approach I confirmed that ELS+ but not ELS- RA synovia implanted in SCID mice release detectable level of human ACPA in the mouse circulation, supporting the notion that lymphoid structures within the rheumatoid arthritis synovia have a direct contribution to RA pathogenesis via ACPA production (85). By using the Hu-RA SCID mice I was also able to provide the first and direct evidence that anti-EBV antibodies (anti-EBNA1 and anti-VCA IgG) are locally produced in the RA synovia characterised by a perturbed EBV infection and ELS formation and correlate with ACPA production. The latter evidence is of particular interest as it was recently demonstrated that circulating ACPA in the blood of RA patients recognize citrullinated EBNA-1 antigenic peptides VCP1 and VCP2(66). Thus, I further analysed the sera of SCID mice engrafted with ELS+ RA synovia, and observed detectable level of antibodies against the citrullinated EBNA1 antigens VCP1 and VCP2 that positively correlate with the level of ACPA. This observation demonstrates that cross-reactive antibodies are actively manufactured by GC in the RA synovium, likely contributing to local inflammation and tissue destruction. It is conceivable that the enzyme peptidyl-arginine deaminase, which is abundantly expressed within the inflamed RA synovium (295) might
be involved in the generation of citrullinated viral and self-antigens. Overall, this data provide the first direct link in the RA synovia between ectopic lymphoid neogenesis, EBV infection, humoral response to the virus and local production of ACPA, one of the most specific markers of RA.

Finally, since latent and lytic EBV proteins are the targets of a cytotoxic immune response, the presence of EBV in RA synovia prompted me to investigate whether EBV might contribute to local inflammation through stimulation of a cytotoxic T cell response. I first observed that in ELS+ RA synovia CD8+ T cells were largely excluded from EBV+ ectopic GC, which suggests that these structures represent immune privileged niches for EBV persistence. Furthermore, the majority of granzyme B+ cells infiltrating ELS+ synovia were CD4+ rather than CD8+ whilst granzyme B+/CD8+ T cells predominated in ELS- RA synovia. These observations are in keeping with the reported preferential accumulation of non-cytotoxic, IFNγ-producing CD8+ T cells in ELS+ RA synovia (274) and impaired cytotoxic function of EBV-specific circulating CD8+ T cells in RA patients, suggesting defective control of EBV infection (235). Accumulation of granzyme B+ CD4 T cells in ELS+ synovia is also consistent with previous studies showing that an uncommon subset of circulating cytotoxic CD4+ T cells is present in chronic viral infections (including EBV) as well as in RA patients (275-278). Taken together, these findings can be interpreted as an attempt of CD4+ T cells to compensate a possible defect of CD8+ T cells in controlling increased EBV loads in RA. Moreover, since I detected a massive accumulation of both CD8+, CD4+ and Granzyme B+ cells in sites where EBV reactivates, it is possible to speculate that viral reactivation in locally differentiated plasma cells contribute to the local chronic inflammation via the recruitment and activation of cytotoxic T cell cells, resulting in an immunopathological process. In support of this hypothesis previous studies
detected CD8+ T cell clones directed against EBV lytic antigens directly into the joints of RA patients (244).

In summary, in this first part of my PhD project aimed at the identification of EBV infection in the RA synovium, I consistently demonstrated that i) EBV perturbation is strongly linked with the latent infection of infiltrating B cells within ELS, ii) EBV reactivation takes place during in situ differentiation of plasma cells within GC-like structures, iii) EBV support the development of autoimmunity either by infecting autoreactive plasma cells or by favouring the generation of cross-reactive self-antigens such as citrullinated EBV proteins and iv) Cytotoxic T cell activity in the RA synovium is defective at sites of EBV infection and may favour EBV persistence.

The results obtained in this part of my PhD significantly advance our understanding of the interplay between EBV infection, loss of immunological self-tolerance and establishment of humoral autoimmunity in RA, and will stimulate further studies on the role of altered EBV-host interactions in RA etiopathogenesis.
7.2 Significance of Epstein-Barr virus infection in Sjogren’s syndrome (SS) salivary glands

Following the demonstration of EBV infection and reactivation in the RA synovia, in the second part of my PhD I moved to a parallel investigation in the salivary glands of SS patients. This investigation was fuelled by several lines of evidence which candidates EBV as an important player in SS pathogenesis. SS is a complex, multifactorial autoimmune rheumatic disease characterised by mononuclear cell infiltration of the exocrine glands and the presence of circulating autoantibodies such as anti-Ro/SSA and anti-La/SSB. A viral involvement in SS etiology has long been suggested, with implication of several pathogens including EBV, HTLV-1 and Cocksackie viruses. Additionally, GC-like structures can be identified in 30-40% of SS salivary gland samples and the presence of ectopic B cell follicles correlates with elevated titres of rheumatoid factors and anti-Ro/La, increased circulating IgG, more severe disease and MALT lymphoma development (296, 297).

