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By

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DECLARATION

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged and the thesis does not exceed 100.000 words.

The studies presented in this thesis were conducted in the department of Experimental Medicine and Rheumatology at the William Harvey Institute, Queen Mary University, London.

The thesis has been written by me and the work presented in this manuscript is the result of my investigation with the following exceptions:

Dr Michele Bombardieri actively contributed in the project development and writing of the papers derived from this Thesis.

Elisa Corsiero and Ken Choi contributed to the FACS staining on isolated lymphocytes.
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Dr Elisa Astorri	Prof Costantino Pitzalis	Dr Michele Bombardieri

ABSTRACT

A continuous process of tissue remodelling and regeneration is a fundamental feature of the homeostatic response of the target organ of several autoimmune diseases. In type 1 diabetes (T1D) the β cell mass is in a constant process of death and renewal in order to regenerate the islets damaged by the autoimmune process. The relationship linking inflammation and regeneration during autoimmunity remains elusive. Reg genes, a multigene family discovered using cDNA libraries derived from rat regenerating islets, have been suggested to play an important role in epithelial regeneration not only in the pancreas but also in the salivary glands (SG) of Sjogren's Syndrome (SS) during autoimmune sialoadenitis.

Both in patients and animal models of T1D and SS, the chronic inflammatory/autoimmune process is heterogeneous and display high immunological variability. In particular, in a sizeable subset of cases, inflammatory lesions display ectopic lymphoid structures (ELS) characterised by T/B cell segregation, follicular dendritic cells networks and differentiation of germinal center B cells. However, there is limited evidence on the cellular and molecular mechanisms underlying ELS formation and their contribution to autoimmunity in the pancreas during autoimmune insulitis and in SG during autoimmune sialoadenitis. In this PhD project, I used the NOD mouse model of T1D and SS in order to investigate i) the cellular and molecular mechanisms regulating ELS formation, ii) the functionality of ELS in supporting in situ autoreactive B cell differentiation and iii) the relationship between formation of ELS and the expression of REG genes.

In this work I showed that ELS formation was preceded by local up-regulation of lymphotoxins ($L\tilde{T}\alpha\beta$) and lymphoid chemokines CXCL13 and CCL19 and that, once formed, ELS were fully functional in promoting autoreactive B cell activation. Importantly, inhibition of the LT-β pathway prevented the formation of ELS and B cell autoimmunity. Finally, I showed that the expression pattern of Reg genes was strictly related to the development of inflammatory infiltrates in NOD

mice and that Reg proteins were target of the autoimmune process itself, as shown by the development of anti-Reg1 antibodies in patients with T1D. Overall, these results suggest that the processes of destruction and regeneration occurring in chronic autoimmune/inflammatory diseases are strongly interdependent whereby autoimmunity may be further enhanced by the attempt to regenerate.

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LIST OF ABBREVIATIONS

Ab	Antibody
AID	Activation induced cytidine deaminase
AMA	Anti-mitochondrial antibodies
ANA	Anti nucleus antibodies
AP	Alkaline phosphatase
APC	Antigen presenting cells
APRIL	A proliferation-induced ligand
BAFF	B-cell-activating factor of the TNF family
BAFF-R	BAFF receptor
BB	Bio Breeding
BCR	B cell receptor
BLC	B lymphocytes chemoattractant
CD	Cluster differentiation
CK	Chemokine
CK-R	Chemokine receptor
CSR	Class switch recombination
DAB	3,3-diaminobenzidine
DC	Dendritic Cell
DM	Diabetes Mellitus
ELN	Ectopic Lymphoid Neogenesis
FDCs	Follicular dendritic cells
Fo	Follicular B cells
FPG	Fasting plasma glucose
GAD	Glutamic acid decarboxylase

GC	Germinal Centres
H&E	Haematoxylin and Eosin staining
Hb	Haemoglobin
HbA1c	Glycated Hb
HCV	Hepatitis C Virus
HEVs	High Endothelial venules
HIP	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HP	Helicobacter Pylori
HRP	Horseradish peroxidase
HTLV-1	Human T cell Leukaemia Virus Type 1
IA-2A	Tyrosine phosphatase IA2
IAA	Autoantibodies to insulin
ICA	Islet cell autoantibodies
IDDM	Insulin dependent DM
IF	Immuno Fluorescence
IFG	Impaired Fasting Glucose
Ig	Immunoglobulin
IGT	Impaired Glucose Tolerance
IHC	Immuno Histo Chemistry
IL	Interleukine
INF	Interferon
LN	Lymph node
LT	Lymphotoxin
LTxβr-Ig	Lymphotoxin β receptor-Ig

MAd-CAM	Mucosal addressin cellular adhesion molecule-1
MHC	Major Histocompatibility Complex
MZ	Marginal zone
NIDDM	Non-insulin dependent DM
NK	Natural Killer
NOD	Non-obese diabetic
OGTT	Oral glucose tolerance test
PAP	Pancreatitis-asssociated protein
PARP	Poly(ADP-ribosil)ated protein
PNAd	Peripheral node addressing
PRRs	Pattern-recognition receptors
RA	Rheumatoid Arthritis
Reg	Regenerating
RF	Rheumatoid factor
SG	Salivary Glands
S1P	Sphingosine 1 Phosphatase
SHM	Somatic Hyper Mutation
SLE	Systemic Lupus Erythematosus
SLO	Secondary Lymphoid Organ
SS	Sjögren's syndrome
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCR	T cell receptor
Th-1	T helper-1
TLR	Toll like receptor
TNF	Tumour necrosis factor

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CHAPTER 1

INTRODUCTION

Autoimmune diseases arise when the immune system turns its defences upon normal components of the body such as insulin-producing pancreatic cells in type 1 diabetes (T1D) or salivary glands in Sjögren's Syndrome (SS). The result of these processes is a sustained immune response that produces local and/or systemic chronic inflammation, tissue destruction and loss of function with progressing clinical symptoms. This first chapter discusses the microarchitecture, molecular determinants and pathogenesis of chronic inflammatory disease focusing in particular on T1D and SS.

1.1 Diabetes mellitus

1.1.1 Introduction

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycaemia. Several distinct types of DM exist and are caused by a complex interaction of genetics and environmental factors. Depending on the aetiology of the DM, factors contributing to hyperglycaemia include reduced insulin secretion, decreased glucose utilization, and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system. With an increasing incidence worldwide, DM will be a leading cause of morbidity and mortality for the foreseeable future.

1.1.2 Classification

DM is classified on the basis of the pathogenic process that leads to hyperglycaemia, as opposed to earlier criteria such as age of onset or type of therapy (Fig. 1.1). The two broad categories of DM are designated as type 1 and type 2 (Table 1.1). Both types of diabetes are preceded by a phase of abnormal glucose homeostasis as the pathogenic processes progresses. T1D is the result of complete or near-total insulin deficiency. T2D is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Distinct genetic and metabolic defects in insulin action and/or secretion give rise to the common phenotype of hyperglycaemia in T2D and have important potential therapeutic implications now that pharmacologic agents are available to target specific metabolic derangements. T2D is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT).

Two features of the current classification of DM diverge from previous classifications. First, the terms insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) are obsolete. Since many individuals with T2D eventually require insulin treatment for the control of glycaemia, the use of the term NIDDM generated considerable confusion. A second difference is that age is not a criterion in the classification system. Although T1D most commonly develops before the age of 30, an autoimmune β cell destructive process can develop at any age. It is estimated that between 5 and 10% of individuals who develop DM after age 30 have T1D. Likewise, T2D more typically develops with increasing age but is now being diagnosed more frequently in children and young adults, particularly in obese adolescents.

T1D is characterized by destruction of the pancreatic β cells of the islets of Langerhans, which subsequently lead to insulin deficiency (1).

Type 1A diabetes (about 90% of Type 1) is due to an autoimmune destruction of the β cells and is associated with anti-glutamic acid decarboxylase, anti-islet cell or anti-insulin antibodies, whereas in Type 1B diabetes (remaining 10% of Type 1), particularly in non caucasian, there is no evidence of autoimmunity (2). T1D represents about 5% of diabetes cases. The incidence of T1D varies according to country, race and age but is increasing with an average of 2-5% per year, worldwide (1).

Past descriptions depict T1D as a disease of childhood and early adulthood, with a sudden, severe presentation - ie, most people being younger than 20 years of age at diagnosis and presenting with diabetic ketoacidosis. More recent data suggest that only about 50–60% of those with T1D are younger than 16-18 years at presentation and that such disease occurs at a low incidence level throughout adulthood. Furthermore, studies of the natural history of T1D in first-degree relatives with positive islet-related antibodies indicate that there is often a long prodrome preceding clinical onset, in which glucose homoeostasis is either normal or only mildly disturbed (3, 4).

Finally, diabetic ketoacidosis is not universal at disease onset: in children, rates of 15–67% have been reported, inversely correlated with the incidence of T1D in the particular geographic location (5).

Figure 1.1

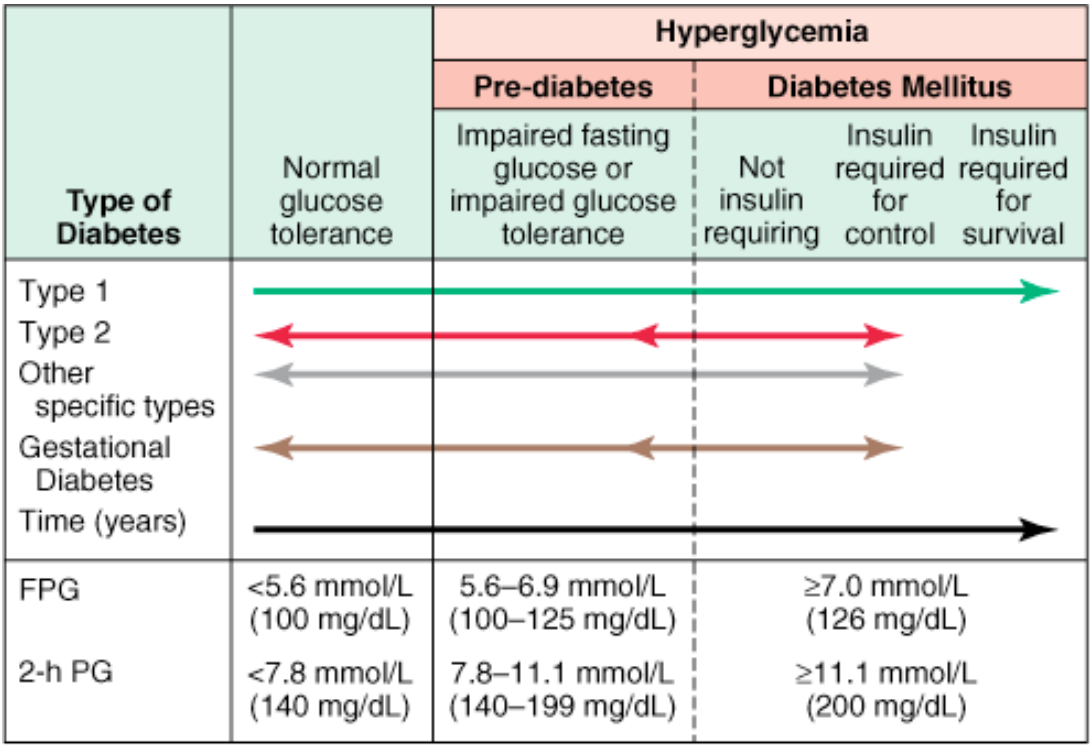

Figure 1.1 Spectrum of glucose homeostasis and diabetes mellitus (DM). The spectrum from normal glucose tolerance to diabetes in T1D, T2D, other specific types of diabetes, and gestational D is shown from left to right. In most types of DM, the individual traverses from normal glucose tolerance to impaired glucose tolerance to overt diabetes. Arrows indicate that changes in glucose tolerance may be bi-directional in some types of diabetes. For example, individuals with T2D may return to the impaired glucose tolerance category with weight loss; in gestational D diabetes may revert to impaired glucose tolerance or even normal glucose tolerance after delivery. The FPG and 2-h PG, after a glucose challenge for the different categories of glucose tolerance, are shown at the lower part of the figure. These values do not apply to the diagnosis of gestational D. Some types of DM may or may not require insulin for survival, hence the dotted line. (Conventional units are used in the figure). (Adapted from the American Diabetes Association, 2007).

Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo dL, Jameson JL, Lo scalzo J.

Table 1.1

Etiologic classification of diabetes mellitus.

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)

A. Immune-mediated

B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance)

III. Other specific types of diabetes

A. Genetic defects of cell function characterized by mutations in:

1. Hepatocyte nuclear transcription factor (HNF) 4 (MODY 1)
2. Glucokinase (MODY 2)
3. HNF-1 (MODY 3)
4. Insulin promoter factor-1 (IPF-1; MODY 4)
5. HNF-1 (MODY 5)
6. NeuroD1 (MODY 6)
7. Mitochondrial DNA
8. Subunits of ATP-sensitive potassium channel
9. Proinsulin or insulin conversion

B. Genetic defects in insulin action

1. Type A insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipodystrophy syndromes

C. Diseases of the exocrine pancreas—pancreatitis, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, fibrocalculous pancreatopathy, mutations in carboxyl ester lipase

D. Endocrinopathies—acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, hyperthyroidism, somatostatinoma, aldosteronoma

E. Drug- or chemical-induced—Vacor, pentamidine, nicotinic acid, glucocorticoids, thyroid hormone, diazoxide, -adrenergic agonists, thiazides, phenytoin, -interferon, protease inhibitors, clozapine

F. Infections—congenital rubella, cytomegalovirus, coxsackie

G. Uncommon forms of immune-mediated diabetes—"stiff-person" syndrome, anti-insulin receptor antibodies

H. Other genetic syndromes sometimes associated with diabetes—Down's syndrome, Klinefelter's syndrome, Turner's syndrome, Wolfram's syndrome, Friedreich's ataxia, Huntington's chorea, Laurence-Moon-Biedl syndrome, myotonic dystrophy, porphyria, Prader-Willi sindrome

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IV. Gestational diabetes mellitus (GDM)

Note: MODY, maturity onset of diabetes of the young.

Source: Adapted from American Diabetes Association, 2007.

1.1.2.1 Other types of DM

Other aetiologies for DM include specific genetic defects in insulin secretion or action, metabolic abnormalities that impair insulin secretion, mitochondrial abnormalities, and a host of conditions that impair glucose tolerance. Maturity onset diabetes of the young (MODY) is a subtype of DM characterized by autosomal dominant inheritance, early onset of hyperglycaemia (usually <25 years), and impairment in insulin secretion. Mutations in the insulin receptor cause a group of rare disorders characterized by severe insulin resistance.

DM can result from pancreatic exocrine disease when the majority of pancreatic islets are destroyed. Hormones that antagonize insulin action can also lead to DM. Thus, DM is often a feature of endocrinopathies such as acromegaly and Cushing's disease. Viral infections have been implicated in pancreatic islet destruction but are an extremely rare cause of DM. A form of acute onset of T1D, termed fulminant diabetes, has been noted in Japan and may be related to viral infection of islets (6).

1.1.3 Epidemiology of T1D and T2D

The worldwide prevalence of DM has risen dramatically over the past two decades, from an estimated 30 million cases in 1985 to 177 million in 2000. Based on current trends, >360 million individuals will have diabetes by the year 2030 (Fig. 1.2). Although the prevalence of both type 1 and T2D is increasing worldwide, the prevalence of T2D is rising much more rapidly because of increasing obesity and reduced activity levels as countries become more industrialized. This is true in most countries, and 6 of the top 10 countries with the highest rates are in Asia. In the United States, the Centers for Disease Control and Prevention (CDC) estimated that 20.8 million persons, or 7% of the population, had diabetes in 2005 (~30% of individuals with diabetes were undiagnosed). Approximately 1.5 million individuals (>20 years) were newly diagnosed with

diabetes in 2005. DM increases with aging. In 2005, the prevalence of DM in the United Sates was estimated to be 0.22% in those <20 years and 9.6% in those >20 years. In individuals >60 years, the prevalence of DM was 20.9%. The prevalence is similar in men and women throughout most age ranges (10.5% and 8.8% in individuals >20 years) but is slightly greater in men >60 years. Worldwide estimates project that in 2030 the greatest number of individuals with diabetes will be 45–64 years of age.

There is considerable geographic variation in the incidence of both type 1 and T2D. Scandinavia has the highest incidence of T1D (e.g., in Finland, the incidence is 35/100,000 per year). The Pacific Rim has a much lower rate (in Japan and China, the incidence is 1–3/100,000 per year) of T1D; Northern Europe and the United States have an intermediate rate (8–17/100,000 per year). Much of the increased risk of T1D is believed to reflect the frequency of high-risk HLA alleles among ethnic groups in different geographic locations. The prevalence of T2D and its forerunner, IGT, is highest in certain Pacific islands, intermediate in countries such as India and the United States, and relatively low in Russia. This variability is likely due to genetic, behavioural, and environmental factors. DM prevalence also varies among different ethnic populations within a given country. In 2005, the CDC estimated that the prevalence of DM in the United States (age > 20 years) was 13.3% in African Americans, 9.5% in Latinos, 15.1% in Native Americans (American Indians and Alaska natives), and 8.7% in non-Hispanic whites. Individuals belonging to Asian-American or Pacific-Islander ethnic groups in Hawaii are twice as likely to have diabetes compared to non-Hispanic whites. The onset of T2D occurs, on average, at an earlier age in ethnic groups other than non-Hispanic whites.

Diabetes is a major cause of mortality, but several studies indicate that diabetes is likely underreported as a cause of death. A recent estimate suggested that diabetes was the fifth leading cause of death worldwide and was responsible for almost 3 million deaths annually (1.7–5.2% of deaths worldwide).

There is also a significant trend towards decreasing age at presentation, particularly in children younger than 5 years (7). This shift to a younger age at diagnosis could indicate either exposure to heavier doses of environmental triggers or perhaps the increasing weight of the population.

Finally, migrating populations take on the incidence rates of their new countries within a short time. For example, incidence rates for T1D in south Asian children in the UK are similar to those of white or other ethnic backgrounds in the same area, which is in striking contrast to the very low rates reported from Asia (8). This convergence of incidence rates for immigrant populations with those of the background population lends support to a strong contribution of environmental factors in causation.

Figure 1.2

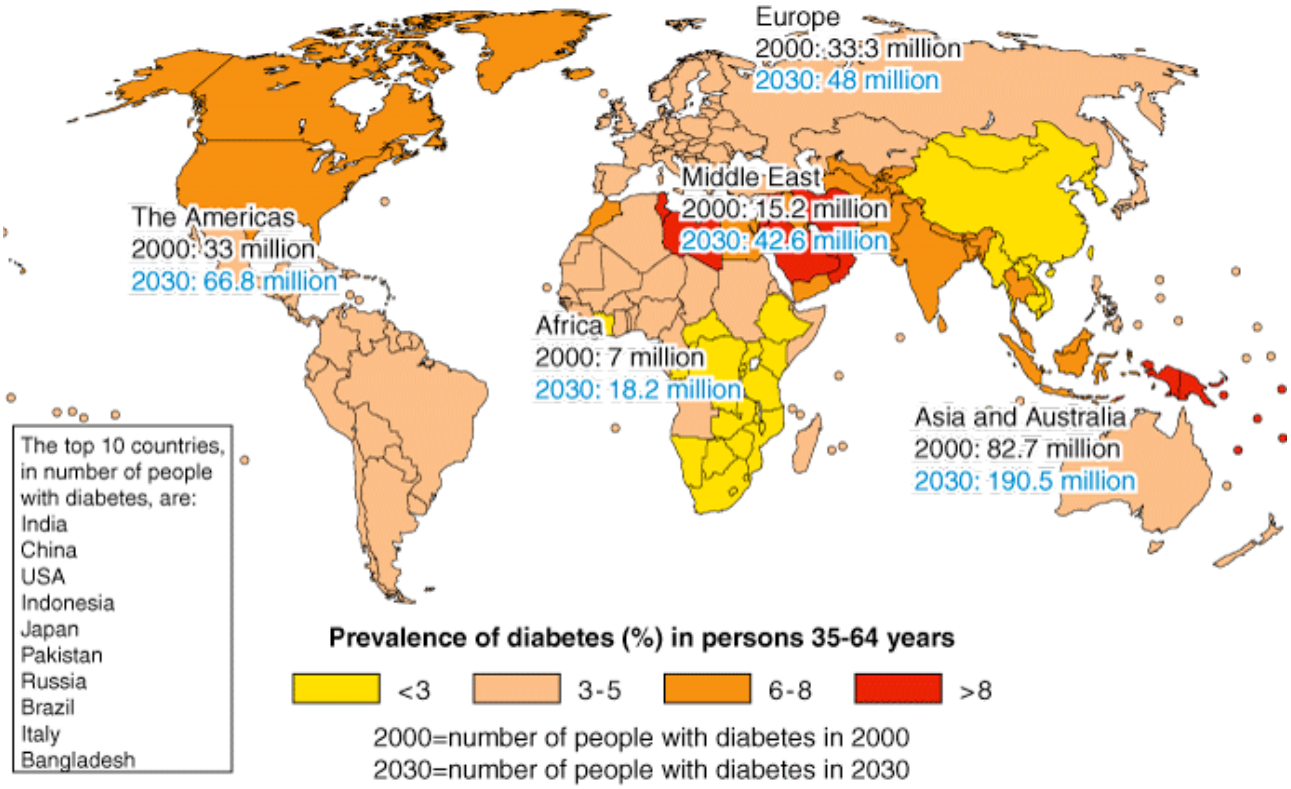

Figure 1.2 Worldwide prevalence of diabetes mellitus. The prevalence of diabetes in 2000 and the projected prevalence in 2030 are shown by geographical region *(adapted from S Wild et al: Diabetes Care 27:1047, 2004).*

1.1.4 Diagnosis

The National Diabetes Data Group and World Health Organization have issued diagnostic criteria for DM (Table 1.2) based on the following premises: (1) the spectrum of fasting plasma glucose (FPG) and the response to an oral glucose load (OGTT - oral glucose tolerance test) varies among normal individuals, and (2) DM is defined as the level of glycaemia at which diabetes-specific complications occur rather than on deviations from a population-based mean. For example, the prevalence of retinopathy in Native Americans (Pima Indian population) begins to increase at a FPG > 6.4 mmol/L (116 mg/dL).

Glucose tolerance is classified into three categories based on the FPG (Fig. 1.1): (1) FPG < 5.6 mmol/L (100 mg/dL) is considered normal; (2) FPG = 5.6–6.9 mmol/L (100–125 mg/dL) is defined as IFG; and (3) FPG 7.0 mmol/L (126 mg/dL) warrants the diagnosis of DM. Based on the OGTT, IGT is defined as plasma glucose levels between 7.8 and 11.1 mmol/L (140 and 199 mg/dL) and diabetes is defined as a glucose>11.1 mmol/L (200 mg/dL) 2 h after a 75-g oral glucose load (Table 1.2). Some individuals have both IFG and IGT. Individuals with IFG and/or IGT, recently designated pre-diabetes by the American Diabetes Association (ADA), are at substantial risk for developing T2D (25–40% risk over the next 5 years) and have an increased risk of cardiovascular disease.

The current criteria for the diagnosis of DM emphasize that the FPG is the most reliable and convenient test for identifying DM in asymptomatic individuals. A random plasma glucose concentration 11.1 mmol/L (200 mg/dL) accompanied by classic symptoms of DM (polyuria, polydipsia, weight loss) is sufficient for the diagnosis of DM (Table 1.2).

Some investigators have advocated the haemoglobin A1C (HbA1C) as a diagnostic test for DM but there is no consensus for the inclusion of this test for diagnosis.

It is important to differentiate between autoimmune β cell destruction and an insulin-resistant state. The clinical presentations of type 1 and type 2 diabetes have become blurred. In the past, it was often assumed that children and adolescents who presented in diabetic ketoacidosis had autoimmune destruction of their insulin-producing pancreatic β cells. These youngsters were classified as T1D. Middle-aged adults with diabetes were assumed to have insulin resistance that resulted in glucose intolerance, and they were classified as T2D.

Unfortunately, obesity has become rampant in the adolescent population, and now there are many young people with insulin resistance and T2D (9). In contrast, occasionally there are adults who have autoimmune-mediated destruction and failure of their β cells (LADA) (10). It is important to determine the type of diabetes so that appropriate therapy can be initiated. Specific autoantibodies to islet cells, insulin, and glutamic acid decarboxylase help identify patients with autoimmune T1D. Antibodies to glutamic acid decarboxylase 65 (GAD65) are considered by many to be the most specific antibodies. 70 to 80% of type 1 diabetics have GAD antibodies prior to or at the onset of disease. GAD65 is detected in less than 3% of control subjects (11). Islet cell antibodies (ICA) are also present in the serum of patients with T1D. When the autoantibody assay for ICA is added to GAD65, it increases autoimmune disease detection to greater than 90%.

Table 1.2

Criteria for the diagnosis of diabetes mellitus.

- Symptoms of diabetes plus random blood glucose concentration 11.1 mmol/L (200 mg/dL)[a]

  or

- Fasting plasma glucose 7.0 mmol/L (126 mg/dL)[b]

  or

- Two-hour plasma glucose 11.1 mmol/L (200 mg/dL) during an oral glucose tolerance test[c]

a Random is defined as without regard to time since the last meal.

b Fasting is defined as no caloric intake for at least 8 h.

c The test should be performed using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water; not recommended for routine clinical use.

Note: In the absence of unequivocal hyperglycaemia and acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day.

Source: Adapted from American Diabetes Association, 2007.

1.1.5 Screening

Widespread use of the FPG as a screening test for T2D is recommended because: (1) a large number of individuals who meet the current criteria for DM are asymptomatic and unaware that they have the disorder, (2) epidemiologic studies suggest that T2D may be present for up to a decade before overt clinical onset and diagnosis, (3) as many as 50% of individuals with T2D have one or more diabetes-specific complications at the time of their diagnosis, and (4) treatment of T2D may favourably alter the natural history of DM. The ADA recommends screening all individuals >45 years every 3 years and screening individuals at an earlier age if they are overweight [body mass index (BMI) > 25 kg/m2] and have one additional risk factor for diabetes. In contrast to T2D, a long asymptomatic period of hyperglycaemia is rare prior to the diagnosis of T1D.

1.1.6 Pathogenesis

1.1.6.1 Type 1 Diabetes

Type 1 diabetes (T1D) is the result of interactions of genetic, environmental, and immunological factors that ultimately lead to the destruction of the pancreatic β cells and insulin deficiency. T1D results from autoimmune β cell destruction and most, but not all, individuals have evidence of islet-directed autoimmunity (3, 12-14). Although some individuals, mostly of African/American or Asian heritage, have the clinical phenotype of T1D but lack immunologic markers indicative of an autoimmune process involving the β cells and develop insulin deficiency by unknown, non immune mechanisms, for the purpose of this dissertation I will refer to the autoimmune pathogenesis of T1D. The temporal development of T1D is shown schematically as a function of β cell mass in Fig.

1.3. Individuals with a genetic susceptibility have normal β cell mass at birth but begin to lose β cells secondary to autoimmune destruction that occurs over months to years. This autoimmune process is thought to be triggered by an infectious or environmental stimulus and to be sustained by a β cell-associated antigen. In the majority of cases, markers of autoimmunity towards pancreatic insulae appear after the putative triggering event but before diabetes becomes clinically overt. Although β cell mass then begins to decline, and insulin secretion becomes progressively impaired, normal glucose tolerance can be maintained for prolonged amount of time. In this regard, the rate of decline in β cell mass varies widely among individuals, with some patients progressing rapidly to clinical diabetes and others evolving more slowly. Features of diabetes do not become evident until a majority of β cells are destroyed (~80%). At this point, residual functional β cells can still exist but are insufficient in number to maintain glucose tolerance. The events that trigger the transition from glucose intolerance to frank diabetes are often associated with increased insulin requirements, as might occur during infections or puberty. After the initial clinical presentation of T1D, a "honeymoon" phase may ensue. During this phase glycaemic control is achieved with modest doses of insulin or, rarely, insulin is not needed (15). However, this fleeting phase of endogenous insulin production from residual β cells disappears as the autoimmune process destroys the remaining β cells, and the individual becomes completely dependent on exogenous insulin.

Figure 1.3

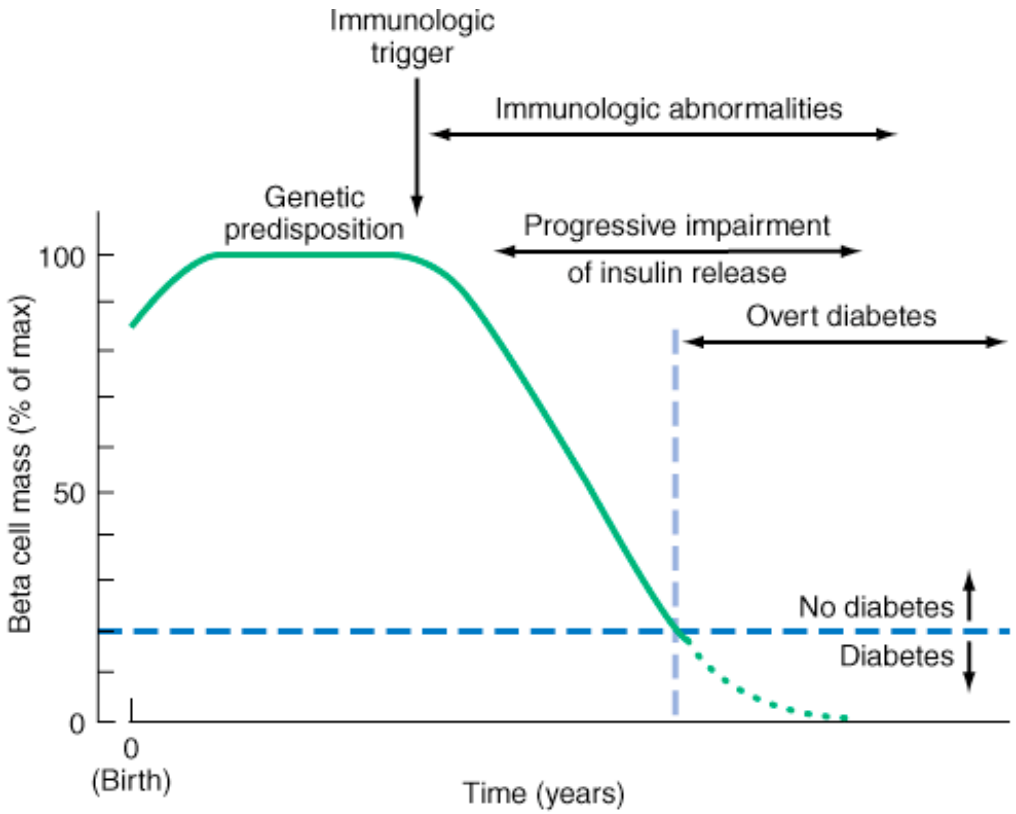

Figure 1.3 Temporal model for development of type 1 diabetes. Individuals with a genetic predisposition are exposed to an immunologic trigger that initiates an autoimmune process, resulting in a gradual decline in β cell mass. The downward slope of the β cell mass varies among individuals and may not be continuous. This progressive impairment in insulin release results in diabetes when ~80% of the β cell mass is destroyed. A "honeymoon" phase may be seen in the first 1 or 2 years after the onset of diabetes and is associated with reduced insulin requirements.
Adapted from Medical Management of Type 1 Diabetes, 3d ed, JS Skyler (ed). American Diabetes Association, Alexandria, VA, 1998.

1.1.6.1.1 Proposed models of T1D development

In the 1980s Eisenbarth (16) proposed a model for the development of the immune form of T1D and although our understanding has progressed significantly since then, the basic aspects of this model remain pertinent. This model postulates that everyone is born with a degree of susceptibility to develop T1D: for some this susceptibility is high, for others very low. Susceptibility is largely inherited, residing predominantly in the HLA genotypes DR and DQ, and to a lesser extent in a host of other genetic loci termed IDDM susceptibility genes. The HLA locus is thought to confer about 50% of the genetic susceptibility, roughly 15% from two other genes - *insulin-VNTR* (*IDDM2*) and *CTLA-4* (*IDDM12*) – with minor contributions from the other IDDM genes (3, 12, 13, 17). The specific contribution of individual HLA haplotypes and other IDDM genes to the genetic susceptibility of T1D are discussed in Section 1.1.6.1.3 below. The next step requires exposure to one or more environmental triggers that alter immune function, thereby initiating β cell destruction. Putative triggers include pathogens, environmental toxins vaccinations etc which will be discussed in section 1.1.6.1.4.

Whatever the initial trigger leading to the autoimmune dysregulation typical of T1D, the final result is an abnormal activation of the T cell-mediated immune system which leads to an inflammatory response within the islets (insulitis) associated with a humoral (B cell) response with production of antibodies to β cell antigens. The histological characteristic and dynamic development of the immune cell infiltration within the pancreatic insulae are discussed in details in section 1.1.6.1.5. The production of autoantibodies against these β cell-associated antigens, such as insulin (IAA), glutamic acid decarboxylase (GADA/GAA), and the protein tyrosine phosphatase IA2 (IA-2AA), is one of the hallmark of the disease and their prevalence and clinical significance are discussed in section 1.1.6.1.6.

Chronic insulitis and immune-mediated destruction of the β cells leads to progressive loss of insulin-secretory reserve with, in order, loss of first phase insulin secretion in response to an intravenous glucose tolerance test, then to clinical diabetes when insulin secretion falls below a critical amount, and finally, in most but not all those with T1D, to a state of absolute insulin deficiency (3, 16).

In addition to the above model, also the hygiene and accelerator hypotheses warrant mention (18, 19). The hygiene hypothesis stems from observations that atopic disorders such as asthma are more common in affluent than in traditional societies, their prevalence rising with increasing modernisation, and that such disorders are less frequent in children in large families or those families receiving day care than in children in small families or those not in day care (19). Similarities exist between these findings and reports of T1D (20). These findings suggest that children in these circumstances are less exposed to infections or other immune challenges early in life that act as protective environmental influences. Gale has proposed that the protective effect is mediated by regulatory T lymphocytes. Although plausible, this hypothesis lacks credible scientific support in the causation of T1D (21).

In the accelerator hypothesis, Wilkin (18) postulates that diabetes is a single disease, rather than two distinct entities, T1D and T2D. He argues that the two types of diabetes are distinguished only by the rate of β cell loss, and the specific accelerators responsible. Wilkin implicates three accelerators: the first is the intrinsic potential for a high rate of β cell apoptosis, an essential but insufficient step in the development of diabetes. The second accelerator is insulin resistance, resulting typically from weight gain and physical inactivity, and is central to the proposed link between the two types of diabetes. Insulin resistance puts pressure on a β cell mass already at risk for accelerated apoptosis, contributing to the expression of clinical diabetes. The third accelerator is

present only in those individuals with genetically determined predisposition to β cell autoimmunity. The metabolically more active β cell, in insulin-resistant individuals who are genetically biased towards a high rate of apoptosis, is at greatest risk for rapid functional deterioration and expression of typical T1D. In the absence of this immune accelerator, apoptosis is slower and progression is towards T2D.

1.1.6.1.2 β cell loss in T1D

As previously mentioned, the clinical onset of T1D occurs when more than 80% of the β cells have been destroyed. Nevertheless, the level of β cell loss might actually be less severe than this value and heterogeneous at disease onset in 40–90% of patients with T1D who are older than 10 years of age. The secretion of insulin in these patients can be also higher than expected even if severe hyperglycaemia is found at diagnosis. Data from studies in NOD mice suggest that inflammatory mediators, such as cytokines, contribute to both functional impairment of β cells and peripheral insulin resistance, as female NOD mice develop a progressive glucose intolerance that parallels the aggravation of insulitis (22). These observations suggest that inflammatory molecules released by infiltrating mononuclear cells induce a reversible inhibition of β cell function, which precedes the actual β cell destruction. In T1D, local, pancreatic islet inflammation (insulitis) contributes to the progressive loss of insulin producing β cells, which eventually renders the patients insulin dependent for life. Although other islet cell types [α cells (glucagon-producing), δ cells (somatostatin-producing), or PP cells (pancreatic polypeptide-producing)] are functionally and embryologically similar to β cells and express most of the same proteins as β cells, they are spared from the autoimmune process. Inflammatory mediators contribute to the induction and amplification of the immune reaction against the β cells and, at later stages, to the stabilization and maintenance of insulitis. Inflammation might contribute not only to β cell destruction but also contributes to prolonged suppression of β cell function, inhibition or stimulation of β cell

regeneration and peripheral insulin resistance. These different roles of inflammation take place during different phases of the course of T1D, and might be influenced by patients' genetic background, which contributes to disease heterogeneity.

I shall next discuss in details the main components of the multi-step hypothesis of T1D development: genetic predisposition, environmental factors and autoimmune dysregulation.

1.1.6.1.3 Genetic susceptibility

Susceptibility to T1D involves multiple genes. The concordance of T1D in identical twins ranges between 30 and 70%, indicating that additional modifying factors are likely involved in determining whether diabetes develops. The major susceptibility gene for T1D is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40–50% of the genetic risk of developing T1D. This region contains genes that encode the class II MHC molecules, which present antigens to helper T cells and thus are involved in initiating the adaptive immune response. The ability of class II MHC molecules to present antigens is dependent on the amino acid composition of their antigen-binding sites. Amino acid substitutions may influence the specificity of the immune response by altering the binding affinity of different antigens for class II molecules. Most individuals with T1D have the HLA DR3 and/or DR4 haplotype. Refinements in genotyping allowed the identification of both high risk (eg, *DR3/4, DQA1*0301-DQB1*0302,* and *DQA1*0501-DQB1*0201*) and protective HLA haplotypes (eg, *DQA1*0102-DQB1*0602,* associated with diabetes resistance, and DRB1*1401, associated with overall protection from diabetes).

These haplotypes are present in 40% of children with T1D as compared to 2% of the normal U.S. population. However, most individuals with predisposing haplotypes do not develop diabetes.

In addition to MHC class II associations, at least 10 different genetic loci contribute susceptibility to T1D (loci recently identified include polymorphisms in the promoter region of the insulin gene, the CTLA-4 gene, interleukin-2 receptor, IFIH1, and PTPN22).

Other genes associated with either rare syndromes including diabetes (eg, *AIRE* and *Foxp3*) or other autoimmune conditions (eg, *PTPN22*) might also provide important insights into the immune pathogenesis of T1D.

Although the risk of developing T1D is increased tenfold in relatives of individuals with the disease, the risk is relatively low: 3–4% if the parent has T1D and 5–15% in a sibling (depending on which HLA haplotypes are shared). Hence, most individuals with T1D do not have a first-degree relative with this disorder.

1.1.6.1.4 Environmental factors

The incidence of T1D has risen steadily in developed countries from the 1950s to the present day, with the recent, alarming prediction that it will double in children under age 5 years by 2020 (23). There seems little doubt that T1D is a "disease of civilization" caused by the numerous alterations to our environment, including sanitation, healthcare, and diet (24, 25). These environmental changes could act in utero, in the first 3 years of life (26) and thereafter, leading to the inflammatory events immediately preceding diagnosis.

During infancy, the immune system may be provoked with a massive rebuilding of the pancreatic islets and their β cells via a naturally programmed cell death or apoptosis. In children genetically susceptible to T1D with reduced immune tolerance to islet antigens including preproinsulin (PPI), these events could be endogenous initiating factors of disease not requiring exogenous factors.

A variety of virus infections could enhance susceptibility to autoimmune β cell destruction and T1D, provided that susceptibility alleles at specific loci are present. Seasonal differences in viral infections, combined with other seasonal effects such as reduced vitamin D levels in more northern countries during the winter months (27), could help explain the well-established seasonality of T1D diagnosis itself. However, it seems unlikely that vaccination against one type of virus will prevent many cases of T1D.

The relationship between virus and susceptibility to T1D has been long investigated and viruses such as Epstein Barr Virus and cytomegalovirus are shown to have great impact (28). Taken into account the timing and profiles of the autoantibody peaks observed in the Finnish DIPP study, enterovirus infections appear to be the most probable trigger of β cell autoimmunity. It has been proposed that pancreatic β cell tropic variants of the coxsackie B virus are present in the general population and that they are able to induce β cell damage in susceptible individuals by easily infecting β cells (29). Recent studies have shown that enterovirus genome or enterovirus proteins can be detected in pancreatic islets of patients affected by T1D (30).

An Australian study reported molecular homology between the VP7 protein of rotavirus and T-cell epitopes in the protein tyrosine phosphatase–related IA-2 molecule and in the GAD65 molecule (31).

Epidemiological studies demonstrated a close association between T1D and Acute Lymphoblestic Leukaemia, caused by HTLV-1 (32).

Numerous environmental events have been proposed to trigger the autoimmune process in genetically susceptible individuals; however, none have been conclusively linked to diabetes. Identification of an environmental trigger has been difficult because the event may precede the onset of DM by several years (Fig.1.3). Putative triggers other than viruses include environmental toxins (eg. nitrosamines) (16), or foods (eg. early exposure to cow's milk proteins, cereals, or gluten) (16, 17). A close relation has been identified with congenital rubella (15). The results of a 2004 study did not accord with a causal relation between childhood vaccination and T1D (18).

Some studies have indicated that the lack of oral vitamin D in infancy increases the subsequent risk of type 1 diabetes (25).

Also, early exposure to cow's milk formula results in an immune response to bovine insulin present in formulas and differing from human insulin by three amino acids: two in the A chain and one in the B chain. Studies show that infants who were exclusively breast-fed at least up to the age of 3 months had substantially lower IgG class antibodies to bovine insulin than those who were exposed to the formula before that age (33).

1.1.6.1.5 Development of insulitis: from innate immunity to tertiary lymphoid structures

Recognition by the mammalian immune system of invading microorganisms depends on innate and adaptive components. The innate immune system recognizes microorganisms by pattern recognition receptors (PRRs), including toll-like receptors (TLRs), riG-i, mDa-5 and receptors of the nucleotide binding oligomerization domain like receptor (NLR) family. Activation of these signalling molecules contributes to the development of various autoimmune diseases, such as systemic lupus erythematosis, rheumatoid arthritis and autoimmune hepatitis (34). Innate immune cells such as macrophages, NK, dendritic cells are likely to be important at this stage. Some components of the innate immunity response, including TLRs, contribute to the development of insulitis and T1D in animal models (35). Studies performed in the BioBreeding Diabetes Resistant (BBDR) and LEW1.WR1 rat models of T1D demonstrate that virus infection leads to islet destruction via mechanisms that may involve TLR9-induced innate immune system activation (36). Data from these studies also show that TLR upregulation can synergize with virus infection to dramatically increase disease penetrance. Reports from murine models of T1D with spontaneous or induced T1D implicate TLR1, TLR2, TLR3, and TLR7 in disease mechanisms (37) (38, 39).

The histological hallmark of T1D is the development of lymphomonocytic infiltrates within the pancreatic insulae, a process termed insulitis. The importance of continuous antigen stimulation in this process is demonstrated by evidence that after destruction of the β cell component and islets atrophy, the inflammatory process commonly abates and most immunological markers disappear. Studies of the autoimmune process in humans and in spontaneous animal models of T1D (i.e. NOD mouse and BB rat) have identified the following abnormalities in the cellular and humoral arms of the immune system: (1) activated T lymphocytes in the islets, peripancreatic lymph nodes, and systemic circulation; (2) Expansion of Ag-specific Th1 lymphocytes that proliferate when stimulated with islet proteins; (3) release of inflammatory cytokines within the insulitis and (4) production of islet cell autoantibodies. The precise mechanisms of the immune-mediated β cell death are not completely elucidated but may involve formation of nitric oxide metabolites, apoptosis, and direct CD8+ T cell cytotoxicity. The islet destruction is primarily mediated by CD8+ T lymphocytes rather than islet autoantibodies, as antibodies do not generally react with the cell surface of islet cells and are not capable of transferring diabetes to animals. Suppression of the autoimmune process (cyclosporine, T lymphocyte antibodies) at the time of diagnosis of diabetes slows the decline in β cell destruction, but the safety of such interventions is unknown. In addition to cell-mediated autoimmunity, β cells seem to be particularly susceptible to the toxic effect of cytokines such as tumor necrosis factor (TNF), interferon γ, and interleukin 1 (IL-1) (40).

Adaptive immune responses within the pancreas in T1D are likely to be fuelled by a series of islet antigens which include insulin, glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the neurotransmitter GABA), ICA-512/IA-2 (homology with tyrosine phosphatases), and phogrin (insulin secretory granule protein). With the exception of insulin, none of the autoantigens are β cell specific. Current theories suggest that the while the initiation of the autoimmune process might be directed towards a dominant β cell antigen (i.e. insulin), the chronicity of inflammation is

associated with the spreading to other islet epitopes, possibly generated during the immune destruction through exposure of a series of secondary autoantigens (41).