Clearly, the main common denominator which prompted this investigation was to test the hypothesis that the presence of ELS in SS salivary glands could identify preferential niches of EBV persistence and reactivation as observed in RA patients in my PhD studies as well as in the target organs of patients with MS (73) and MG (72).

Thus, the overarching hypothesis was that EBV latency and reactivation is a common factor in different autoimmune diseases which shared B-cell dysregulation and ELS formation as hallmarks. In addition, the evidence that EBV has its normal site of latency in salivary and lacrimal glands identified SS as a unique platform to investigate the complex interactions between infection of
epithelial cells and development of local inflammatory foci which have a typical periductal distribution in SS lesions.

Previous investigations strongly support a role for EBV in SS pathogenesis. First, antibodies against EBV antigens are higher in serum of SS compared to healthy patients (238, 298, 299). Also, several examples of molecular resemblances between self-antigens in SS and EBV antigens have been identified; anti-La autoantibodies precipitate proteins that are complexed with EBV-encoded small RNAs (EBER1 and EBER2) (33). Moreover, serum from SS, but not from other autoimmune diseases or from healthy controls, cross-reacted with lipocardin (a protein highly expressed in tear and saliva and recently suggested as a novel auto-antigen in SS), α-fodrin (a cytoskeleton protein considerate an important auto-antigen in SS) and EBV proteins (254). It has also been recently demonstrated that the saliva of patients with SS can activate EBV (256), reinforcing the pathological link between the virus and the disease.

Furthermore, a more functional link between EBV and the pathogenesis of SS was proved by Miyasaka et al with an in vivo study on humanized mice whereby they transferred B cell lines generated from SS patients into SCID mice and observed an induced monoclonal lymphoproliferative disorder resembling those arising in SS (255).

Despite the above observations, similar to RA, it is still somewhat controversial whether EBV proteins and nucleic acids are detected more frequently and at higher level in salivary and lacrimal glands from SS compare to healthy patients and whether the accumulation of EBV in the salivary glands is simply an epiphenomenon of the chronic inflammatory process or plays an active role in the pathogenesis (74, 257-259, 300). Moreover, the majority of the studies identified EBV proteins, RNA and DNA in epithelial cells of SS salivary and
lacrimal glands while only one study reported EBV+ lymphocytes infiltrating the SS salivary glands (74). Notably, similarly to RA, no attempt has been made to correlate the presence of EBV with the degree of B cell infiltration, functional activation and the formation of ELS in the salivary glands. With these limitations in mind, in the second part of my PhD project I therefore aimed to clarify whether; i) EBV proteins/nucleic acids are expressed in SS salivary glands, ii) there is a correlation between EBV expression and ELS formation and iii) EBV is associated with the development of autoimmunity to SS-specific antigens.

For this purpose I first assessed in a semiquantitative score the degree of infiltration of B, T and plasma cells as well as the presence of CD21+ FDC networks in SS and NSCS (as disease control) salivary glands. By these means I was able to precisely characterise SS samples as ELS+FDC+, ELS+FDC- or with diffuse inflammation. Then, I analysed EBV latent and lytic expression using the same experimental strategy and array of techniques optimised in the RA study, i.e. IHC, IF, ISH and real-time RT-PCR.

Using this approach I could demonstrate without any reasonable doubt that ectopic B cell follicles forming in the salivary glands of SS patients are a main site of EBV persistence and reactivation.

Using IHC and IF I clearly demonstrated that EBV latent antigens (i.e. LMP2A and LMP1) are expressed by B cells only within ELS and preferentially in the context of highly organised follicles with FDC network differentiation. Conversely B cells in SS samples with diffuse inflammation or NSCS salivary glands were EBV-. The evidence of EBV latency in SS salivary glands and its close association with ELS formation was further confirmed by ISH for EBER. I detected EBER+ cells only in ELS+ SS salivary glands and exclusively in the B cell and plasma cell compartment of the inflammatory infiltrate. Similarly to RA, it
appears that B cell and plasma cells in SS display a different latency programme whereby B cells express both LMP1/LMP2A and EBER whilst plasma cells express EBERs but not LMP1/LMP2A. As argued before, this discrepancy may however be dependent on differences in the sensibility of the two techniques linked with diverse level of expression of EBV proteins and RNA in the two cell types examined. Selective EBER expression in ELS+ SS salivary glands was also confirmed by quantitative RT-PCR analysis which demonstrated a close association of EBER with the degree of B cell infiltration (CD19), functional activation (AID) and differentiation (BLIMP-1). Overall, these data allowed me to conclude that EBV latency is mainly expressed by activated B cells in SS salivary glands and significantly correlate with ELS formation, substantiating a direct link between EBV infection and B cell dysregulation in SS.