Chronic inflammation in T1D is associated with the continuous recruitment and positioning of immune cells within the islets. These processes are largely dependent on chemokines, which are signal molecules that direct leukocyte migration and activation during the early stages of an innate immune response and contribute to the transition to adaptive immunity (42). The specificity and complexity of the chemokine system derives from both the release of specific chemokines in various inflammatory reactions, and the regulated expression of their receptors in leukocytes, which varies in different immune responses (43). Expression of chemokines and their receptors is altered in several autoimmune diseases, as is also the case for T1D. Increased levels of T helper 1 (Th1) associated chemokines (CCL3, CCL4 and CXCL10) are present in patients with T1D at diagnosis (44). Indeed, most of the pathological processes that occur in the early phases of T1D take place in the islet microenvironment and in pancreatic draining lymph nodes. These tissues are difficult to access in humans and locally generated inflammatory signals are diluted in the general circulation making it difficult to dissect the relevance of specific pathways.

Thus, animal models of T1D have been instrumental in understanding the mechanisms regulating immune cell recruitment and localization during autoimmune insulitis. Studies in NOD mice have shown increased levels of CXCL10, CCL2 and CCL20 in pancreatic islets during the pre-diabetic stage. During the course of diabetes, macrophages/antigen presenting cells are the first to infiltrate the islets of NOD mice and BB rats, and depletion or inactivation of macrophages prevents the development of the disease (45). As both CXCL10 and CCL2 can attract macrophages, their early expression in the islets of NOD mice likely contributes to macrophage recruitment during the early stages of insulitis.

At a later stage of insulitis development in T1D, characterized by stabilization and maintenance of insulitis, the adaptive immune response is dominant. Studies of cellular dynamics in NOD revealed that B and T lymphocytes invade the islets simultaneously (46) and that CD4+ T cell priming and

proliferation occur in draining pancreatic lymph nodes before the onset of insulitis (47, 48). Interestingly, recent evidence suggests that with the progression from peri-insulitis to intra-insulitis, B and T cells can organize into tertiary lymphoid structures (TLS) characterised by a central T cell-rich area surrounded by B cell aggregates (49). The mechanisms regulating the formation, maintenance and function of TLS in comparison with secondary lymphoid organ (SLO) development and homeostasis are described in section 1.4. Although it has long been identified that autoimmune diabetes is associated with an imbalance between T-helper (Th)1 cells (producing IL-2 and IFNγ) and Th2 cells (expressing IL-4 and IL-5) (50) and mediated by pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which have cytotoxic, cytostatic, or cytocidal actions towards pancreatic islets (51), an increasing role for B lymphocytes in promoting the disease has been suggested. B cells are required for the disease to occur in the NOD model (52), must have autoreactive specificities, must express appropriate MHCII alleles (53), and are essential for the processing and presentation of at least one islet antigen (Ag) (54). Thus, the key pathogenic role for B cells in T1D is most likely related to Ag presentation, which is strongly favoured by the presence of an organized lymphoid setting in order to promote T-B cell interactions (55). Interestingly, B lymphocytes in TLS express a different Ig L chain gene repertoire compared to the draining pancreatic lymph nodes, supporting the hypothesis that an Ag-driven somatic hypermutation takes place within inflamed islets and is consistent with the presence of a germinal center (GC) response. These findings indicate that TLS in pancreatic islets offer a site at which B lymphocytes undergo affinity maturation and clonal selection independently from SLO.

1.1.6.1.6 Humoral autoimmunity and autoantibodies in T1D

Islet cell antibodies were the first described, but have been supplanted by more specific autoantibodies, which are easily detected by sensitive radioimmunoassay (3).

Islet cell autoantibodies (ICAs) are a composite of several different antibodies directed at pancreatic islet molecules such as GAD, insulin, and IA-2/ICA-512 and serve as a marker of the autoimmune process of T1D. Assays for autoantibodies to GAD-65 are commercially available. Testing for ICAs can be useful in classifying the type of DM as type 1 and in identifying non-diabetic individuals at risk for developing T1D. Importantly, the presence of one or more type of antibody can precede the clinical onset of T1D by years or even decades and the persistence of positive testing to multiple antibodies increases the likelihood of progression to clinical disease (56). ICAs are present at diagnosis in the majority of individuals (>75%) with new-onset T1D, in a significant minority of individuals with newly diagnosed T2D (5-10%), and occasionally in individuals with GDM (<5%). ICAs are present in 3-4% of first-degree relatives of individuals with T1D. In combination with impaired insulin secretion after IV glucose tolerance testing, they predict a >50% probability of developing T1D within 5 years. Without an associated impairment in insulin secretion, the sole presence of ICAs predicts a 5-year probability of <25%. Based on these data, the risk of a first-degree relative of developing T1D is relatively low (3, 56).

1.1.6.2 Type 2 Diabetes

Insulin resistance and abnormal insulin secretion are central to the development of T2D. Although the primary defect is controversial, most studies support the view that insulin resistance precedes an insulin secretory defect but that diabetes develops only when insulin secretion becomes inadequate (57).

1.1.6.2.1 Genetic considerations

T2D has a strong genetic component. The concordance of T2D in identical twins is between 70 and 90%. Individuals with a parent with T2D have an increased risk of diabetes; if both parents have T2D, the risk approaches 40% (58). Insulin resistance, as demonstrated by reduced glucose utilization in skeletal muscle, is present in many non-diabetic, first-degree relatives of individuals with T2D. The disease is polygenic and multifactorial since in addition to genetic susceptibility, environmental factors (such as obesity, nutrition, and physical activity) modulate the phenotype. The genes that predispose to T2D are incompletely identified, but recent genome-wide association studies have identified several genes that convey a relatively small risk for T2D (relative risk of 1.1-1.5). Most prominent is a variant of the transcription factor 7-like 2 gene that has been associated with T2D in several populations and with impaired glucose tolerance in one population at high risk for diabetes. Genetic polymorphisms associated with T2D have also been found in the genes encoding the peroxisome proliferators-activated receptor-γ, inward rectifying potassium channel expressed in β cells, zinc transporter expressed in β cells, IRS, and calpain 10. The mechanisms by which these genetic alterations increase the susceptibility to T2D are not clear, but several are predicted to alter insulin secretion.

1.1.6.2.2 Pathophysiology

T2D is characterized by impaired insulin secretion, insulin resistance, excessive hepatic glucose production and abnormal fat metabolism. Obesity, particularly visceral or central (as evidenced by the hip-waist ratio), is very common in T2D. In the early stages of the disorder, glucose tolerance remains near-normal, despite insulin resistance, because the pancreatic β cells compensate by increasing insulin output. As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. IGT, characterized by elevations in postprandial glucose, then develops. A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycaemia. Ultimately, β cell failure may ensue (59, 60).

1.1.6.2.3 Metabolic abnormalities

Abnormal muscle and fat metabolism.

Insulin resistance, the decreased ability of insulin to act effectively on target tissues (especially muscle, liver, and fat), is a prominent feature of T2D and results from a combination of genetic susceptibility and obesity (57). Insulin resistance is relative, however, since supernormal levels of circulating insulin will normalize the plasma glucose. Insulin dose-response curves exhibit a rightward shift, indicating reduced sensitivity, and a reduced maximal response, indicating an overall decrease in maximum glucose utilization (30–60% lower than in normal individuals). Insulin resistance impairs glucose utilization by insulin-sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycaemia. Increased hepatic glucose output predominantly accounts for increased FPG levels, whereas decreased peripheral glucose usage results in postprandial hyperglycaemia. In skeletal muscle, there is a greater impairment in nonoxidative glucose usage (glycogen formation) than in oxidative glucose metabolism through glycolysis. Glucose metabolism in insulin-independent tissues is not altered in T2D.

The precise molecular mechanism leading to insulin resistance in T2D has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia and are not a primary defect. Therefore, "postreceptor" defects in insulin-regulated phosphorylation/dephosphorylation may play the predominant role in insulin resistance. For example, a PI-3-kinase signalling defect may reduce translocation of GLUT4 to the plasma membrane (61). Other abnormalities include the accumulation of lipid within skeletal myocytes, which may impair mitochondrial oxidative phosphorylation and reduce insulin-stimulated mitochondrial ATP production. Impaired fatty acid oxidation and lipid accumulation within skeletal myocytes may generate reactive oxygen species such as lipid peroxides (62). Of note, not all insulin signal transduction pathways are resistant to the effects of insulin (e.g., those controlling cell growth and differentiation using the mitogenic-activated protein kinase pathway). Consequently, hyperinsulinemia may increase the insulin action through these pathways, potentially accelerating diabetes-related conditions such as atherosclerosis. The obesity accompanying T2D, particularly in a central or visceral location, is thought to be part of the pathogenic process (62). The increased adipocyte mass leads to increased levels of circulating free fatty acids and other fat cell products. In addition to regulating body weight, appetite, and energy expenditure, adipokines also modulate insulin sensitivity. The increased production of free fatty acids and some adipokines may cause insulin resistance in skeletal muscle and liver. Adipocyte products and adipokines also produce an inflammatory state and may explain why markers of inflammation such as IL-6 and C-reactive protein are often elevated in T2D (63).

1.1.6.2.4 Impaired Insulin Secretion

Insulin secretion and sensitivity are interrelated. In T2D, insulin secretion initially increases in response to insulin resistance to maintain normal glucose tolerance. Initially, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion. The response to other

nonglucose secretagogues, such as arginine, is preserved. Eventually, the insulin secretory defect progresses to a state of grossly inadequate insulin secretion.

The reason(s) for the decline in insulin secretory capacity in T2D is unclear. The assumption is that a second genetic defect - superimposed upon insulin resistance - leads to β cell failure. Islet amyloid polypeptide or amylin is cosecreted by the β cell and forms the amyloid fibrillar deposit found in the islets of individuals with long-standing T2D. Whether such islet amyloid deposits are a primary or secondary event is not known. The metabolic environment of diabetes may also negatively impact islet function. For example, chronic hyperglycaemia paradoxically impairs islet function ("glucose toxicity") and leads to a worsening of hyperglycaemia. Improvement in glycaemic control is often associated with improved islet function. In addition, elevation of free fatty acid levels ("lipotoxicity") and dietary fat may also worsen islet function.

Increased Hepatic Glucose and Lipid Production.

In T2D, insulin resistance in the liver reflects the failure of hyperinsulinemia to suppress gluconeogenesis, which results in fasting hyperglycaemia and decreased glycogen storage by the liver in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, though likely after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle.

1.2 Sjogren's Syndrome

1.2.1 Definition and epidemiology

In 1932, Henrik Sjögren, a Swedish ophthalmologist described a triad of keratoconjunctivitis sicca, xerostomia and Rheumatoid Arthritis (RA). This was the first report of the disease, today known worldwide with the name of Sjögren's Syndrome (SS).

SS is a chronic autoimmune disease, characterized by exocrine gland involvement leading to glandular dysfunction, aberrant autoantibody production and extra glandular systemic features, including fatigue, arthritis and diverse organ involvement (64). Approximately one-third of patients present with systemic manifestations; a small but significant number of patients may develop malignant lymphoma. The disease presents alone (primary SS) or in association with other autoimmune rheumatic diseases (secondary SS).

Middle-aged women (female-to-male ratio, 9:1) are primarily affected, although it may occur in all ages, including childhood. The prevalence of primary SS is approximately 0.5-1.0%, while 30% of patients with autoimmune rheumatic diseases suffer from secondary SS (64).

The main targets of the inflammatory process in SS are the exocrine glands (minor and major salivary glands, lachrymal etc), determining the hallmark clinical feature: the *sicca syndrome* (64).

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 onset and the progression of the disease has prompted their inclusion as a critical criterion for the diagnosis of SS.

1.2.2 Clinical aspects

SS exocrine glands are typically characterised by a chronic autoimmune process leading to severe tissue damage and glandular dysfunction. The ductal epithelium is broadly believed to represent the main target of this process and currently SS is defined as "*autoimmune epithelitis*" (65).

SS patients commonly complain of dry mouth and dry eyes (*sicca syndrome)* together with problems in swallowing, taste alterations and a broad range of ocular problems such as photosensitivity and fluctuating vision. Hallmark of the ocular involvement in SS is the development of a keratoconjunctivitis sicca. SS patients are also reported to have a high incidence of dental caries, mucositis and oral candidiasis (66). All together, these symptoms have been related to the lack of saliva and qualitative and quantitative alterations in the ocular tear flow, but it is still debated whether the loss in the secretory component of the glands represents the only cause of the glandular dysfunction. At present, most authors believe that autoantibodies, present in the blood of SS patients might contribute to this process by interfering with neurological stimuli regulating glandular secretion (66).

Similarly to many other autoimmune diseases SS might affect diverse organs: joints, gut, respiratory tract, skin, neurological system can be involved during the disease course, diversely influencing disease morbidity and prognosis (Table 1.3).

The osteoarticular involvement in SS is common (42 to 83% of SS patients) and more diffuse in the female population. The spectrum of articular manifestations is large and in one third of the patients articular manifestations develop before *sicca syndrome* (66). 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. Rarely the development of a proper arthritis has been reported (67). Musculoskeletal involvement in SS also includes the development of myalgia, association with Fibromyalgia and symptoms of diffuse weakness, usually refereed as fatigue (66).

SS patients may develop haematological abnormalities: anaemia, lymphopenia, monoclonal gammopathy and lymphoproliferative disorders have been described with various prevalence in different populations (68).

The skin is often involved in SS: skin xerosis is common (between the 23% and the 67% of SS patients) most probably related to an impaired sebaceous and sweet glands glandular secretion (69). Common cutaneous manifestations include vasculitis (palpable or non palpable purpura), urticaria vasculitis and annular lesions (70) (71). One third of SS patients presents Raynaud's phenomenon (72) often associated with articular involvement.

Neurological involvement includes central-nervous-system involvement (demyelinating disease) and the development of peripheral neuropathies (66).

A broad range of respiratory involvement has been described in SS with a prevalence of respiratory involvement in SS affected population of 2 to 75% (73-75). Nasal cavity involvement with bleeding and hyposmia, xerotrachea and bronchial hyper reactivity has been described (66, 76) (73).

The whole gastrointestinal tract might be involved in SS, most probably due to the diffuse distribution of exocrine glands in the gut. Up to 90% of the patient refers the occurrence of dysphagia (77).

A significant association between SS and primary biliary cirrhosis has been described being sicca syndrome more frequent in primary biliary cirrhosis patients (47-73% of the patients), with anti hepatocytes, anti-mitochondrial antibodies (AMA) frequently found in SS patients (78).

Histologically chronic active hepatitis with prevalent periductal involvement and portal fibrosis have been detected in the liver of SS patients (78). Finally, liver involvement during Hepatitis C virus (HCV) infection has been correlated with the development of a sicca syndrome (64), and hence is one of the exclusion criteria for the diagnosis of SS (79), presence of ANA, mixed cryoglobulinemia, AMA, anti-Ro/SS-A, RF (80) and with the development of salivary gland infiltrates similar to primary SS. Interestingly, in SS patients the prevalence of anti HCV antibodies is higher compared to the healthy population (4 to 19% of the patients) (81).

The exocrine pancreas might be involved in SS, with development of acute pancreatitis, malabsorption and calcification (82).

Renal involvement in SS is not frequent, usually asymptomatic and manifests itself only in a small percentage of patients (2-6%). Mild proteinuria is present in 44% of the patients. Defects in urine concentration have been described in 22 to 44% patients with renal involvement in SS, while clear diabetes insipidus with polydipsia and hypotonic urine is less frequent. Infrequently distal and proximal (Fanconi's syndrome) tubular defects have been described. All together tubular defects might lead to chronic metabolic acidosis, hypokalemia, muscle weakness, hyporeflexia, increased calcium mobilisation with osteopenia, hypouricemia, hypophosphatemia, nephrolitiasis and calcinosis, proteinuria. Rarely renal failure has been described. In symptomatic patients common histological finding is the presence of interstitial nephritis (55%) with prevalent T lymphocyte infiltrate, tubular atrophy, calcinosis, interstitial oedema and fibrosis. Glomerular involvement is rare with the development of a mesangio-proliferative or a membranous-proliferative glomerulonephritis.

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 (66).

Interstitial cystitis is common in SS. Finally autonomic bladder dysfunction has been described in association with anti-muscarinic receptors antibody production.

Gynaecological symptoms are often refereed and in particular dryness and development of gynaecological infection related to impaired gland secretion (66).

A strong association between SS and thyroid gland dysfunction has been reported in several studies, the most common association being between SS and with Hashimoto thyroiditis (83).

In the population of SS patients the presence of peculiar clinical features and laboratory findings has been associated with the increased risk to develop a NH B cell lymphoma (84).

Table 1.3

Incidence of Extraglandular Manifestations in Primary Sjögren's Sindrome.

Clinical Manifestation	Percent
Arthralgias/arthritis	60
Raynaud's phenomenon	37
Lymphadenopathy	14
Lung involvement	14
Vasculitis	11
Kidney involvement	9
Liver involvement	6
Lymphoma	6
Splenomegaly	3
Peripheral neuropathy	2
Myositis	1

1.2.3 Diagnosis and differential diagnosis

The diagnosis of primary SS is obtained if the patient presents with eye and/or mouth dryness, the eye tests disclose keratoconjunctivitis sicca, the mouth evaluation reveals the classic manifestations of the syndrome, and the patient's serum reacts with Ro/SS-A and/or La/SS-B autoantigens. Labial biopsy is needed when the diagnosis is uncertain (i.e. in the absence of anti-Ro/La) or to rule out other conditions that may cause dry mouth or eyes or parotid enlargement. Validated diagnostic criteria have been established by a European study and have now been further improved by a European-American study group (Table 1.4). Hepatitis C virus infection should be ruled out since, apart from serologic tests, the remainder of the clinicopathologic picture is almost identical to that of SS.

Table 1.4

Revised International Classification Criteria for Sjögren's Syndrome *a,b,c*

I. Ocular symptoms: a positive response to at least one of three validated questions.

1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than three times a day?

II. Oral symptoms: a positive response to at least one of three validated questions.

1. Have you had a daily feeling of dry mouth for more than 3 months?
2. Have you had recurrent or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry foods?

III. Ocular signs: objective evidence of ocular involvement defined as a positive result to at least one of the following two tests:

1. Shirmer's I test, performed without anesthesia (≤5 mm in 5 min)
2. Rose Bengal score or other ocular dye score (≥4 according to van Bijsterveld's scoring system)

IV. Histopathology: In minor salivary glands focal lymphocytic sialoadenitis, with a focus score ≥ 1.

V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result to at least one of the following diagnostic tests:

1. Unstimulated whole salivary flow (≤1.5 mL in 15 min)
2. Parotid sialography
3. Salivary scintigraphy

VI. Antibodies in the serum to Ro/SS-A or La/SS-B antigens, or both

[a] Exclusion criteria: Past head and neck radiation treatment, hepatitis C infection, AIDS, preexisting lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs.

[b] Primary Sjögren's syndrome: any four of the six items, as long as item IV (histopathology) or VI (serology) is positive, or any three of the four objective criteria items (items III, IV, V, VI).

[c] In patients with a potentially associated disease (e.g., another well-defined connective tissue disease), the presence of item I or item II plus any two from among items III, IV, and V may be considered as indicative of secondary Sjögren's syndrome.

Source: From Vitali C et al.

1.2.4 Pathology of SS

Despite being considered a systemic disease, SS can be easily included within the organ-specific autoimmune disorders. Data on the dynamic formation of the typical inflammatory aggregates (inflammatory foci) in SS glands have been mainly derived by studies on the salivary glands (minor and major). Salivary glands (in particular minor salivary glands) have been chosen as histological readout for SS because of their common involvement in the disease course and easy accessibility to their tissue with minor surgical procedures (i.e. minor glands or parotid biopsy).

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 myoephithelial cells), striated and excretory. Typically SS lymphomonocytoid inflammatory infiltrate is organized around the excretory ducts. Histologically the diagnosis of SS is based on 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 (85). Chisholm and Mason histological score is shown in Table 1.5.

Table 1.5

Chisholm and Mason histological score.

Grade 0	No lymphocytes
Grade 1	Slight infiltration
Grade 2	Moderate lymphocytic infiltration (less than 1 focus)
Grade 3	Presence of one focus
Grade 4	Presence of more than one focus

Diverse cell populations have been demonstrated to interact at glandular level and in the last few years a critical role in the initiation and progression of the inflammatory process has been conferred to the ductal epithelium (86). During the disease course the immune cells infiltrate the epithelium giving rise to the formation of the lymphoepithelial lesions “LESA”. In the latest stages of the disease the epithelial structures are completely subverted and only residual ducts can be observed in the glands as shown in Fig 1.4.

The aetiology of SS is unknown. However, similarly to the majority of autoimmune disorders, a combination of genetic background, gender, immunological abnormalities, environmental factors and infectious antigens etc. has been implicated in disease development.

Figure 1.4

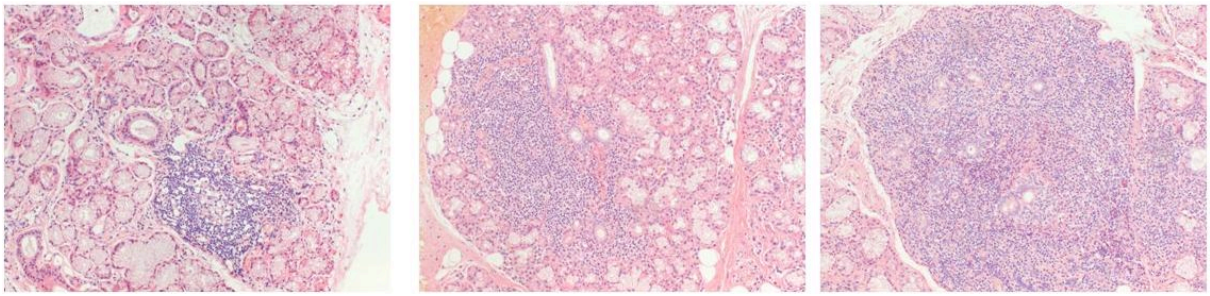

Figure 1.4 Histological features of SS. Haematoxylin and Eosin staining of minor salivary gland sections from patients with SS (samples were biopsied at Barth’s and the London Hospital, Rheumatology Department). The staining represents lymphocytic infiltrates (blue cells) showing the typical periductal organization of the foci. Original magnification X100.

1.2.4.1 Genetic factors

Higher frequency of selected histocompatibilty antigens, in particular HLA B8, DR3 e DRw52 has been observed in patients affected by primary SS compared with normal subjects. In particular, it has been reported an association between the HLA DRB1*03-DQB1*02 aplotypes and the production 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 (87).

More recently cytokine and pro-inflammatory gene polymorphisms have been described in SS and implicated in the disease pathogenesis (88).

1.2.4.2 Pathogen infection

Evidences for a viral role in the pathogenesis of SS have been supplied from a large number of studies; however, at present, no agreement on a single infectious agent responsible for SS development has been achieved.

Cytomegalovirus, Hepatitis viruses C (HCV), Human Immunodeficiency virus (HIV) and Human T Cell Leukemia Virus type 1 (HTLV-1) have been often associated with development of a sialoadenitis and the occurrence of a SS-like syndrome in humans and in mouse models. Similarly, Epstein-Barr 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) (64). The description of a SS like syndrome in Human T-lymphotropic virus type 1 (HTLV-1) transgenic mice implicated a direct role for HTLV-1 infection in SS, further supported by the evidence of a higher prevalence of SS in areas endemic for HTLV-1 infection (89).

Upon Human Immunodeficiency Virus (HIV) infection between 2 and 5% of patients develops a salivary gland infiltrates similar to primary SS, nonetheless 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 (90).

Among the other viruses, the recent evidence of a cross reaction between a linear B cell epitope of Ro60-kd autoantigen and a protein of the Coxsackie virus 2B, strongly implicates Coxakievirus infection in SS pathogenesis (91).

Diverse mechanisms have been advocated to dissect the relationship between viral infection and SS development. The presence of molecular mimicry between viral antigens (i.e. the retroviral gag protein) and self epitopes (SS-B/La) has been widely supported (92). More recently, it has 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 perpetuating the inflammatory process.

1.2.5 Pathogenesis

In contrast to the unknown aetiology, the pathogenesis of SS has been largely defined. At present it is believed that, apart from the pathogen infection, other factors interact in the disease pathogenesis both systemically and at glandular level.

I shall discuss hereunder some of these aspects with particular emphasis on the factors involved in the aberrant activation of the immune system leading to the systemic production of poly-reactive

antibodies and to the local development of highly organized immune stations within the inflamed glands.

1.2.5.1 B cell distribution and autoantibody production

In SS there is large body of evidences supporting the theory of a systemic B cell deregulation leading to poly-reactive autoantibody production. A decreased number of circulating memory CD27+ B cells (predominantly CD5+) has been reported in several studies in SS patients compared to either normal controls or patients with SLE (93, 94). Conversely, an enlarged number of CD27- naïve B cells and germinal center (GC) founder cells (CD38++/IgD+/CD27-) were detected in the blood of SS patients (95-97). More recently, the presence of B cell precursors or transitional type I B cells, usually detected only within the spleen, has been described in SS patient circulation (98). Whether these data reflect a constitutive impairment in the B cell development in SS or a selective distribution of these populations under inflammatory conditions (95-97) is still debated. However, recent data on the increased serum levels and salivary gland expression of BAFF (a molecule involved in B cell maturation and survival) during SS, strongly support the first hypothesis, suggesting that B cell distribution in SS reflects a real defect in the system regulating the B homeostasis (99, 100). The specific role of BAFF in B cell development and SS pathogenesis will be discussed later in this Chapter.

Regardless of their distribution in the tissue or in the circulation, B cells of patients with SS appear to be strongly activated. Systemically, this activation is reflected in the elevated serum levels of polyclonal immunoglobulins and autoantibodies such as anti-Ro/SSA, anti-La/SSB, RF, anti-muscarinic receptors and anti-alpha fodrin. Approximately 60–80% of SS patients are positive for anti-Ro and 40–60% is positive for anti-La antibodies. Local auto-antibody production has also been reported at glandular level (66) and will be further discussed in this thesis. Autoantibody

levels have been described to correlate with clinical manifestations such as *sicca syndrome* (IgA anti-Ro and anti-La), neurological involvement (anti-Ro) and congenital heart block development (anti-Ro and anti-La) (66). However, as mentioned above, the direct involvement of autoantibodies in glandular loss of function and organ involvement in SS is still debated (101, 102).

Autoantibody production in SS seems to reflect a breakage in the immune tolerance towards auto-antigens, 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 antigen/autoantigens can favour the development of autoreactive B cells, whose survival and spreading is supported by the excess of antigens presented by the local antigen presenting cells (APC) and by the aberrant expression of B cell survival factors within the glands. These mechanisms will be further discussed in relation to the role of BAFF in the survival of autoreactive B cells.

1.2.5.2 Immune system dys-regulation

SS patients present several abnormalities in the number and function of the immune cell components that are believed to contribute to the autoimmune background of the disease.

Firstly the aberrant activation of the immune system in SS is reflected in the imbalance between pro inflammatory and anti inflammatory cytokines detected in the blood of SS patients. Increased levels of IL-1, IL-10, IL-13 and IL-6, correlating with disease duration and autoantibody production, have been described in SS, together with reduced levels of the anti-inflammatory cytokines such as TGF-β (103-105).

Decreased number of the total number of dendritic cells (DC) and in particular of the CD11c+CD11a+ has been described in the blood of SS patients, while the CD11c+/CD11a-/CXCR4+ (myeloid DCs) were unchanged as compared to normal. Since in the inflamed glands of

the same patients a large number of mDCs has been demonstrated, it has been suggested that the decrease in specialized APCs in SS patients' blood is secondary to their selective recruitment within the glands (106).

An impairment in the number and activity of cell-mediated natural cytotoxicity has also been reported (107). These data are interesting given the direct implication of the cytoxic-mediated response to viral infection. It is broadly believed that this impairment plays a role in the lack of clearance of the exogenous antigen/s, contributing to SS chronicization.

On the contrary, no evident alterations in the profile of the immunoregulatory T cells have been detected in SS, excluding a direct implication of this system in SS pathogenesis.

1.2.5.3 The ductal epithelium

The ductal epithelium has been implicated in diverse phases of SS pathogenesis such as the immune cell recruitment within the glands, the process of antigen presentation and the direct stimulation of the immune cells (108).

Epithelial cells extracted from the salivary glands of SS patients have been demonstrated capable to secrete molecules involved in immune cells recruitment and organization (109). This suggests that, upon antigen stimulation the ductal epithelium provides the first signals for leukocyte migration.

During the disease course ductal epithelial cells have been suggested to actively participate to the organization of the immune response by presenting the antigen/s and providing the costimulatory molecule capable to activate the infiltrating lymphocytes. Primary cultures of ductal epithelial cells of SS patients appear to retain the expression of costimulatory molecules such as HLA-DR/DP/DQ (110), CD80/CD86 (111) and CD40 (112) and adhesion molecules such as VCAM-1 and ELAM-1 also in vitro, supporting the active role of the ducts in the antigen presentation process in vivo.

Ductal epithelial cells can also directly stimulate the infiltrating immune cells by releasing pro-inflammatory cytokines (IL-1, IL-6, and TNF-a) and metalloproteinase (113).

Finally an increased apoptosis rate of the ductal epithelium related to the aberrant expression of Fas and Fas ligand (114) has been implicated in the release of surface blebs containing autoantigens implicated in the break of tolerance towards autoantigens and in the perpetuation of the inflammatory process.

Recently an important role of the ductal epithelium in the production of B cell survival factors has been demonstrated and will be later discussed in this dissertation (115).

However, whether the activation of the ductal epithelium is a constitutive feature of SS salivary glands or reflects a secondary event due to pathogen infection is still debated.

In the most severe cases of SS, the ductal epithelium can be infiltrated by the immune cells giving rise to the formation of areas of lymphoepithelial proliferation or lymphoepithelial sialoadenitis (LESA). The presence of this hystopathological lesion is believed to specifically associate with the development of the mucosa associated lymphoid tissue (MALT) type B-cell lymphomas.

1.2.5.4 The inflammatory infiltrate

The immune focal infiltrate within the glands is mainly composed by T lymphocytes, B cells, CD8+ cytotoxic cells (~ 20%), dendritic cells and few macrophages (116).

The T cells infiltrating SS salivary glands display a mature, activated phenotype, being mostly CD4+/CD45RO+ (Gordon and Reichlin, 1997) and expressing HLA-DR and IL-2R. Infiltrating T lymphocytes closely interact with the ductal epithelium and with the infiltrating B cells via costimulatory molecules (i.e. CD40/CD40L) and the BAFF axis etc.

It is currently believed that the formation of these aggregates reflects an antigen- specific immune response directed towards exogenous or autoantigens abundantly presented within the inflamed

glands. Evidence in support of this theory were firstly derived from the analyses of T-cell receptors (TCR) of the T cells and the B-cell receptors (BCR) present on T and B cells infiltrating SS salivary glands.

The studies focused on the TCR analysis of T cells extracted from the salivary glands of patients with SS showed clonal expansion and a clear bias in the usage of the Variable (V) family repertoire among diverse patients affected by SS. Moreover within the hyper variable region complementarity-determining region 3 (CDR3) the presence of conserved amino acid motifs was often demonstrated (117). Altogether these data suggests that the polyclonal populations of T cells infiltrating SS salivary glands are selected for a limited number of antigens or for antigens with high degree of homology.

Additional aspects of this antigen driven immune response will be discussed in the section on lymphoma development.

A Th-1 immune response seems to predominate within the salivary glands. In this regards interferon gamma (INFγ) appears to be the cytokine mostly produced by the activated mDCs, together with Tumor necrosis factor (TNF), lymphotoxin α (LTα), IL-1α, IL-6, IL-10 and transforming growth factor (TGF)-β (106, 118). However during the disease course the aberrant systemic activation of the humoral response is reflected at glandular level by the increased production of Th-2 type cytokines.

Similarly to insulitis, the process of lymphocyte recruitment within the salivary glands is actively regulated by the expression of chemokines. These molecules, released by the same epithelium, stromal cells or immune cells are responsible of the migration and organization of the immune infiltrate, among those CCL3, CCL4, CCL5, CXCL13 and CCL19 (109, 113). The specific role of some of these molecules in SS pathogenesis will be discussed in more detail later in this dissertation.

Recently a large number of studies have been focused on the characterization of the B cells infiltrating SS salivary glands. This interest has been supported by the strict association of SS with

the development of B cell lymphomas and the possible implication of molecules involved in the formation of the inflammatory infiltrate (such as BAFF) with the malignancy development (119). Some of these studies will be later discussed in the paragraph on the role of BAFF in SS lymphoma genesis.

Within SS infiltrates, B cells are often organized in follicle-like structures that share many of the structural and architectural features of the follicles described in secondary lymphoid organs (120). These structures are present in a variable percentage of SS patients (121) and are characterized by the presence of T-B cell segregation, follicular dendritic cell (FDC) networks and formation of germinal centers (CG) like structures (120).

In the last few years evidence has emerged providing support for the *in situ* production of autoantibodies in association with the ectopic formation of these structures and there are data in the literature suggesting a pathogenetic link between the formation of ectopic GCs and expansion of B cell clones related to the development of lymphoproliferative disorders in SS (122). Thus in the next section I shall discuss the mechanisms of physiologic and pathologic lymphoid neogenesis.

1.3 The NOD mouse model of type 1 diabetes and Sjögren's syndrome

1.3.1 General features

The NOD mouse strain is an excellent model of autoimmune disease and an important tool for dissecting tolerance mechanisms. The strength of this mouse strain is that it develops spontaneous autoimmune diabetes, which shares many similarities to autoimmune diabetes (T1D) in human subjects, including the presence of pancreas-specific autoantibodies, autoreactive CD4+ and CD8+ T cells, and genetic linkage to disease syntenic to that found in humans.

NOD mice are also prone to developing other autoimmune syndromes, including autoimmune sialitis (123), autoimmune thyroiditis, autoimmune peripheral polyneuropathy (124), a systemic lupus erythematosus–like disease that develops if mice are exposed to killed mycobacterium, and prostatitis (in male mice).

To date no inducible models of SS in normal mouse strains have been described. Most of the information from animal models of SS derives from studies of inbred mouse strains or mice carrying specific genetic defects that spontaneously develop autoimmune sialoadenitis, such as NZB/W, MRL/lpr and NOD mice (125).

For the work of this thesis I focused on the NOD mouse model of T1D and SS because of the numerous clinical and hystopathological features that this model shares with the human diseases.

1.3.2 Strain origins and characteristics

Makino and colleagues (126) originally developed the NOD strain in Japan during the selection of a cataract-prone strain derived from the outbred Jcl:ICR line of mice. During the selection of this

cataract-prone strain, the NOD strain was established, through repetitive brother-sister mating, as a subline that spontaneously developed diabetes. The incidence of spontaneous diabetes in the NOD mouse is 60% to 80% in females and 20% to 30% in males (127). Interestingly, the incidence of disease is highest when mice are maintained in a relatively germ-free environment but dramatically decreases when mice are maintained in conventional "dirty" housing facilities (128, 129). The basis for this effect is unclear, but it has been suggested that it reflects the fine-tuning of the immune system that occurs during exposure to foreign proteins and protects the individual from allergy, autoimmunity, and other diseases of immune dysregulation (130).

Diabetes onset typically occurs at 12 to 14 weeks of age in female mice and slightly later in male mice. Histological studies have shown that few immune cell infiltrates are noted in islets until approximately 3 to 4 weeks of age, when both male and female mice begin to demonstrate mononuclear infiltrates that surround the islet (peri-insulitis). These infiltrates progress and invade the islets (insulitis) over the subsequent few weeks, such that most mice demonstrate severe insulitis by 10 weeks of age. The finding that the reduced incidence in male mice occurs in spite of similar levels of early insulitis suggests that late regulatory events control disease progression. Thus, the autoimmune process in the pancreas of NOD mice includes two checkpoints: checkpoint 1, or insulitis, which is completely penetrant; and checkpoint 2, or overt diabetes, which is not completely penetrant (131). The islet mononuclear infiltrates are complex in their makeup. The majority of cells are CD4+ T cells, and although CD8+ T cells, NK cells, B cells, dendritic cells, and macrophages can also be identified in the lesions (126, 127), NOD disease is primarily dependent on CD4+ and CD8+ T cells (132, 133). Evidence for this includes the ability to transfer disease with purified CD4+ and CD8+ T cells from NOD donors, the ability of individual T cell clones (both class I and class II restricted) derived from NOD islets to passively transfer disease (134, 135), and the fact that T cell modulating therapies inhibit disease incidence (136, 137). Whereas diabetes can be transferred from affected animals by passive transfer of splenocytes, it

cannot be transferred by autoantibodies from new onset diabetic donors, although B cells are also clearly important for the development of the disease (138).

1.3.2.1 NOD Genetics

Multiple loci control the genetic susceptibility to diabetes in this mouse. NOD mice harbour a unique major histocompatibility complex (MHC) haplotype, termed H-2g7, that is essential and is the highest genetic contributor for disease susceptibility (139, 140). This MHC haplotype does not express an I-E molecule because of a defective Eα locus. Moreover, the unique I-A molecule contains a non-aspartic acid substitution at position 57 of the β chain that substantially alters the repertoire of MHC binding peptides presented by this allele. Strikingly, this substitution is also seen in human T1D MHC susceptibility loci in the DQ β chain (141). Several studies that examine the MHC requirement in NOD mice for the development of insulitis and diabetes conclude that homozygosity of the H-2g7 haplotype may be necessary for diabetes development and that dominant protection may be provided by some MHC manipulations including introducing a functional I-E or non-I-Ag7 allele but not others (142, 143). The major contributor to autoimmune diseases susceptibility is the MHC class II molecule itself. Its unique structure, its ability to bind an array of low affinity peptides, and its shared structural features in humans susceptible to autoimmune disease suggests that targeting this gene product, both in terms of genetic screening and potential therapy, remains a high priority. Finally, it should be noted that multiple genes are encoded within the MHC loci, many of which have been associated with immune functions. Possibly, the high susceptibility endowed by the H-2g7 MHC may be caused, in part, by polymorphisms in other genes, such as TNF-α, encoded within this chromosomal segment.

In addition to the MHC locus, many other loci contribute to disease development and are termed Idd loci. To date, over 20 potential Idd loci have been identified (140, 144), but in most cases, the

exact structural or regulatory elements that lie within these loci still await identification. Researchers have discovered some clues about the nature of some of these genetic susceptibilities. In the case of the Idd5 locus (which may encode two regulatory elements), a unique polymorphism in the CTLA-4 gene was determined that affects gene splicing. Interestingly, CTLA-4 is also a candidate gene in humans susceptible to a variety of autoimmune diseases, although the structural basis for CTLA-4 dysfunction is distinct (145).

Candidate genes have been suggested in other Idd loci as well. Vav 3 polymorphisms may account for Idd18, CD101 for Idd10, and the IL-2 or IL-21 genes for Idd3 and so on. The existence of multiple susceptibility loci in the NOD strain again highlights the inherent complexity of the autoimmune process and supports the hypothesis that multiple tolerance networks are defective and interact in this strain. In fact, the spontaneous incidence of diabetes in the NOD mouse strain is likely to be a consequence of the absence of protective genes, as well.

1.3.3 Pathogenesis of T1D in NOD Mice

The NOD mouse has evolved as an important tool that allows researchers to gain insights into tolerance pathways that are active both in the thymus and the periphery.

Autoimmunity in this mouse likely proceeds via the summation of multiple defective tolerance pathways. Thymic development of the NOD T cell repertoire likely harbors defects that allow islet-reactive T cells to escape deletion and prevent the proper generation of regulatory T cells. In the periphery, islet-reactive T cells likely see their antigen in the draining lymph nodes of the pancreas and are inappropriately primed to become effectors. The milieu of cytokines, APCs, innate immune cells, and costimulatory molecule interactions that are present during this step likely leads to the initial priming and expansion of an anti-islet response that later manifests itself as insulitis. Subsequently, this anti-islet response proceeds over an extended period in a complex

immunoregulatory battle involving (but not limited to) Treg cells, NK cells, and Th1 effector skewing that ends towards an improper effector response to the islets and the development of diabetes. Autoimmunity is an incredibly complex process, and the NOD mouse has proved to be an important tool for dissecting both the central and peripheral tolerance mechanisms that contribute to spontaneous autoimmune disease.

NOD mice exhibit a number of immune defects that may contribute to their expression of autoimmunity. Although much of the emphasis in the field has been on the role of T cells in the development and progression of the disease, the immune system in NOD mice harbours defects in multiple subsets of leukocytes. These include defective macrophage maturation and function (146), low levels of natural killer (NK) cell activity (147), defects in NKT cells (148), deficiencies in their regulatory CD4+CD25+ T cell population (149), and the absence of C5a and hemolytic complement.

1.3.3.1 NK cells

Recent studies have shed some light on NK function, which is impaired in these mice. NK cell-mediated cytotoxicity is reduced in cells from NOD mice compared to other strains when assayed against NK-sensitive targets (150). In addition, the Idd6 locus maps to a region of the genome containing the multiple NK cell-associated genes (the NK gene complex) on mouse chromosome 6 (151). At least one mechanism contributes to this NK cell defect. Investigators examining the NK activating receptor, NKG2D, which is expressed on the surface of NK cells, γ δ T cells, activated CD8+ T cells, and NKT cells (147), noted that activated NK cells from NOD mice expressed both NKG2D and its ligand(s), RAE-1. This “inappropriate” upregulation of RAE-1 caused the NKG2D receptor to be internalized through ligand interactions with RAE-1, compromising NK activity, which contributes to the NK cell defect observed in NOD mice.

1.3.3.2 B cells

Although autoimmune diabetes is a T cell-driven disease, studies using the NOD mouse (46, 152), and other models (153, 154), have shown that B cells, as well as T cells, participate in pancreatic inflammation. In transgenic mice with islet-specific T cells, but no B cells, T cells are primed in the pancreatic lymph node but fail to enter the pancreas. Reconstitution of the B1 cell population by adoptive transfer permits extensive T cell pancreas infiltration (155). Recent evidence suggests that B cells play an important role in the development of autoreactivity in NOD T1D. NOD mice rendered B cell deficient using B cell depleting treatments with monoclonal antibodies are protected from the development of insulitis and diabetes: targeting B cells with anti-CD22/cal monoclonal antibody therapy delays diabetes onset in prediabetic NOD mice and restores normoglycaemia in new-onset hyperglycaemic NOD mice.

Also genetic evidence highlights the importance of B cells in NOD T1D. B cell specific deletion of the MHC class II I-Ag7 (Idd1) prevented spontaneous autoimmune diabetes in NOD mice, highlighting the essential function of MHC class II expression on B cells for the development of T1D (156). In addition, MHC-independent genetic loci promote the activation of autoreactive B cells. Genes on proximal chromosome 1 (*Idd*5) and distal chromosome 4 (*Idd*9/11) were both found to directly control diabetes development by regulating B cells in NOD mice, specifically by preventing the induction of B cell anergy following low-avidity BCR engagement by self-antigen (52). Furthermore, another locus that mapped to a region on chromosome 1 in the vicinity of the *Idd*5 locus, controls TACI (transmembrane activator and calcium-modulating and cyclophilin ligand interactor, which is bound by both BAFF and APRIL) expression on B cells. Finally, a locus on chromosome 8 near *Idd*22 temporally regulates the expression of TACI on NOD B cells (157).

B cells could promote autoimmunity in T1D by several mechanisms including: production of autoantibodies with consequent generation of immune complexes (IC), antigen presentation to generate primary autoreactive T cell responses, contribution to the maintenance of $CD4^+$ T cell

memory, or production of pro-inflammatory cytokines. Although autoantibodies alone may directly mediate certain autoimmune pathologies, some autoimmune diseases, such as T1D, are B cell-dependent, yet independent of antibody production. In these cases B cells may contribute to disease via activation of autoreactive T cell responses or the maintenance of T cell memory (53, 54). In addition, B cells produce a vast array of cytokines that can regulate the development, expansion or differentiation of Th1 and Th2 cells, as well as antibody production (158). The importance of B cells in the spontaneous development of autoimmune diabetes in NOD mice was clearly established using NOD.Igμ^{null} mice that lack B cells (138). These mice are resistant to disease induction. In-vivo depletion of B cells by anti-IgM antibody treatment prevented the development of insulitis and sialitis in NOD mice (53). Furthermore, passive treatment of NOD Igμ^{null} mice with immunoglobulin from overtly diabetic NOD donors did not induce disease or insulitis. T1D did not develop in experiments in which transmission of maternal autoantibodies to NOD pups was prevented, suggesting that autoantibodies may play some indirect licensing role in disease (159).

Although autoantibodies may not be directly pathogenic, their existence is indicative of an ongoing antigen-specific autoimmune response. It is, therefore, predicted that the unique ability of B cells to capture and internalize antigens through cell surface Ig allows them to more efficiently activate diabetogenic T cells, compared with other APC populations in NOD mice (155) (52).

On the basis of the requirement for B cell-specificity in T1D, it is easy to understand how T cell-specific responses may be altered in disease, whether B cells contribute to disease by initial activation of autoreactive T cells or by ensuring the quality of the CD4$^+$ T cell memory response.