An interesting observation which I reported in these studies is that only rare ductal epithelial cells displayed EBV latent infection in SS but also in NSCS samples as demonstrated by LMP2A/LMP1 expression. EBV latency was independent from the presence of periductal inflammation, suggesting that latent EBV infection in ductal epithelial cells may not be directly related to the generation of an autoimmune response in the salivary glands. However, it may also be possible that occasional reactivation (which however I was unable to detect) within the epithelial compartment might trigger a local immune response contributing to the chronicity of the inflammatory process.

Since EBV reactivation in SS salivary glands can have important pathogenic consequence, i.e. can provoke an attack from the immune system contributing to local inflammation, I also analysed whether evidence of EBV reactivation could be observed in SS salivary glands. Similarly to what I observed in the RA synovia, BFRF1 and BMRF1, two EBV lytic antigens, were almost exclusively
expressed by plasma cells in ELS+ SS salivary glands. BFRF1+ and BMRF1+ plasma cells mostly localised at the border of B-cell follicles, again suggesting that EBV reactivated in locally differentiated plasma cells. Thus, in this scenario, on one side ELS provide B cell niches for EBV latent persistence and on the other side they allow the reactivation of the virus in the context of a functional germinal centre response giving rise to locally differentiated plasma cells.

A crucial discovery of my PhD work is that these locally differentiated EBV+ plasma cells are not only frequently autoreactive, but they display immunoreactivity towards autoantigens specifically associated with the autoimmune disease in question. Thus, a large subset of CD138+/BFRF1+ plasma cells in SS were found to be reactive with Ro52, a major autoantigen in SS, but not citrullinated proteins (and vice versa in RA), strongly suggesting that different local autoantigenic triggers fuel the autoimmune process in the different target organs. Several studies have demonstrated an increased humoral response to EBV in the blood of SS patients compared to healthy controls (238, 298, 299), but none investigated local production of anti-EBV antibodies in the SS salivary glands. Here, using SCID mice engrafted with SS salivary glands, I demonstrated that anti-VCA and anti-EBNA1 antibodies can be locally produced in the SS salivary glands and significantly correlate with the production of SS-associated autoantibodies, such as anti-Ro52/SSA and anti-La48/SSB. These findings strongly suggest that EBV can contribute to stimulate the intra-glandular humoral immune response in SS patients. Additionally, it is conceivable that this phenomenon may contribute to breach of self tolerance and trigger/contribute to the development/maintenance of autoimmunity in SS via molecular mimicry and epitope spreading. In particular, EBNA1 shares antigenic epitopes with the human autoantigen Ro52 (81, 301) which might
explain the observed association between the production of anti-Ro52 and anti-EBNA1 antibodies in the SS salivary glands engrafted into SCID mice. Thus, although I do not provide a direct evidence of such cross-reactivity in this study, my data further support the possibility that EBV can contribute to humoral autoimmunity in SS salivary glands.

In the final part of my PhD I investigated the relationship between cytotoxic cells and EBV reactivation in SS salivary glands. CD8+ T-cell-mediated immunopathology is one of the major determinants of tissue destruction in EBV-associated diseases and it has long been suggested to contribute to tissue damage and exocrine dysfunction in SS patients.

By analysing the infiltration of Granzyme B+ cytotoxic cells and their characterization with common T cell markers I was able to demonstrate that a significant accumulation of cytotoxic cells was preferentially associated with areas of EBV reactivation, mostly in association with peri-follicular BFRF1+ plasma cells suggesting that EBV reactivation might contribute to chronic inflammation via recruitment and activation of cytotoxic cells. Interestingly, as observed in the RA synovia, in ELS+ SS salivary glands the majority of the CD8+ cells did not display evidence of cytotoxicity whilst the majority of granzyme B+ T cell were characterised as activated Th cells and possibly T follicular helper cells as demonstrated by expression of PD1+, although further characterization is needed in order to substantiate this finding.

Nevertheless, it is possible to speculate that in ELS+ EBV+ SS salivary glands, EBV latently infected cells may be allowed to accumulate within B cell-follicles due to the follicular exclusion of CD8+ T cells which might not be fully compensated by the acquisition of a cytotoxic phenotype within the CD4 Th compartment.
In conclusion, results obtained in the study of EBV in SS salivary glands support a complex role for EBV in the SS pathogenesis through its ability to establish a persistent infection in ELS+ salivary glands, promote local differentiation of autoreactive plasma cells and stimulate production of SS-associated autoantibodies.
CHAPTER 8

Scientific questions to be addressed and future plans
8.1 Scientific questions to be addressed and future plan

In the present work, I clearly demonstrated that a perturbed EBV infection is a common feature of RA synovia and SS salivary glands characterised by ELS formation. In addition, the results obtained during my PhD project strongly support a pivotal role of EBV in promoting autoimmunity either by directly providing proliferative advantage to infiltrating autoreactive B cell/plasma cells or via molecular mimicry and the induction of cross-reactive autoantibodies. Although the above evidence is extremely compelling, there are several unknowns which should be addressed and would prompt for further investigation into the factual pathogenic role of EBV in RA, SS and more in general, the formation of ectopic lymphoid neogenesis.

First of all, it should be noted that a mechanistic evidence of a direct role of EBV in the pathogenesis of RA or SS is extremely difficult to obtain because of the absence of a suitable animal models. EBV fails to infect murine B cells or epithelials cells and a murine equivalent of EBV is not available, thus in vivo studies of EBV pathogenesis have been very limited. Whereas Old World primates have been found to be refractory to EBV infection, New World species, including the common marmoset (Callithrix jacchus) and the cottontop tamarin (Sanguinus oedipus oedipus) (302), have been found to be susceptible to infection by EBV. Common marmosets have been infected by direct intramuscular or intraperitoneal injection. Persistent EBV infections can be detected in this model, but EBV-associated lymphomas do not develop (303). Cottontop tamarins that are injected with EBV develop multifocal large-cell lymphomas. Despite their usefulness as in vivo models of EBV infection, New World primates represent endangered species and can only be used in small
numbers because they are rare and costly. An alternative model that has been used to study EBV-associated B-cell lymphoproliferative disease is the SCID mouse (304, 305). SCID mice lack functional B and T cells due to a deficiency in double-stranded DNA break repair activity involved in the proper joining of V(D)J coding sequences of B- and T-cell antigen receptors during lymphocyte maturation (306). Because of their lack of T and B cells, SCID mice are not capable of rejecting human cells. SCID mice can be reconstituted with human cells transplanting human hematopoietic stem cells (HSC) into preconditioned mice (307). The fact that the majority of human cells in recipient SCID mice are B cells makes them ideal targets for infection with EBV. Unfortunately, the transplantation of human HSC into SCID mice results in relatively low levels of reconstitution with human cells. In contrast, non-obese diabetic SCID (NOD/SCID) mice also support the growth and differentiation of human HSC and result in significantly higher levels of engraftment and reconstitution with human cells.

Therefore in order to study the direct involvement of EBV in the RA pathogenesis I am planning to use a humanised mouse model by transplanting SCID mice with cord blood derived CD34+ stem cells and with ELS+ RA synovia or SS salivary glands. In this context, gain of function experiments with the inoculation of high doses of EBV (308)together with loss of function approaches with antiviral therapies (i.e. acyclovir or Cydofovir or Acyclovir, Ganciclovir alone or in combination with gemcitabine or doxorubicin) (309-311) would allow to clarify the direct role of EBV in synovial/salivary gland inflammation, B cell activation, ELS function and development of autoimmunity.

A second objective of my future plan of investigation is to provide direct evidence in in vitro studies that EBV promotes the preferential survival of
autoreactive cells in SS and RA blood circulation. Previously, Shimaoka et al (312) observed that after 4-weeks of culture of RA PBMC, surviving B cells were invariable EBV infected. Starting from this finding I aim to investigate if RA and SS B cells that resist to apoptosis after a long culture are not only EBV infected but also display an autoreactive phenotype. For this purpose I am planning to isolate B cells from the blood and tissues of RA and SS patients and culture them for up to 4 weeks. At the end of the experiments, lymphoblastoid cells will be collected, in order to investigate the expression of EBV latent/lytic genes together with the assessment of the production of autoreactive antibodies (anti-CCP/RF in RA and anti-Ro/SSA and anti-La/SSB in SS).