1.3.3.3 T cells

The most proximal and well-studied pathogenic responses are mediated by T cells. Early studies suggested that both CD4+ and CD8+ T cells participate in the development and progression of the

disease. CD4+ T cells are essential both early and late in disease development. Several groups have shown that CD4+ T cells can be used to transfer disease and that anti-CD4 mAb therapy prevents diabetes onset in NOD mice (137). Interestingly, Fathman and colleagues (160) have shown that the antigen-specific T cells reside in the CD4 high T cells subpopulation in the pancreatic draining lymph nodes. Although, CD4+ T cells are very much involved in the pathogenesis of disease and can directly mediate islet cell destruction, CD8+ T cells can promote the disease as well. Initial studies pointed to a role for CD8+ T cells early in disease development, perhaps by causing sufficient islet cell destruction to prime the more robust CD4+ T cell response. The antigens recognized by CD4+ and CD8+ include insulin, GAD, insulinoma-associated protein 2 (IA-2), and heat shock protein 60 (Hsp60), all of which are produced in pancreatic islets.

Naive T cell priming events are generally thought to occur in the SLOs, where antigen is presented by dendritic cells that have migrated from the tissue into the organ. After this priming event, newly activated T cells change their surface receptors and homing pattern and gain access to non-lymphoid compartments where their antigen is located. In the NOD system, several studies have demonstrated that the initial priming events occur in the peri-pancreatic lymph nodes. Selective removal of the pancreatic lymph node before 3 weeks of age completely protects against the development of diabetes in NOD mice, an effect that was not seen with splenectomy (47). The timing of this activation event appears to be related to a wave of β cell death in the islets that occurs at this time.

An increasing number of studies suggest that cell trafficking may also be intimately involved in disease progression. Regulatory CD4+CD25+ T cells capable of preventing diabetes express high levels of the chemokine receptors CCR7, CCR6, and CCR4. Furthermore, the development of insulitis and progression to disease correlates with expression of CCL2, CCL3, CCL4, CCR5, RANTES (regulated on activation, normal T cell expressed and secreted), CCR3, CCL12, and IFN-inducible protein-10 (CXCL10) (161).

Given the overwhelming evidence that T cells are intimately involved in the pathogenesis of diabetes in NOD mice, a large body of work has examined whether defects in thymic selection and development are the major cause of autoimmunity (162). Work on the Aire gene highlighted the significance of thymic selection of ectopically expressed antigens and autoimmune endocrine diseases. Aire controls the ectopic expression of many self-proteins (including insulin) in medullary epithelial cells of the thymus. In the absence of Aire, there is a defect in the negative selection of organ-specific T cells (163). Investigators have also examined the role of GAD65 in the thymus. This autoantigen was originally identified in human subjects with autoimmune diabetes (164).

1.3.4 Pathogenesis of SS in NOD Mice

Autoimmune sialoadenitis in NOD mice has an incidence similar to insulitis, being more frequent in female as compared with male NODs (approximately 70-80% in females and 30% in males). Conversely, dacryoadenitis develops more often in male mice.

Initial immune cell infiltrates in NOD submandibular glands are observed around 10 weeks of age in females, several weeks after the development of the insulitis, which is observed from week 4 onwards. Importantly, decrease in exocrine secretory function follows the inflammatory process being evident normally after 16 weeks of age with reduced salivary flow. This feature is rather unique of NOD mice in comparison with other animal models of SS, in which autoimmune sialoadenitis is not followed by functional impairment of the salivary glands. Thus, NOD mice offer the possibility to investigate not only the mechanisms leading to breach of tolerance and autoimmunity, but also to understand the events that links chronic inflammation in the target organ to reduction of exocrine function.

Interestingly, different loci have been associated with the development of diabetes and sialoadenitis, with the Idd3 and Idd5 intervals, on chromosomes 3 and 1 respectively, being more strongly

involved in conferring susceptibility to SS-like disease (165). This suggests that the autoimmune process in the pancreas and in the salivary glands of NOD mice occur independently. Thus, differently from other mouse strains in which autoimmunity is driven by single genetic defects (i.e. MRL/lpr), NOD mice represent a more reliable model of the immune deregulation that leads to human autoimmunity and chronic inflammation in the target organs.

1.3.4.1 Role of the cytokines

Knockout or transgenic mice in the NOD background represented a valid model to evaluate the specific role of single cytokines in the disease pathogenesis. More recently, the use of cytokine blocking compounds has provided further insight into the complex networks of pro- and anti-inflammatory mediators regulating salivary gland inflammation. It is important to underline that because of the redundancy of the cytokine system, with the differential and often paradoxical role of cytokines in different phases of the immune responses, a full understanding of the pathogenic relevance of each cytokine system in NOD mice is extremely difficult. In this contest it is also interesting to underline the differences in terms of disease development and progression in the sialoadenitis and the diabetes in the NOD mice transgenic or knockout for specific cytokines.

NOD.IFNγ-/- do not develop inflammatory infiltrates in the salivary glands and do no present loss of salivary flow. Conversely, these animals develop diabetes and dacryoadenitis with lachrymal gland dysfunction with similar incidence and severity as in NOD original strain (166). This data suggests that IFNγ may exert differential roles in the diverse anatomical districts possibly in combination with local factors. In this regard, IFNγ has been shown to be able to directly activate ductal epithelial cells to produce pro-inflammatory cytokines and express class II MHC in NOD mice (167). Whether these events occur in an early phase of the salivary gland disease process, as

suggested (168), and to what extent they are able to influence the development of the sialoadenitis, is still unclear.

NODIL-4-/- do not show secretory loss, although inflammatory infiltrates in the salivary glands still develop with B/T cell infiltration similar to the original strain (169). This effect is possibly related to the systemic and local reduction in (auto)antibody production influenced by the absence of IL-4.

The role of IL-10 in sialoadenitis has been originally investigated by transgenic expression of the murine IL-10 gene under the salivary amylase promoter in C57BL/6 mice. In these mice IL-10 induced apoptosis of glandular epithelial cells via up-regulation of FasL expression on T lymphocytes (170). Accordingly, NOD mice deficient for IL-10 develop normal disease progression (168). Conversely, administration of an adeno-associated virus (AAV) expressing IL-10 via retrograde cannulation of submandibular glands in NOD mice resulted in reduced glandular inflammation and preservation of the salivary flow. Similarly controversial results have been described in the insulitis of NOD mice where IL-10 exerts a pro-inflammatory role in early phases (171) while treatment with IL-10/Fc fusion protein protects mice from developing diabetes (172). Thus, it is likely that IL-10 may exert different net effects depending on different phases of the autoimmune process and diverse local milieu of cytokine expression.

1.3.4.2 Apoptosis

In analogy with human SS, apoptosis of acinar and ductal salivary gland epithelial cells is an important feature of the autoimmune process in the sialoadenitis of NOD mice, although to what extent increased epithelial cell apoptosis is relevant in mediating exocrine dysfunction in NOD mice is still unclear.

It has been suggested that apoptosis in NOD salivary glands occurs at a very early stage and even before the development of cellular infiltrates. Early apoptotic events, possibly as a result of

abnormal salivary gland homeostasis developing before or concomitant to the onset of immune cell infiltrates, is thus believed to induce break of tolerance towards salivary gland-associated antigens and trigger the autoimmune exocrinopathy in NOD mice (173).

1.3.4.3 B cell activation and autoantibodies production in NOD mice

B cell activation and autoantibody production is one of the hallmarks of human SS. Similarly, NOD mice display features of B cell abnormalities that share several characteristics with SS.

Hypergammaglobulinemia has been described in the serum of female and male NOD mice with approximately 1.5 fold increase of circulating IgG as compared to control BALB/c mice. Around 70% of diabetic NOD females aged >25 weeks also display anti-nuclear reactivity at IIF on Hep-2 cells, a similar prevalence as compared to human SS (174). Conversely, anti-Ro52 autoantibodies are infrequently observed in the sera of NOD mice with a prevalence of around 10%. Anti-Ro60 and anti-La antibodies are non-detectable (175).

More recently NOD mouse sera have been demonstrated to react with a described neo-autoantigen of SS: the alpha-fodrin, a non erythroid spectrin that becomes antigenic following the cleavage by calpains to a 120Kd occurring during the apoptosis process. Serum anti-alpha-fodrin antibodies are detectable by western blot in NOD mice already at 12 weeks of age and their levels seems to correlates with salivary gland inflammation until 30 weeks of age (176). Anti-alpha fodrin B cell reactivity is likely to represent a T cell dependent antigen-driven process with splenic Th1 lymphocytes responding to antigenic challenge with recombinant alpha-fodrin (as assessed by T cell proliferation and Th1-related cytokine expression) (176). The possibility that the source of antigenic alpha-fodrin resides within the salivary gland microenvironment is suggested by the demonstration that ductal epithelial cells in NOD, but not control mice, express the 120-Kd

fragment of alpha-fodrin. However, evidence that a population of antigen-specific anti alpha-fodrin T cells localises within NOD salivary glands has not been provided.

Finally, another important autoantigen in NOD mice that acts as a target of humoral immune response is muscarinic receptor M3, these antibodies have been suggested to be the more important antibody specificity in mediating exocrine dysfunction in submandibular glands in NOD mice (174). In this regard, NOD.Igμ null, which lack B lymphocytes, do not develop decrease in salivary flow despite the presence of focal lymphocytic infiltrates in the submandibular glands, moreover transfer of human IgG from SS patients into NOD.Igμ null mice is able to induce secretory loss (102). Similarly, transfer of IgG from old NOD mice into young NOD mice in the absence of focal salivary glands infiltrates resulted in impaired secretory function (177). All together these data strongly implicate autoantibody production in development of sialoadenitis in NOD mice. Thus a better understanding of the mechanism behind the production of these autoantibodies would represent an important step towards the treatment of this disease in humans.

1.3.4.4 NOD mice: histological and functional characteristics of the immune cell infiltrates in NOD salivary glands

Tissue expression of various cytokines in NOD salivary glands have been described as a result of local activation of immune cells. On the other hand the aberrant production of these molecules results in the continuous recruitment of inflammatory cells in an amplificatory loop resulting in disease chronicization.

Normally, increase in local expression of pro-inflammatory cytokines in NOD mice is observed in parallel with the development of initial focal infiltrates around 10-12 weeks of age. Conversely, expression of Th2-related cytokines, such as IL-4 and IL-5, is normally absent in the target organ of NOD mice. The pattern of cytokine expression in NOD sialoadenitis is reminiscent of that observed

in salivary glands of SS patients and related to a Th1-mediated inflammation. Accordingly, high levels of mRNA of TNFα, IL-1β, IFNγ, IL-2, IL-6, IL-10, IL-12 have been detected in NOD submandibular glands (173, 178). Information on local cytokine expression in NOD is limited however to a qualitative or, at best, semiquantitative evaluation, and no precise evaluation of the dynamic expression of cytokine transcripts during the course of autoimmune sialoadenitis in NOD mice has been reported.

The immune cells infiltrating the submandibular glands of NOD mice are organised in focal aggregates similar to the ones observed in the human salivary glands. Most often these aggregates surround a central ductal structure or a blood vessel. Inflammatory foci are mainly composed by CD4+ T cells with a minority of B220+ B cells and CD8+ cytotoxic T cells. Interestingly, an organization of T and B cells in separate and distinct areas within the gland has been demonstrated, similar to human SS (163). However, whether in NOD salivary glands a true phenomenon of ectopic lymphoid neogenesis takes place, with presence of follicular dendritic cell networks, ectopic expression of lymphoid CKs and in situ production of autoantibodies, has not been described as yet. Evidence that T cells may be activated in an antigen-driven process within the submandibular glands of NOD mice has been suggested by the demonstration that NOD extracted salivary gland T cells (similarly to the human counterpart) display a preferential TCR Vβ usage, with predominance of Vβ6 and Vβ8 expression by focal T lymphocytes (173). DC and activated macrophages have been described in NOD submandibular glands, suggesting the possibility that local antigen presentation is taking place within focal inflammatory infiltrates.

1.3.4.5 Role of chemokines and B cell survival factors in NOD mice sialoadenitis

At present no definitive data on the presence of ectopic lymphoneogenesis, expression of CKs and B cell survival factors have been provided in NOD mouse sialoadenitis.

The presence of lymphoid features associated with ectopic lymphoneogenesis has been reported in NOD mice salivary glands. However no studies on the relationship between the appearance of these features and disease development and progression have been performed as yet. Similarly the expression of CCL21 and lymphotoxin has been reported in NOD pancreas, in association with the presence of inflammation of the islet (179) but not in the salivary or lachrymal glands.

More recently the expression of BAFF and other molecules related to B cell activation has been reported by microarray analysis in salivary glands of 8-12 weeks old NOD mice (180). Despite representing the first attempt to define the implication of these molecules in NOD sialoadenitis, this study has not been performed in the contest of a hystopathological evaluation of the inflammatory infiltrate and on a time course base and therefore cannot assess the specific role of these molecules in the dynamic formation of the inflammatory aggregates in NOD salivary glands.

1.4 Ectopic lymphoid neogenesis

1.4.1 General aspects

It is a common finding that tissues that harbour the target antigen(s) of chronic immune responses are infiltrated by cellular effectors of the immune system, mainly T cells and macrophages, but also dendritic cells (DCs), B cells and plasma cells. Intriguingly, it has long been observed that these cellular elements often organize themselves anatomically and functionally as observed in SLOs, leading to the de novo formation of B-cell follicles and T-cell areas within the target organ (181-183). This phenomenon has been termed ectopic lymphoid neogenesis or TLS formation (179, 184). B- and T-cell priming, clonal expansion, antigen retention, somatic hypermutation, affinity maturation, immunoglobulin class switching, B-cell-receptor revision and maintenance of peripheral tolerance are crucial processes that take place in SLOs and can also occur in TLSs, probably contributing to the exacerbation of chronic inflammatory diseases. Lymphoid neogenesis has therefore received increasing attention and the list of diseases in which it has been observed has grown longer (Table 1.6).

In SS, it has been demonstrated that between 20-40% of patients exhibit, within the inflamed salivary glands, lymphoid-like structures characterized by the formation of ectopic GCs (121).

The possibility that during chronic inflammation the same pathways implicated in the physiological development of lymphoid organs could be aberrantly re-activated and determined to the formation of TLS gave rise to a growing interest in the dissection of the pathways and regulatory systems involved physiologic lymphoid neogenesis.

Table 1.6

Human chronic inflammatory diseases with lymphoid neogenesis.

Disease	Target tissue	Percentage of patients with ectopic follicles that contain CD21$^+$ CD35$^+$ FDCs or GCs	T-cell aggregates with CCL19$^+$CCL21$^+$stromal cells, DCs and HEV-like vessels	Antigen recognized by antibodies generated in ectopic GCs	References
Autoimmune diseases					
Rheumatoid arthritis	Diarthrodial joints	10–35%	Present; PNAD$^-$CCL21$^+$ blood vessels and PNAD$^+$CCL21$^+$ HEVs	Rheumatoid factor (?)	6,14,17,18, 41,45,71,80
Hashimoto's thyroiditis (hypothyroidism)	Thyroid gland	100%	Present; HECA-452$^+$CCL21$^+$ HEVs	Thyroglobulin, thyroperoxidase	1,4,15,43,44
Graves' disease (hyperthyroidism)	Thyroid gland	54–63%	Present; HECA-452$^+$CCL21$^+$ HEVs	Thyroglobulin, thyroperoxidase	1,44,15,43,44
Myasthenia gravis	Thymus	Mainly patients with early-onset myasthenia gravis	Present	Nicotinic acetylcholine receptor	2,5,78,79
Sjogren's syndrome	Salivary glands	17%	Present; PNAD$^+$CCL21$^+$ HEVs	SSA/Ro SSB/La	16,55,58,81
Multiple sclerosis	Central nervous system	30–40% of patients with secondary progressive multiple sclerosis	Absent	Not determined	3,46, R. Magliozzi, personal communication
Cryptogenic fibrosing alveolitis	Lungs	83–90%	Not determined	Not determined	83,84
Primary sclerosing cholangitis and primary biliary cirrhosis	Liver	None	Present; CCL21$^+$MADCAM1$^+$HEVs	Not determined	42,56
Other chronic inflammatory diseases					
Ulcerative colitis*	Gut	27%	Present; CCL21$^+$PNAD$^-$ blood vessels	Not determined	53,71,123,124
Crohn's disease*	Gut	Not determined	Present; CCL21$^+$PNAD$^-$ blood vessels	Not determined	53,71,123,124
Atherosclerosis	Arteries	32%	Present; HECA-452$^+$ HEVs	Not determined	106,107
Infectious diseases					
Chronic hepatitis C	Liver	33–85%	Not determined	Not determined	98,99
Helicobacter pylori-(or *Campylobacter pylori*)-induced gastritis	Stomach	27–100%	Present; PNAD$^+$ HEVs	Bacterial antigens	40,57,60,63,97
Chronic Lyme disease	Joints	17%	Present	Not determined	102,103
Tumours					
Ductal breast carcinoma	Breasts	33–100%	Not determined	Tumour-associated and normal breast tissue antigens	108,109

*In inflammatory bowel diseases, it is more difficult to distinguish between lymphoid neogenesis and hyperplasia of mucosa-associated lymphoid tissue. CCL21, CC-chemokine ligand 21; DC, dendritic cell; FDC, follicular dendritic cell; GC, germinal centre; HECA-452, high endothelial cell antigen-452; HEV, high endothelial venule; MADCAM1, mucosal addressin cell-adhesion molecule 1; PNAD, peripheral node addressin; SSA/Ro, Sjogren's syndrome antigen A (ribonucleoprotein autoantigen); SSB/La, Sjogren's syndrome antigen B (autoantigen La).

Source: Aloisi et al. Nature Reviews Imm 2006.

1.4.2 Microarchitecture of tertiary lymphoid organs

The complex microarchitecture of SLOs – characterized by distinct B-cell (follicle) and T-cell (paracortex) areas, and specialized vascular and canalicular systems - is essential for regulating leukocyte traffic and compartmentalization.

It therefore optimizes the efficiency of antigen sampling by naive lymphocytes and allows the appropriate activation and differentiation of antigen responsive B and T cells (Fig. 1.5). Microanatomical and immunohistochemical analyses indicate that lymphoid neogenesis is a dynamic process during which sparse lymphocytic infiltrates evolve into aggregates that eventually organize in secondary B-cell follicles with germinal centres (GCs) and distinct T-cell areas containing DCs and high endothelial venules (HEVs) (121, 179, 185, 186).

The most organized structures are generally found in highly infiltrated tissues, which indicate that TLS induction requires extensive local activation of immune cells. Ectopic GCs comprise proliferating B cells and networks of follicular dendritic cells (FDCs), which are essential for B-cell maturation owing to their ability to retain antigens on their membrane in the form of immunocomplexes, and to stimulate proliferation and prevent apoptosis of GC B cells (187, 188). Remarkably, naive B cells, centroblasts, centrocytes, memory B cells and plasma cells have all been detected in ectopic GCs or in the nearby biological fluids, which indicates that a complete B-cell maturation process takes place in ectopic GCs. T cells are also a regular component of ectopic GCs, probably providing cognate T-cell help, which is required for the progression of the GC B-cell response.

Although the basic cellular constituents of B- and T-cell areas in SLOs and TLS are similar, the overall structure of TLS differs markedly from that of conventional SLOs. Unlike lymph nodes, TLS are not supplied by afferent lymph vessels and are not encapsulated, which implies that they are directly exposed to signals, such as stimulating antigens and cytokines, from the inflamed

environment. This incomplete development of TLS could result in unrestricted access of DCs, lymphocytes and macromolecules to the TLS, favouring abnormal B- and/or T-cell activation.

Figure 1.5

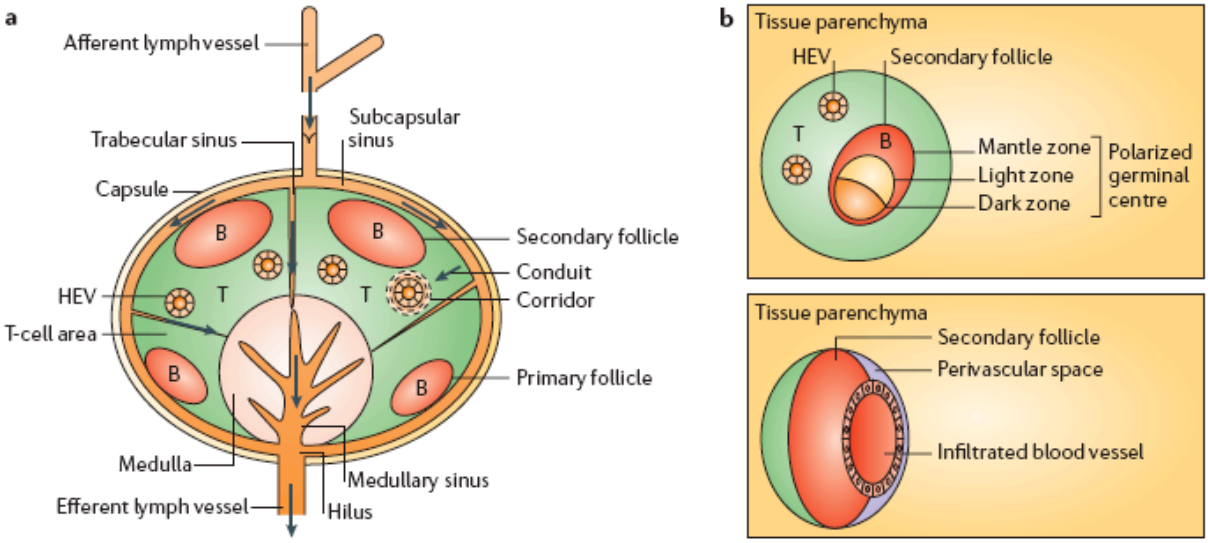

Figure 1.5 Basic structure of secondary and tertiary lymphoid organs. A. The main structural components of a lymph node (SLO) are shown. The cortex contains T-cell areas and B-cell areas that consist of primary follicles and, after antigen challenge, secondary follicles that contain germinal centres (GCs). Afferent lymph vessels carry interstitial fluid and antigen-loaded dendritic cells (DCs) into the lymph node, and high endothelial venules (HEVs) regulate the extravasation of naive B and T cells. **B.** A schematic depiction of TLOs. Secondary B-cell follicles, which are surrounded by T-cell aggregates with HEVs, are found in the tissue parenchyma, and in some tissues, such as the thymic medulla in myasthenia gravis and the brain meninges in multiple sclerosis, they arise as perivascular expansions. Although atypical GC structures have been described, the architecture of ectopic GCs in TLOs is markedly similar to that of secondary B-cell follicles in SLOs. Detailed immunohistochemical analysis carried out in thyroid autoimmune disease has revealed the presence of polarized intrathyroidal GCs with a dark zone containing proliferating lymphocytes (centroblasts), surrounded by a light zone containing small lymphocytes (centrocytes) and enriched in follicular dendritic cells (FDCs), a mantle zone with T cells and mature DCs, and scattered plasma cells. In most pathological tissues that have been analysed, except for multiple sclerosis, the inflamed endothelia acquire a HEV phenotype.

Source: Aloisi et al. Nature Review Imm. 2006.

1.4.3 Chemokines and Chemokines Receptors

1.4.3.1 Lymphoid chemokine and chemokine receptors: structure, function, and mode of action

Chemokines (CK), chemoattractant cytokines, are small heparin-binding proteins that are produced locally in tissues and function to direct the migration of circulating leukocytes throughout the body to sites of inflammation or damage. Thus, they play a key role in inflammation and in the immune response, and contribute to the pathogenesis of a number of diseases.

There are approximately 50 human chemokines, which are divided into four families on the basis of their different structure and functions. The largest family is represented by the CC CKs whose name, in the official nomenclature, derives from the position of the first two of the four cysteine residues, which are adjacent to each other (42).

The new systematic nomenclature classifies chemokines according to this criterion, together with their binding properties. This family of chemokines attracts mononuclear cells to sites of chronic inflammation. CKs are secreted molecules that act in a paracrine or autocrine fashion, being released constitutively or upon stimulation by a large body of immune and non immune cells. Upon release, CKs bind to the matrix glycosaminoglycans (GAGs) with different affinity (189). CKs can be actively transported and exert their effects in areas diverse from their site of production. In this regard, CK active transcytosis on the lumen surface of the vascular endothelium has been demonstrated (190). Within the tissues, gradients of CK concentration have also been demonstrated and it has been shown that lymphocytes are capable to respond to these gradients, acquiring appropriate tissue localization (191). CKs exert a wide range of homeostatic and inflammatory functions being involved in immune cell maturation and migration (192, 193), lymphoid organ development and homeostasis, angiogenesis, organization of the inflammatory process and cancer growth and metastatization (194). CK mediate their effects on cells by binding to cell surface

receptors. These receptors consist of a family of more than 20 seven-transmembrane-domain glycoprotein receptors coupled to a G-protein signalling pathway (G-protein-coupled receptors). CK receptors are named according to the class of CK ligand they bind, such as the CC receptor (CCR) for the CC CK family. CK-R have different chemokine specificity, and the specificity can overlap within a chemokine class, thus a CK-R can have multiple ligands and vice versa.

On binding to their receptors, CKs activate a signalling cascade leading to activation of phospholipase and the production of inositol-trisphosphate and DAG. An increase in intracellular calcium and PKC activation has also been noted. Many kinases participate in this cascade, such as the MAPKs. Ultimately, this signalling cascade leads to actin rearrangement and changes in cell shape and motility.

Differently from inflammatory CKs, lymphoid CKCXCL13, CCL21, CXCL12 and CCL19 have been demonstrated to display an important role in lymphoid organogenesis and homeostasis and regulate diverse physiological as well as inflammatory functions(195). In the next section, I will focus on their role in lymphoid organ development and homeostasis.

1.4.3.2 Role of Chemokines in lymphoid organs homeostasis

In this paragraph I shall discuss the role of the CKs involved in the maintenance of SLO in the adult life.

SLOs are characterized by a strict organizational structure and the CKs CXCL13, CCL19, CCL21 and CXCL12 have been demonstrated critical for the maintenance of this organization. Lymphoid cell segregation in areas of T and B cell rich areas has been demonstrated to be functional for the correct lymphocyte interaction, being the lack of organization involved in the generation of impaired immune responses.

In SLOs, the large T cell area is characterized by the presence of a high number of dendritic cells (DCs), specialized vascular structures characterized by high endothelium (HEVs) expressing vascular addressing (PNAd or MAdCaM) and lymphatic vessels. The CKs CCL21 and CCL19 are selectively produced in this area, bind to the CK-R CCR7 mainly expressed by naïve T cells and are generally referred as T cell CKs (115). Both CCL21 and CCL19 have also been involved in the migration of mature DCs to SLOs trough afferent lymphatic vessels (196).

It has been described that the co-ordinated signal of CCL21 and CXCL12 through their receptors CCR7 and CXCR4 mediates the ingress of naïve lymphocytes lymph nodes and Peyer's Patches trough HEVs (197). Recently, it has been demonstrated a novel role for CCL21 not only in the chemoactraction of naïve lymphocytes trough HEVs but also in the activation of the first immunological synapses between lymphocytes and DCs (198). The role of CCL19 in the T/DC interaction has been also established (199).

The B cell area of lymphoid organs presents a follicular organization, being mainly characterized by aggregates of naïve B cells in primary follicles. CXCL13 is strongly and selectively expressed in these areas by FDCs and mediates the homing of B cells carrying the CK-R CXCR5 (200). CXCL13 has been demonstrated to be necessary for the ingress of B cells in to lymphoid organs (200).

Upon antigen presentation and cognate activation by DCs, antigen specific T cells up-regulate CD69 (198) and down regulate CCR7 moving towards the follicular area. Similarly, B cell activation by (via BCR) causes B cell re-localization at the T cell/B cell boundary (mediated by the down regulation of CXCR5) where they can interact with the T cells. Once the B cell have received the information on the specific antigen and the cognate help from the newly migrated T cells they start to proliferate (201) giving rise to the maturation of the primary follicle in to secondary follicle. The structure of the secondary follicles is complex and tightly regulated. In the inner part of the follicle a dense network of CD21 positive FDCs is usually detected (light zone). This network is in physical contact with B cells just migrated from a highly proliferating area (dark zone). Dark and

light zone are surrounded by a dense ring of naïve B lymphocytes forming the mantle zone, populated by the residual component of the naïve B cells constituting the primary follicle before the antigenic challenge. The mantle zone is surrounded by the large T cell area enriched in DCs and HEVs previously described. As mention before, the T/B cell segregation mainly reflect the selective expression of CCL21 and CCL19 in the CCR7+/T cell areas and of CXCL13 in the CCR5+/B cell area and impaired migration of the T and B lymphocytes have been detected in mouse models respectively defective for either CCR7 or CXCR5 (196, 200, 201).

Finally in mouse models it has been demonstrated that the cooperative effect of CXCL12 and CXCL13 regulates the organization of the GCs in dark and light zone and that mice defective for these CKs show a severe GC disorganization. A more detailed report on the events occurring in the GCs will be discussed in the section relative to the B cell maturation.

Antigen experienced B cell exit from the GC, upon CXCR5 down regulation, and are dismissed in the circulation as memory B cells, effector B cell or plasma cells. Plasma cells localize in the bone marrow and their migration to this site appears to be mainly mediated by CXCL12/CXCR4 interaction.

1.4.3.3 Lymphoid organogenesis and maturation of secondary lymphoid organs

Physiologically, the formation of SLOs such as lymph node (LN), spleen and Peyer's patches is a complex process that begins early in embryonic life. Primordial LNs are represented by clusters of haematopoietic CD3-/CD4+/CD45+ "inducer cells" expressing the interleukin-7 receptor-α (IL-7Rα). Signalling through IL-7Rα induces expression of the membrane bound lymphotoxin α1β2 heterotrimer (LTβ), which has been shown, by elegant gene deletion experiments, to be crucial for lymphoid organogenesis (202, 203). The activation of lymphotoxin β receptor (LTβR) signals for

the induction of ICAM-1+/VCAM-1+ “organizer cells” and their production of Chemokines (CKs) CXCL13, CCL21, CCL19 and CXCL12 (203).

Upon LTβ-R stimulation, the increased expression of CXCL13 and CCL21 establishes a positive feedback loop with a progressive clusterization of CD3-/IL-7Rα+ cells bearing CXCR5 and CCR7 (respectively CXCL13 and CCL21/CCL19 receptors). Once sufficient cell clustering has occurred, the presence of LTα producing cells (up-regulating CK production) contribute to the perpetuation of the amplificatory loop (204). The subsequent lymphoid organization would then proceed with the formation of HEVs displaying on their surface PNAd or MAdCAM-1 functional for lymphocytes recirculation in a LTβ dependent pathway, the organization of compartmentalized T and B cell areas and development of FDC networks (205).

By using gene knockout strategies additional pathways have been demonstrated to be involved in the maturation of lymphoid organs: among those TNFRp55, TNFRp75, RANK/RANKL, LIGHT, IKAROS, ID2, and RORγ (203). The specific contribution of these pathways will not be further discussed in this thesis.

All together the animal studies on physiological lymphoneogensis established the critical role of CXCL13/CXCR5 interaction in the development of lymphoid organs. In turn, CXCL13 expression and the consequent organization of lymphoid follicles have been demonstrated to be strictly dependent on the presence of TNF, TNF receptor 1 and LT (204).

Figure 1.6 shows a diagram representing the cellular interactions regulating the physiological formation of SLOs.

In differentiated lymphoid tissue, B cells, and to a lesser extent T cells, are the main source of LTα1β2, which together with TNF is required for adhesion-molecule and lymphoid chemokine expression by HEVs and stromal cells, the induction of FDC differentiation and function, and lymphocyte and DC homeostasis (206).

The most probable sources of LTα1β2 at inflammatory sites are infiltrating natural killer (NK) cells, B cells and T cells (mainly of the T helper 1 type) (207, 208).

Remarkably, a correlation between the expression of LTα or LTβ and/or lymphoid chemokines and lymphoid tissue organization has been reported in human autoimmune lesions, which supports a causative involvement of these mediators in TLS formation (185, 208, 209).

However, it was also noted that lymphoid CKs can be expressed in inflamed tissues without leading to the formation of lymphoid structures (208). These data indicate that lymphoid CKs, which are probably induced upstream of lymphoid tissue organization, are not sufficient to drive the complete process. TLS induction and/or stabilization depend, at least in part, on the local availability of the same molecules that mediate physiological SLO organization. SLO development is mainly induced by an LT- and/or chemokine-driven positive-feedback loop that leads to the accumulation and compartmentalization of naive lymphocytes. These lymphocytes will only subsequently encounter their activating antigens and, in the case of B cells, will enter GC reactions. By contrast, TLS formation under pathological conditions probably requires previous as well as persistent antigen exposure. The idea that antigen is crucial for maintaining TLS organization is supported by the finding that ectopic GCs generate plasma cells that produce antibodies specific for antigens that are expressed in the target tissue (121). GCs are dynamic structures that evolve quickly during the primary immune response but are more stable under chronic antigenic stimulation, such as in the tonsil and probably in the TLSs (210).

Figure 1.6

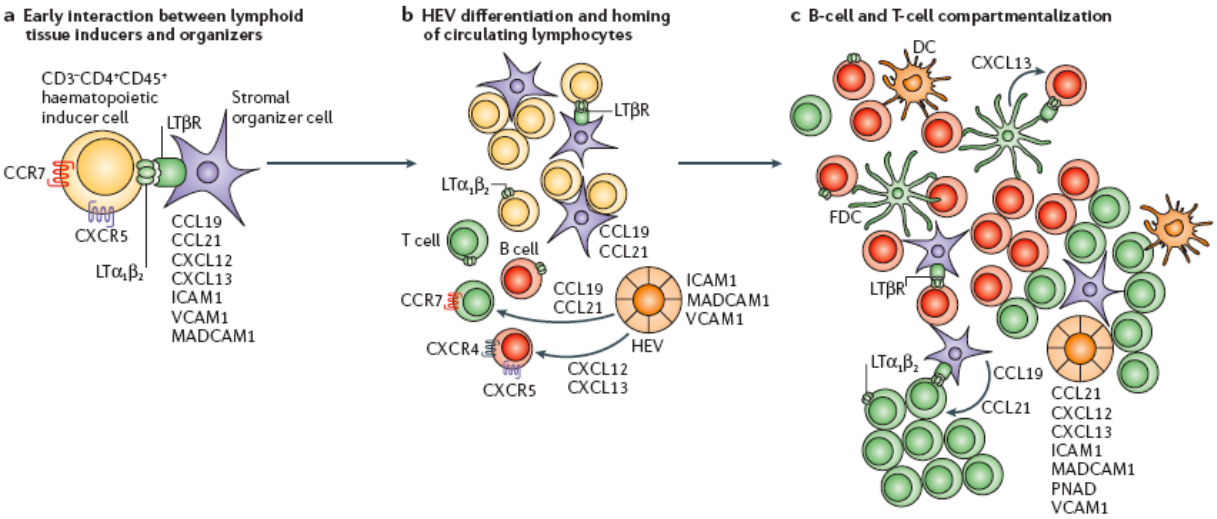

Figure 1.6 Diagram representing the cellular interactions regulating the physiological formation of secondary lymphoid organs.

Source: Lymphoid neogenesis in chronic inflammatory diseases by Aloisi et al.

1.4.3.4 Role of Chemokines in ectopic lymphoneogenesis in T1D

Not much is known about pancreatic lymphoidneogenesis in T1D (49) but the role of CKs has been dissected deeply in the disease.

Studies in NOD mice have shown increased levels of CXCL10, CCL2 and CCL20 mRNAs and/or proteins in pancreatic islets during the prediabetic stage (211).

As mentioned before, during the course of diabetes, macrophages/APCs are the first cells to infiltrate the islets of NOD mice and early expression of CXCL10 and CCL2 contributes to macrophage recruitment during the early stages of insulitis. Transgenic expression, which leads to high production of CCL2 by β cells, causes insulitis and autoimmune diabetes (211). Of interest, high, basal CCL2 production by human islets correlates with a poor clinical outcome following islet transplantation in patients with T1D (212).

In the adaptive immune response, diabetogenic Th1 cells in NOD mice express the CCR5 receptor and its ligands (CCL3, CXC10), as well as CXCL1, CCL2, CCL7 and CCL12 (213). Deletion of CCL3 in NOD mice ameliorates symptoms of insulitis and prevents autoimmune diabetes, (213) whereas deletion of CCR5 leads to a switch from a Th1 response to a Th2 response, which delays islet allograft destruction in mice (214).

A relevant role for locally produced cytokines and CKs was also observed in the mouse model of virus induced autoimmune diabetes. In these mice, the blockade of CXCL10, but not that of CCL5, prevented the development of autoimmune diabetes after infection with lymphocytic chorio meningitis virus (215). Conversely, overexpression of CXCL10 accelerated the onset of T1D (215). The absence of CXCR3-the receptor for CXCL10, CXCL9 and CXCL11-delayed, but not prevented, the onset of insulitis and diabetes, which suggests that these CKs mainly have a role in the early stages of the disease.

An important source of CKs during insulitis are the β cells themselves (216). This suggest the possibility of a dialog between immune cells and the target β cells during the course of insulitis, where activated macrophages, natural killer (NK) cells and T cells produce cytokines, such as IFNγ, IL1β and TNF, which induce β cells to further release CKs and inflammatory cytokines. These molecules will attract more mononuclear cells that also release multiple chemokines. If this vicious circle is not interrupted, it will evolve to progressive accumulation of activated macrophages and T cells around and inside the islets and may be the starting drive for lymphoid neogenesis in pancreatic tissue.

1.4.3.5 Role of Chemokines in ectopic lymphoneogenesis in Sjögren's syndrome

Although TLS have been described in various chronic inflammatory disorders, they are more frequently observed in chronic diseases affecting epithelial or mucosal tissues. In this regard, ectopic lymphoneogenesis has been described in the thyroid gland during Hashimoto's disease, in the gut affected by inflammatory bowel disease and in the thymus of patients with myasthenia gravis. These data strongly advocate a role for the epithelium in the formation of lymphoid like structures.

The relationship between the epithelium and the formation of the immune infiltrate in SS has been previously discussed. As mentioned above, SS epithelium is capable to provide chemoactractive stimuli for the recruitment of immune cells in the glands. In particular epithelial production of CXCL13 and CCL19 mRNA and CXCL12 transcript has been detected in SS salivary glands (CXCL12 expression was also detected in control sialoadenitis) (109, 120).

Within the inflammatory aggregates in SS, ectopic expression of some of these molecules has been also detected. In particular the presence of CXCL13, CCL21 (and CXCL12) in association with characteristic lymphoid structure ectopic formation (FDCs network and germinal centres GCs) has

been described (120, 217). These data, while suggesting a possible role of the epithelium in the organization of SS foci, strongly support the relevance of the ectopic production of these homeostatic/lymphoid CKs in ectopic lymphoneogenesis during SS.

More recently a role for these ectopic GCs in the aberrant production of autoantibody-producing plasma cells has been also advocated. It has been shown that in salivary glands of SS patients AID is invariably expressed within FDC networks but is not detectable in SGs in the absence of ectopic GC, suggesting that FDC networks play an essential role in sustaining the Ag-driven B cell proliferation within SS-SGs. These results strongly support the notion that ectopic lymphoid structures in SS-SGs express the molecular machinery to support local autoantibody production and B cell expansion and may play a crucial role toward lymphomagenesis (218).

All together these data support a driving role for ectopic lymphoneogenesis and therefore for the ectopic expression of the lymphoid CKs in the pathogenetic events leading to SS. In this regard it is vital to understand the relationship between these ectopic organs and the aberrant B cell maturation and activation observed in SS. This will be discussed in the next section.

1.4.4 Modulating TLS formation for immunotherapy

Given the many shared features of SLOs and TLS, modulation of the signalling pathways that regulate lymphoid tissue organization can be thought of as a useful approach to counteract or promote lymphoid neogenesis. Disruption of established TLS or prevention of TLS formation could be advantageous in autoimmune diseases and all inflammatory conditions in which TLS are suspected to have adverse effects (such as transplant rejection and allergic diseases). Conversely, promoting TLS formation at sites of infection or tumour growth could facilitate the eradication of infectious agents and tumours, but this approach could increase the risk of autoimmunity.

TLS-associated molecules and pathways that might be blocked for therapeutic intervention include: adhesion molecules (such as MADCAM1 and PNAD), and lymphoid chemokines (such as CCL21), to suppress lymphocyte migration and compartmentalization; the LTα1β2–LTβR pathway or downstream chemokine-driven signalling (such as CXCL13) to inhibit lymphoid organization and the formation of FDC networks; FDC signalling molecules that promote B-cell survival and proliferation in GCs (such as BAFF and IL-15), to counteract dysregulated B-cell homeostatic processes; and the CD40–CD40L and CD86–CD28 pathways to block B-cell–T-cell interactions that are required for the activation and/or maturation of B cells.

Several tools, including neutralizing antibodies and decoy receptors, that interfere with these signalling pathways are now available and can be tested in animal models. Among these, the LTβR–immunoglobulin fusion protein, which acts as a decoy receptor and blocks the LTα1β2–LTβR pathway, suppresses pathogenic immune responses in experimental autoimmune disease, and this approach is being tested in preclinical trials (206). Notably, in NOD mice, treatment with LTβR–immunoglobulin fusion protein at a late stage of disease reverses insulitis and causes disassembly of already formed pancreatic lymphoid aggregates (219).

Of the lymphoid chemokines, only the blockade of CXCL13 has been evaluated in an experimental therapeutic setting, and this has been shown to be effective in reducing the severity of collagen-induced arthritis in mice and GC formation in splenic and synovial tissues (220). Injection of TACI (transmembrane activator and CAML (calcium-modulating cyclophilin ligand) interactor)–immunoglobulin fusion protein, which blocks binding of BAFF to its three receptors

(TACI, BCMA (B-cell maturation antigen) and BAFF receptor), inhibits the production of collagen-specific antibodies and disease progression in a mouse model of rheumatoid arthritis (221).

However, the use of systemic compounds that target the lymphoid tissue could have serious limitations in chronic inflammatory diseases, owing to a generalized suppressive activity on SLOs and other microanatomical tissue compartments, such as MALT.

1.4.5 B Cells

Mature B cells constitute 10–15% of human peripheral blood lymphocytes, 20–30% of lymph node cells, 50% of splenic lymphocytes, and ~10% of bone marrow lymphocytes. B cells express on their surface intramembrane Ig molecules that function as B cell receptors (BCRs) for antigen in a complex of Ig-associated and signalling molecules with properties similar to those of T cells. Unlike T cells, which recognize only processed peptide fragments of conventional antigens embedded in the notches of MHC class I and class II antigens of APCs, B cells are capable of recognizing and proliferating to whole unprocessed native antigens via antigen binding to B cell surface Ig (sIg) receptors. B cells also express surface receptors for the Fc region of IgG molecules (CD32) as well as receptors for activated complement components (C3d or CD21, C3b or CD35). The primary function of B cells is to produce antibodies. B cells also serve as APCs and are highly efficient at antigen processing. Their antigen-presenting function is enhanced by a variety of cytokines. Mature B cells are derived from bone marrow precursor cells that arise continuously throughout life.

B lymphocyte development can be separated into antigen-independent and antigen-dependent phases. Antigen-independent B cell development occurs in primary lymphoid organs and includes all stages of B cell maturation up to the sIg+ mature B cell. Antigen-dependent B cell maturation is driven by the interaction of antigen with the mature B cell sIg, leading to memory B cell induction, Ig class switching, and plasma cell formation. Antigen-dependent stages of B cell maturation occur in SLOs, including lymph node, spleen, and gut Peyer's patches. In contrast to the T cell repertoire that is generated intra-thymically before contact with foreign antigen, the repertoire of B cells expressing diverse antigen-reactive sites is modified by further alteration of Ig genes after stimulation by antigen - a process called somatic mutation - which occurs in the GCs of SLOs.

During B cell development, diversity of the antigen-binding variable region of Ig is generated by an ordered set of Ig gene rearrangements that are similar to the rearrangements undergone by TCR α,

β, γ and δ genes. For the heavy chain, there is first a rearrangement of D segments to J segments, followed by a second rearrangement between a V gene segment and the newly formed D-J sequence; the C segment is aligned to the V-D-J complex to yield a functional Ig heavy chain gene (V-D-J-C). During later stages, a functional or light chain gene is generated by rearrangement of a V segment to a J segment, ultimately yielding an intact Ig molecule composed of heavy and light chains.

The process of Ig gene rearrangement is regulated in a way that results in a single antibody specificity produced by each B cell, with each Ig molecule comprising one type of heavy chain and one type of light chain. Although each B cell contains two copies of Ig light and heavy chain genes, only one gene of each type is productively rearranged and expressed in each B cell, a process termed allelic exclusion.

There are ~300 V genes and 5 J genes, resulting in the pairing of V and J genes to create >1500 different light chain combinations. The number of distinct light chains that can be generated is increased by somatic mutations within the V and J genes, thus creating large numbers of possible specificities from a limited amount of germ-line genetic information. In heavy chain Ig gene rearrangement, the VH domain is created by the joining of three types of germ-line genes called VH, DH, and JH, thus allowing for even greater diversity in the variable region of heavy chains than of light chains.

The most immature B cell precursors (early pro-B cells) lack cytoplasmic Ig (cIg) and sIg. The large pre-B cell is marked by the acquisition of the surface pre-BCR composed of heavy (H) chains and a pre-B light chain, termed LC. Pro- and pre-B cells are driven to proliferate and mature by signals from bone marrow stroma-in particular, IL-7. Light chain rearrangement occurs in the small pre-B cell stage such that the full BCR is expressed at the immature B cell stage. Immature B cells have rearranged Ig light chain genes and express IgM. As immature B cells develop into mature B cells, sIgD is expressed as well as sIgM. At this point, B lineage development in bone marrow is

complete, and B cells exit into the peripheral circulation and migrate to SLOs to encounter specific antigens.

Random rearrangements of Ig genes occasionally generate self-reactive antibodies, and mechanisms must be in place to correct these mistakes. One such mechanism is BCR editing, whereby autoreactive BCRs are mutated to not react with self-antigens. If receptor editing is unsuccessful in eliminating autoreactive B cells, then autoreactive B cells undergo negative selection in the bone marrow through induction of apoptosis after BCR engagement of self-antigen.

After leaving the bone marrow, B cells populate peripheral B cell sites, such as lymph node and spleen, and await contact with foreign antigens that react with each BCR. Antigen-driven B cell activation occurs through the BCR, and a process known as somatic hypermutation takes place whereby point mutations in rearranged H- and L-genes give rise to mutant sIg molecules, some of which bind antigen better than the original sIg molecules. Somatic hypermutation (SHM), therefore, is a process whereby B cells undergo affinity maturation with selection of high affinity antibodies. In addition to SHM, B cells undergo a further process defined class switch recombination (CSR) whereby they synthesize IgG, IgA, and IgE.

1.4.5.1 B cell subsets in mouse and humans

In the dynamics of the immune system B cells play a critical role: by producing antigen-specific antibodies the B cells represent the more elegant arm of the adaptive immune system.

B cell ontogenesis has been described in mice and more recently in humans. Despite the numerous similarities between the two species, important differences in the phenotypes of the diverse B cell subtype have been identified (222). The most striking being the presence only in mice (but not in humans) of a specific B cells population deputised to the defence of mucosal cavities and likely to be implicated in autoimmunity development: the B1a cells. This subset is generated in the foetal

liver, inhabits the peritoneum and produces natural antibodies. Other important differences are related to the diverse responses of human versus mouse B cells at the point of pathogen stimulation i.e. murine B cells respond to bacteria LPS through TLR4 while human B cells primarily express TLR7 and TLR9 (223). It is necessary to evaluate these differences when data derived from mouse models are used to understand the pathogenesis or treatment of human conditions.

In humans B cells are generated in the bone marrow and their development from a pluripotent stem cell to a naive B cell requires the rearrangement of a functional B cell receptor (BCR). In the bone marrow, within functional niches, B cells are supplied with survival factors necessary for their development and maturation by radiation resistant stromal cells (224). CXCL12 has been demonstrated critical the for correct B cell development, trapping B cells within the bone marrow niches until full development, thus, CXCL12 prevents the premature release of myeloid and B-cell precursors. Once a functional BCR is rearranged, B cells down regulate CXCR4 (ligand of CXCL12), leave the bone marrow and are released in the bloodstream (224). At this stage of development B cells express CXCR5, CCR7 and CCR6, CK-Rs that allow B cells to enter into SLOs and in particular in the spleen where important steps for their maturation take place.

The B cell population dismissed from the bone marrow, named transitional type 1 or newly formed transitional (NF-T) B cells, express high levels of CD24+ and are negative for IgD and CD23 and express very low levels of CD21 (CD20+CD5+CD10+/−$CD21^{low}$ $CD23^{low}IgM^{high}IgD^{low}$CD38+). At this stage, the expression of CXCR5 drives this population within the lymphoid follicular areas of the spleen. Within the splenic white pulp NF-T1 B cells have been demonstrated to differentiate in T2 B cells (CD20+CD5+/−$CD21^{int/high}$ $CD23^{high}IgM^{high}IgD^{high}$CD38+/−). Because of their strong expression of IgD and CD23 this latter population is believed from some authors to be the direct precursors of follicular (Fo) B cells and therefore the reference to this population as transitional type 2 follicular precursors (T2-FP). Data derived from knockin and knockout experiments suggest that T2-FP are also precursors of another splenic population: the marginal zone B cell precursors (MZ-P) which are defined as CD20+CD5+/−$CD21^{high}$CD23+IgM ^{high}IgD ^{high}CD38+/−. These two

populations inhabit diverse areas in the spleen: respectively the follicular area and the marginal zone, at the border between the white and red pulp. Other authors believe that T2-FP B cells represent a non proliferative compartment, therefore not capable to give rise to Fo B cells or MZ B cells (225). In an animal model of arthritis these cells have been demonstrated (following adoptive transfer) to prevent disease onset and ameliorated already established disease by secretion of suppressive cytokines, such as IL10. These data would suggest that among the B cells population this subset plays an immunoregulatory role, thus the new term of regulatory B cell (Breg) has been proposed (226).

In humans transitional B cells have a similar phenotype to mice T1 and express CD21 and CD23 (though to a lower extent as compared to mature B cells) and high levels of IgM and CD24. Transitional B cell Ig genes are mainly germ line-encoded and this population respond poorly to BCR stimulation.

1.4.5.2 Follicular B cells and the germinal centre reaction

Fo B cells are long-lived, resting lymphocytes (half-life of approximately 4 to 5 months) that preferentially localize within the follicles of SLO. This selective localization is mediated by the high expression of CXCR5 (receptor for CXCL13). On the basis of the relative expression of CD38 and IgD, in humans, Fo B cells have been classified in naïve (CD38−IgD+) or Bm1 (B cells that have not encounter the antigen), activated Bm2 (CD38+IgD+) and Bm2′ GC founder (CD38++IgD+) B cells. GC founder B cells populate the GC and upon appropriate antigen stimulation give rise to the GC reaction (227). Very recently a novel role for Fo B cells has been advocated. Migrant Fo B cells have been identified to be the migrant cells capable of picking up antigens from sub capsular sinus macrophages and to bring them to the centre of the follicles, where these antigens displayed on FDCs become the target of the GC reaction (228, 229).

Within the GC, the GC founder B cells or Bm2' cells (IgD+,CD38++), receive and integrate multiple stimulatory signals derived by direct contact with the antigen-challenged DCs and T cells and by the soluble molecules secreted by these and other populations (227). Once the first phase of antigen presentation has occurred, antigen challenged B cells establish a second immunological synapse with the T cells. At this stage B cells can undergo to rapid transformation in plasma blasts and generate an extra follicular immune response (mainly producing germ-line encoded autoantibodies) or migrate back to the follicle and give rise to the GC reaction. The GC reaction consists in a T cell dependent rapid expansion of B cells followed by BCR diversification ultimately resulting in the production of antigen-specific B cell clones. Following antigen presentation and T cell stimulation GC B cells start to proliferate and acquire the phenotype of centroblasts (CD38++IgD− Bm3) (227). In the GC dark zone the blast in B cell proliferation is accompanied by the process of somatic hypermutation (SHM), consisting in the introduction of single base-pair substitutions into the variable regions of antibody gene segments. The enzyme activation-induced cytidine deaminase (AID) regulates somatic hypermutation, with the result of approximately one mutation introduced into the BCR at each cell division (230). Following SHM, the BCR presented on the surface of the centrocytes (Bm4) is selected on the base of its affinity for the antigen presented in the light zone of the GC by the FDCs. Centrocytes that do not bind the receptor die in situ by apoptosis and apoptotic debris are phagocytated by specific monocyte-derived cells: the tingible body macrophages (TBMs).

After BCR selection, B cells undergo class switch recombination also under the regulation of AID. Class switch recombination is the phenomenon that regulates the usage of the immunoglobulin isotype in order to produce the immunoglobulin subtype more appropriate for the type and the site of the immune response (i.e. usage of IgA in the gut) (230). Diverse cytokines have been implicated in the regulation of the class switch recombination process, in particular IL-4 has been demonstrated to drive the switch to IgG1 and in a stepwise manner through IgG1 to IgE, IL-21

favours the production of IgE, IFN-γ is implicated in the switch to the IgG2a subclass and TGFβ, IL-2 and IL-5 modulates the expression of IgA etc.

Upon selection and exit from the GC, centrocytes can re enter in the dark zone for further rounds of expansion and somatic hypermutation. The strength of the BCR binding to the antigen has been strongly implicated in the termination of the GC reaction and in the commitment of the post-GC B cells. GC B cells bearing high affinity BCR strongly express Bcl-6 and only down regulate this molecule once their cycles of proliferation are terminated. Once Bcl-6-is down regulated strong expression of Blimp-1 represses Bcl-6 and determined the irreversibility of the B cell commitment towards the plasma cell fate. GC B cells bearing intermediate-affinity BCR are committed to become memory B cells; they do not express Blimp-1 and maintain a low level of Bcl-6 expression (though sufficient to inhibit plasma cell differentiation). Finally, low-affinity BCR GC B cells cannot compete for the survival signal, die by apoptosis and are engulfed by tingible body macrophages in the GC.

In humans post GC B cells are divided in early memory B cells (CD38+IgD−), later differentiating in to mature memory B cells (CD38−IgD−)Bm5 and the plasmablasts (CD38++IgD−) that migrate in bone marrow where they differentiate in long-lived PCs following CXCL12 gradient (231).

1.4.5.3 Marginal zone B cells

Murine MZ B cells express high levels of IgM, CD21 (complement receptor type II), CD1d, CD38, CD9, CD25 (the α chain of the IL-2 receptor), LFA-1, the α4β1 and B7integrin and low levels of IgD. MZ B cells have been demonstrated to be long lived cell and from some authors even believed to be immortal (232). MZ B cells are potent activators of naive CD4+ T cells both in vitro and in vivo and are readily activated in a T-cell independent fashion. This population provides a first line response differentiating into plasmablasts producing natural antibodies towards lipid antigens and

encapsulated bacteria in extra follicular areas i.e. the splenic MZ (233, 234). In this context, the interaction between MZ macrophages and MZ B cells appears to be essential for MZ response to blood-borne pathogens. Some authors believe that MZ B cells can be directly stimulated by T-independent antigens through the engagement of the complement receptor CD21 or the TLRs (235). The localization of this population in the MZ area of the spleen is mediated by the interaction of sphingosine 1-phosphate (S1P) with his receptors S1P1 and S1P3 expressed on the cellular surface of MZ B cells. Nonetheless, it has been demonstrated that upon activation by T-dependent antigens, MZ B cells can also shuttle into the follicles and contribute to the GC reaction in a process regulated by down regulation of S1P1 and upregulation of CXCR5 (236).

Naive MZ B cells in mice do not recirculate and are trapped in the spleen. Human MZ B cells recirculate freely, are found in many anatomical sites other than the spleen andhave been identified in the somatically mutated, circulating IgM+CD27+ memory cells. Human MZ B cells express high level of CD24 and CD27 and are negative for CD38 and express CD1c and not CD1d (murine MZ B cells) (237). About half of this population expressed an isotype-switched BCR and there is a large consensus in assuming that MZ B cells in humans are a post germinal center population although they display low frequency (2%) of somatic mutations.

There is no consensus on the site where MZ B cell mutate their Ig receptors. The lack of AID expression in the MZ area seems to suggest that in humans there are no naïve MZ B cells and that MZ B cells derived from a T-dependent response taking place in GC. Thus MZ B cells would be a post GC B cell population escaped from the GC after SHM but before CSR. Others suggest that MZ B cells can mutate their BCR in cryptic areas not yet identified (238).

1.4.5.4 B cell survival factors

It has been speculated that the complex system integrating signals received from antigens, BCR and survival factors has been designed from the immune system in order to rescue B cells that react very poorly with self-structures and place them in areas where they can deal with pathogen-derived structures showing little resemblance to self blood-borne pathogens (235).

I shall discuss the signals mediated by BAFF and its family of ligands and receptor in more details in the following section.

1.4.5.4.1 BAFF, APRIL and their receptors

BAFF (B cell-activating factor belonging to the TNF family, also called BLyS, TALL-1, THANK, and ZNF-41–5) is a member of the TNF family and has been demonstrated to promote B cell survival and differentiation in vitro and in vivo (239). BAFF is expressed as transmembrane protein by myeloid cell lineage, CD11c+ DCs, FDCs in GCs, activated T cells and CD34+ cells from cord blood. BAFF expression has been detected in the cytotrophoblast and in the radiation-resistant stromal cells in the bone marrow. Moreover BAFF expression in epithelial cells has recently been reported.

A proliferation-inducing ligand or APRIL (also named TALL-2, TRDL- 1 and TNFSF13a) is characterized by a close homology to BAFF, exerting similar functions in vitro and in vivo by interacting with receptors belonging to the BAFF family. APRIL production has been detected in monocytes, macrophages, DCs, T cells and more recently on epithelial cells (240, 241).

In order to exert its biological functions BAFF has to be proteolytically processed by a member of the furin family and released from the cellular membrane as a soluble molecule. Upon release, BAFF assembles as a trimer similar to other molecule of the large TNF family. An alternative splice

isoform of BAFF has been described, *Δ*BAFF, with negatively regulative functions on BAFF activity (242).

APRIL is processed intracellularly and can be expressed or as a cell surface fusion protein, linked to the membrane protein TWEAK (TWE-PRIL) or as soluble molecules bound to BAFF (BAFF/APRIL).

Three diverse receptors for the BAFF/APRIL family have been described: the transmembrane activator and CAML interactor (TACI), the B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R or BR3).

BAFF can bind all three receptors, while APRIL has been demonstrated capable to bid only TACI and BCMA, but not BAFF-R. Another possible receptor for APRIL on tumour cells has been suggested but not identified as yet (243). TACI, BCMA and BAFF-R have been demonstrated to be expressed on B cells at diverse stages of maturation. In particular BAFF-R appears to be expressed by all peripheral B cells from the T2 stage with higher expression been detected in Fo B cells, followed by the MZ cell population, while low levels of BAFF-R have been detected on GC B cells. TACI is expressed on T2 cells, though higher level of this receptor has been found on CD27+ memory B cells and MZ B cells. Finally, BCMA is highly expressed by plasma cells, plasmablasts and GC B cells (244).

1.5 Reg genes and regeneration

1.5.1 Reg genes family and structure

Reg genes (i.e. regenerating gene) were discovered by screening a rat cDNA library derived from regenerating islets from 90% depancreatized animals (245). Further studies isolated several Reg genes, which are, not detected in normal islets, from human, rat and mouse, and revealed that they constitute a multigene family (Table 1.7). Based on the amino−acid sequence homology among proteins encoded by Reg genes, the members of the family can be grouped into four subclasses, type I, II, III and IV. In humans, the Reg family genes are ordered in the 95−kbp DNA region of chromosome 2p12 (246) as follows: HIP/PAP − Reg 1α − Reg 1β − PAP IB − Reg2. Human Reg 4 gene is the only one located on chromosome 1. The same chromosomal organization was found in the rat at 4q33−q34 (247). Mouse Reg genes instead, were assigned to chromosome 6 (248, 249) and are disposed in the following order: 5' − Reg 3β − Reg 3δ − Reg 3α − Reg 2 − Reg 1 − Reg 3γ − 3' (250). Mouse Reg 4 was assigned to chromosome 3. These data suggest that the Reg family genes are derived from a common ancestor gene by several gene duplications, and have reached divergence in expression and function in the process of genetic evolution.

Table 1.7

The Reg gene family.

Type	*Human*	*Rat*	*Mouse*	*Hamster*	*Cow*
I	Reg Iα	Reg I	Reg I		
	Reg Iβ				
II	Reg II		Reg II		
III	HIP/PAP	Reg III/PAP II	Reg IIIα	INGAP	PTP
	Reg IIIγ	PAP I	Reg IIIβ/PAP		
	Reg IIIδ	PAP III	Reg IIIγ		
			Reg IIIδ		
IV	Reg IV		Reg IV		

HIP, hepatocellular carcinoma, intestine and pancreas; **PAP**, pancreatitis-asssociated protein; **PTP**, pancreatic thread protein; **INGAP**, islet neogenesis-associated protein.

1.5.2 Tissue expression & function

Human Reg genes are mainly expressed in pancreas, intestinal tract and brain. They are associated with several malignancies of the human gastrointestinal tract.

Human Reg1α gene maps to the short arm of chromosome 2 near the centromere at band 2p12 (251). It spans -3.0 kb, encodes a 166-amino acid protein including a 22-amino acid signal peptide and its molecular weight is 19 kDa (252).

It is expressed in high levels in foetal and infant brain. In adults, it is expressed, in low levels, also in the brain. In addition, Reg1α is expressed in Langerhans islets during β-cell regeneration; increased serum levels were observed in acute pancreatitis and chronic renal failure. Higher levels of urinary Reg1α were found in patients with diabetic nephropathy. Together with HIP/PAP, Reg1α is co-expressed in intestinal Paneth cells and in the brain of Alzheimer patients. The overexpression of Reg1α is associated with several cancer diseases, e.g. pancreatic cancer derived from acinar or ductal cells, cholangiocarcinomas, cancer derived from colon, gastric and enterochromatin-like cells.

Generally, Reg1α may act as mitogenic, antiapoptotic or anti-inflammatory factor; it can promote bacterial aggregation or increase resistance to antitumoral agents (252). Reg1α may be a sensitive marker for mucosa at risk for the development of neoplasia (253); increased secretion may reflect renal tubular dysfunction (254, 255).

Human Reg 1β gene displays another pattern of expression: is found in pancreas, colon and total brain in the foetus; in pancreas, jejunum, colon and pituitary gland in the adult (256).

Human Reg 3, HIP/PAP (human counterpart of mouse Reg 3 α and β) is expressed in normal Paneth cells, pancreas and hepatocellular carcinomas (257). HIP/PAP has a possible involvement in antiapoptosis in a pancreatic acinar cell line, AR4-2J cells (247). HIP and PAP are simply different

names for a single protein derived from the same gene (247, 258). Members of the RegIII family of intestinal C-type lectins are directly antibacterial proteins that play a vital role in maintaining host-bacterial homeostasis in the mammalian gut, yet little is known about the mechanisms that regulate their biological activity. The antibacterial activities of mouse Reg3γ and its human ortholog, HIP/PAP, are tightly controlled by an inhibitory N-terminal prosegment (259). INGAP (islet neogenesis-associated protein) (260) may be a hamster homologue of type III Reg. In the rat, the HIP/PAP gene shows a comparable tissue-specific expression pattern. It is overexpressed in the pancreas during acute pancreatitis, and also highly expressed in the columnar epithelial cells of ileum, jejunum and duodenum (261).

This protein from the Reg family contains a putative IL-6 response element: HIP/PAP becomes over expressed in human diabetic islets because of the local inflammatory response.

Human Reg 3γ gene, also known as PAP IB, is expressed almost only in the pancreas (262). A faint expression was observed in the placenta and PAP IB is absent in the small intestine. The peculiarity of this protein is its high homology with Reg 1α: 50% sequence identity between the two proteins. The common protein fold and the sequence identity explain why these two proteins also share the same specific functions.

Human Reg 3δ was not detected in normal islets but mainly expressed in exocrine pancreas. Although the other type III Reg genes are expressed in the gastrointestinal tract, Reg 3δ is not (263).

Human Reg 4 gene is the only Reg gene located on a different chromosome: chr1, 1p13.1-p12. This gene, also named RELP, is involved in inflammatory and metaplastic responses of the gastrointestinal epithelium and is up-regulated in malignancies of the human gastrointestinal tract (264). Reg 4 in fact is a potent activator of the EGF receptor/Akt/AP-1 signalling pathway in colorectal carcinoma and overexpression of Reg 4 may be an early event in colorectal carcinogenesis (265). Overexpression of Reg 4 is also associated with pancreatic cancer and with hormone refractory metastatic prostate cancer.

In the mouse, Reg genes have a common gene structure with 6 exons and 5 introns and encode homologous 165-175 aa proteins. Both Reg 1 and Reg 2 mRNAs are detected in the exocrine pancreas and hyperplastic islets of aurothioglucose-treated mice, but not in the normal islets. Reg 3α, Reg 3β and Reg 3γ are expressed weakly in pancreas, strongly in the intestinal tract, but not in hyperplastic islets (248, 250). Reg 3δ displays a widespread occurrence: exocrine pancreas and hyperplastic islets (together with Reg 1 and Reg 2) and intestine or colon (together with the other Reg 3 subtypes) (249).

Mouse Reg 3β was shown to act as a Schwann cell mitogen associated with the regeneration of motor neurons. From these reports, it is reasonable to assume that Reg proteins, especially type III Reg proteins, act as growth factors in alimentary tract, liver, and pancreatic acinar cells and neuronal cells, as type I Reg protein acts on pancreatic β-cells. Mouse Reg 4 was assigned to a different chromosome, like Reg 4 in humans, chr 3.

In summary, the main functional role of Reg genes in different tissues appears to be the promotion of tissue homeostasis and regeneration upon tissue injury by a wide range of insults. In this regard, β-cells have been shown to be susceptible to damage from numerous agents such as immunological abnormalities, virus infections, irradiation, and chemical substances, leading to local inflammation in and/or around pancreatic islets. In particular, the close relationship between β-cells damage, resulting inflammation and consequent up-regulation of Reg genes in the pancreas has been recently dissected, with a prominent role for in situ IL-6 production during the inflammatory process.

1.5.2.1 Reg receptor

Although much is known on the genetic, transcriptional regulation and tissue expression of Reg genes, very little information is currently available on the receptor(s) utilization and intracellular signalling activated by Reg genes in the target cells. A receptor for Reg protein that mediates the growth signal of Reg proteins for β-cell regeneration has been identified (266). The expression of

the Reg receptor, however, is not increased in regenerating islets as compared with that in normal islets; this observation suggests that the regeneration of pancreatic β-cells is primarily regulated by the increased expression of Reg genes (267).

The only known Reg receptor has been identified with EXTL3 (268).

Hereditary Multiple Exostoses (HME) is an autosomal dominant disease that determines disorganized growths of chondrocytes leading to ossification. Several genes are mutated in the disorder: EXT1 (60%), EXT2 (30%), EXT3 (10%) (269). It has been demonstrated a close homology between EXT genes and EXTL3 (EXT-like) only in the C-ter region; the N-ter region contains the membrane-spanning-domain that determines the presence on the cell surface (270).

EXT genes code for transmembrane proteins; key enzymes in heparan sulphate (HS) synthesis and glycosyltransferases. HS proteoglycans act as coreceptors for several growth factors and morphogenic proteins (FGF, BMP, Wnt). Lack of HSPGs in HME determines aberrant bony growths (269).

EXTL3 is also a cell surface receptor that binds to Reg proteins and a modulator of NF-κB activity in response to TNF-α (270); the Reg protein receptor cDNA was isolated from a rat islet library.

Reg receptor is a cell surface-transmembrane 919-aa protein expressed in: pancreas (islets, ductal cells of embryos and adults), brain, intestine, stomach, liver (271).

A very close homology was found to human EXTL3/EXTR1 gene (over 97% amino-acid identity).

Studies demonstrate that the expression of Reg receptor was not increased in regenerating islets as compared with normal islets, suggesting that β-cell regeneration and proliferation are only regulated by the expression of Reg proteins.

1.5.3 Mechanisms of induction

It was found that IL-6 plays a role in the activation of Reg genes and that PARP inhibitors such as nicotinamide and 3-aminobenzamide enhanced the induction. IL-6 stimulation induces the formation of an active transcriptional complex for Reg, in which PARP is involved. PARP was shown to bind the IL-6-responsive element of Reg gene, forming the active transcriptional DNA/protein complex for Reg gene expression.

The formation of the active transcriptional complex was further enhanced by the inhibition of the autopoly(ADP-ribosyl)ation of PARP. When the PARP is not poly(ADP-ribosyl)ated in the presence of PARP inhibitors, the transcriptional complex is stabilized and the Reg gene transcription is maintained (272, 273). Reg protein then produced in β-cells acts as an autocrine/paracrine growth factor on β-cells via the Reg receptor. DNA replication in β-cells occurs and the β-cell regeneration is achieved.

In addition to regenerating islets in 90% depancreatized rats receiving PARP inhibitors (274), Reg gene expression was also observed in the phase of transient β-cell proliferation such as in pancreatic islets of Bio Breeding (BB) rats during the remission phase of diabetes (275), in islets of NOD mice during active diabetogenesis (276) and pancreatic ductal cells, which are thought to be progenitor cells of β-cells, during differentiation and proliferation in a mouse model of autoimmune diabetes (277), and inflammation in and/or around islets was involved in these cases.

1.5.4 Reg proteins as T and B cell autoantigens

Despite up-regulation of Reg genes in the pancreas is a prominent feature in the course of the autoimmune insulitis, it is clear that regeneration of the β cells induced by Reg genes is not capable of preserving β cell function, as patients with T1D and animal models progress towards β cell

destruction and overt diabetes. Thus, it has been suggested that additional mechanisms are implicated in impairing the attempt of Reg genes to restore the β cell mass upon inflammation-mediated injury. Recent evidence suggests that an autoimmune process directed against the Reg genes themselves might be responsible for the impaired function of Reg genes. In this regard, it has been shown that Reg might act as novel autoantigens in T1D and thus become a target of the autoimmune process, possibly further promoting chronic inflammation and contributing to the disease pathogenesis.

In this regard, it has been demonstrated that the presence of auto-antibodies against Reg1 in Asiatic diabetic patients (278) with 24.9% of T1D and 14.9% of T2D patients testing positive for anti-Reg1α antibodies. No data on Caucasian patients is available.

Data in literature demonstrate that NOD mice display spontaneous T-cell (CD4+) responses to HIP/PAP. These T-cells can home to the pancreatic islets and can transfer disease when coinjected with CD8+ T-cells from diabetic NOD mice. It has been suggested that these T-cells require the cytokine/chemokine signals produced by islet-infiltrating CD8+ cells in order to leave the pancreatic lymph nodes and migrate to and remain in the islets. Alternatively, islet-infiltrating CD8+ cells might cause upregulation of production/secretion of Reg from the islets, which then activate T-cells resting in the pancreatic lymph nodes, causing them to accumulate in the islets.

It has also been demonstrated that IL-6 mediates upregulation of Reg production/secretion from isolated human islets. If such a mechanism indeed plays a role in vivo, it could be possible to imagine a scenario where during the progressive islet inflammatory process, increased amounts of IL-6 are released either from the infiltrating cells or (when triggered by their cytokines, such as γ-interferon) from the islets themselves (251, 279). This would then lead to upregulation of Reg expression; potentially, proliferation of T-cells recognizing the autoantigen HIP/PAP; and perhaps a progressive acceleration of the disease process. The fact that islets respond with upregulation of Reg expression/secretion when injured by inflammation would fit the role the Reg family has been reported to play. Its function in response to islet inflammation might be to support islet regeneration

and to protect the islet from inflammatory damage. If this was the case, overexpression of a putative islet regeneration protein that has the potential to act as an autoantigen (capable of generating an autoimmune response against it) could create a vicious cycle, accelerating the immune process leading to diabetes. Consistent with this hypothesis, the presence of autoantibodies against Reg has been demonstrated in diabetic patients, as described above (278). Although confirmatory studies are needed, evidence accumulated suggests that a continuous process of destruction/regeneration take place in the pancreas during autoimmune insulitis and that Reg genes might play an important role not only in the attempt to regenerate the reducing β cell mass, but also as a target of the autoimmune process itself.

CHAPTER 2

RATIONALE FOR THE THESIS AND AIMS

2.1 Aims of the project

Both in patients and animal models of T1D and SS, the chronic inflammatory/autoimmune process is heterogeneous and displays high immunological variability. In particular, in a sizeable subset of cases, inflammatory lesions display ectopic lymphoid structures characterised by T/B cell segregation, follicular dendritic cells networks and differentiation of germinal center B cells. However, there is limited evidence on the cellular and molecular mechanisms underlying the formation of these structures and their contribution to autoimmunity in the pancreas during autoimmune insulitis and in SG during autoimmune sialoadenitis.

Thus, **the first aim** of my PhD project was to better characterize the cellular and molecular mechanisms regulating the formation of ectopic lymphoid structures in the pancreas and SG of the NOD mouse model of T1D and SS.

During several organ-specific autoimmune diseases, a continuous process of tissue remodelling and regeneration takes place and is a fundamental feature of the homeostatic response of the tissues target of the chronic inflammatory process. It has been recently hypothesised that Reg genes might play an important role in this balance between immune-mediated destruction and regeneration not only in the pancreas during autoimmune insulitis, but also in the salivary glands of patients and animal models of SS. Thus, **the second main aim** of my project was to evaluate the expression pattern and the physiopathological role of Reg genes and proteins in cell regeneration in the target organs of NOD mice as a model of chronic inflammation during T1D and SS. A parallel aim was also the evaluation of the clinical utility of the determination of circulating Reg proteins and anti-Reg autoantibodies as bio-marker of residual tissue function/regeneration and disease activity, respectively in patients with T1D and T2D.

The specific objectives of this PhD thesis can be summarized as follows:

- To characterise the cellular and molecular evolution of TLS in the pancreas and SG by using the NOD mouse model of T1D and SS. To evaluate the development of inflammatory infiltrates, T/B cell segregation, the formation of FDC networks and ectopic GCs within the aggregates.

- To characterise the dynamic expression of local up-regulation of lymphotoxins (LTαβ) and lymphoid chemokines and their specific receptors in the context of TLS formation and functionality in the pancreas and SG of NOD mice.

- To determine the functionality of TLS in supporting in situ autoreactive B cell differentiation in NOD pancreas and SG by evaluating the expression of AID expression and the in situ production of disease-specific autoantibodies. This analysis will allow a better understanding on whether TLS contribute to the induction and maintenance of autoimmunity over and above secondary lymphoid organs.

- To characterize which B cell subsets localise within the lymphoid aggregates with particular focus on the definition of the main mature B cells subpopulation in mice: follicular and marginal zone B cells.

- To investigate whether inhibition of the LT-β dependent pathway is sufficient to prevent/delay the formation in of TLS and the generation of local B cell autoimmunity in both pancreas and SG of NOD mice.

- To evaluate the relationship between the formation of ELS and the expression of REG genes in both NOD pancreas and SG: to assess whether the expression pattern of Reg genes is related to the development of inflammatory infiltrates in NOD mice and whether Reg proteins were target of the autoimmune process itself.

- To examine the relevance of serum Reg proteins levels and anti-Reg auto-antibodies as bio-marker of damage/regeneration and disease activity in patients with different types of DM and SS.

CHAPTER 3

MATERIALS AND METHODS

List of the buffers, solutions, medium and recipes used in this project can be found in Appendix at the end of the Thesis.

3.1. Animal studies

3.1.1 Mouse samples

NOD mice came from the NOD/Ba colony, established in 1987 at St. Bartholomew's Medical College, London, UK, originally derived from Dr E. Leiter's laboratory (Bar Harbor, ME, USA) (280). There is a stable cumulative incidence of diabetes of approximately 80% in female and 15% in male mice at 30 weeks of age (22). The colony is housed in a purpose-built area and maintained strictly according to international (281) and United Kingdom guidelines for animal care. All mice are maintained under pathogen free conditions. Breeding and all procedures are carried out according to the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* (PPL 70/6109) (282). The diabetic status of the mice was initially diagnosed by monitoring urinary glucose level (Diabur Test 5000, Boehringer Mannheim, Germany) and then confirmed by measuring blood glucose levels (≥11.5 mmol/L) using Accu-chek AVIVA Blood Monitor (Roche Diagnostics GmbH, Mannheim, Germany). Balb/c mice were obtained from Charles River Laboratories (Charles River Laboratories, Wilmington, ME, USA).

A total of 126 female NOD mice were sacrificed at different ages ranging from 4 to 38 weeks. All mice were culled within 1 week from high blood glucose detection except mice <8 weeks which were non diabetic.

Pancreas, salivary glands and spleens were extracted and used for immunohistochemistry and RT-PCR analysis. The samples taken for histology and gene analysis were divided in equal parts. One part was frozen in RNALater and the other embedded in OCT (optimal cutting temperature) and stored in -80°C freezer until use.

A total of 20 female Balb/c mice ranging from 5 to 30 weeks were included in the study as controls. All mice were sacrificed by cervical dislocation.

3.1.2 Histology

All materials were purchased from Dako (Glostrup, Denmark), Sigma Aldrich (Poole, UK) and VWR (Poole, UK) unless otherwise stated.

3.1.2.1 Sample processing

3.1.2.1.1 Sample embedding

Mouse samples: pancreas, peri-pancreatic lymph nodes, spleen, submandibular glands, periglandular lymph nodes and Peyer's patches (used as control) were collected for the purpose of this study and processed as described below.

Tissues were collected freshly immediately after animal culling using cervical dislocation. Following removal, a drop of OCT (optimal cutting temperature) was placed on the top of a labelled small piece of cork, and the sample was arranged on the OCT. More OCT was poured over the sample in order to cover completely the tissue. A small plastic Becker filled with isopentane (2-

methyl butane) was placed within the liquid nitrogen, bringing the isopentane at its freezing point (-160°C). The cork with the embedded sample was then immersed into the ice-cold isopentane, until completely frozen. The sample was then wrapped in aluminium foil and transferred within a falcon tube in liquid nitrogen for transportation. Embedded samples were stored in -80°C freezer until used.

3.1.2.1.2 Samples cutting

Four microns sequential cryostat sections were obtained by cutting the specimens in the cryostat (Leyka) and mounted on glass slides (SuperFrost Plus). Slides were left overnight at room temperature to dry. The day after, each slide was individually wrapped in aluminium foil, placed in boxes and stored at -80ºC until use. Each slide was allowed to come to room temperature before being unwrapped and used for immunohistochemistry.

3.1.2.1.3 Sample fixation

Fixatives stabilize cells and tissues, make the antigen insoluble (therefore detectable) and protect the tissue from the processing and the osmotic damage caused by the solutions used during the staining procedures.

Frozen samples were fixed before the stainings with cold acetone (-20°C for 10 minutes). Acetone fixes the tissue by coagulating the proteins and inducing their denaturation (similar effect can be obtained by methanol fixation).

3.1.2.2 Haematoxylin and Eosin (H&E) staining

In order to visualize the microscopic structure of the tissue to study, Haematoxylin and Eosin (H&E) staining was performed on all the samples.

The H&E staining, by using two diverse dyes, allows us to visualize and differentiate the presence of cellular nuclei and cytoplasms under light microscopy. Haematoxylin stains the chromatin within the nucleus, with a deep purplish-blue colour, while Eosin stains the cytoplasmic material, connective tissue and collagen with a pinkish colour.

3.1.2.2.1 H&E protocol

After acetone fixation and air-drying, frozen sections were placed in jars containing filtered Mayer's Haematoxylin for 5 minutes. Slides were then rinsed in tap water and placed in 1% Acid Alcohol for a few seconds. Acid alcohol passage removes the excess of Haematoxylin from the cytoplasm, allowing Haematoxylin to stain only in the nuclei.

The slides were then washed again in tap water and placed in Eosin for 5 minutes. Finally the slides were washed in distilled water, dehydrated and mounted in organic solvent soluble mounting medium (DePeX).

3.1.3 Immunohistochemistry

The immunohistochemistry technique allows the localization of a specific antigen/s on tissue sections. This system is based on the recognition of a specific antigen/s by a conjugated (direct method) or un-conjugated (indirect method) primary antibody (first layer).

The first layer is followed by one or more incubations with a secondary antibody linked to enzymes (commonly horseradish peroxidase HRP or alkaline phosphatase AP) or a biotinylated antibody followed by enzyme-linked Avidin complexes. The use of a substrate solution and a chromogen, while reacting with the used enzyme, allows the precipitation of the colour (colorimetric methods) on the site of antibody-antigen binding.

3.1.3.1 Colorimetric methods

The two more diffuse colorimetric methods used in immunohistochemistry are based on the activity of two enzymes, the horseradish peroxidase (HRP) and alkaline phosphatase (AP). The HRP reacts with diverse chromogen substrates (3,3'-diaminobenzidine or DAB, 3-Amino 9-ethylcarbazole AEC). I used the DAB that develops in a brown end product, insoluble in alcohol and other organic solvents. Since endogenous peroxidase activity is present in many tissues and can be non-specifically detected with the DAB substrate, pre-treatment of the tissue section with hydrogen peroxide or other peroxidase blocking solutions was performed prior to incubation of the primary antibody in order to avoid non specific staining.

Alkaline phosphatase catalyzes the hydrolysis of phosphate–containing products in the basic pH range. Its enzymatic activity is visualized by using a purple or blue chromogen in an alkaline buffer. The chromogens can be either soluble in organic solvent or permanent. Endogenous alkaline phosphatase is diffusely expressed in human and mouse tissue, therefore the inhibition of the endogenous enzyme is mandatory in order to avoid unspecific staining in the section.

The chromogen used was the Vector Red (Vector Lab) according to the manufacturer's instruction. Levamisole was added to the substrate solution in order to inhibit the endogenous activity of alkaline-phosphatase.

3.1.3.2 Staining procedures

3.1.3.2.1 Avidin-Biotin Complex (ABC) Method

The Avidin-Biotin Complex (ABC) method is based on the strong affinity that Biotin (a small molecular weight vitamin) displays for Avidin (a large glycoprotein of 68 kilo Dalton). Avidin presents four binding sites for biotin providing the backbone of a macromolecular amplificatory complex. Avidin can be labelled with diverse enzymes (horseradish peroxidase or alkaline-phosphatase) and fluorochrome, allowing diverse methods of detection to be used. In this study I used Avidin-Biotin Complex (ABC) linked to horseradish peroxidase and alkaline phosphatase. The method requires three layers: the first layer is unlabelled primary antibody, the second layer is the biotinylated secondary antibody and the third layer is the enzyme HRP or AP-Avidin conjugate. The used enzyme is then visualized by the use of the appropriate chromogen and substrate. I used in this work HRP and AP conjugated Avidin-Biotin kits from Dako.

Briefly, after fixation a water-repellent circle was drawn around the tissue sections using DAKO Pen, in order to inhibit the dispersion of the solutions used and to avoid tissue desiccation. In order to avoid non specific binding to endogenous biotin (present in diverse tissues) pre-treatment with unconjugated avidin saturated with biotin was performed on each slide. In my work endogenous biotin activity was inhibited using the Dako Biotin Blocking System. After a first incubation for 10 minutes with the avidin solution, slides were rinsed and incubated with the biotin solution for 10 minutes. Then slides were washed and left in TBS before application of the primary antibody.

All primary antibodies for immunohistochemistry in bright field were unconjugated, diluted in the antibody diluents and incubated for 1 hour unless otherwise stated. Appropriate biotinylated secondary antibodies were used. Single staining was performed either by indirect alkaline phosphatase (AP) avidin-biotin complex or by indirect horseradish peroxidase (HRP) avidin-biotin

complex. Colour development for both AP and HRP was checked under the light microscope and enzymatic reaction blocked as appropriate in distilled water.

Once the slides were stained, accordingly to the substrate used for colour development, they were either mounted with aqueous mounting medium or dehydrated trough passages in alcohol and xylene and mounted in xylene-based mounting medium.

3.1.3.3 Image capture

Images for IHC were captured by using a Nikon digital camera linked to a motorized light microscope (BX60) from Olympus (London, UK). Image were analysed by using the analysis system software from Soft Imaging System (Münster, Germany). This software provides a fully automated image capture and analysis system that delivers high quality images. Both quantitative and qualitative analysis was performed. Qualitative analysis was based on the detection of specific fluorescence on tissue and its localization. Quantitative analysis was performed by highlighting differently stained areas on tissue. PhotoShop software was used to save and reduce the size of the images.

3.1.4 Histological Characterization of Pancreas and Salivary Glands of NOD mice

3.1.4.1 Histological characterization of pancreatic infiltrates

Immune cells infiltrates organization and evolution in pancreas islets were characterized by immunohystochemistry and immunofluorescence staining of sequential sections. Four microns

sequential cryostat sections were cut and mounted on glass slides (SuperFrost Plus). Sections underwent routine staining with haematoxylin and eosin (H&E) in order to define the histological quality of tissue, prevalence and pattern of islets insulitis (peri vs intra-insulitis). Spleen and peri-pancreatic lymph node sections were used as positive controls.

3.1.4.2 Histological characterization of submandibular gland infiltrates

Immune cells infiltrates organization and evolution in submandibular glands (SUBM) were characterized by immunohystochemistry and immunofluorescence staining of sequential sections. Only right submandibular glands were chosen for the study in order to minimize the variation between diverse animals. Four microns sequential cryostat sections were cut and mounted on glass slides (SuperFrost Plus). Sections underwent routine staining with haematoxylin and eosin (H&E) in order to define the histological quality of tissue, prevalence and pattern of inflammatory infiltrates. Spleen and peri-glandular lymph node sections were used as positive controls.

3.1.4.3 Antibodies

A list of primary and secondary antibodies used in this study is reported Table 3.1 below.

Table 3.1

Primary and secondary Abs used for immunofluorescence.

Clone/Name	Specificity	Conjugation	Host	Dilution	Source
Primary Abs					
1B1	CD1d	PE	Rat	1/50	Cambridge biosciences
B3B4	CD23	PE	Rat	1/100	Cambridge biosciences
11-26C.2a	IgD	FITC	Rat	1/100	Cambridge biosciences
R6-60.2	IgM	PE	Rat	1/100	BD Biosciences
7G6	CD21/CD35	FITC	Rat	1/100	BD Biosciences
RA3-6B2	B220	PE	Rat	1/100	BD Biosciences
GL7, Ly-77	GL7	FITC	Rat	1/100	BD Biosciences
500-A2	CD3	Biotinylated	Rat	1/70	BD Biosciences
FDC-M1	FDC	Unconjugated	Rat	1/100	BD Biosciences
EK2-5G9	AID	Unconjugated	Rat	1/20	Ascenion
281-2	CD138	Biotinylated	Rat	1/100	BD Biosciences
2G8	CXCR5	PE	Rat	1/100	BD Biosciences
MECA-79	PNAd	Biotinylated	Rat	1/100	Biolegend
TEC-3	Ki67	Unconjugated	Rat	1/20	Dako
	Reg3β	Unconjugated	Sheep	1/200	R&D Systems
	Reg3δ	Unconjugated	Rat	1/100	R&D Systems

Secondary Abs:

Rabbit anti-Rat biotin Ig	Rabbit	1/300	DakoCytomation
Streptavidin ALEXA 488		1/300	Invitrogen
Streptavidin ALEXA 555		1/300	Invitrogen
Rabbit anti-sheep-HRP	Rabbit	1/100	DakoCytomation
Chicken anti-rat-HRP	Chicken	1/100	DakoCytomation

3.1.4.4 Histological evaluation of T/B cell segregation

Doubled staining for CD3/B220 was used to analyze T/B cell segregation and characterization of infiltrates. For immunofluorescence, frozen sections were fixed in cold acetone for 10', air dried and washed in TBS. Endogenous biotin was blocked using the avidin-biotin blocking system (DakoCytomation), while non specific binding blocked using the Dako protein block for 10'. Biotinylated anti-CD3Ab (dilution 1:70) incubation was carried out for 1h at room temperature and followed by 10' washing in TBS 1X and 1h incubation with streptavidin Alexa-488 (dilution 1:300). After washing, B220-PE Ab (dilution 1:100) incubation was then carried out for 1h. Slides were stained with DAPI for 10', washed and mounted with Mowiol.

3.1.4.5 Histological evaluation of functional ectopic GC-like structures

Staining for GL7 (marker for mouse germinal center B cells) and FDC-M1 (follicular dendritic cell networks) were performed on sequential sections of NOD pancreatic tissue and submandibular glands in order to evaluate the presence of GCs. Such stainings were correlated with the histological characterization of the lymphoid aggregates. Frozen sections were processed as above and GL7-FITC Ab (dilution 1:100) incubation was carried out at 4°C overnight. For FDC identification, FDC-M1 Ab (1:100) incubation was carried out for 1h and followed by 1h incubation with the biotinylated rabbit anti-rat Ig secondary Ab (dilution 1:300) and streptavidin Alexa-555 (dilution 1:300) 1h incubation. FDC networks were also evaluated using standard immunohistochemistry with peroxidase staining in order to confirm the localization of the cells on bright field. In this case, after the secondary biotinylated Ab, slides were washed and incubated with streptavidin HRP-ABC

complex (Dako) for 30'. After washing, colour reaction was developed using DAB solution (Dako), slides counterstained with hematoxilin, dehydrated and mounted in DePex.

As AID is expressed transiently and exclusively in B cells undergoing SHM/CSR, staining for AID was carried out to evaluate the functionality of the TLS. Acetone-fixed and avidin-biotin-blocked sections were incubated with AID Ab (1:20 dilution) for 1h, followed by 1h incubation with the biotinylated rabbit anti-rat Ig secondary Ab (antibody dilution 1:300) and the streptavidin Alexa-555 for 1h.