The third long term aim which spans directly from this project is to investigate the persistence of EBV infection in the RA synovium and/or SS salivary glands of patients undergoing B cell depletion with the monoclonal antibody Rituximab. On the basis of the data obtained during my PhD, one would predict that local B cell depletion should result in a strong reduction/abolition of EBV signals with disappearance of EBV+ B cells. However, there is increasing evidence that ELS in RA synovium or SS salivary glands might escape depletion at target tissue level due to their ability to rescue B cells from apoptosis via the release of B cell trophic factors. In this context, EBV may play a further role through the provision of strong survival signals delivered by the activation of the latent viral programme (i.e. LMPs and EBNAs). Patients with RA undergoing Rituximab treatment (and possibly also SS patients) and US-guided synovial biopsies before and after treatment will be made available in my Institution providing a unique platform to test thus hypothesis.

Finally, another extremely interesting and surprising aspect which has emerged from my work is the evidence that Th cell subsets acquire cytotoxic capacity in
the context of EBV+ ELS in both the RA synovia and SS salivary glands. Preliminary work in SS salivary glands suggests that these cells may represent a unique subset of T follicular helper (Tfh) cells. However, because no single marker is sufficient to characterise Tfh, a short-term objective of my future work is to combine staining with Granzyme B/perforins together with Tfh markers such as CD4, CD3, PD1, ICOS, CXCR5, CD45Ro and IL-21 using multicolour confocal microscopy as well as FACS analysis. Once I identify the cytotoxic cells, the next step would be to characterise their functional properties in controlling EBV infection in in vitro experimental settings.
APPENDICES
APPENDIX I

Immunohistochemistry

4% paraformaldehyde

4g of paraformaldehyde were added to 100 ml of TBS. The solution was warmed up and stirred until the paraformaldehyde was dissolved.

10X Tris (hydroxymethyl)methylamine-buffer saline (TBS)

61 g of Trizma Base (0.5 M) and 90g of NaCl (9%) were dissolved into distilled H2O. To adjust the PH to 7.6, 1M HCl was added until needed. Solution was stored at room temperature and diluted 1:10 with distilled water before use.

Acid Alcohol

Add 1ml of concentrated Hydrochloric Acid to 99 ml of 70% Alcohol.

Tris-HCl

24.22 g of Trizma base were diluted in 800 ml of distilled water and adjusted to pH 8.2 within 3N HCl. Distilled water was added up to 1000 ml and solution was stored at room temperature.

HRP Avidin-Biotin Complex (DAKO)

10 µl of Solution were added to 1 ml of TBS pH 7.6, and mixed well. Another 10 µl of solution 2 were added and mix well. The ABC complex was prepared at least 30’ before use.
**APPENDIX II**

**Molecular Biology Buffers and Reagents**

**Ethylenediaminetetraacetic acid (EDTA 9.5 M pH 8.0):**
18.6 g of EDTA were dissolved in 80 mL H2O. NaOH was used to adjust the pH. Distilled water was added to the final volume of 100 ml. The solution was stored at room temperature.

**Tris Borate EDTA (TBE buffer 5x):**
27g of Tris base, 13.75 g of boric acid and 10 ml of EDTA 0.5 M (pH 8.0) were dissolved in 400 ml of distilled water. Distilled water was added to the final volume of 500 ml and the solution was stored at RT.

**Tris Acetic acid EDTA (TAE buffer 5x):**
12.1 g of Tris base, 2.86 ml of acetic acid and 5 ml of EDTA 0.5 M (pH 8.0) were dissolved in 50 ml of distilled water. Distilled water was added to the final volume of 55 ml and the solution was stored at RT.

**Loading dye (Bromophenol-blue dye 6X):**
300 ml of 30% glycerol and 400 ml of EDTA 0.5 M (pH 8.0) were added to 300 ml of deionised H2O and mixed well. 2 mg of bromophenol-blue were added to the solution and dissolved by inversion. Solution was stored at -20°C and diluted to working solution at the time of use.
List of submitted/published original paper directly related to the work presented in this PhD dissertation


2. Cristina Croia, Barbara Serafini, Aloisi Francesca, Costantino Pitzalis, Michele Bombardieri. Epstein-Barr virus infection in Sjogren’s syndrome salivary glands: association with the ectopic B cell follicles, cytotoxicity and autoreactive plasmacells (under revision).
This work has also been presented as oral or poster presentation at several meetings:

1. Poster presentation. 2010 BSI, Liverpool
2. Poster presentation. 2011, BSR, Brighton
5. Poster presentation. 2011, Germinal Centre congress, Birmingham
6. Poster presentation, 2011, ACR, Chicago
7. Oral presentation, 2012, BSR, Glasgow
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