3.1.4.6 Characterization of B cell subsets within the infiltrates

Immunofluorescence was carried out in order to evaluate the subset of B cells infiltrating the pancreatic islets and the submandibular gland infiltrates. Spleen sections were first used in order to validate the immunofluorescence technique for the identification of the different B cell subsets based on well identified markers. Follicular mature B (FoB) cells in the spleen are situated in white pulp follicles and are characterized by a $B220^{+}$, IgD^{high}, $CD23^{high}$, $CD21^{low}$, IgM^{low}, $CD1d^{low}$, $CXCR5^{high}$ phenotype. Conversely, marginal zone B (MZB) cells are located between the white and red pulp, close to the marginal sinuses and exposed to blood antigens. These cells are characterized by a $B220^{+}$, IgD^{low}, $CD23^{low}$, $CD21^{high}$, IgM^{high}, $CD1d^{high}$, $CXCR5^{low}$ phenotype (283). Thus, to identify whether FoB and/or MZB cell were present within infiltrates, double IF stainings on sequential sections were performed for IgD/B220, IgD/Cd1D, CD21/CD23, IgD/IgM, CXCR5/B220. Following validation in the spleen, the staining was then performed on pancreatic and submandibular gland tissue.

3.1.4.7 Pancreatic in situ antibodies production

In order to localize the presence of anti-insulin antibodies producing cells within the pancreatic infiltrates, single staining with FITC-conjugated insulin (Invitrogen, 100μg/1ml PBS PH7.5) (dilution 1:10) was carried out for 1h on acetone-fixed slides. Prior to incubation, slides were blocked for 1h with Dako protein block. To confirm specificity of the binding, sections were pre-incubated with unstained insulin prior to the FITC-conjugated insulin incubation. Followed washing in TBS 1X, DAPI 10' incubation and final fixation with Mowiol. Peri-pancreatic lymph nodes and infiltrated salivary glands were included as positive (284) and negative controls, respectively. In order to identify anti-insulin producing cells, double stainings for PE-B220/FITC-conjugated-insulin (B cells) and for CD138/FITC-conjugated insulin (plasma cells) were performed on sequential sections. Biotinylated anti-CD138 Ab incubation (dilution 1:100) was carried out overnight at 4°C on slides previously incubated in avidin-biotin and protein block. Alexa-555 (dilution 1:300) incubation was performed for 1h.

3.1.4.8 Reg protein tissue localization

Frozen sections were processed as above, except for an additional blockade of endogenous peroxidase prior to incubation with anti-mouse Reg3β and Reg3δ (R&D Systems) antibodies for 1h (dilution 1:50; 1:10). Slides were then washed and incubated with secondary rabbit anti-sheep-HRP or chicken anti-rat-HRP antibodies, respectively, for 1h (dilution 1:100) and developed with DAB substrate (Dako, UK).

3.1.5 FACS analysis

3.1.5.1 Isolation of mononuclear cells from mouse spleen, pancreas and submandibular glands

3.1.5.1.1 Spleen

After collecting spleen from the mouse, the tissue was conserved in PBS1X + 0.5% FBS + 2mM EDTA (Buffer 1).

The same solution was poured into a Petri dish where the tissue was mashed. The organ was placed on a nylon cell strainer and crush through with the plunger from a 2 ml syringe.

The mashed cells were collected from the Petri dish into a 50 ml tube and centrifuge at 1500 g for 5 min. Supernatant was discarded and pellet resuspended in 5 ml of red cell lysis buffer for 5 min. A 70μm pore-size cell strainer (BD Falcon, Erembodegem, Belgium) was placed over a new 50 ml tube to filter the solution. The cell strainer was washed with buffer 1.

At this point the solution was centrifuged at 1.500 g for 5 min. After centrifugation, the pellet formed an opaque layer between the top buffer and the bottom layer, because of the different components' densities (separation by a density gradient centrifugation method). The lymphocytes were then collected from the opaque layer using a Pasteur pipette. The supernatant was discarded and pellet resuspended in appropriate buffer 1. Finally it was possible to proceed with FACS staining.

3.1.5.1.2 Pancreas and submandibolar glands

After collecting pancreas or salivary glands from the mouse, tissue was conserved in PBS1X + 0.5% FBS + 2mM EDTA (Buffer 1). The same solution was poured into a Petri dish where the tissue was mashed. The organ was placed on a nylon cell strainer and crush through with the

plunger from a 2 ml syringe. The mashed cells were collected from the Petri dish into a 50 ml tube and 20 ml of Buffer 1 were added. Collagenase (0.5% final) was further added to the solution and left for 1 h in shaking incubator at 37 °C. The solution was then filtered with a 70μm pore-size cell straniner (BD Falcon, Erembodegem, Belgium) in a new 50 ml tube and the cell strainer washed with buffer 1.

The solution was then centrifuged at 1.500 g for 5 min and the pellet collected as described above prior to FACS staining.

3.1.5.2 Principles of cytofluorimetry

Flow cytometry is a technique that allows analyzing and sorting complex cellular suspensions in terms of both physical and biochemical features using a laminar cell flux excited by a laser of the appropriate length.

Different parameters of a single cell passing through the luminal flux can be evaluated depending on the number of the lasers and the combination of the fluorochrome used to label the cells. The presence of fluorescent detectors either in line with the light beam (Forward Scatter) or perpendicular to it (Side Scatter) allows the collection of the light reflected by the single particle. The combination of the information derived by the diverse detectors defines the physical and chemical structure of each individual particle. The elaboration of the diverse signals allows the elaboration of a dispersion diagram or cytogram functional for the discrimination of diverse populations. The data generated by detectors can be plotted in a single dimension (histogram) or in two dimensional dot plots or even in three dimensions. The regions obtained in these plots can be separated, based on fluorescence intensity, creating a series of subset extractions, called "gates". Fluorescence-activated cell-sorting (FACS) allows the sorting of a heterogeneous mixture of cells

on the base of the cells specific characteristics. Physical separation of the cell of interest can be obtained with the cell sorter (Figure 3.1).

Figure 3.1

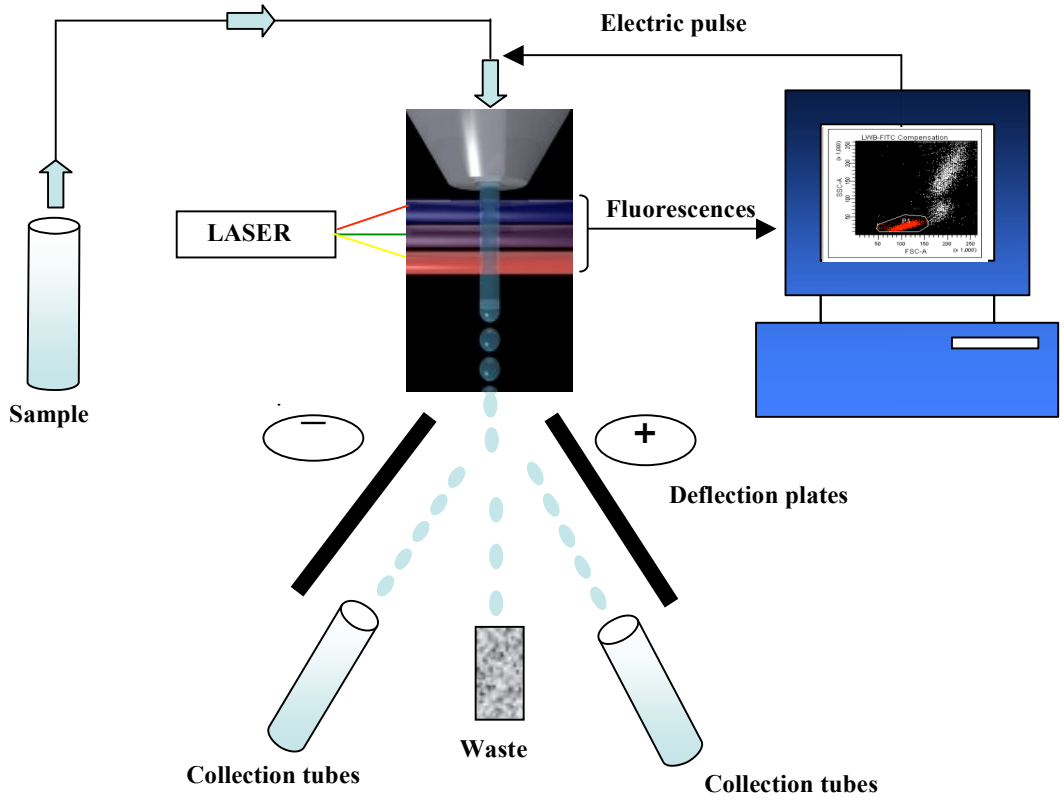

Figure 3.1 Schematic representation of a FACS machine with a cells sorter. Diagram illustrating the component of a FACS machine with a cell sorter. The sample is run into the machine and accordingly to the physical features and the presence of the staining the population/s of interest are isolated in collection tubes.

3.1.5.3 Cell staining procedure

All reagents were kept at 4°C during all procedures.

Mononuclear cells were divided (100µl/well) into appropriate round bottom wells of a 96 microwell plate. Cells were then centrifuged for 4 minutes at 1200 g in a refrigerated centrifuge (4°C), supernatant was removed. Cells were washed with 10 ml FACS Buffer and resuspend in 50/100 µl of FACS Buffer (PBS1X + 2% FBS with or w/o NaN_3). Cells were counted and for each staining $5x10^5$ to $3x10^6$ cells per sample were used.

Stainings were performed in the round bottom 96 microwell plates by adding the antibodies and washing volume used was 250µl (3 times). Incubation was carried out for 30 minutes at 4 °C (on ice) in the dark. After the staining, cells were centrifuged at 1.200 g for 4 minutes, supernatant discarded and 200 µl of FACS buffer added. Finally cells were transferred in FACS tubes and 300 µl of PBS1X was gently mixed before acquisition.

Dead cells were excluded from analysis by side/forward scatter gating and or PI staining.

PI staining:

10µl propidium iodide (PI) solution (stock at 10 µg/ml) was added to the cell suspension just before the analysis. Cells were then analyzed. PI was read with FT2 or FT3.

3.1.5.4 Antibodies

All antibodies used were conjugated and are listed below:

Anti mouse CD19 Cy5.5 (Clone 1D3)

Anti mouse CD23 PE-Cy5 (Clone B3B4)

Anti rat and mouse CD21/35 FITC (Clone 7G6)

Proper concentration for each antibody was tested before the final experiments. The concentration which better separates positive from negative cells was chosen for each antibody.

3.1.5.5 FACS Calibur acquisition

The Facs Loader

The Loader option on FACSCalibur was used for automated sample collection and analysis. This option could be used manually to acquire samples or in conjunction with Worklist Manager software. Loader Manager software was also used to perform the cleaning (FACSClean and ddH2O) procedure automatically. Carousel with samples were placed on loader. The drawer was pushed in completely and the Loader cover replaced. Samples were mixed by pressing the MIXING key for ~5 sec. for a high-energy mix, or by pressing the MIXING key for 1 second for a low energy mix.

The cytometer was placed in RUN mode and the sample acquired. The same procedure was carried out to all tubes until all samples had been acquired.

Acquisition of data using Cellquest Pro

CELLQuest Pro was launched from the Apple menu to open a new untitled experiment document.

The computer was then "Connected to Cytometer" from the "Acquire menu". A new acquisition document was created with FSC and SSC plotted as the x and y parameters. An acquisition histogram plot was created for a single fluorescent parameter (FL1, 2, or 3) and/or an acquisition density plot for dual parameter acquisition (e.g. FL1 vs. FL2) was created. Negative and single positive control samples were always needed.

Detectors/Amps, Threshold, and Compensation from the Cytometer menu were chosen. In general the instrument settings menus are dealt with in order, i.e. first detectors/amps are adjusted, then threshold, then compensation). Samples were placed on the Loader-carousel, mount to the flow cytometer, the cytometer set to RUN and the acquisition began. While acquiring, the FSC amplifier, SSC detector in the detectors and amps window were adjusted to find our populations of cells. The FSC and SSC amplifiers in general should be linear. The voltages and amp gain on the FSC and SSC detectors were adjusted so that our cells would appear in the middle of the FSC vs SSC dot plot. The Threshold was set to eliminate extraneous events, noise, or debris. Thresholding is electronic gating. Events below a threshold value are electronically excluded from acquisition. Using the negative control tube, the threshold level was set to remove most of the debris without cutting off the population of interest.

For investigation of subpopulations during acquisition or to set the counting to the relevant population, a region was defined by drawing around the events of interest. A plot was selected and the gate turned on in the plot source menu. The background fluorescence was adjusted for each fluorescent parameter.

Fluorescence Compensation was necessary for appropriate multi-color analysis. For a given setup of cells this was performed once initially.

It was then possible to acquire and save the sample-data. Data-Analysis using Cell Quest could be performed at the offline workstation and at the FACSCalibur. An analysis dot plot was created, a data file included and FSC and SSC were put as the x and y parameters with no gate. An analysis histogram plot with a data file and the appropriate fluorescent parameter (FL1, 2, 3, or 4) was created or an analysis dot plot, an analysis density plot, an analysis contour plot for dual parameter acquisition (e.g. FL1 vs FL2) were created. A region was defined and a gate applied.

3.1.6 Detection of circulating murine autoantibodies

3.1.6.1 Collection and storage of mouse serum

Following cervical dislocation, blood was immediately collected from NOD and Balb/c mice by means of cardiac puncture using fine needle insulin syringes. An average of 400µl of blood was collected in microcentrifuged tubes and left undisturbed for 2h at room temperature to allow clot formation. Sera were then separated from the precipitate following centrifugation at 10.000 g in a microcentrifuge for 10 min and stored at -20°C until use.

3.1.6.2 ELISA for anti-single-stranded DNA, anti-chromatin, anti-histone and anti-Ro/La Abs

Nunc multisorb ELISA plates (VWR, Poole, UK) with a 96 wells layout were coated for three hours at 37°C or at 4°C overnight with the appropriate antigen diluted in 50µl volume per well of BBS (Boric acid 100mM, Na tetraborate.10H_2O 25mM and NaCl 75mM at pH8.3-8.5). In order to quantify the non specific binding happening between the serum sample and plastic plate, each third row of the plate was incubated with BBS only. The following antigen concentrations were used:

- 10µg/mL of ssDNA prepared in this way: dilute calf thymus DNA (Sigma D-1501) with BBS to a final concentration of 0.5 mg/ml, mix overnight at 4° C on magnetic stirrer, pour into 10 ml Pyrex tubes (5 ml/tube) and place in boiling water bath for 10', cool rapidly in ice-methanol bath, check concentration by spectrophotometer, aliquot and store at -70°C.

- 5μg/mL of chromatin prepared in this way: dilute Nucleohistone (Roche UK) with BBS to a final concentration of 400μg/ml, mix overnight at 4° C on magnetic stirrer, pass through an orange needle and add equal volume of glycerol, check concentration by spectrophotometer, aliquot and store at -20°C.

- 5μg/mL of histone (Roche UK)

- 5μg/mL of recombinant Ro52Kd or La48Kd (ProspecBio, Israel)

After the coating step, plates were washed three times with PBS 1x [NaCl, KCl, $Na_2HPO_4(12H_2O)$ and KH_2PO_4, pH 7.4] and saturated at room temperature with 100μL/well of PBS 0.5%BSA (PAA, UK) NaN_3. Samples were screened in triplicates (duplicate plus non specific binding) at 1/100 dilution for IgG anti-ssDNA (total IgG and subclasses) and 1/500 for IgG anti-chromatin and anti-histone antibodies respectively. Pooled serum from MRL/Mp.*lpr/lpr* mice was used as a standard positive starting at 1/400 dilution for the anti-ssDNA and 1/100 for the anti-chromatin and anti-histone Abs; a standard curve made of 12 serial dilutions was included only in the first ELISA plate of each assay and control samples were used to quantify the intra-assay variability. All dilutions were performed in PBS supplemented with 2% BSA, 0.05% Tween 20, 0.02% NaN3. Samples were incubated for at least 5 hours at 4°C and then plates washed five times with PBS. Bound antibodies were detected with alkaline phosphatase (AP) conjugated goat anti-mouse IgG (γ-chain specific) (Sigma-Aldrich, Dorset, UK) diluted 1/3000 for the anti-ssDNA and 1/1000 for the anti-chromatin and anti-histone Abs. For the anti-ssDNA IgG subclasses the following AP conjugated anti-mouse Abs were used: IgG1 diluted 1/800, IgG2a and IgG2b diluted 1/500, and IgG3 diluted 1/1000 (all from Southern Biotechnology Associates, Inc Birmingham, AL, USA). All dilutions were performed in PBS 2% BSA. After 5 hours at 4°C, plates were washed five times and the bound AP-

conjugated antibody was detected using a P-nitrophenylphosphat tablet sets (Sigma FAST N-2770). Plates were read at 405nm (Multiskan Ascent, Thermo Electron Corporation) at different time points and data recorded. Results were expressed as arbitrary ELISA units (AEU) relative to the standard curve extrapolated from a sigmoidal dose-response equation where Y is the optical density reading (OD) and X the logarithm of the concentration (GraphPad Software, San Diego, CA).

3.1.6.3 ELISA for anti-double-stranded IgG Abs

Nunc multisorb ELISA plates were sensitized for three hours at 37°C or at 4°C overnight with 50µL/well of 1µg/mL of Streptavidin (Streptavidin Roche, UK) in BBS. After the washing and saturation steps previously described biotinylated dsDNA at 200ng/mL concentration in PBS was applied as antigen. dsDNA biotinylation was performed as follows: dilute the plasmid DNA (ΦX174RF Promega D1531) with TE (10mM Tris, 1mM EDTA) to a concentration of 0.5µg/µl, mix equal volumes of plasmid and photobiotin (Photoprobe biotin Vector #SP-1000) and cover with mineral oil, incubate at 95°C for 12'. Biotinylated DNA extraction was carried out in this way: for a 40µl volume add 40µl double distilled water, 80µl Tris pH9.5, 160µl 2-butanol, vortex and spin 5', remove upper layer and discard. Repeat and after add 10µl 10M NH_4 acetate, 2µl 1M $MgCl_2$ and 125µl ice cold (-20°C) 100% Ethanol. Mix and incubate for 15' at -20ºC, spin 15' in the cold, remove supernatant and add 70% ice cold ethanol. Spin 3', remove supernatant and let evaporate the rest. Resuspend at the desired concentration and store at -70°C. After a further washing cycle and saturation step samples and conjugate antibody were applied following the same protocol described in 3.4.1 as follows: standard 1/400 dilution, samples 1/100 dilution and conjugate antibody at 1/3000 dilution for total IgG and 1/800 for IgG1, 1/500 for IgG2a and IgG2b, and 1/1000 for IgG3. Analysis of the data was performed as above.

3.1.7 Quantitative TaqMan real-time PCR

3.1.7.1 Extraction of total RNA from mouse tissues

RNA was extracted using the Quiagen RNeasy mini kit according to manufacturer' instructions. Tissues stored at -80°C in RNA Later were defrost in ice, weighted and then cut in order to obtain approximately 20mg (30mg of tissue is the maximum amount suggested in the protocol to avoid reduction in RNA yield and purity). RNA was also extracted from Peyer's plaques and spleens as positive control.

Tissues were placed in a sterile, RNAse free tube containing microspheres and 600 ***µ***l of buffer RLT (containing denaturing guanidine thiocyanate and with the addition of 10***µ***l of fresh β-Mercaptoethanol/ml of RLT buffer) were added. Tissues were homogenised using an automatic homogenizer until the sample was uniformly homogeneous (3 cycles at max strength). Tissue lysates were centrifuged for 3 minutes at maximum speed (13,000 g) in a microcentrifuge and supernatant was transferred to a new RNAse free 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***µ***l) 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 g) to allow the RNA to bind to the silica column. The flow-through was discarded and the remaining sample (approximately 500***µ***l) added to the RNeasy mini column and centrifuged as above.

To avoid any possible DNA contamination, a DNase step was included according to manufacturer' instruction. 350 ***µ***l of Buffer RW1 were added to the column, centrifuged for 15 s at ≥8000 x g (≥10,000 g) and the flow-through discarded. 10 ***µ***l of DNAse I stock solution (previously prepared by dissolving solid DNase I (1500 Kunitz units) in 550 µl of RNase-free water) were added to 70***µ***l

of Buffer RDD, gently mixed and added to the RNeasy mini column silica-gel membrane. Following 15 min incubation at room temperature (RT), 350 µl of Buffer RW1 were added to the column, centrifuged for 15 s at ≥8000 x g (≥10,000 g) and the flow-through discarded.

The RNeasy column was transferred into a new 2 ml RNase free collection tube. In order to wash away contaminants (residual DNA and proteins) in the organic phase, 500 µl of Buffer RPE were placed onto the RNeasy column and centrifuged for 15 s at ≥8000 x g (≥10,000 g) to wash the column. The flow-through was then discarded and another 500 µl Buffer RPE added to the RNeasy column. The tube was centrifuged for 2 min at ≥8000 x g (≥10,000 g) to dry the RNeasy silica-gel membrane and the flow-through discarded. To eliminate any chance of possible Buffer RPE carryover the tube was centrifuged again for 1 min at full speed (13,000 g).

For elution, the RNeasy column was transferred to a new RNAse free 1.5 ml collection tube, and 30 µl of RNase-free water were pipetted directly onto the RNeasy silica-gel membrane and the tube centrifuged for 1 min at ≥8000 x g (≥10,000 g). The elution was immediately frozen at -80°C until required.

3.1.7.2 Quantification of total RNA

The concentration of RNA isolated with RNeasy Kits can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. Absorbance readings should be greater than 0.15 to ensure significance. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 = 40 µg/ml). This relationship is valid for measurements in water. Therefore, RNA was diluted in water to quantify it spectrophotometrically.

3.1.7.3 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 Acetic Acid EDTA (TAE) buffer was boiled in a microwave for 2 minutes, then the ethidium bromide (a fluorescent dye that binds to nucleic acids) was added and the solution poured into a minigel tray where a well-comb was inserted. After polymerisation, 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 dye (1:6) to monitor migration, loaded into the gel wells and run at 80V (voltage) for 30 minutes. RNAs were visualized by fluorescence of the incorporated ethidium bromide using a UV light transilluminator and examined for RNA degradation and DNA contamination.

RNA should demonstrate two 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 (Fig. 3.2). If the 18S band appears more intense than 28S band, or a smear is present, RNA degradation is likely. Extra bands at high molecular weight normally indicate genomic DNA carry-over.

Figure 3.2

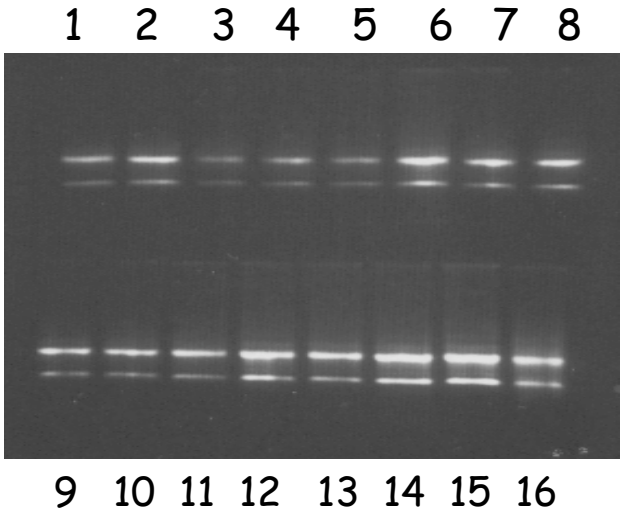

Figure 3.2 Assessment of RNA quality. Representative example of 0.8% agarose gel electrophoresis to assess RNA quality/integrity. RNAs extracted from 16 mouse submandibular glands are reported.

3.1.7.4 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, cat#11146-016). Briefly, 1µg of extracted total RNA from each sample was mixed with 1 µl of Oligo(dT)20 Primers (50 µM) and 2 µl of 10 mM dNTP mix and brought to a 12 µl volume reaction with DEPC-treated water in a 0.5ml 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 RNaseOUT™ Ribonuclease Inhibitor (40 units/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 1h 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 1µl of E. coli RNase H (2 units/µl) at 37°C for 20 min. Finally, the completed cDNA strand was diluted to a final concentration of 10ng/µl with DEPC-treated water and stored until used.

3.1.7.5 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 the reaction.

For quantitative TaqMan real-time evaluation of mRNA expression levels of human and mouse 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 Biosystems) with an equal loading of 10ng of cDNA/well and detected using the ABI PRISM 7900HT Instrument. The thermal cycling conditions used comprised a 2 min UNG activation step at 50 ºC, a 95 ºC Taq polimerase enzyme activation step for 10 min, and cycles of 95 ºC denaturation for 15 sec and 60 ºC anneal/extension for 60 sec. Results were then analysed after 40 cycles of amplification using the ABI PRISM 7900HT Sequence Detection System Version 2.1 (SDS 2.1). cDNA from mouse spleen or Peyer's patches were used as calibrators. Relative quantification was measured using the Comparative Ct (Threshold Cycle) Method. Two different endogenous controls (mouse beta-actin and mammalian 18S) were used to normalize for the cDNA of each sample. The ΔCt for each of the triplicate (Ct of the target gene minus Ct of the endogenous control) and then the average ΔCt 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 the ΔΔCt, the ΔCt of each sample was subtracted to the chosen reference sample (usually a salivary gland or pancreas from a control mouse). The relative quantity was then calculated following the equation $RQ= 2^{-\Delta\Delta CT}$ 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. As shown in a representative example in Figure 3.3, a difference of 5 Ct was observed between each dilution, demonstrating optimal PCR efficiency.

Figure 3.3

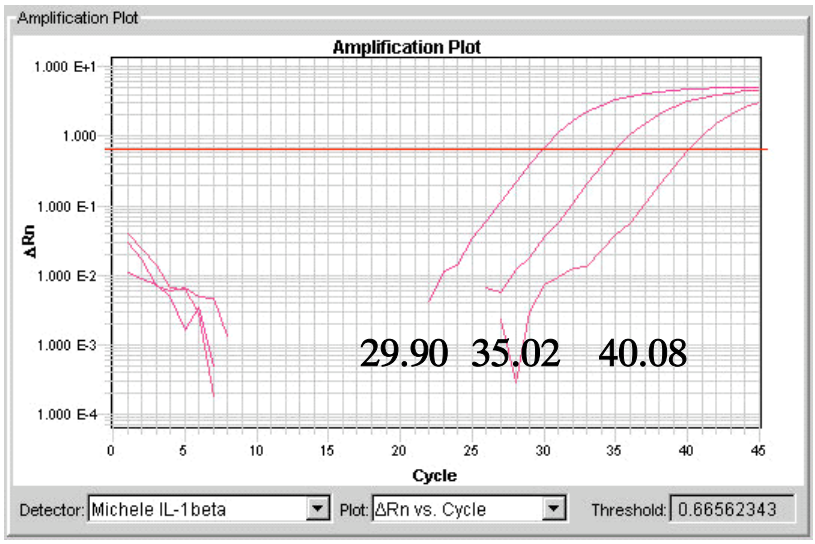

Figure 3.3 Evaluation of efficiency of Taqman real-time PCR. Taqman real-time curves of amplification of mouse IL-1b at 2^5 dilutions are shown. The y axis represents the detected level of fluorescence from each sample while the x axis represents the number of cycles of amplification. As expected, a difference of 5 cycles in reaching the threshold cycle (red line) was observed comparing the undiluted sample, the 1:32 and the 1:1024 dilutions of a cDNA sample obtained from mouse Peyer's patches.

3.1.7.6 Quantitative Taqman real-time evaluation of target gene expression

Right submandibular glands, pancreas, speen and lymph nodes of 126 female NOD mice ranging from 4 to 38 weeks of age and from 20 control Balb/c ranging from 5 to 30 weeks were stored in individual vials at -80C in RNA later until used. RNA was extracted from approximately 30mg of tissue using the RNA Easy mini kit (Quiagen) as described above and reverse transcribed to cDNA using the Thermoscript kit (Invitrogen). cDNA from NOD spleens and Peyer's patches was used as positive tissue control in order to optimise the real-time PCR conditions and to validate multiplexing with the endogenous controls 18S and mouse β-actin. The expression levels of the following target genes were tested: B cells survival factors (April, BAFF, BAFFR), lymphotoxins (LTα, LTβ), lymphoid chemokines (CXCL12, CXCR4, CXCL13, CXCR5, CCL19, CCR7), AID and cytokines involved in regulating B cell class-switching (IL-4, IFNγ, IL-13), Reg genes (Reg1, Reg2, Reg3α, Reg3β, Reg3γ, Reg3δ and Reg4) and IL-6. The complete list of primers and probes used to detect mRNA expression levels of the above-mentioned target genes is reported in Table 3.2. In order to determine the relationship between stage of disease and the expression of target genes, mice were grouped based on their age. The mean expression levels for each target and each group of age was then calculated.

Table 3.2

Genes, Specific Primers, and Probes used for RT-PCR

Gene product	RefSeq	Gene Expression Assay Id
Mouse IL-4	NM_021283	Mm00445259_m1
Mouse IL-13	NM_008355	Mm00434204_m1
Mouse AID	NM_009645	Mm00507774_m1
Mouse CXCL13	NM_018866	Mm00444533_m1
Mouse CXCR5	NM_007551	Mm00432086_m1
Mouse CCL19	NM_011888	Mm00839967_g1
Mouse CCR7	NM_007719	Mm00432608_m1
Mouse CXCL12	NM_013655	Mm00445552_m1
Mouse CXCR4	NM_009911	Mm01292123_m1
Mouse BAFF	NM_033622	Mm00446347_m1
Mouse BAFFR	NM_028075	Mm00840578_gl
Mouse APRIL	NM_023517	Mm00840215_gl
Mouse LTα	NM_010735	Mm00440227_m1
Mouse LTβ	NM_008518	Mm00434774_g1
Mouse IFNγ	NM_008337	Mm00801778_ml
Mouse beta actin	NM_007393	Part N 4325341E
Eukaryotic 18S	X03205	Part N 4319413E
Mouse Reg1	NM_009042.1	Mm00485651_m1
Mouse Reg2	NM_009043.1	Mm00485653_m1

Mouse Reg3α	NM_011259.1	Mm00441121_m1
Mouse Reg3β	NM_011036.1	Mm00440616_g1
Mouse Reg3γ	NM_011260.1	Mm00441127_m1
Mouse Reg3δ	NM_013893.2	Mm00516553_g1
Mouse Reg4	NM_026328.2	Mm00471115_m1
Mouse IL-6	NM_031168.1	Mm00446191_m1

Source of all primers and probes:

Applied Biosystems.

3.1.8 Modulation of TLS with Lymphotoxin-β Receptor-Ig fusion protein

Three distinct trials were carried out on three separate groups of NOD mice. Mice were ear marked and randomised in separate cages. All mice were weighed. Mice were culled one week after detection of high blood glucose.

In the first trial 28 mice were involved. 11 NOD mice were injected intra-peritoneally with 100μg of Lymphotoxin-β Receptor-Ig (LTβR-Ig, a kind gift from Dr J Browning, Biogen, Cambridge, MA, USA) weekly from week 12 for 5 weeks. 17 NOD mice used as controls were injected with 100μg MOPC-21 isotype control. In the second trial 10 NOD mice were involved: 5 mice were injected with LTβR-Ig and 5 with MOPC-21.

In the third trial 10 NOD mice were treated just like in the second trial but before the first injection and before culling the mice, salivary function was also assessed. In order to collect saliva from the mice, a specific protocol was performed. Mice were briefly exposed to isoflurane inhalational anaesthesia and injected IP with pentobarbital. As soon as the animals were sedated, pilocarpine was IP injected according to their weight (0.5μg per gram). Animals were then put mouth-down in a falcon tube under the microscope. Saliva was collected 4 minutes after pilocarpine injection, for 5 minutes. Saliva was weighed in pre-weighed tubes.

Mice were then put on a warm surface slightly facing down to avoid saliva obstructing their throats while recovering from anaesthesia.

At the end of the trial, mice were culled and salivary glands, pancreas, spleen and peri-salivary-glands lymph nodes collected for analysis. Quantitative TaqMan real-time evaluation of mRNA expression levels in salivary glands, peri-gland lymphnodes, pancreas and spleen of NOD mice treated with Ltxβr-Ig and isotype control was performed. mRNA gene expression was evaluated by

real time PCR for the following genes: CXCL13, CXCR5, CCL19, CCR7, CXCL12, CXCR4, Ltxα, Ltxβ, Ltxβr, AID, Bact, 18S.

Histology was performed to evaluate inflammatory cells infiltrates and TLOs formation.

Histological characterization of salivary glands infiltrates in NOD mice treated with Ltxβr-Ig and isotype control was carried out by H&E staining, immunofluorescence staining for CD3/B220, immunofluorescence staining for CD21 as described above.

Sequential sections were also stained for FDC-M1 by peroxidase staining, marker for follicular dendritic cell networks and GL7 marker for germinal centers. Peri-gland lymphnodes from NOD mice treated with Ltxβr-Ig and isotype control were stained with GL7 to evaluate the presence and quantity of germinal centers.

3.1.9 Statistical analysis

Differences in quantitative and semiquantitative variables among multiple groups were first analysed by Kruskal-Wallis test followed by Dunn's post-hoc test to compare the differences between each variable. Spearman's rank test was used to assess the correlation between quantitative variables. χ^2 with Yates' correction when required or Fisher's exact test when appropriate was used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA USA). A p value <0.05 was considered statistically significant. Data are presented as the mean+SEM.

3.2 Human studies

3.2.1 Patients enrolment

Subjects with DM enrolled in this study have been identified in previous studies through the IMDIAB network (285-289). Patients were all Caucasian and came from the same geographical area of continental Italy, ensuring the important feature of a homogeneous cohort. Blood samples were collected and stored for analysis. Serum levels of Reg1α protein were measured in the following patient groups (Tab.3.3): 87 type 1 diabetes subjects with different disease duration (31 newly diagnosed and 56 long standing), 63 type 2 diabetes subjects, 80 normal subjects sex/age matched (a control group of 20 for newly diagnosed type 1 diabetes, a control group of 20 for long-standing type 1 diabetes and a control group of 40 for type 2 diabetes). Diagnosis for type 1 diabetes and type 2 diabetes was assessed according to the ADA criteria (290, 291). All type 1 diabetes patients, newly diagnosed and long standing, enrolled in this study were positive at diagnosis for either GAD or IA2. In my casistic anti-GAD and anti-IA2 were present respectively in 82% and 32% of type 1 diabetic patients at diagnosis. For the present investigation, I also studied sera from a cohort of 39 SLE subjects attending the Rheumatology Division of Sapienza Università di Roma. All patients were diagnosed according to the American College of Rheumatology revised criteria for the classification of SLE (292, 293). Table 3.3 shows the clinical features of subjects enrolled in the study.

Table 3.3

Clinical features of subjects enrolled in the study.

Total Number of Subject	**No.** **269**	**Sex**	**Mean age ±SD (yrs.)**	**Mean duration of disease ± SD (yrs.)**
Newly diagnosed Type 1 Diabetes	31	19 M - 12 F	18.8 ± 7.4	< 1 month
Long-standing Type 1 Diabetes	56	33 M - 23 F	36.8 ± 11.8	13.5 ± 7.3
Type 2 Diabetes	63	48 M - 15 F	63.1 ± 10.4	15.6 ± 8.7
Normal Subjects match for newly diagnosed type 1 diabetes	20	12 M - 8 F	19.5 ± 5.4	NA
Normal Subjects match for long-standing type 1 diabetes	20	13 M - 7 F	37.3 ± 7.7	NA
Normal Subjects match for type 2	40	30 M - 10 F	60.7 ± 7.8	NA
Systemic Lupus Erythematosus	39	5 M - 34 F	39.1 ± 11.3	8.4 ± 7.7

3.2.2 Sjogren's patients' serum and salivary glands collection

Sera were obtained from 37 patients (30 females and 7 males with mean age 53.65; range 19-80; mean disease duration 138 months, range 6-180) affected by primary SS fulfilling the revised criteria of the American-European Consensus Group (79). The presence of other underlying autoimmune diseases or HCV infection was carefully excluded. As negative control population, sera from twenty-one normal healthy subjects matched for sex and age were studied, while sera from 42 patients with RA classified according to ARA criteria were used as disease controls. From each patient and control a blood sample was taken and sera were collected at –20°C until tested. Patients with SS were also analysed for the presence of extraglandular manifestations such as arthralgia/arthritis, cryoglobulinemia, Raynaud's phenomenon and hepatic, pulmonary or renal involvement. Twelve patients with SS had extraglandular manifestations (4 arthralgia/arthritis, 3 Raynaud's phenomenon, 2 pleuritis, 2 cutaneous vasculitis, 1 renal involvement).

3.2.2.1 Minor salivary gland biopsies

Minor salivary gland samples of primary SS patients were obtained from the tissue bank of the Oral Pathology Department at Guy's Hospital. Specimens were selected among samples presenting the histological criteria for the diagnosis of SS (85) with severe cellular infiltration presenting a focus score>1. Salivary gland biopsies were carried out for routine diagnostic purposes following patient's consent.

3.2.3 Reg1α protein detection in serum

The protein was measured using a Human Reg1α ELISA assay (BioVendor Laboratory, Heidelberg, Germany), which is a double polyclonal sandwich enzyme immunoassay for quantitative measurement of human Reg1α protein in serum and plasma. Serum samples were diluted 1:50 with Dilution Buffer in two steps as follows: 10 µl of sample were added to 90 µl of Dilution Buffer (dilution 1:10); 50 µl of dilution 1:10 were pipetted into another 200 µl of Dilution Buffer to prepare the 1:50 final dilution. In the BioVendor Human Reg1α ELISA, 100 µl of calibrators, quality controls and samples were pipetted, in duplicates, and incubated with polyclonal anti-human Reg1α antibody coated in microtitration wells. 100 µl of Dilution Buffer as Blank was pipetted into the wells. The plate was incubated at room temperature (25°C) for 1 hour, shaking at 300 g on an orbital microplate shaker. Wells were washed 3-times with Wash Solution (0.35 ml per well).

Polyclonal anti-human Reg1α antibody labelled with horseradish peroxidase (HRP) was added to the wells and incubated with captured Reg1α. Reading was done at 450nm with a spectrophotometer. A standard curve was obtained by plotting absorbance values versus Reg1α concentrations of calibrators. The results are reported as the Reg1α (ng/ml) concentration in samples. The actual amount of Reg1α in the original blood sample has been assessed by multiplying the assay result by dilution factor 50 (e.g. 13.5 ng/ml x 50 gives 675 ng/ml).

Sensitivity of the assay is assessed by the Limit of Detection (LOD), defined as concentration of analyte giving absorbance higher than mean absorbance of blank (Dilution Buffer is pipetted into blank wells) plus three standard deviations of the absorbance of blank. LOD is calculated from the real Reg1α values in wells and is 0.094 ng/ml. The antibodies used in this ELISA are specific for human Reg1α with no detectable cross reactivities to recombinant human REG 1β, PAP and REG IV.

3.2.4 Western Blot for anti-Reg1 autoantibodies

Western blotting was used to estimate the presence of autoantibodies anti Reg1α protein in sera. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) involves separation of proteins, the blotting of these proteins onto a nitrocellulose membrane and their detection using specific antibodies. The polyacrylamide gels are run in the presence of the anionic detergent SDS that denatures and binds to proteins in a constant-weight ratio leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size and not charge.

3.2.4.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Reg1α proteins migrated in response to an electrical field through the pores of a SDS polyacrylamide gel matrix toward the anode, under denaturing conditions. A discontinuous buffer system was used, with buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel.

Polyacrylamide gels form after polymerisation of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by N, N'-methylenebisacrilamide. The discontinuous polyacrylamide gel consisted of an upper 4% stacking gel with large pore size where proteins were concentrated and a 10% lower separating gel with smaller pore size where they were separated.

The gel, Invitrogen NuPAGE 4-12% Bis-Tris Gel, 1.5mm x 15 wells, was assembled in the electrophoresis tank according to the manufacturer's instructions (Bio-Rad) and electrophoresis running buffer poured into both the inner and outer chambers.

The running buffer (pH 7.7) composition is 50mM MOPS, 50 mM Tris Base, 0.1% SDS, 1mM EDTA. Equal amounts of protein samples (~5µg) were denatured by five minutes boiling at 95°C in LDS buffer, H_2O and the reducing agent β-mercaptoethanol in the following quantities: LDS buffer 2.5µl, reducing agent 1µl, 10µl Reg 1α protein and H_2O in different quantity to a maximum of 15µl total.

The protein samples were loaded into the gel lanes and then separated by electrophoresis at 120V for 90 minutes.

A pre-stained molecular weight marker containing labelled proteins of specific molecular weights was run in parallel and used to visualise protein migration and calculate protein molecular weight.

3.2.4.2 Blotting onto nitrocellulose membrane

Following electrophoresis, proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane, with 0.45µm pore size, using a mini transblot apparatus (Bio-Rad). Membrane, gel, two pieces of filter paper and two fibre pads per gel were soaked in the transfer buffer (pH 7.2), containing 25 mM Bicine, 25 mM Bis-Tris and 1 mM EDTA, to equilibrate for 10 minutes.

A transfer cassette was assembled consisting of a sandwich containing layers of fibre pad, filter paper, gel, membrane, filter paper and fiber pad. The cassette was then placed in the blotting tank according to the manufacturer's instructions, all fully covered with transfer buffer and with an ice block to prevent excessive heating of the buffer and consequent protein denaturation.

Electrophoretic transfer of negatively charged proteins from the gel to the membrane was achieved by the application of a 100V current for 60 minutes.

Equal protein loading and transfer efficiency to the membrane was verified using the protein dye Ponceau S. The membrane was rocked gently with 0.1% Ponceau S staining solution for 10 minutes

and then destained with water until pink protein bands appeared on the membrane. To completely remove the staining the membrane was then washed 3 times with PBS+0,1% Tween solution.

3.2.4.3 Immunodetection of proteins

Prior to immunodetection the membrane was blocked in a solution of Blocking buffer containing PBS/2%Skim Milk/0.1% Tween 20, for 1hr at room temperature on a rocker platform to minimize the non specific binding of the antibody.

The membrane was then incubated with patients' sera, eventually containing antibodies against Reg1α, diluted in Blocking buffer on a rocker platform for 2hrs.

Following three 5 minutes washes of the membrane in PBS+0.1% Tween 20, to remove unbound antibody, the membrane was incubated at room temperature for 1hr on a rocker platform with goat anti-human-IgG secondary antibody diluted in Blocking buffer. The membrane was again washed, as above, to remove unbound secondary antibody. Finally three 5 minutes washes of the membrane in PBS+0.1% Tween 20, to remove unbound antibody. The membranes were then incubated with Super Signal West Dura Extended Duration Substrate Antibodies (Pierce) for 5 minutes and then developed.

3.2.4.4 Enhanced Chemiluminescent (ECL) detection

Immunocomplexed bands were detected by enhanced chemiluminescence.

The membrane was exposed, under red light, to light-sensitive autoradiography film (Hyperfilm ECL) for various time periods. The film was than washed in the Developer and Fixer buffers. If antibodies are present in the sera a black band shows on the film.

The presence of anti-Reg1α antibodies was confirmed upon evidence of a specific band at 17.8kDa. (Fig.7.5 a). A second band at 30kDa was present in all samples and it is most likely an artefact band of E.coli degradation (Fig.7.5 b), which frequently occurs in this kind of analysis using recombinant proteins expressed in E.coli (176).

3.2.5 C-peptide measurement

The correlation between C-peptide (marker of β cell function) and Reg1α (marker of β-cell regeneration) was studied.

Baseline C-peptide was measured fasting in the morning using a radioimmunoassay method using a commercially available kit (Bio-Rad Laboratories). The reference range of fasting C-peptide stabilised in 64 control subjects (matched for age and sex, with no family history of T1D) was > 0.4 nmol/l with intracoefficient and intercoefficient variability between 10 and 15%, respectively.

Our laboratory has participated in the world-wide standardization of C-peptide assay and contributed to the definition of the standard. The comparison between laboratories in single specimens, expressed as %CV, ranged from 12.8 to 33.4% (our laboratory 16%).

3.2.6 HbA1c measurement

I investigated the correlation between HbA1c, duration of disease and Reg1α (marker of β cell regeneration). Glycated haemoglobin (normal range 4.0–7.0%) was measured centrally (Bio-Rad Laboratories, Milan, Italy). The chromatographic assay uses an HPLC instrument and ion exchange or affinity column to separate HbA1c molecules from other haemoglobin molecules.

The HbA1c content is calculated based on the ratio of HbA1c peak area to the total hemoglobin peak areas.

The High Performance Liquid Chromatography (HPLC) method gives better precision over the immunoassay method, but it is much more expensive per test. HPLC is considered the "Gold Standard" technology in the follow-up of the plasma glucose concentration of diabetic patients over time, via the measurement of HbA1c.

3.2.7 HLA and non-HLA genes polymorphisms

From the genetic point of view this study evaluated the association with candidate genes that have significant effect on β-cell including HLA, CTLA4, PTPN22, INS; these genes have been selected based on their association with type 1 diabetes (145, 294-297).

Genomic DNA was extracted from peripheral EDTA-blood lymphocytes using the QIAamp DNA Blood Kit (QIAGEN Genomics Inc., Bothell, WA). Samples were analysed using the Applied Biosystem Snapshot and/or Taqman technologies, two of the cheapest and fastest methods of genotyping available today.

HLA DRB1 polymorphism was analysed using a SSO Reverse Line Blot method. Briefly, a set of unlabelled oligonucleotide probes was immobilized onto backed nylon membrane sheets in a series of parallel strips. Cross-sections of the sheets could then be hybridised to PCR products labelled with biotin. A colorimetric detection system, which utilized the substrate tetramethyl benzidine (TMB), was used to detect hybridization.

Non HLA genes, CTLA-4, PTPN22 and INS SNPs were detected with Taqman Allelic Discrimination: SNPs were typed using the TaqMan Allelic Discrimination (AD) Assay Applied Biosystem (ABI,Foster City, CA).

3.2.8 Statistical analysis

The Mann-Whitney U test (for comparison of 2 groups) and the Kruskal-Wallis test with Dunn's post test (for 3 or more groups) were used to compare quantitative variables. Spearman's rank test was used to investigate the significance of correlations between variables. Genotypic and allelic distributions were compared using the χ^2 test (with Yates' continuity correction) or Fisher's exact test when the criteria of chi-square test were not fulfilled. The effect of gene polymorphism on the quantitative variables was investigated using multiple linear regression. Unless otherwise indicated, *P* values of less than 0.05 were considered significant. Statistical analysis was performed using SPSS statistical software, version 12 (SPSS, Illinois, USA).

Data are presented as the mean+SEM.

CHAPTER 4

RESULTS

Evolution of Ectopic Lymphoid Neogenesis and in situ autoantibodies production in autoimmune diabetic NOD mice: cellular and molecular characterization of tertiary lymphoid structures in pancreatic islets.

4.1 Histological characterization of pancreatic inflammatory infiltrates development

I first characterized the prevalence and the progression of inflammatory infiltrates within pancreatic islets of NOD mice between 4 and 38 weeks of age. H&E and CD3/B220 staining were performed to identify progression from peri- to intra-insulitis and presence of T/B cell aggregates, respectively (Fig.4.1 A,B). As expected, initial infiltrates characterized by T and B lymphocytes were observed from week 6 onwards and were mostly organized as peri-islets aggregates (*peri-insulitis* Fig.4.1 A,C). With increasing age, larger T and B cells aggregates developed and progressively colonized the whole islet with disappearance of β-cells (*intra-insulitis* Fig.4.1 B,C). As expected, infiltrates evolution displays a trend from prevalent peri-infiltration to prevalent intra-infiltration throughout mice life/disease, although different stages of infiltration coexist within the same pancreas.

Figure 4.1

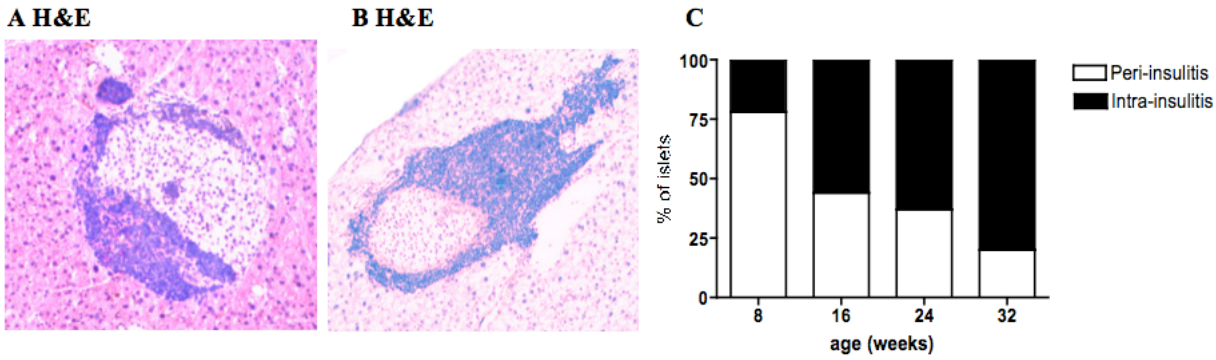

Figure 4.1 Histological characterization of pancreatic islet infiltrates in NOD mice. A-B: H&E staining of pancreatic islets showing peri (A) and intra (B) insulitis. **C)** Percentage of islets with peri-insulitis vs intra-insulitis amongst all islets in relation to age populations of NOD mice.

A total of 126 female NOD mice were sacrificed at different ages ranging from 4 to 38 weeks; mice were grouped according to age in 4 groups: 4 to 8 weeks (N=28); 9 to 20 weeks (N=34); 21 to 32 weeks (N=34); >32 weeks (N=30). All mice were culled within 1 week from high blood glucose detection except mice <8 weeks which were non diabetic. Original magnification X100.

4.2 Pancreatic islets infiltrates progressively acquire features of TLS: T/B cell compartmentalization and formation of FDC network

I next evaluated the progressive acquisition of features of SLOs such as T/B cell segregation and differentiation of FDC networks (218).

Sequential section analysis of pancreatic infiltrates was performed using immunostaining for CD3/B220 and FDC-M1 in order to assess the presence of T/B cell compartmentalization and FDC networks. As shown in Fig.4.2, inflammatory aggregates clearly display various degrees of cellular organization with initial infiltrates mostly characterized by absence of T/B cell segregation followed by the development of highly organized lymphoid structures with T and B lymphocytes localizing in distinct areas (Fig.4.2 A,B,C). Thus, inflammatory cells enter the islets first with a non-segregated pattern (*non-segregated insulitis*) and then they gradually gain an organized segregated disposition within the islets (*segregated insulitis*). In the context of segregated infiltrates a further degree of lymphoid organization, characterized by the differentiation of FDC networks was observed during the evolution of insulitis (Fig.4.2 E,F). FDC networks closely colocalized with the B cell rich areas as shown by sequential section analysis (Fig.4.2 D,E,F). Interestingly, the analysis of the prevalence of FDC positive infiltrates in different age groups of NOD mice demonstrates that early-onset diabetic mice displayed the highest percentage of FDC+ aggregates (>65%) whilst mice developing diabetes at a later stage displayed a progressive decrease in the prevalence of FDC networks (Fig.4.2 F, G), despite a similar incidence of *intra-insulitis*. Such data is consistent with the presence of a more aggressive phenotype, characterized by evolution of TLS.

Figure 4.2

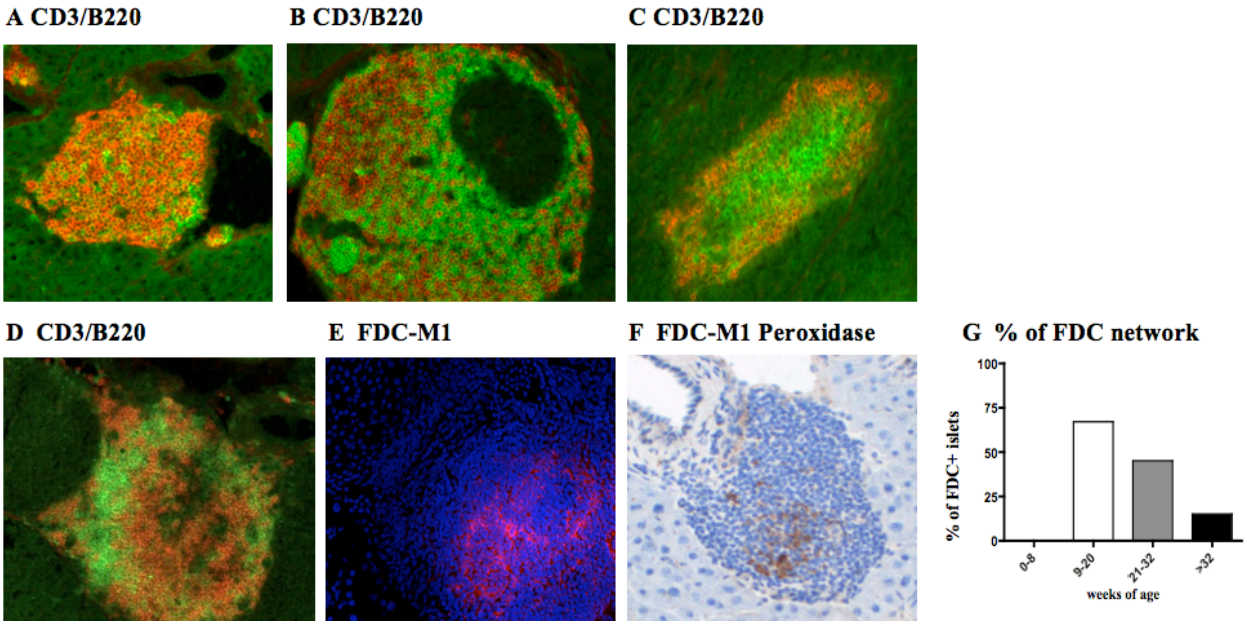

Figure 4.2 Development of ectopic lymphoid structures characterized by T/B cell segregation and FDC networks within pancreatic infiltrated islets of NOD mice. A-C, Immunofluorescence staining for CD3 (T cells, **green**)/B220 (B cells, **orange**) in pancreatic islet showing progressive acquisition of T/B cell segregation. Intra-insulitis with: non-segregated T/B cells (A); partially segregated T/B cells (B); complete segregation of T/B cells (C). D-F: representative microphotographs of sequential sections of NOD pancreas showing co-localization of ectopic lymphoid structure markers. **D**) Immunofluorescence staining for CD3 (T cells, **green**)/B220 (B cells, **orange**) showing an islet with intra-insulitis characterized by T/B cell segregation. **E-F**: sequential sections stained with FDC-M1 (FDC network) using immunofluorescence (E, **red**) and peroxidase on bright field (F, **brown**) showing exclusive co-localisation of FDC networks within the B cell area. **G**) Graph enumerating the prevalence of mice displaying FDC+ islets in the different age groups. A total of 126 female NOD mice were sacrificed at different ages ranging from 4 to 38 weeks; mice were grouped according to age in 4 groups: 4 to 8 weeks (N=28); 9 to 20 weeks (N=34); 21 to 32 weeks (N=34); >32 weeks (N=30). All mice were culled within 1 week from high blood glucose detection except mice <8 weeks which were non diabetic. Interestingly the younger the mice develop diabetes the more T/B cell segregation and FDC networks were observed. Original magnification X100.

4.3 AID expression and differentiation of germinal center B cells support functionality of TLS developing within pancreatic islets

Following demonstration of acquisition of features of TLS, I next examined whether ectopic lymphoid structures in the pancreas of NOD mice display characteristics of functionality typical of SLOs.

In SLOs, B cells enter the GC where they undergo affinity maturation and clonal diversification followed by differentiation into memory B cells and plasma cells that leads to the production of high-affinity antibodies. The above phenomena are critically dependent on the processes of somatic hypermutation (SHM) and class-switch recombination (CSR) of the Ig genes. Both processes require the presence of the enzyme activation-induced cytidine deaminase (AID), which initiates these processes (298). Because AID is exclusively and transiently expressed in B cells undergoing SHM and/or CSR, identification of AID+ B cells allows the evaluation of their functionality (218, 299). Thus, in order to characterize the functionality of TLS in NOD insulitis, I performed analysis for GL7 (marker for mouse germinal centre B cells) and AID on sections sequential to those stained for FDC. As shown in Fig.4.3 aggregates characterized by FDC network invariably display the presence of GL7+ GC B cells expressing high levels of AID. Conversely, no AID expression was observed in B cells localizing outside FDC networks. In agreement with the evidence that AID expression requires FDCs, quantitative gene expression analysis (Fig.4.3D) demonstrated that the highest expression of AID synchronised with the peak of FDC prevalence (Fig.4.2 F).

Overall these data strongly suggest that TLS developing during the course of autoimmune insulitis support a GC response characterized by B cell activation and differentiation. Importantly, as expected no organised follicular structures or expression of AID were detected in the pancreas of Balb/c control mice.

Figure 4.3

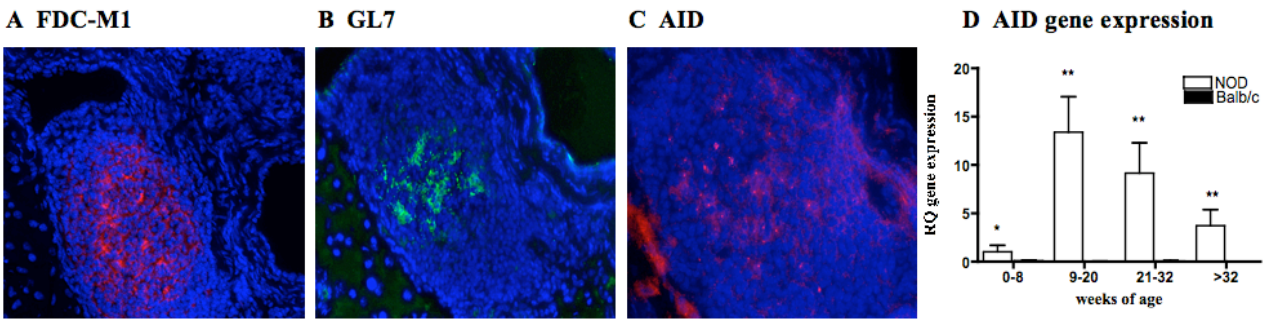

Figure 4.3 TLS in NOD islets display a GC response with expression of AID as marker of functionality. A-C: Immunofluorescence staining of pancreatic sequential sections of NOD mice. **A)** Staining for FDC-M1 (**red**). **B)** Staining for GL7 (**green**), marker for mouse germinal center B cells. **C)** Staining for AID (**red**), which is required for and exclusively expressed in B cells undergoing SHM and/or CSR. **D)** Quantitative RT-PCR for AID gene expression in NOD and Balb/c mice pancreas. A total of 126 female NOD mice were sacrificed at different ages ranging from 4 to 38 weeks; mice were grouped according to age in 4 groups: 4 to 8 weeks (N=28); 9 to 20 weeks (N=34); 21 to 32 weeks (N=34); >32 weeks (N=30). All mice were culled within 1 week from high blood glucose detection except mice <8 weeks which were non diabetic.
(* = $p<0.05$; ** = $p<0.01$). Original magnification X100.

4.4 Progressive evolution of TLS is associated with local over-expression of genes regulating ectopic lymphoid neogenesis and B cell functionality

Evolution of TLS is dependent on the expression and function of a critical set of genes such as lymphotoxins and lymphoid chemokines (300).

In addition, B cell survival, proliferation and function require the presence of a number of cytokines/growth factors, which bind to specific receptors on the cell surface of B lymphocytes. Thus, I examined whether development of TLS in NOD mice pancreas was associated with a concomitant upregulation of such genes. A shown in Fig.4.4, lymphoid chemokines and their cognate receptors CXCL13/CXCR5, CCL19/CCR7 as well as CXCL12/CXCR4, known to regulate localization of GC B cells (217), were all upregulated in NOD mice, as compared to Balb/c, already at early stages of the autoimmune process and preceded disease onset. Consistent with the histological detection of TLS, the highest expression level of these molecules was observed in the pancreas of NOD mice with earlier onset of diabetes. In agreement with the upregulation of CXCL13 and CCL19, both lymphotoxin α and β, which are known to be critical for the induction of lymphoid chemokines, were highly expressed in NOD but not Balb/c mice and displayed a similarly consistent trend (Fig.4.4 G,H).

Development of B cell rich aggregates in the pancreas of NOD mice was associated with the in situ upregulation of the B cells survival and proliferating factors BAFF and April (Fig.4.5 A,B,C) as well as of IL-4, which are critical activators of AID and class switching in B cells (Fig.4.5 D). This is consistent with the observed pancreatic expression of AID at both mRNA and protein levels (Fig.4.3 C,D).

Figure 4.4

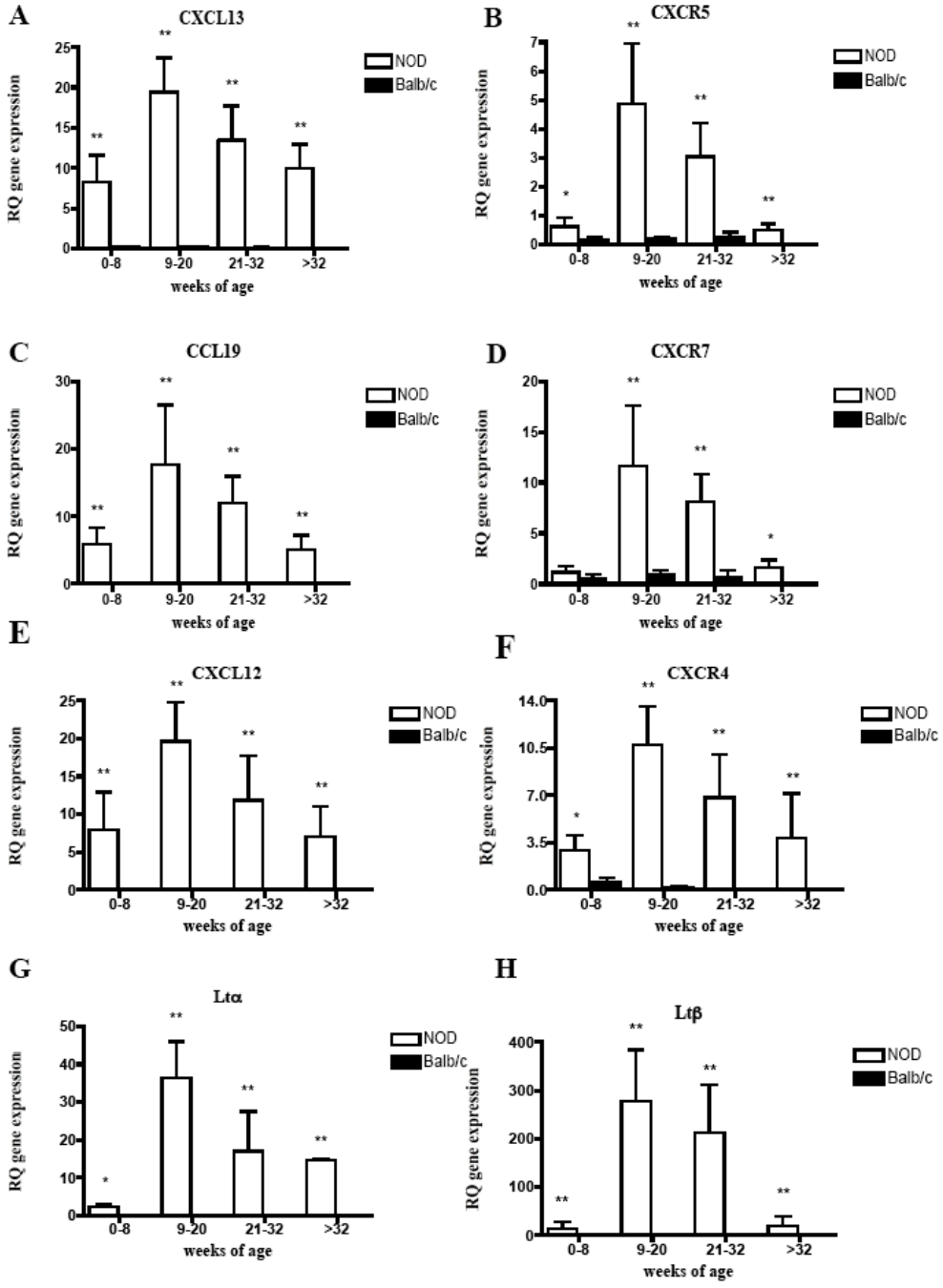

Figure 4.4 Quantitative mRNA expression of TLS-related genes in NOD pancreas. Quantitative TaqMan real-time evaluation of mRNA expression levels of TLS-related genes in pancreas of NOD and Balb/c mice. Lymphoid chemokines and their specific receptors CXCL13/CXCR5 (**A,B**), CCL19/CXCR7 (**C,D**), CXCL12/CXCR4 (**E,F**) as well as lymphotoxins α (**G**) and β (**H**) are shown. Mice were grouped according to age: 4 to 8 weeks (N=28); 9 to 20 weeks (N=34); 21 to 32 weeks (N=34); >32 weeks (N=30). Data are presented as the mean+SEM. *p<0.05, **p<0.01 between NOD and Balb/c.

Figure 4.5

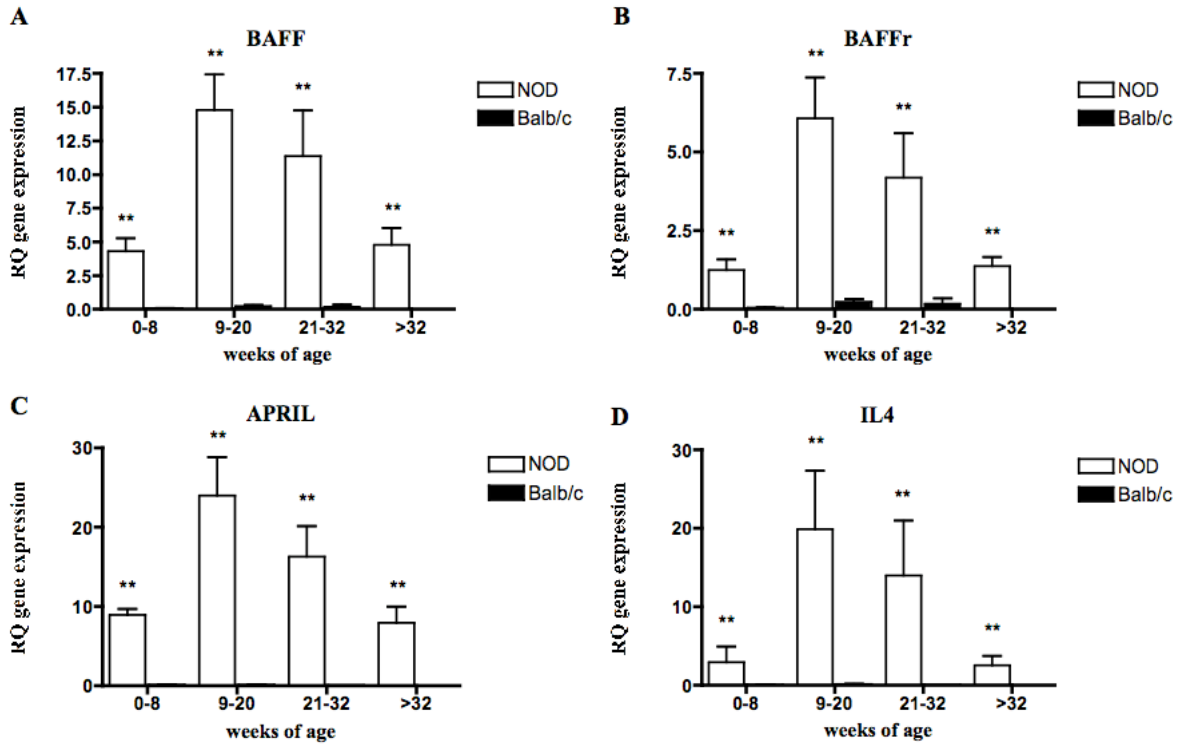

Figure 4.5 Evaluation B cells proliferating and survival factors genes expression in NOD mice pancreas. Quantitative TaqMan real-time evaluation of pancreatic mRNA expression levels in NOD and Balb/c mice. Gene expression was evaluated for BAFF (**A**), BAFFR (**B**), April (**C**) and IL-4 (**D**). Mice were grouped according to age: 4 to 8 weeks (N=28); 9 to 20 weeks (N=34); 21 to 32 weeks (N=34); >32 weeks (N=30). Data are presented as the mean+SEM. *p<0.05, **p<0.01 between NOD and Balb/c.

4.5 CXCR5+ follicular B cells are the main subset infiltrating pancreatic islets and localize within ectopic follicles

Two main subsets of mature B cells have been identified in rodents. Follicular B cells (FoB), which localize within B cell follicles in the spleen and other SLOs, mainly undergo a classical T-dependent GC reaction during which diversification and affinity maturation of the BCR take place allowing the generation of a high-affinity response to cognate antigens. Conversely, Marginal Zone B cells (MZB) localize at the periphery of the B cell follicles in association with the marginal sinus of the spleen where they provide a T-independent first line 'innate' defence against blood borne pathogens (283, 301). A number of cell surface markers allow the identification of these B cell subsets being FoB $B220^{+}/IgD^{high}/CD23^{high}/CD21^{low}/IgM^{low}/$ $CD1d^{low}/CXCR5^{high}$, while MZB are $B220^{+}/IgD^{low}/CD23^{low}/CD21^{high}/IgM^{high}/$ $CD1d^{high}/CXCR5^{low}$ (283). Thus, in order to characterize the B cell subsets infiltrating pancreatic islets, I performed double staining in sequential sections for IgD/B220, IgD/Cd1D, CD21/CD23, IgD/IgM and CXCR5/B220. Stainings were first performed on spleen sections in order to validate the procedure. As shown in Fig.4.6, double and sequential staining for IgD/Cd1D and CD21/CD23 clearly identify the two populations in NOD spleen. Sequential sections analysis of pancreatic tissue demonstrated that the majority of B cells infiltrating TLS were characterized by a B220+/IgD+/CD23+/CD21- phenotype consistent with a FoB cell subset. CD21 expression in the pancreas was restricted to FDC networks as demonstrated by positive sequential staining for GL7 (Fig.4.6 M). Interestingly, only in occasional large infiltrates it was possible to identify a subset of IgD-/CD1d+ B cells localizing at the periphery of the B cell area, possibly consistent with a transitional 2/MZ phenotype (Fig.4.6 J,K,L).

Consistent with the prevalent FoB cell phenotype, the vast majority of islet-infiltrating B220+ cells also displayed high expression of CXCR5, with a pattern highly reminiscent of that observed in the

follicular area of the spleen (Fig.4.7). This suggests that the ectopic expression of CXCL13 is functional in attracting CXCR5+ cells in the infiltrated islets.

Figure 4.6

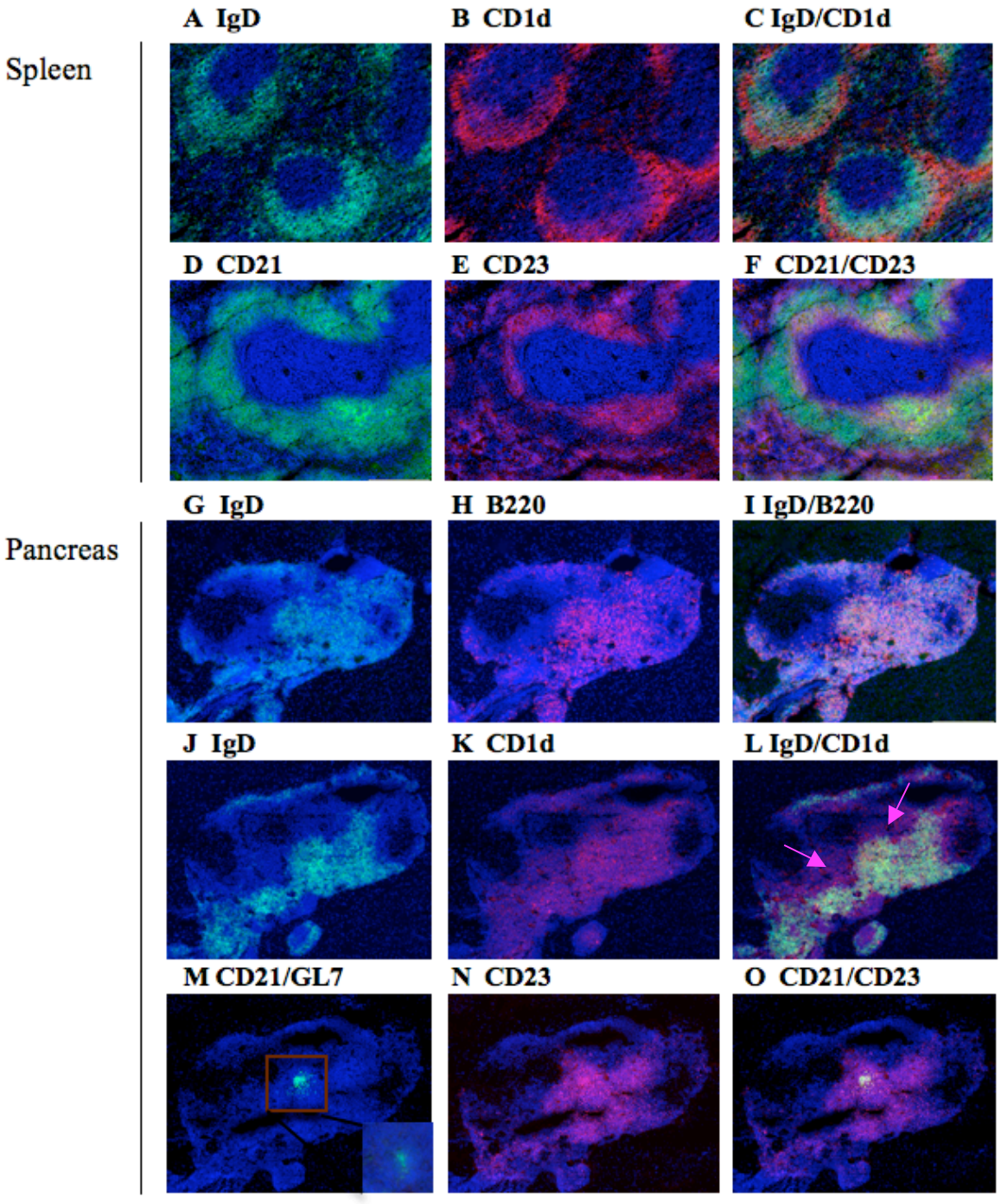
A IgD
B CD1d
C IgD/CD1d
Spleen
D CD21
E CD23
F CD21/CD23
G IgD
H B220
I IgD/B220
Pancreas
J IgD
K CD1d
L IgD/CD1d
M CD21/GL7
N CD23
O CD21/CD23

Figure 4.6 Characterization of B cells subsets within the pancreatic infiltrates. Immunofluorescence sequential double stainings were carried out in order to evaluate the subset of B cells infiltrating pancreatic islets. Staining was first optimised on spleen sections (**A-F**). FoB cells in the spleen are situated in follicles within the white pulp and are characterized by a $B220^{+}$, IgD^{high}, $CD23^{high}$, $CD21^{low}$, IgM^{low}, $CD1d^{low}$, $CXCR5^{high}$ phenotype (**green** area in A-C, **red** area in E-F). MZB cells are located between the white and red pulp and are characterized by a $B220^{+}$, IgD^{low}, $CD23^{low}$, $CD21^{high}$, IgM^{high}, $CD1d^{high}$, $CXCR5^{low}$ phenotype (**red** area in A-C, **green** area in E-F). Sequential doublestaining was performed in pancreatic islets for the same specific markers (**G-O**). The majority of B cells infiltrating the islets are characterized by a B220+/IgD+/CD23+/CD21- phenotype consistent with a FoB cell subset. CD21 expression in the pancreas was restricted to FDC networks as demonstrated by positive sequential staining for GL7 (**M right corner, green**). Interestingly, only in occasional large infiltrates it was possible to identify a subset of IgD-/CD1d+ B cells localizing at the periphery of the B cell area, possibly consistent with a transitional 2/MZ phenotype (**K,L red area identified with arrows**). Original magnification X100.

Figure 4.7

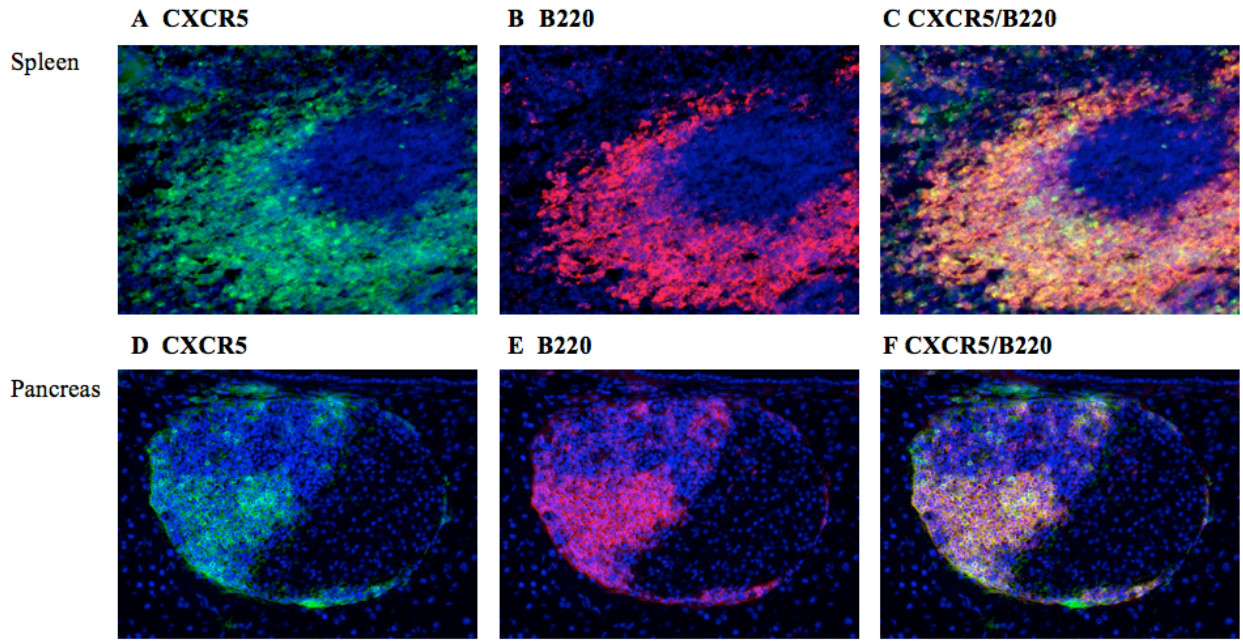

Figure 4.7 Infiltrating B cells display CXCR5 within NOD inflamed islets. A-C: Double immunostaining for CXCR5 (**A, green**), B220 (**B, red**) and CXCR5/B220 (**C,** superimposed) in NOD spleen sections. **D-E**: Double immunostaining for CXCR5 (**D, green**), B220 (**E, red**) and CXCR5/B220 (**F,** superimposed) in NOD pancreas with intra-insulitis. Original magnification X100.

4.6 TLS sustain in situ production of anti-insulin antibodies

Recent evidence demonstrated that B cells isolated from NOD pancreas (and peri-pancreatic lymph nodes) display reactivity of the BCR against insulin suggesting that autoreactive B cells are preferentially recruited to the inflammatory site (49). However, it is not known whether these autoreactive cells are actually generated and localized in association with functional TLS. In order to provide direct evidence of this possibility, I performed staining with FITC-conjugated insulin and demonstrated specific binding to infiltrating cells in close association with the B cell area of the aggregates (Fig.4.8 A,B). Of relevance, no binding was ever observed in non-infiltrated islets of both NOD and Balb/c mice nor in highly inflamed salivary gland aggregates of NOD mice, suggesting that generation of an anti-insulin response in this strain takes place exclusively during insulitis (Fig.4.8 C,D respectively). Conversely, as expected, anti-insulin reactivity was detected in reactive peri-pancreatic lymph nodes of NOD mice (Fig.4.8 E,F). In all cases, pre-incubation with unconjugated insulin strongly reduced immunoreactivity (data not shown). In order to characterize the cells displaying autoreactivity against insulin, I performed double staining for B220 and CD138 (Fig.4.8 B,G), markers of B cells and plasma cells respectively. My analysis demonstrated that anti-insulin autoreactive cells are of plasma cell origin (CD138+ and B220-) and are closely associated with the B cell area, suggesting local differentiation in the context of TLS. This suggests that TLS can promote humoral autoimmunity against islet antigens over and above SLOs.

Figure 4.8

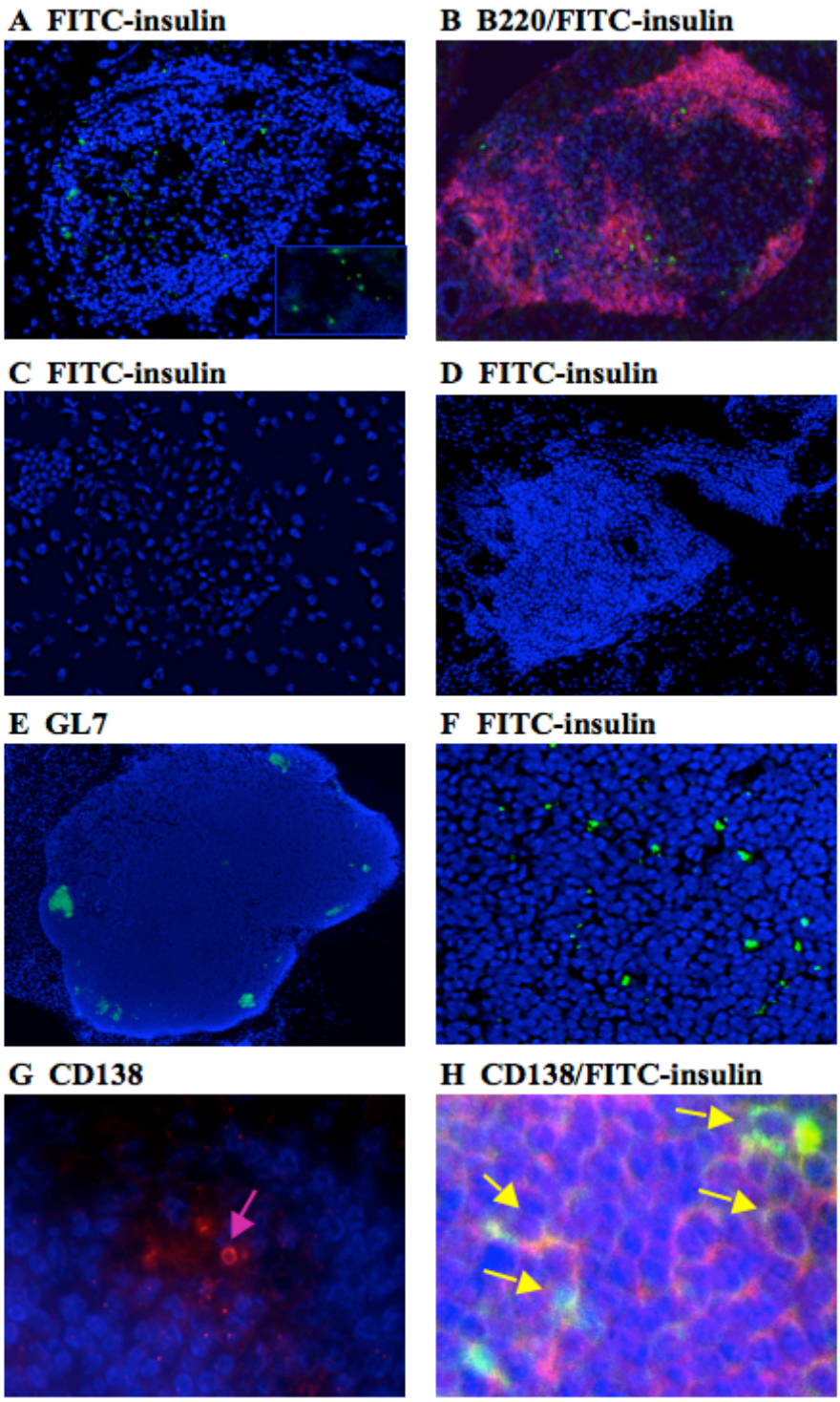
A FITC-insulin
B B220/FITC-insulin
C FITC-insulin
D FITC-insulin
E GL7
F FITC-insulin
G CD138
H CD138/FITC-insulin

Figure 4.8 In situ autoantibody production in NOD mice pancreas islets infiltrates. Stainings of NOD mice pancreas, submandibular glands (as negative control for islet-associated autoimmunity within TLS) and peri-pancreatic lymph nodes (positive control for autoreactive B cells) with FITC-conjugated-insulin. **A-B:** Representative example of TLS+ pancreatic islets, showing cells with reactivity for FITC-conjugated-insulin alone (A**, green**) and in double staining with B220 (B**, red**). **C-D:** Representative examples showing that neither pancreatic islets without inflammatory cells (C), nor submandibular glands with large inflammatory aggregates (D) displayed reactivity against FITC-conjugated-insulin. **E-F**: Reactive peri-pancreatic lymph node showing several GL7+ GCs (**E, green**) and displaying numerous FITC-insulin reactive cells (**F, green**). **G-H:** Sequential staining in NOD islets for CD138 (**G, red**) and CD138/FITC-conjugated-insulin (**H, red/green**) showing colocalization of the reactivity towards FITC-insulin with CD138+ plasma cells. Original magnification X100 (A-E) and X200 (F-H).

CHAPTER 5

RESULTS

Molecular and cellular evolution of functional tertiary lymphoid structures in salivary glands of NOD mice

5.1 Dynamic development of TLS in the submandibular glands of NOD mice: progressive acquisition of T/B cell segregation and differentiation of FDC networks

I first characterized the prevalence and the progression of inflammatory infiltrates within salivary glands of NOD mice between 4 and 38 weeks of age. H&E and CD3/B220 staining were performed to identify progression from small to large infiltrates and presence of T/B cell aggregates, respectively. Sequential section immunostaining for CD3/B220 and FDC-M1 was performed in order to assess the presence of T/B cell segregation and FDC networks. Inflammatory infiltrates in NOD sialoadenitis typically develop surrounding ductal epithelial structures and are initially dominated by T cells with progressive B cell infiltration. As shown in Fig.5.1, similar to insulitis, inflammatory aggregates in the salivary glands clearly display progressive degrees of cellular organization with early infiltrates mostly characterized by absence of T/B cell segregation followed by the development of highly organized lymphoid structures with T and B lymphocytes localizing in distinct areas (Fig 5.1 A and B). Initial infiltrates characterized by T and B lymphocytes in submandibular glands were observed at a later stage compared to insulitis (from week 8 onwards as compared to 4 weeks) and displayed progressive features of TLS with over 80% of lymphoid aggregates displaying B/T cell segregation after 28 weeks of age (Fig 5.1 E).

In the context of segregated aggregates, during the evolution of sialoadenitis, it was possible to indentify the development of FDC networks (Fig. 5.1 C,D). Which closely co-localized with the B cell rich areas. Interestingly, the analysis of the prevalence of FDC positive cells in salivary gland infiltrates in different age groups of NOD mice demonstrates that the percentage of mice displaying FDC networks progressively increases with the age of the mouse, with around 75% of mice displaying FDC networks (Fig. 5.1 F). A trend clearly different compared with that of insulitis (see Fig. 4.2 for comparison). Overall, these data suggest that in sialoadenitis, differently from NOD

insulitis in which TLS recede with the destruction of islets, the persistence of a chronic antigenic stimulus fuels the continuous and progressive development of TLS.

Figure 5.1

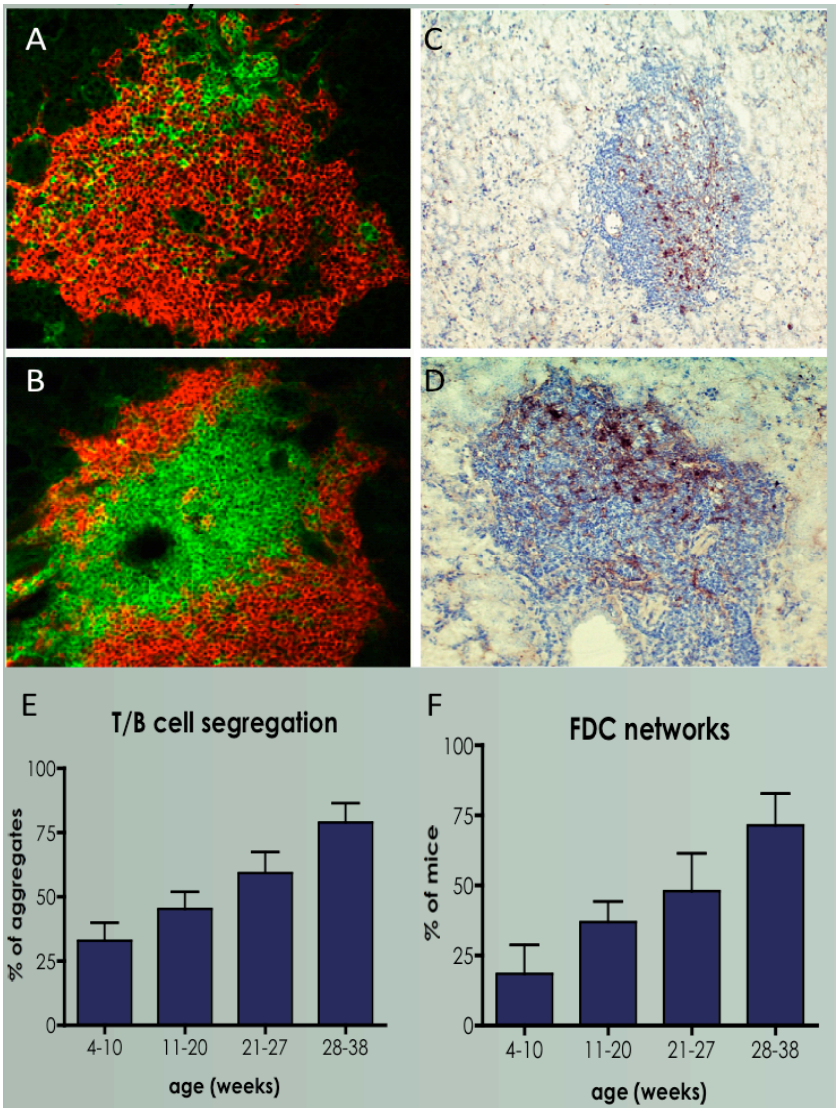

Figure 5.1 NOD submandibular glands progressively acquire features of SLOs. A-B: Representative double IF staining demonstrating absence (A) and presence (B) of T/B cell compartmentalization in NOD SG. **C-D:** Representative IHC evaluation of FDC networks in periductal inflammatory infiltrates in NOD SG. **E-F:** Enumeration of the prevalence of segregated vs non-segregated aggregates (mean ± SEM) and of FDC networks at different time points.
Mice were grouped according to age: 4 to 10 weeks (N=30); 11 to 20 weeks (N=32); 21 to 27 weeks (N=28); >32 weeks (N=36). Original magnification X100.

5.2 Differentiation of AID+ germinal center B cells supports functionality of TLS within submandibular glands

Similarly to the work in NOD insulitis, I next examined whether ectopic lymphoid structures in the salivary glands of NOD mice develop functional SLOs, as defined by the capacity to support the differentiation of GC B cells and promote AID expression as a marker of ongoing affinity maturation, clonal diversification and class switching.

For this purpose, I next investigated whether periductal aggregates in NOD submandibular glands express markers of GC (GL7) and expression of AID. As shown in Fig. 5.2, periductal aggregates characterized by FDC networks invariably supported the differentiation of GL7+ GC B cells. Importantly, sequential staining clearly showed that the presence of GL7+FDC was sufficient to induce strong expression of AID, with exact colocalization within the B cells area. Interestingly, in comparison with NOD insulitis, late lymphoid aggregates in the salivary glands displayed significantly larger FDC networks and stronger AID expression. Transcript analysis of AID salivary gland expression, demonstrated that a bimodal up-regulation of AID is present in these mice with an initial modest up-regulation until around week 20, followed by a strong peak of expression (Fig 5.2 E) in parallel with the development of FDCs (Fig.5.1 F) and with a striking peak in salivary gland IL-4 mRNA (Fig.5.2 F).

Figure 5.2

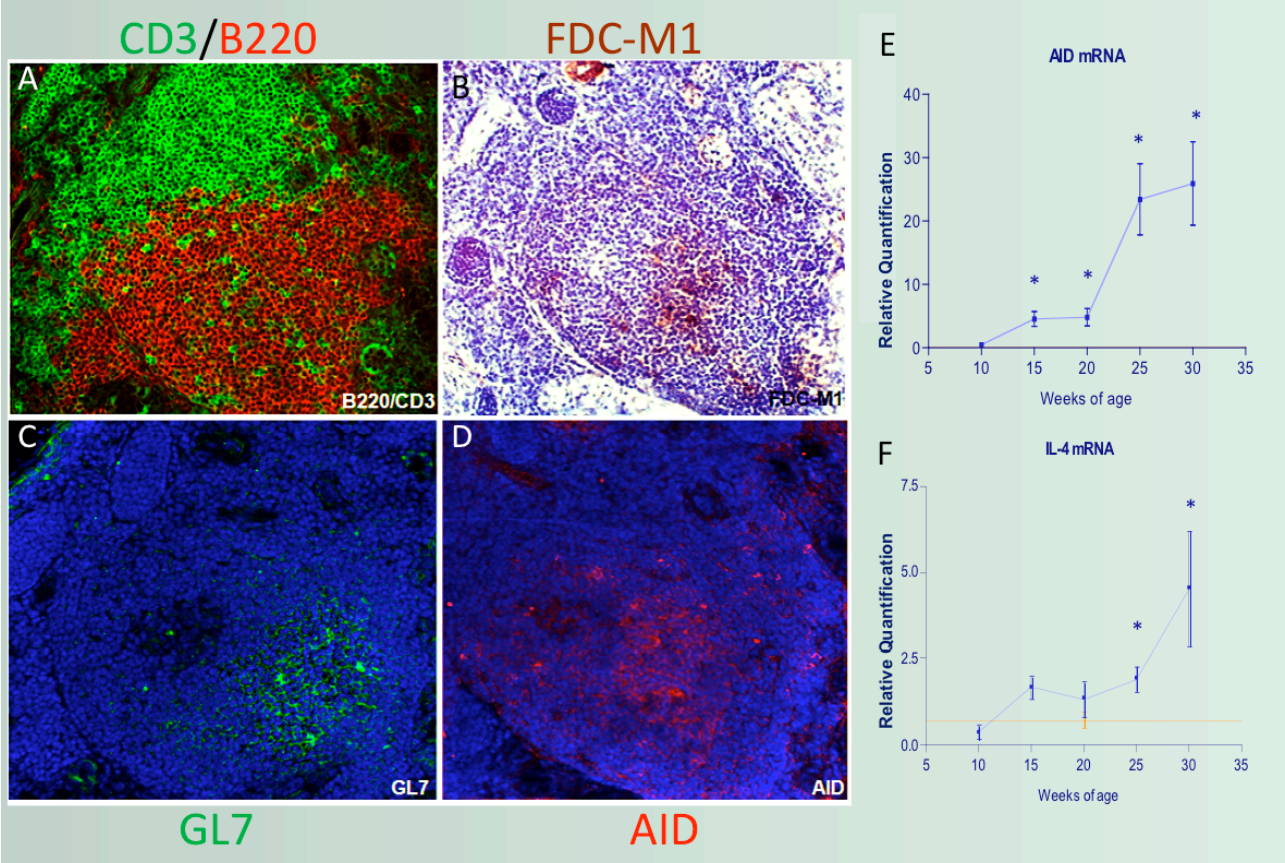

Figure 5.2 Evidence of functionality of ectopic germinal centre in NOD SG. A-D: Sequential IF/IHC staining demonstrating that aggregates characterised by T/B cell segregation (A) and FDC networks (B) support differentiation of GC-B cells (C) and in situ somatic hypermutation/class switch recombination (AID) (D). **E-F:** AID mRNA (E) is significantly increased in the SG of older NOD mice and is associated with local increase in IL-4 mRNA expression. (**Blue** line NOD, **Orange** line Balb/c). Mice were grouped according to age: 4 to 10 weeks (N=30); 11 to 20 weeks (N=32); 21 to 25 weeks (N=25); 26 to 35 weeks (N=35). Data are presented as the mean+SEM. *p<0.05 between NOD and Balb/c. Original magnification X100.

5.3 Progressive over-expression of genes regulating ELN and B cell functionality characterise disease evolution in salivary glands of NOD mice

As described elsewhere in this thesis, the evolution of TLS requires the expression of a critical set of genes which regulates the lymphoneogenetic programme (300) while B cell functionality is critically dependent on the *in situ* expression of B cell survival and proliferating factors. Accordingly, and similarly to NOD insulitis, submandibular glands display progressive expression of the CXCL13/CXCR5, and CCL19/CCR7 axis, but not, interestingly of CXCL12. This result is in keeping with evidence that this CK is constitutionally expressed by ductal salivary gland epithelial cells and is not modulated during the development of TLS in the salivary glands. In contrast, the expression of lymphoid CKs and their cognate receptors closely overlaps with and precedes the acquisition of histological features of TLS (Fig 5.3), suggesting that these pathways act upstream in the series of events leading to the formation of TLS. In particular, it was evident that CXCL13 is one of the first genes to show differential expression, as early as 6 weeks of age, in NOD salivary glands as compared to Balb/c mice of the same age.

Of relevance, the acquisition of functional features of TLS was also associated with the high expression of the B cell survival and activating factor BAFF and its receptor, with the latter likely reflecting the increased rate of B cell infiltration within the glands (Fig.5.4).

Figure 5.3

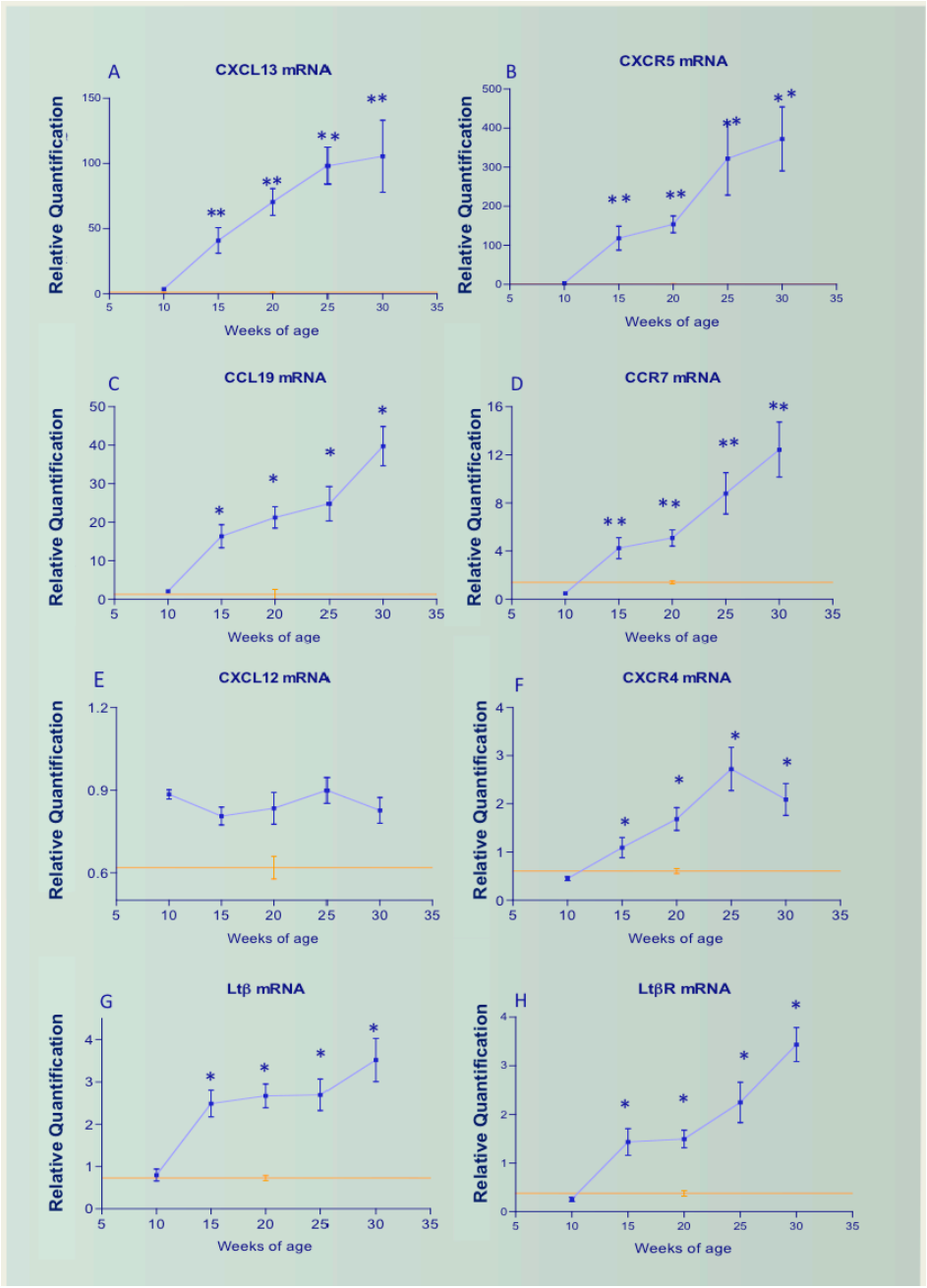

Figure 5.3 Development of aggregates in NOD SG is preceded and maintained by up-regulation of genes regulating ELS. Quantitative TaqMan gene expression analysis demonstrates progressive up-regulation of lymphoid chemokines CXCL13 (A) and CCL19 (C) and their specific receptors CXCR5 (B) and CCR7 (D), but not homeostatic chemokine CXCL12 (E), in NOD SG in parallel with increase in the LTβ/LTβR axis (G-H). (**Blue** line NOD, **Orange** line Balb/c). Mice were grouped according to age: 4 to 10 weeks (N=30); 11 to 20 weeks (N=32); 21 to 25 weeks (N=25); 26 to 35 weeks (N=35). Data are presented as the mean+SEM. *p<0.05, *p<0.01 between NOD and Balb/c.

Figure 5.4

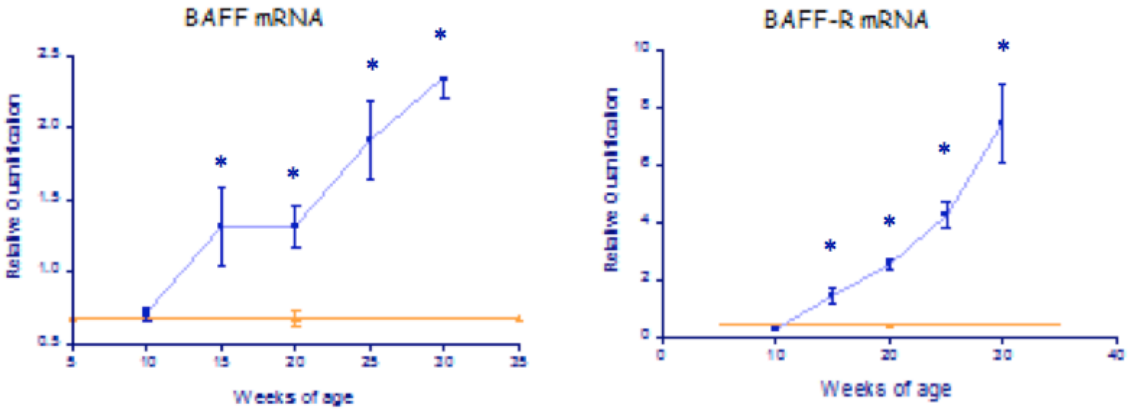

Figure 5.4 Development of B cell rich aggregates in the salivary glands of NOD mice was associated with the in situ upregulation of the B cells survival and proliferating factors BAFF and BAFF-r.

(**Blue** line NOD, **Orange** line Balb/c). Mice were grouped according to age: 4 to 10 weeks (N=30); 11 to 20 weeks (N=32); 21 to 25 weeks (N=25); 26 to 35 weeks (N=35). Data are presented as the mean+SEM. *$p<0.05$ between NOD and Balb/c.

5.4 IgD+ CXCR5+ Follicular B cells are the main subset infiltrating the salivary glands in NOD mice

The role of different B cell subsets in the pathogenesis of autoimmune sialoadenitis is a matter of controversy. In particular, while the role of Fo B in contributing to the formation of ectopic follicles has been clearly demonstrated in patients with SS (218, 302), the contribution of Fo B and MZ B cells in NOD sialoadenitis is currently unknown. This is of particular interest because a prominent role for MZ B cells in promoting autoimmunity in general, and sialoadenitis in particular, is suggested by several studies: 1) MZ B cells in the spleen provide a reservoir of autoreactive B cells (235); 2) MZ-like B cells infiltrate the SG of SS patients (303); 3) animal models of autoimmune sialoadenitis, such as non-obese diabetic (NOD) mice and BAFF-Tg mice display expansion of the MZ B cell population (99), whereas MZ-deficient BAFF-Tg mice are protected from sialoadenitis but not nephritis (304); and 4) parotid MALT-L in SS derive from transformed autoreactive MZ-like B cells (84) that frequently display rheumatoid factor activity (305, 306). Thus, in order to dissect the contribution of Fo B and MZ B cells I performed sequential sections stainings for the same markers previously described in Chapter 4. As shown in Fig. 5.5, double and sequential staining for IgD/Cd1D and CD21/CD23 clearly identified the two populations in NOD spleen (see Fig. 4.6) and in SG (Fig. 5.5). Sequential sections analysis of submandibular gland tissue demonstrated that the vast majority of B cells infiltrating TLS were B220+/IgD+/CD23+/CD21- consistent with a FoB cell subset. Differently from insulitis, I could not detect a population of MZ-like B cells surrounding B cell follicles. The dominant presence of FoB cells in NOD salivary glands was further confirmed by FACS analysis using combined staining for CD19/CD21/CD23 whereby FoB cells are $CD23^{high}/CD21^{low}$ and MZ B cells are $CD23^{low}/CD21^{high}$. While both populations could be easily identified in spleen mononuclear cell preparation, the only detectable B cell subset in NOD salivary gland was consistent with Fo B cells (Fig 5.6).

Further confirming the strong prevalence of the FoB cell phenotype, immunostaining for CXCR5 demonstrated that virtually all infiltrating B220+ cells also displayed high expression of CXCR5, (Fig.5.7). This further suggests that CXCL13 is a master regulator of B cell recruitment and positioning within TLS in the glands.

Figure 5.5

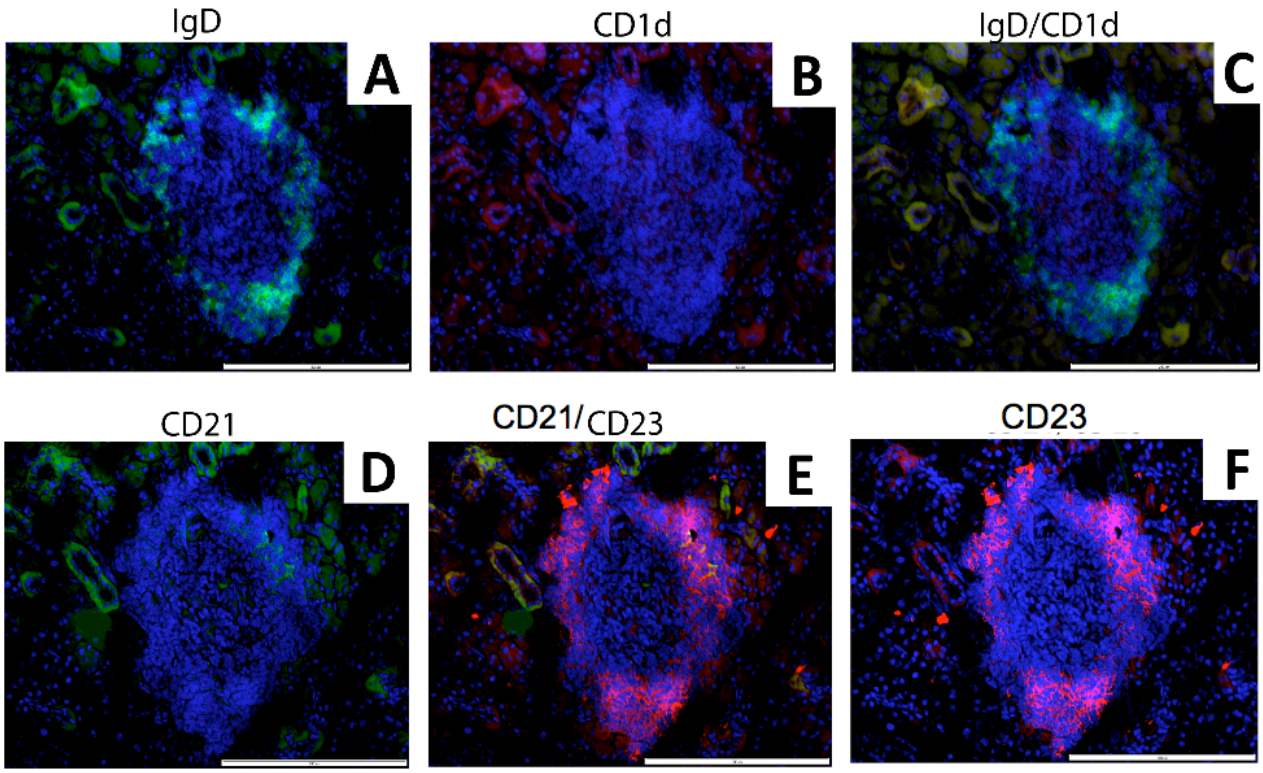

Figure 5.5 Characterization of B cell subsets within the SG infiltrates in NOD mice. Immunofluorescence sequential double stainings were carried out in order to evaluate the subset of B cells infiltrating SG infiltrates. The majority of B cells infiltrating the SG are characterized by a B220+/IgD+/CD23+/CD21- phenotype consistent with a FoB cell subset. It was not detected a population of MZ B cells surrounding B cell follicles. Original magnification X100.

Figure 5.6

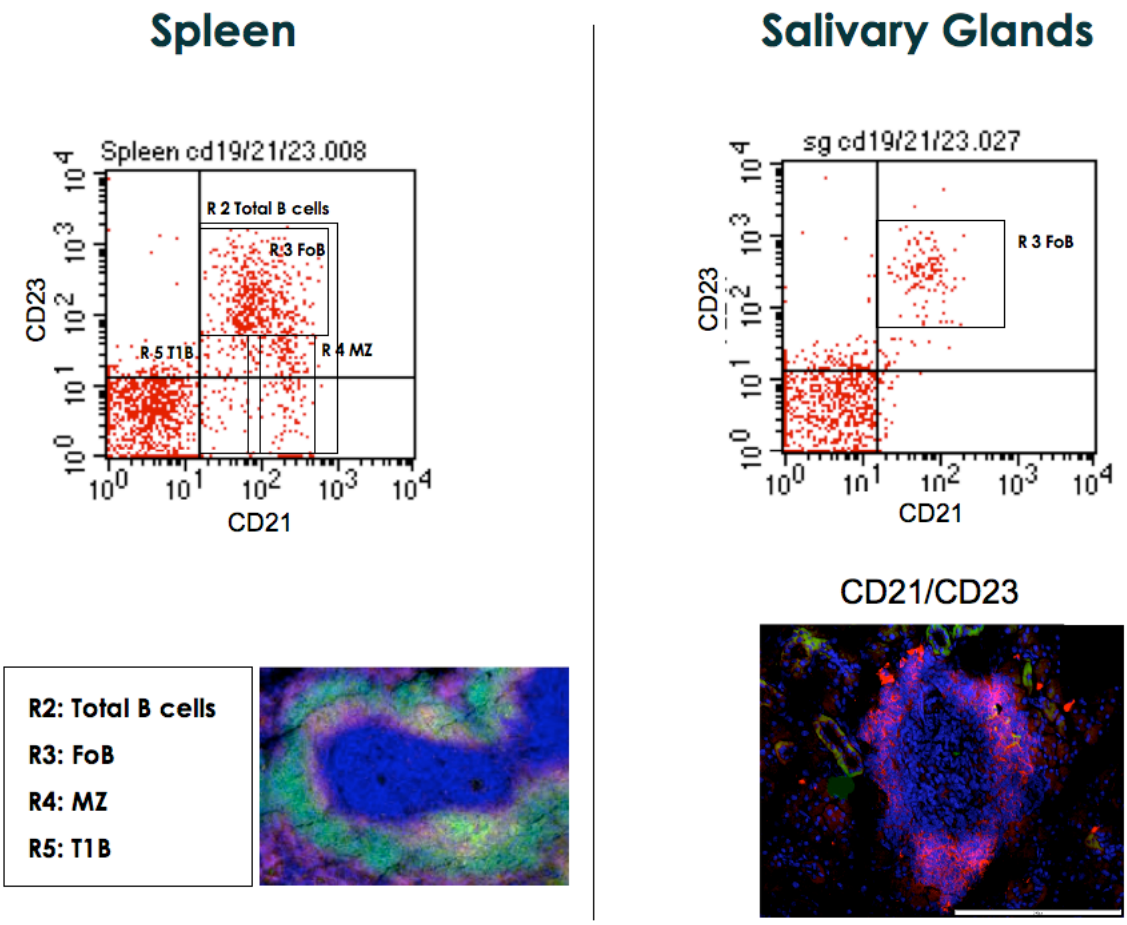

Figure 5.6 FACS analysis of B cell subpopulations in Spleen and SG glands of NOD mice. The dominant presence of FoB cells in NOD salivary glands was further confirmed by FACS analysis using combined staining for CD19/CD21/CD23 whereby FoB cells are $CD23^{high}/CD21^{low}$ and MZ B cells are $CD23^{low}/CD21^{high}$. While both populations could be easily identified in spleen mononuclear cell preparation, the only detectable B cell subset in NOD salivary gland was consistent with Fo B cells.

Figure 5.7

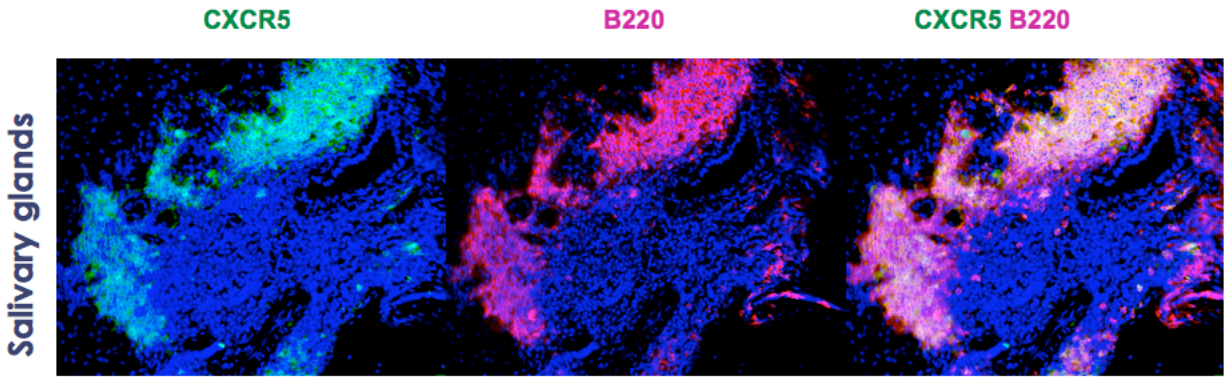

Figure 5.7 CXCR5 expression in SG glands of NOD mice. Consistent with the prevalent FoB cell phenotype, the vast majority of islet-infiltrating B220+ cells also displayed high expression of CXCR5, with a pattern highly reminiscent of that observed in the follicular area of the spleen (shown in Fig. 4.7). Original magnification X100.

5.5 Progressive increase of Sjogren-associated circulating autoantibodies accompanies the evolution of TLS in NOD mice

I next evaluated the dynamic of the appearance of autoantibodies in the circulation of NOD mice. Because a dominant autoantigen in humoral autoimmunity in NOD sialoadenitis has not been identified, I analysed the reactivity towards well established nuclear autoantigens which are commonly associated with autoimmunity as well as the prevalence of autoantibodies towards the ribonucleoprotein Ro/SSa and La/SSB which are normally associated with SS rather than T1D in patients. As shown in Figure 5.8 autoantibodies against ssDNA and dsDNA appeared extremely early in the course of the disease in NOD mice, and progressively increased with the increasing age of the mouse. This suggests that these autoantibodies mark a general autoimmunity trait in NOD mice highlighting the early activation of autoreactive B cells as a pivotal feature of this model. Conversely, the SS-associated anti-Ro/SSA and anti-La/SSB IgG autoantibodies were virtually undetectable before 20 weeks of age, following which their production was prominent and progressive. Remarkably, their appearance and titre closely paralleled the development of AID+TLS in the salivary glands (but not in the pancreas), indirectly suggesting the salivary gland TLS contribute towards breach of tolerance towards SS-associated autoantigens and development of class-switched antibodies (Fig.5.8).

Figure 5.8

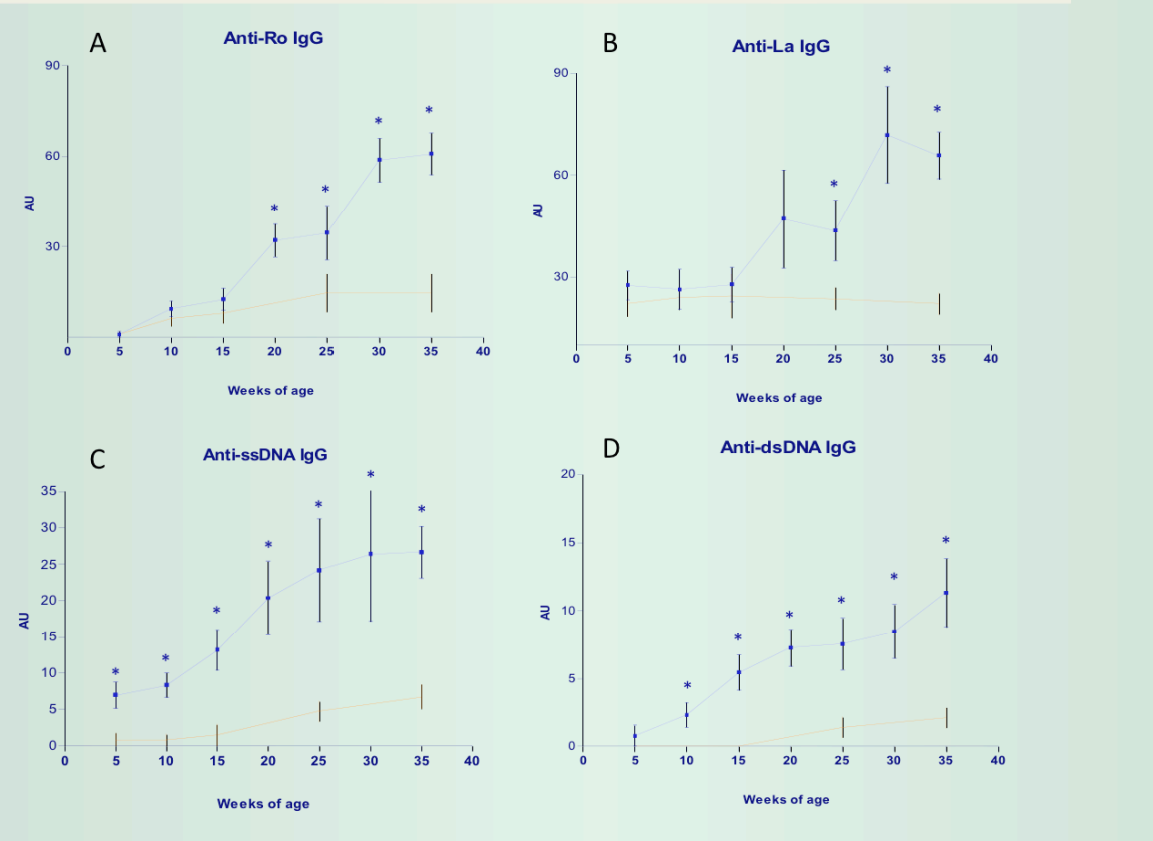

Figure 5.8 Progressive increase in circulating autoantibodies in NOD mice. Quantitative evaluation of serum autoantibodies in NOD mice. The detection of the SS-associated anti-Ro (A) and anti-La (B) antibodies in the serum of female NOD mice is concomitant with the formation of ELS in the SG and is significantly delayed compared to the onset of circulating anti-ssDNA (C) and anti-dsDNA (D) antibodies.

(Blue line NOD mice, Orange line C57/BL6 mice. * = $p<0.05$).

CHAPTER 6

RESULTS

Lymphotoxin-βR-Ig disrupts TLS development

in the NOD mouse model of T1D and SS

6.1 Functional and structural impairment of TLS following short-term treatment with LTβR-Ig in NOD mice

I previously showed in this thesis that B-cells can be activated within TLS in the pancreas and submandibular glands of NOD mice and are able to promote disease-specific in situ autoantibody responses. In this study I carried out a trial in NOD mice in order to inhibit the LT-β pathway as a potential therapy for T1D and SS. Because previous work extensively demonstrated the efficacy of this therapeutic approach in preventing the development of insulitis and the onset of diabetes in NOD mice, in this part of my PhD project I primarily focused on the modulation of TLS in the salivary glands. In addition, effect on SLOs, such as the spleen and periglandular lymph nodes was evaluated. NOD mice were injected intra-peritoneally with 100μg/mouse LTβR-Ig weekly from week 12 for 5 weeks. Furthermore, NOD mice used as controls were injected with equal amounts of MOPC-21 isotype control. Mice were ear-marked and randomised to either treatment.
Strikingly, after 5 weeks none of the mice treated with LTβR-Ig developed hyperglycaemia. The histological analysis of the pancreas from these mice displays normal islets and no inflammatory infiltrates (data not shown). All the mice treated with MOPC-21 isotype control developed overt diabetes and 13 mice either died or had to be culled within 1 week of diabetes development.

Mice completing the trial were culled and salivary glands, pancreas, spleen and peri-salivary glands lymph nodes were collected for analysis. Samples from the same mice were either snap frozen in OCT for immunophenotypical characterization or stored in RNA later for gene expression profiling. I first investigated whether the development of TLS was disrupted following blockade of LTβ by performing sequential double immunofluorescence analysis. As shown in Figure 6.1, only rare and poorly organized inflammatory infiltrates were observed in mice treated with LTβR-Ig, in striking contrast with mice treated with the isotype control that clearly showed large infiltrates, T/B cell

segregation, FDC networks and GL7+ GCs (Figures 6.2, 6.3). Moreover, no AID expression was detectable at protein level in the salivary glands of treated mice (not shown). In addition to TLS, LTβR-Ig also impaired GC responses in periglandular lymph nodes as demonstrated by a striking reduction in the formation of GL7+ GC as compared to the treatment group (Fig. 6.4).

I next evaluated whether the disruption of TLS formation was related to the down-regulation of the key set of genes known to regulate the development, maintenance and function of ectopic lymphoid neogenesis, with particular focus on CXCL13/CXCR5, CCL19/CCR7, CXCL12/CXCR4, LTα, LTβ, LTβR and AID. Quantitative TaqMan real-time evaluation of mRNA expression levels in salivary glands of NOD mice treated with LTβR-Ig and isotype control is shown in Figure 6.5 and 6.6. A striking reduction in the expression levels of lymphoid CKs and their receptor was clearly observed, compared with mice treated with the isotype control (Fig 6.5). In particular, the CXCL13/CXCR5 axis was strongly down-modulated, reflecting the non-redundant upstream role of LTβ in the induction of CXCL13 and the subsequent reduced recruitment of CXCR5+ B cells. In keeping with the histological data, treatment impaired not only the architecture, but also the function of TLS, as demonstrated by the significant reduction of AID mRNA expression.

Evaluation of LTs and B cells proliferating and survival factors gene expression was also carried out (Fig. 6.6). Interestingly, LTβR-Ig did not affect the expression of LTα nor induced down-regulation of LTβ itself. In contrast, there seems to be an upregulation of LTβ, possibly as a compensatory mechanism. Similarly, an increase in BAFF mRNA was observed and was accompanied by a parallel down-regulation of BAFF-R, most likely related to the reduced recruitment of B cells withing the glands. In order to investigate whether the effects of LTβR-Ig were also at systemic level, I evaluated the mRNA expression levels of lymphoid neogenesis-related genes in the spleens of the same animals. As shown in Figure 6.7 and 6.8, significant decrease in the mRNA expression levels of TLS-related genes was also observed in the splenic compartment of LTβR-Ig -treated mice as compared with mice treated with the isotype control.

Figure 6.1

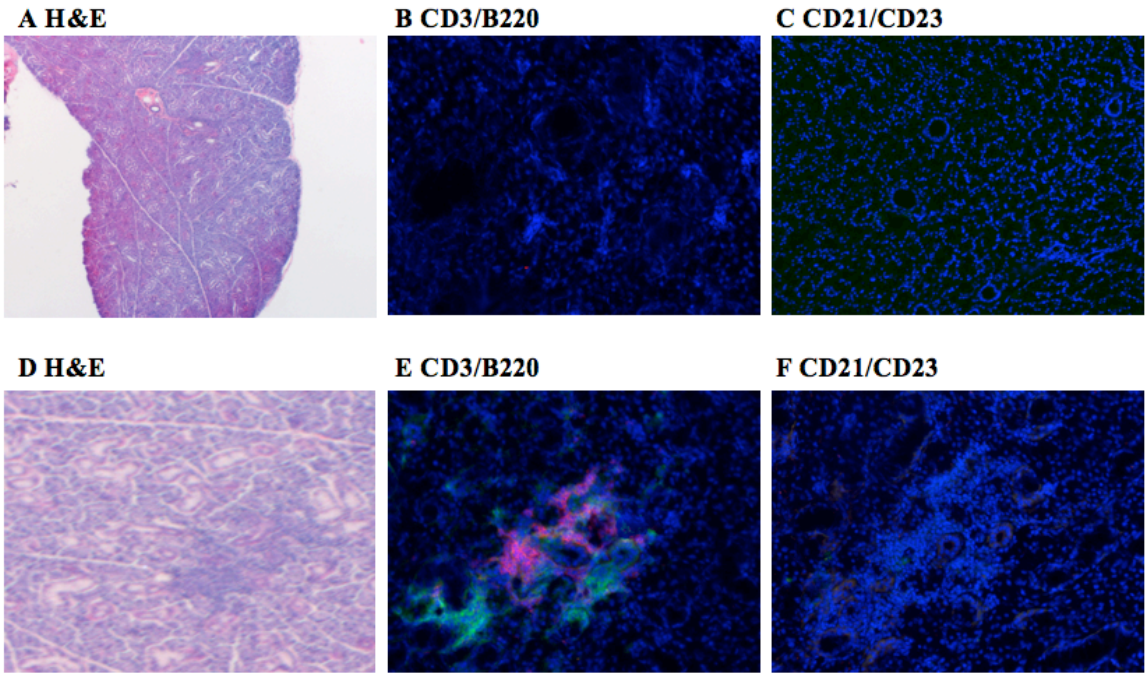

Figure 6.1 Histological characterization of salivary glands infiltrates in NOD mice treated with Ltxβ r-Ig. A-D: H&E staining **B,E**: Immunofluorescence staining for CD3 (T cells, **green**)/B220 (B cells, **pink**). **C,F**: Immunofluorescence staining for CD21 (**green**)/CD23 (**pink**). Only rare and very small inflammatory cells infiltrates can be observed in mice treated with Ltxr-Ig. Original magnification X40 (A) and X100 (B-F).

Figure 6.2

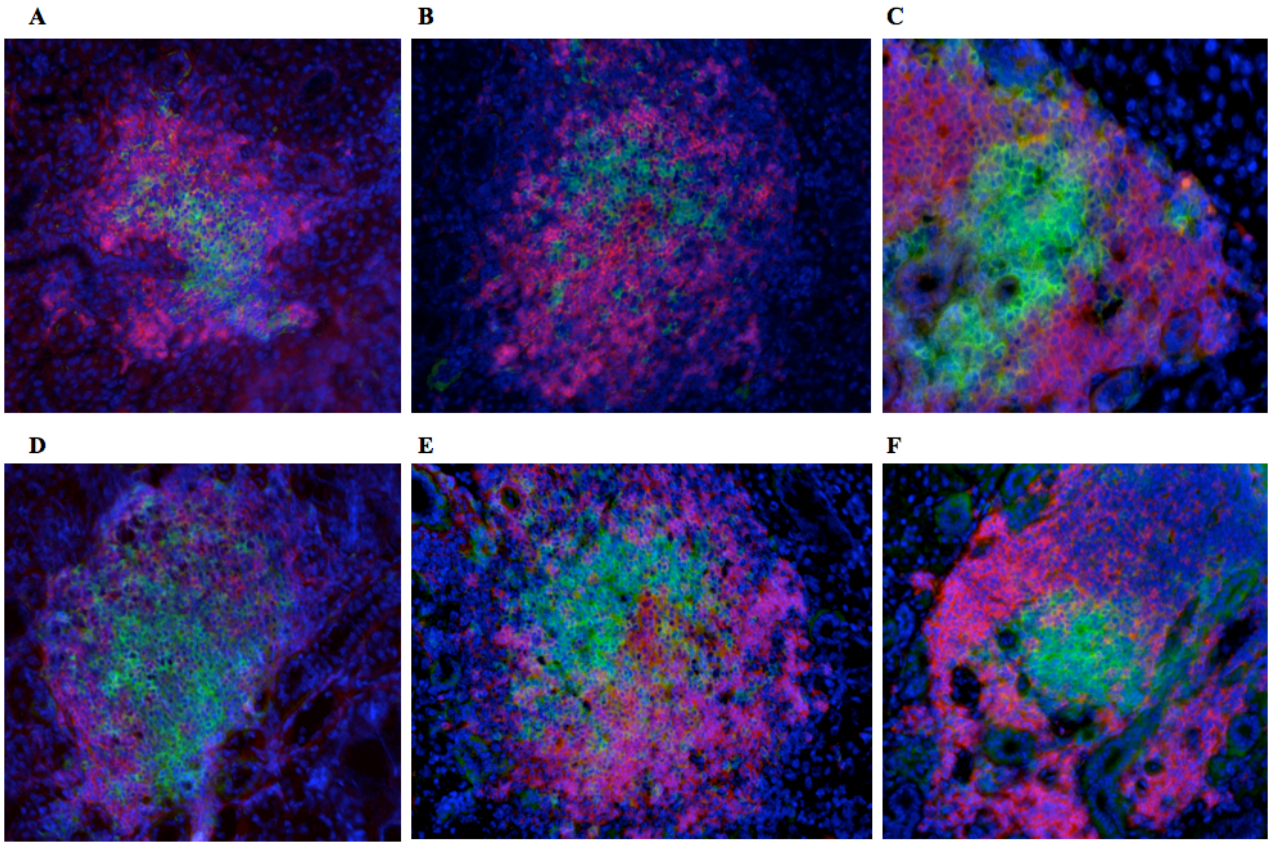

Figure 6.2 Histological characterization of salivary glands infiltrates in NOD mice treated with isotype control showing very large infiltrates. A-F: Immunofluorescence staining for CD3 (T cells, **green**)/B220 (B cells, **pink**). Original magnification X100.

Figure 6.3

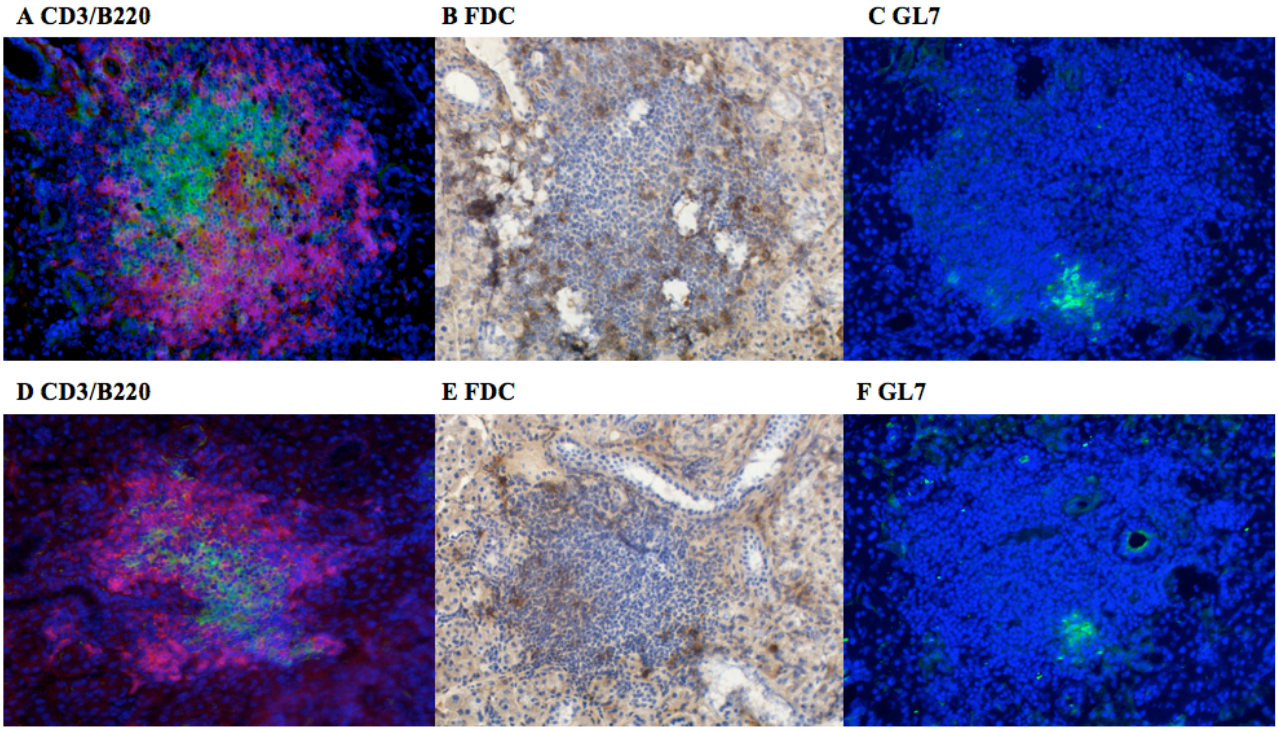

Figure 6.3 Histological characterization of salivary glands infiltrates in NOD mice treated with isotype control. A-F: Sequential sections stained for (A,D) immunofluorescence staining for CD3 (T cells, **green**)/B220 (B cells, **pink**); (B,E) FDC-M1 peroxidase staining, marker for follicular dendritic cell networks (**brown**); (C,F) GL7 marker for germinal centers (**green**). Original magnification X100.

Figure 6.4

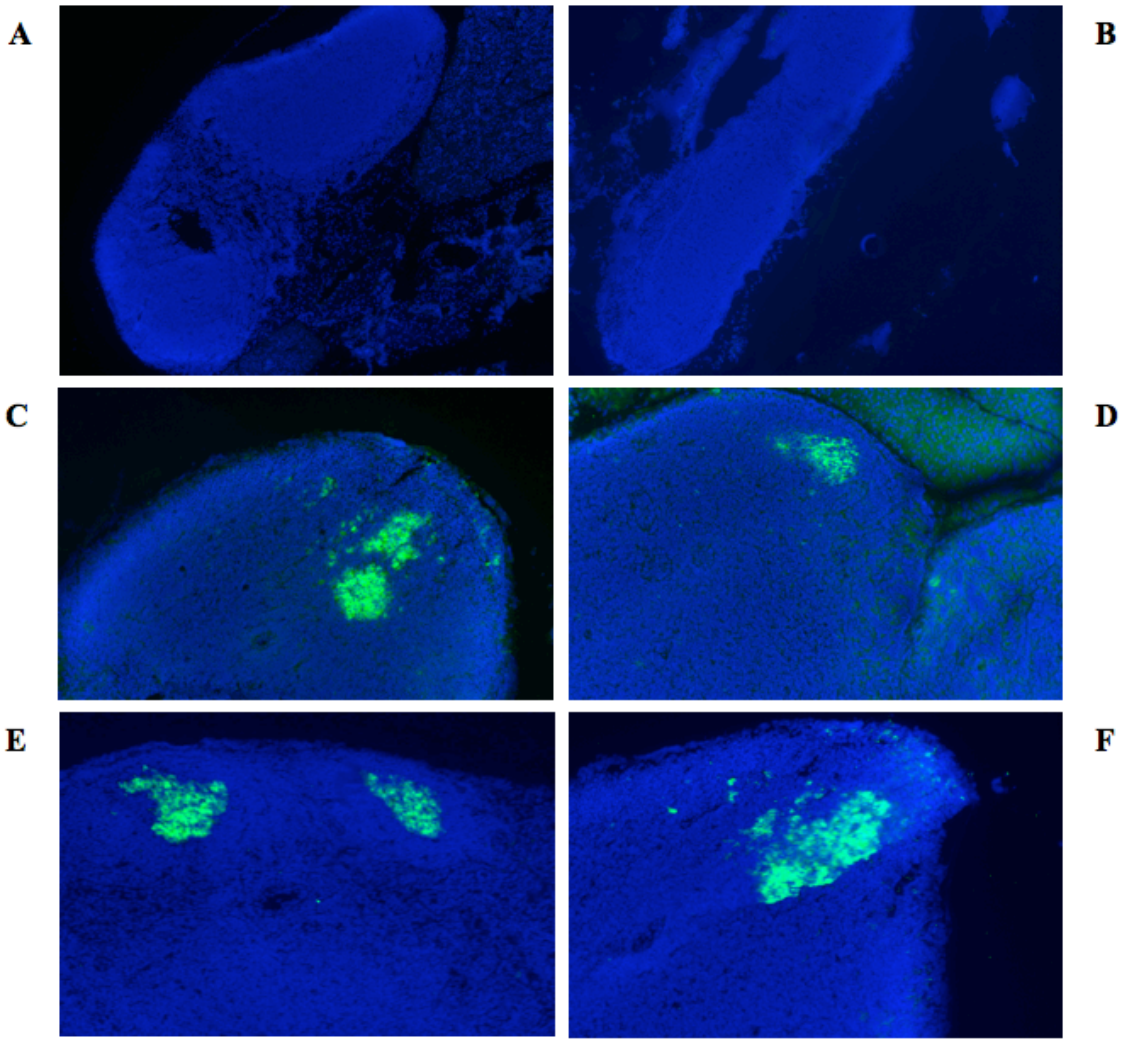

Figure 6.4 Peri-gland lymph nodes stained with GL7. A,B. Lymph nodes from mice treated with Ltxβr-Ig do not display GL7+ germinal centers; **C-F** Lymph nodes from mice treated with isotype control display GL7+ germinal centers. Original magnification X100.

Figure 6.5

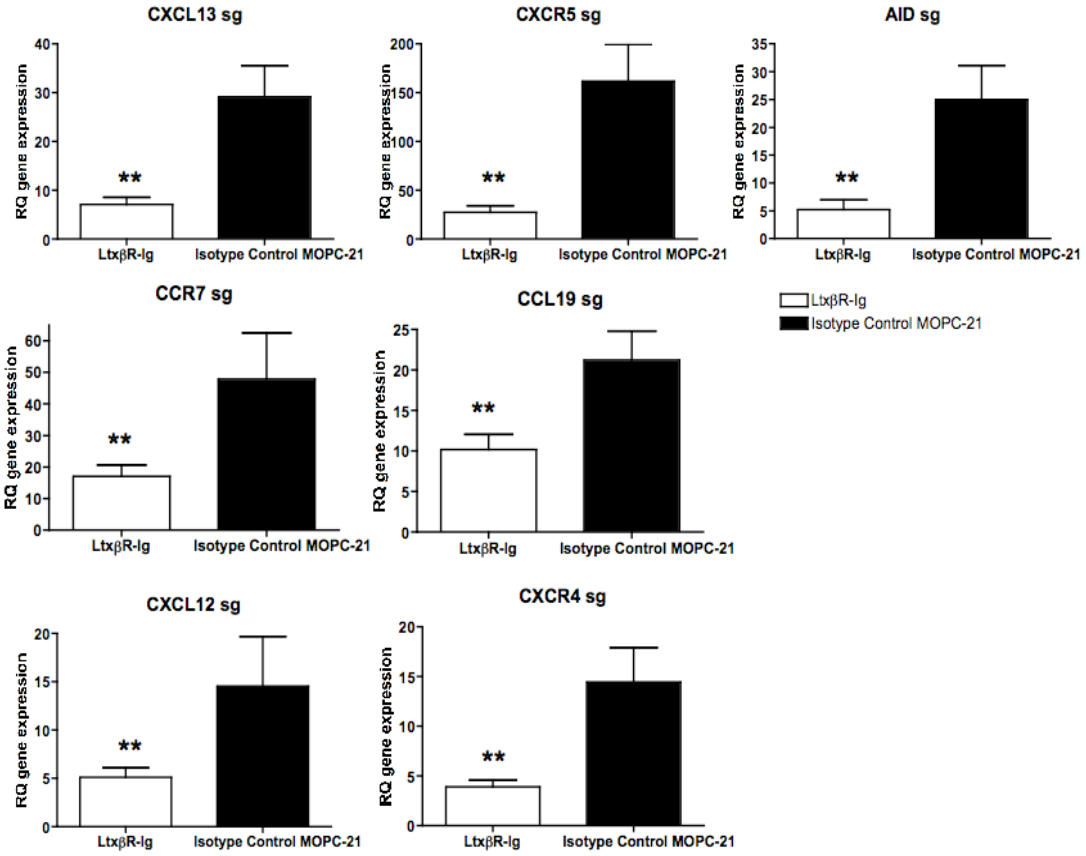

Figure 6.5 Evaluation of mRNA expression of lymphoid neogenesis related genes and AID gene in salivary glands. Quantitative TaqMan real-time evaluation of mRNA expression levels in salivary glands of NOD mice treated with Ltxβr-Ig (**white**) and isotype control (**black**). Significant decrease in mRNA gene expression can be observed in mice treated with Ltxβr-Ig compared with mice treated with the isotype control. Mice treated with Ltxβr-Ig N=21; mice treated with isotype control N=27. *p<0.05, **p<0.01.

Figure 6.6

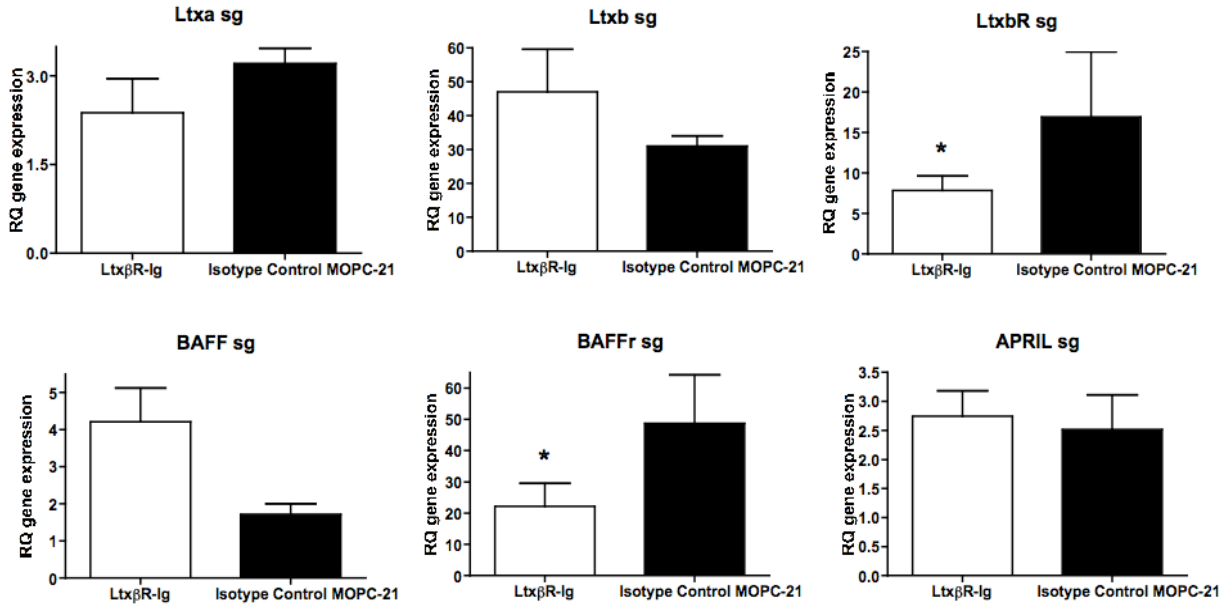

Figure 6.6 Evaluation of lymphotoxins and B cells proliferating and survival factors mRNA gene expression in salivary glands. Quantitative TaqMan real-time evaluation of mRNA expression levels in salivary glands of NOD mice treated with Ltxβr-Ig (**white**) and isotype control (**black**). Compensatory upregulation of Ltxβ and BAFF genes can be observed in mice treated with Ltxβr-Ig compared with mice treated with the isotype control. Mice treated with Ltxβr-Ig N=21; mice treated with isotype control N=27. *$p<0.05$, *$p<0.01$.

Figure 6.7

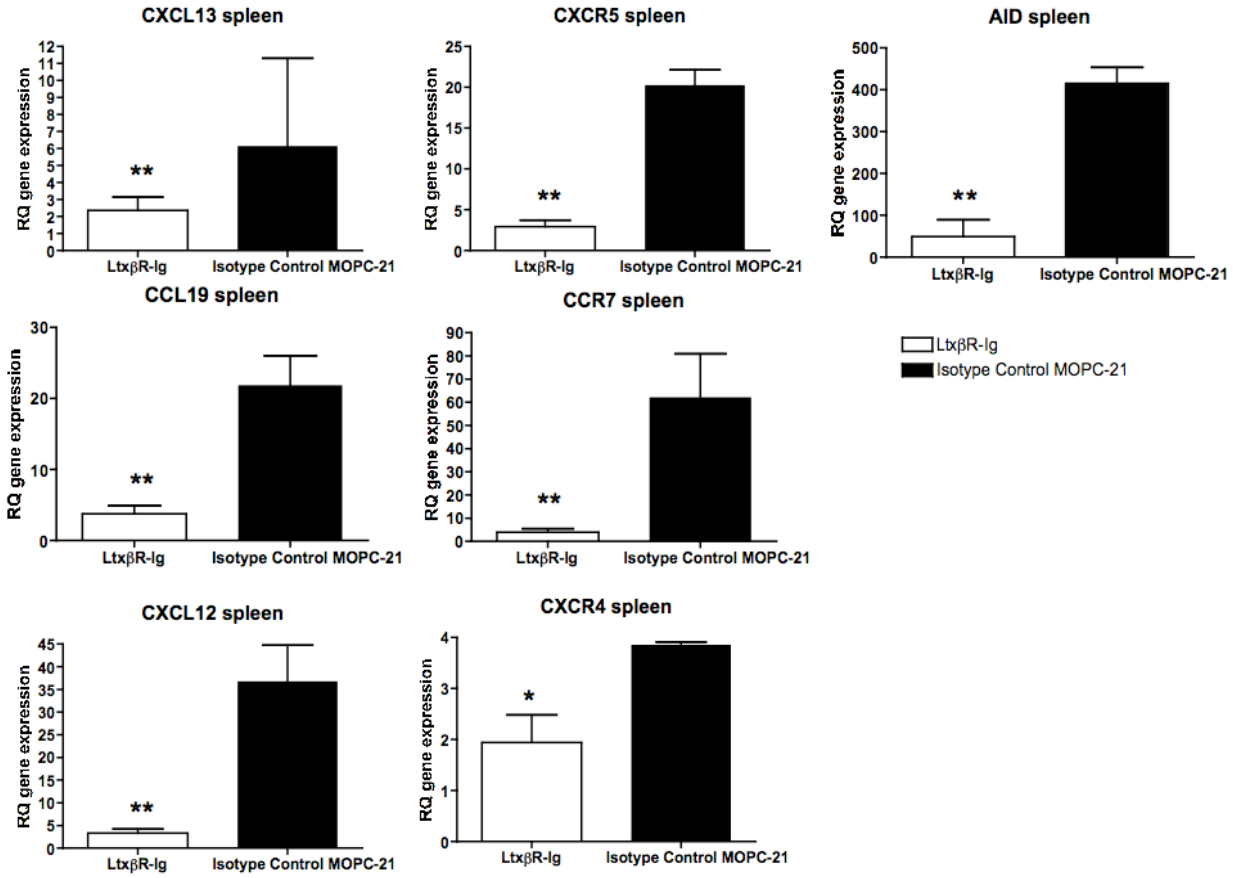

Figure 6.7 Evaluation of mRNA expression of lymphoid neogenesis related genes and AID gene in spleen. Quantitative TaqMan real-time evaluation of mRNA expression levels in spleen of NOD mice treated with Ltxβr-Ig (**white**) and isotype control (**black**). Significant decrease in mRNA gene expression can be observed in mice treated with Ltxβr-Ig compared with mice treated with the isotype control. Mice treated with Ltxβr-Ig N=21; mice treated with isotype control N=27. *p<0.05, *p<0.01.

Figure 6.8

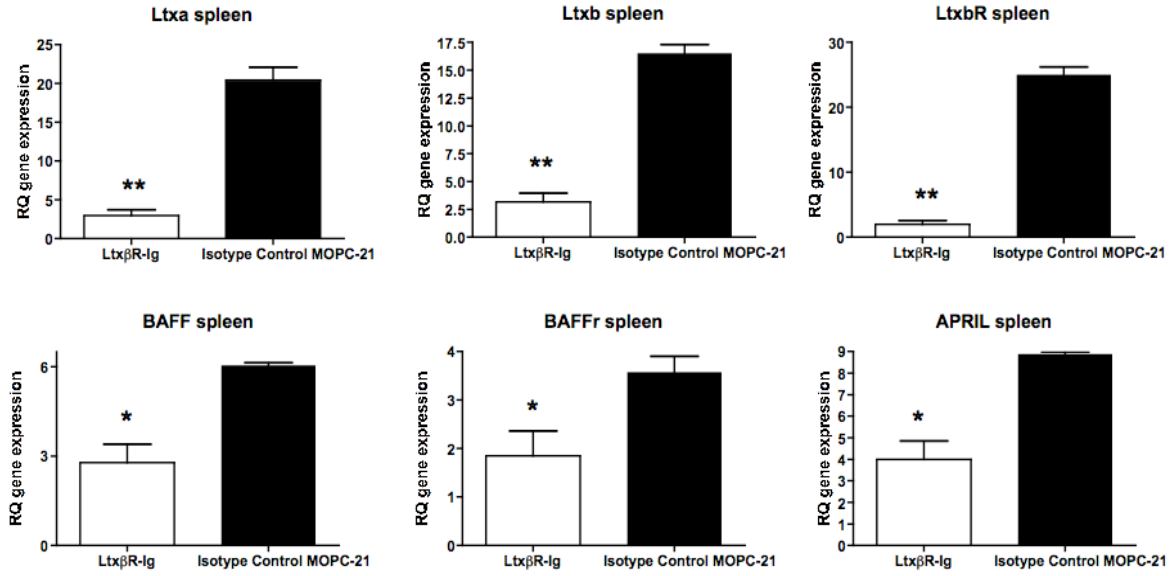

Figure 6.8 Evaluation of lymphotoxins and B cells proliferating and survival factors genes expression in spleen. Quantitative TaqMan real-time evaluation of mRNA expression levels in spleen of NOD mice treated with Ltxβr-Ig (**white**) and isotype control (**black**). Significant decrease in mRNA gene expression can be observed in mice treated with Ltxβr-Ig compared with mice treated with the isotype control. Mice treated with Ltxβr-Ig N=21; mice treated with isotype control N=27. *$p<0.05$, *$p<0.01$.

CHAPTER 7

RESULTS

Expression of Reg genes in autoimmune insulitis and sialoadenitis in the NOD mouse model of and in patients with T1D and SS

7.1 Reg genes expression during autoimmune insulitis in NOD mice

7.1.1 Reg genes display a bimodal expression profile in NOD but not Balb/c mice pancreas

The development of insulitis was first analyzed. As expected, initial infiltrates were observed from week 4 onwards and were mostly organized as peri-islets aggregates (*peri-insulitis* Fig.7.1 A). With increasing age, larger T and B cells aggregates developed and progressively colonized the whole islet followed by disappearance of β-cells (*intra-insulitis* Fig.7.1 B,C). The percentage of peri-insulitis vs intra-insulitis in NOD mice pancreas is reported in Table 7.1.

I next investigated the expression of all known Reg genes at different time points in both strains of mice. At baseline (4 weeks), both NOD and Balb/c mice showed high pancreatic expression of Reg1, Reg2, Reg3α, Reg3β, Reg3γ and Reg3δ at 4 weeks (Fig.7.2 A-F). No expression of Reg 4 was observed at any time point analyzed. Interestingly, at baseline only Reg1, Reg3β, andReg3δ were significantly increased (~2-fold) in NOD as compared to Balb/c mice (Fig.7.2 A,D,F). Between 4 and 8 weeks a dramatic decrease (between 200 and 600-folds) in all Reg genes was observed in both NOD and Balb/c, with some of the Reg genes becoming undetectable even by sensitive Taqman rt-PCR. Strikingly, while in Balb/c mice all Reg genes remained low/undetectable, NOD mice displayed a clear bimodal expression profile with a consistent and sharp de novo expression in parallel with the clinical onset of disease (12 to 15 weeks). This overexpression was preceded by a rapid increase in local IL-6 mRNA, which peaked in correspondence of the peak in Reg, and was sustained until around 20 weeks of age after which it was followed by a progressive and rapid decline in line with complete disappearance of islets and inflammatory infiltrates and return to normal IL-6 levels (Fig.7.2 G).

Figure 7.1

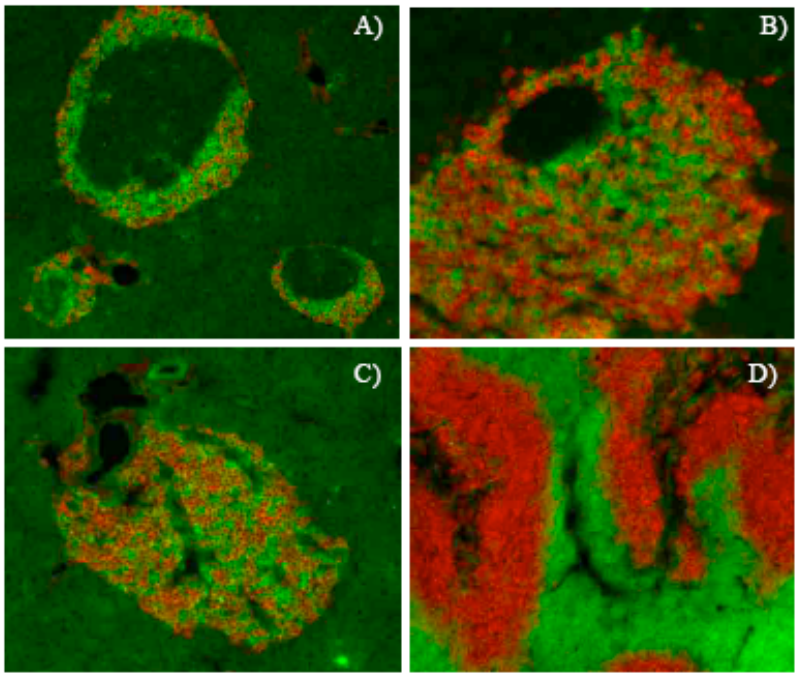

Figure 7.1 Characterization of inflammatory cells infiltration in NOD mice pancreas. Immunofluorescence staining for B cells (CD20 **red**) and T cells (CD3 **green**). A. Periinsulitis; B. intra-insulitis; C. complete infiltration of an islet; D. spleen tissue as positive control. Original magnification X100 (A-C) and X40 (D).

Table 7.1.

Percentage of peri-insulitis vs intra-insulitis in NOD mice pancreas. Data from the same cohort of mice, shown in Fig. 4.1 C.

Age	**Percentage of islets**	
	Peri-insulitis	**Intra-insulitis**
8 weeks	78	22
16 weeks	44	56
24 weeks	37	63
32 weeks	20	80

Figure 7.2

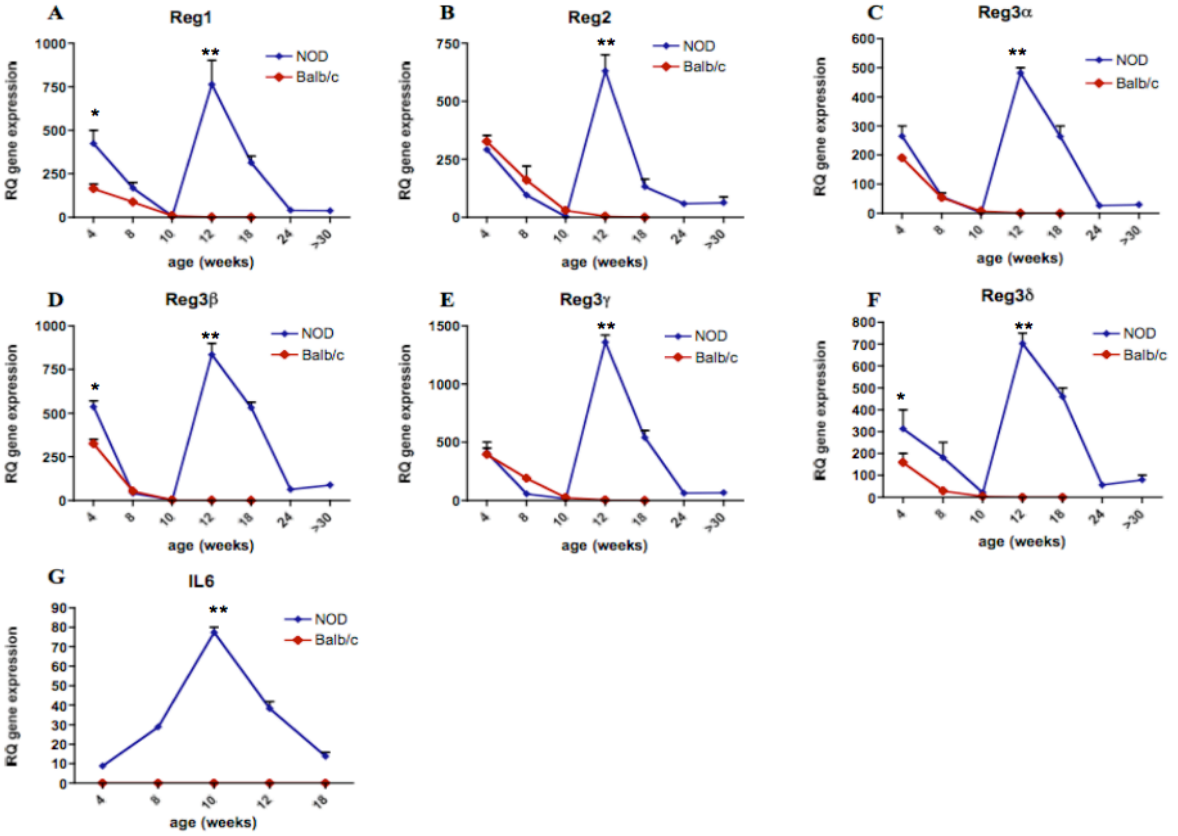

Figure 7.2 RQ gene expression in NOD (blue line) and Balb/c (red line) mice pancreas. RT-PCR was carried out for Reg1 (A), Reg2 (B), Reg3α (C), Reg3β (D), Reg3γ (E), Reg3δ (F), Reg4 (non expressed) and Il-6 (G). Mice were grouped according to age: 4 to 6 weeks (N=12); 7 to 9 weeks (N=12); 10 to 11 weeks (N=16); 12 to 16 weeks (N=18); 17 to 22 weeks (N=18); 23 to 29 weeks (N=16); >30 weeks (N=18). Data are presented as the mean+SEM. *p<0.05, **p<0.01 between NOD and Balb/c.

7.1.2 Tissue localization of Reg3β and Reg3δ within the pancreas

I next examined the expression of Reg3β and Reg3δ at protein level by immunohistochenistry. Consistent with the gene expression profiling, both NOD and Balb/c young mice (4 weeks) showed extremely high levels of Reg3β and Reg3δ positive cells. In addition to islets, Reg3β was widely expressed by ductal rather than acinar epithelial structures in the exocrine pancreas (Fig.7.3 A,E). In comparison, Reg3δ expression was more restricted to the islets except fewer positive cells outside the islets (Fig.7.3 C,H). At 8 weeks both NOD (Fig.7.3 F,I) as well as Balb/c (not shown) displayed an almost complete absence of expression of both Reg3β and Reg3δ, with the exception of few residual scattered cells which were only observed in NOD mice. However, and again consistently with the RNA data, after disease onset I observed a sharp increase of both Reg3β and Reg3δ protein expression in NOD mice (Fig.7.3 G,J). Interestingly, re-expression of Reg3δ was mostly confined to the islets, while Reg3β displayed a similar pattern of expression as observed at earlier time point. No re-expression of Reg3β nor Reg3δ was observed in Balb/c mice (Fig.7.3 B,D).

Figure 7.3

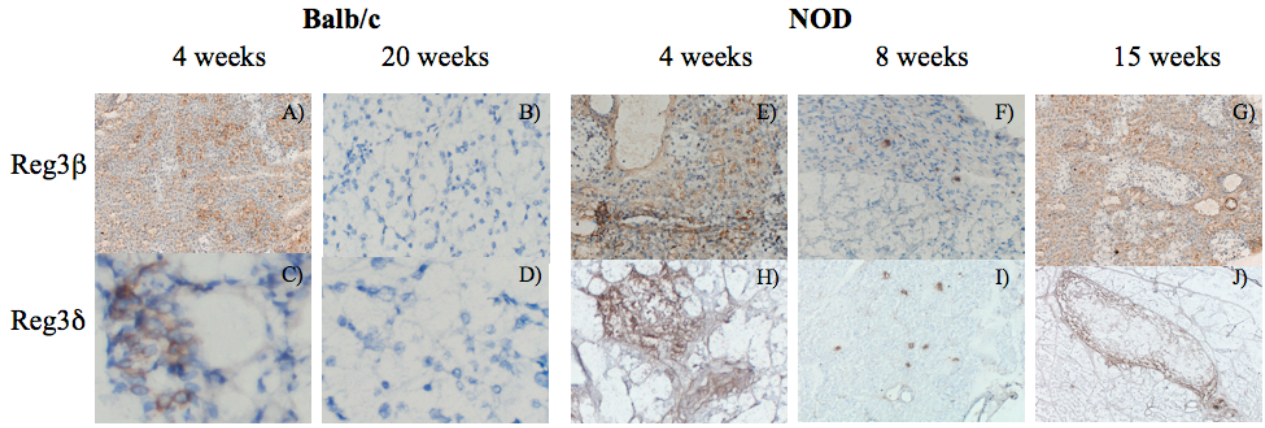

Figure 7.3 Localization of Reg expression in NOD and Balb/c mice pancreas. Staining for Reg3β (peroxidase) and Reg3δ (peroxidase and immunofluorescence) at different ages. Balb/c Reg3β at 4 weeks (A) and 20 weeks (B); Reg3δ at 4 weeks (C) and 20 weeks (D). NOD Reg3β at 4 weeks (E), 8 weeks (F) and 15 weeks (G); Reg3δ at 4 weeks (H), 10 weeks (I) and 18 weeks (J). Original magnification X40 (A, E, F, G), X100 (B, H, I, J) and X200 (C, D).

7.2 Circulating Reg1α Proteins and Auto-Antibodies to Reg1α Proteins as Biomarkers of β Cell Regeneration and Damage in Type 1 Diabetes

7.2.1 Circulating levels of Reg1α protein in different types of DM

Pancreatic β-cells are one of the most important sources of insulin, but they have a limited capacity for regeneration which is a predisposing factor for the development of an autoimmune disease such as type 1 diabetes. Strategies for influencing the replication and growth of the β-cell mass are therefore important for the prevention and/or treatment of type 1 diabetes.

There is evidence that the β-cell mass of an adult normal pancreas is not static but in a constant process of death and renewal (regeneration/self-duplication) and Reg proteins stimulate the replication of pancreatic β-cells resulting in the amelioration of diabetes. Therefore, in order to characterize circulating Reg1α proteins as biomarkers of β-cell regeneration, I measured serum levels of Reg1α protein in several patient groups (Tab. 3.3).

Increased levels of Reg1α protein were observed in newly diagnosed type 1 diabetes patients compared to controls ($p=0.002$), in long standing type 1 diabetes patients compared to controls ($p=0.001$) but no difference was found between newly diagnosed and long standing type 1 diabetes patients ($p=0.22$). Type 2 diabetes patients also showed higher levels of the protein compared to controls ($p<0.001$).

Levels of Reg1α protein in SLE patients were comparable to those found in healthy controls and significantly lower than those reported in diabetic patients ($p<0.001$) (Fig. 7.4).

Figure 7.4

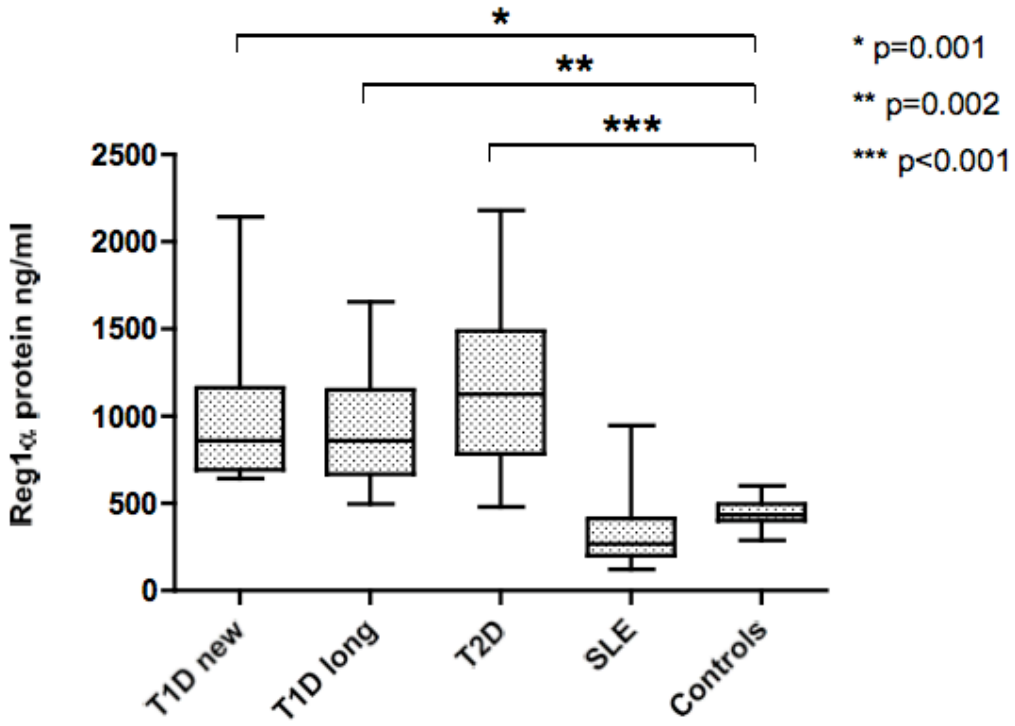

Figure 7.4 Human Reg1α ELISA assay (BioVendor Laboratory). Quantitative measurement of human Reg1α protein in serum. Increased levels of Reg1α protein were observed in newly diagnosed type 1 diabetes patients compared to controls (p=0.002), in long standing type 1 diabetes patients compared to controls (p=0.001) but no difference was found between newly diagnosed and long standing type 1 diabetes patients (p=0.22). Type 2 diabetes patients also showed higher levels of the protein compared to controls (p<0.001). Levels of Reg1α protein in SLE patients were comparable to those found in healthy controls and significantly lower than those reported in diabetic patients (p<0.001). T1D, type 1 diabetes; T2D, type 2 diabetes; SLE, systemic lupus erythematosus. The number of patients enrolled is: 87 T1D subjects with different disease duration (31 newly diagnosed and 56 long standing), 63 T2D subjects, 80 normal subjects sex/age matched, 39 SLE.

7.2.2 Reg1α autoantibodies in T1D and T2D

Autoantibodies were detected in 47.1% of type 1 diabetes subjects, 3.1% of normal subjects and none of type 2 diabetes subjects (Fig.7.5). By classifying type 1 diabetes patients according to their antibodies subtype (GAD+/IA2-; GAD-/IA2+; GAD+/IA2+), it was possible to observe a strong correlation between anti-Reg1α antibodies and the presence of both GAD/IA2 autoantibodies, while patients displaying only IA2 had a very low prevalence of anti-Reg1α (Tab.7.2). The high levels of serum Reg1α protein in type 2 diabetes but the absence of autoantibodies suggest that Reg proteins represent the target of an autoimmune response only in type 1 diabetes.

Figure 7.5

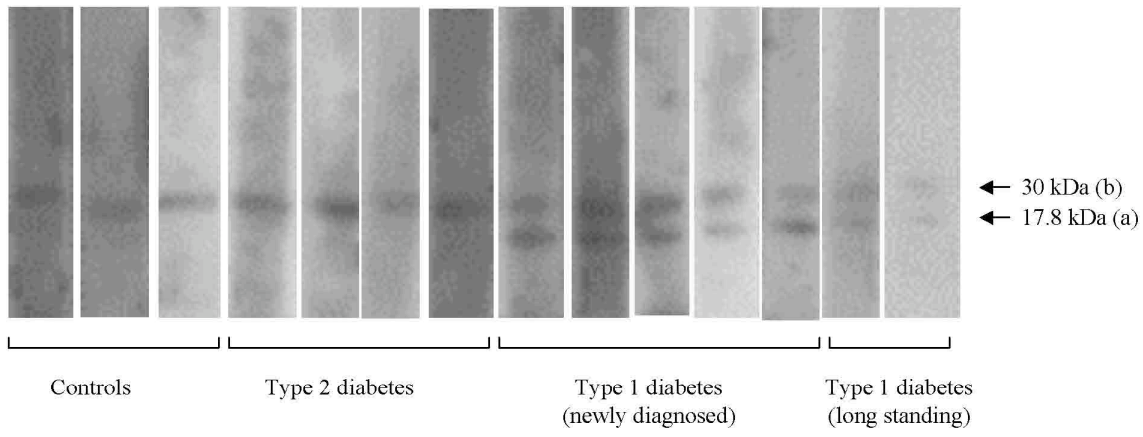

Figure 7.5 Western Blots for Reg1α antibodies with sera from controls, type 1 and type 2 diabetes patients. 10μl of Reg1α protein (BioVendor 17.8kDa) were loaded in each well and, if antibodies were present in the sera a black band was observed on the film at 17.8kDa (**a**). A second band (**b**) is an artefact band of E.coli degradation.

Table 7.2

Anti-Reg1α antibodies in type 1 diabetes in relation to the presence of GAD and IA2.

Type 1 diabetes patients antibodies			Reg1α antibodies		
	N	**%**	**N**	**% in Total**	**% in each subgroup**
GAD+/IA2-	59	67.8	26	29.9	44.1
GAD-/IA2+	15	17.2	3	3.4	20.0
GAD+/IA2+	13	15.0	12	13.8	92.3
Total	87	100	41	47.1	47.1

7.2.3 Metabolic and Genetic correlations.

Circulating Reg1α protein levels were grouped as above (High) and under (Low) the mean (Tab. 7.3). No correlation was found between Reg1α serum levels, fasting C-peptide levels, HbA1c or age in both long-standing patients and newly diagnosed. In long-standing type 1 diabetes patients, correlation between disease duration and protein levels showed a significant decrease of Reg1α in years ($p<0.0176$; Spearman r -0.36) (Fig.7.6). The relation between Reg1α levels and type 1 diabetes related gene polymorphisms (HLA, INS, PTPN22, CTLA4) did not show any significant association.

Figure 7.6

Correlation between disease duration and circulating Reg1α protein in long-standing type 1 diabetes patients.

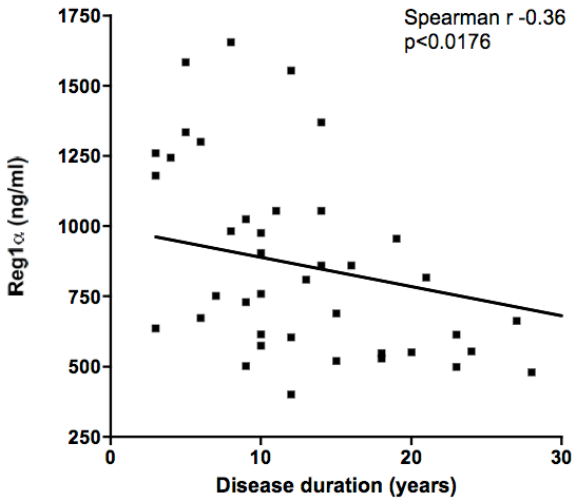

Table 7.3

Reg1α protein correlation with clinical parameters. Reg1α protein values were grouped as above (High) and under (Low) the mean in Long-standing and Newly diagnosed Type 1 Diabetes patients.

Type 1 Diabetes	**Reg1α protein High**	**Reg1α protein Low**
Long-standing	Mean±SD	Mean±SD
Age (years)	36.2 ± 11.6	37.3 ± 12.2
Disease duration (years)	12 ± 9.8	14 ±7.5
C peptide (nmol/l)	0.39 ± 0.69	0.14 ± 0.10
HbA1c (%)	7.5 ± 1.2	7.2 ± 0.8
Type 1 Diabetes	**Reg1α protein High**	**Reg1α protein Low**
Newly diagnosed	Mean±SD	Mean±SD
Age (years)	20 ± 8.7	17 ± 5.8
C peptide (nmol/l)	0.83 ± 0.46	0.71 ± 0.52
HbA1c (%)	10.2 ± 1.2	10.7 ± 2.7

P values were not significant.

7.3 Restricted Reg1 and Reg3γ gene expression in the salivary glands of NOD mice

I also investigated the expression of all known Reg genes at different time points in both strains of mice in salivary glands. Only Reg1 and Reg3γ were expressed in submandibular glands in NOD, Balb/c and C57BL/6 mice. The trend of expression was different not only in the two strains but also in the two genes. Reg1 gene expression peaked at 20 weeks in NOD mice but then decreased rapidly (Fig.7.7 A). Balb/c submandibular glands showed a low expression of the gene at all ages and no expression in C57BL/6 mice (Fig. 7.7 B), demonstrating a possible connection between gene expression and disease onset. Reg3γ on the contrary showed an expression pattern that increased gradually with age in NOD, Balb/c and C57BL/6 mice (Fig.7.7 C). This could be explained by a constitutive gene expression related to the growth of the organ.

Figure 7.7

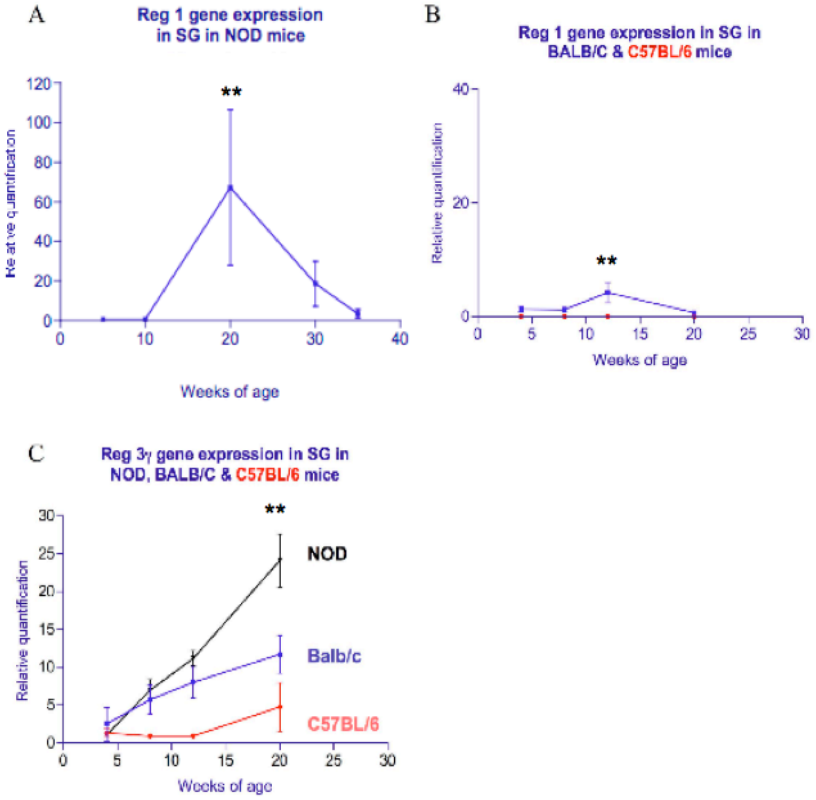

Figure 7.7 RQ gene expression in NOD, Balb/c and C57BL/6 mice salivary glands. RT-PCR was carried out for all Reg genes, only Reg1 and Reg3γ were expressed. Mice were grouped according to age: 4 to 8 weeks (N=20); 9 to 16 weeks (N=18); 17 to 24 weeks (N=20); 25 to 32 weeks (N=18); >32 weeks (N=12). Data are presented as the mean+SEM. **p<0.01

7.4 Circulating Reg1α and salivary gland expression of Reg genes in patients with primary SS

Quantitative measurement of human Reg1α protein was carried out in serum of patients affected by other autoimmune diseases such as SS, RA and SLE. Increased levels of Reg1α protein were observed in SS patients and in RA patients compared to controls. Levels of Reg1α protein in SLE patients were comparable to those found in healthy controls. Results are shown in Fig. 7.8 where circulating levels of the protein are compared to control subjects.

Furthermore, I evaluated the mRNA expression of Reg genes and Reg receptor EXTL3 in human tissues from patients affected by SS and RA (Table 7.4). I examined synovia and fibroblasts from RA patients, salivary glands from SS patients and chondrocytes. Salivary glands showed expression of Reg3γ; chondrocytes express Reg1β and Reg3α mRNA; synovia and fibroblasts on the contrary did not express any Reg gene. Interestingly, all tissues expressed high levels of EXTL3 (Table 7.4). It is possible that regeneration in different organs may be regulated by receptor modulation or by exogenous Reg proteins.

Fig. 7.8

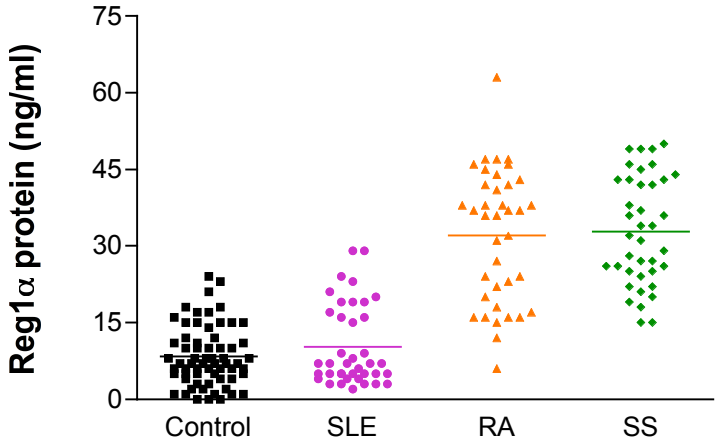

Figure 7.8 Human Reg1α ELISA assay in autoimmune disorders (BioVendor Laboratory). Quantitative measurement of human Reg1α protein in serum. Increased levels of Reg1α protein were observed in SS patients and in RA patients compared to controls. Levels of Reg1α protein in SLE patients were comparable to those found in healthy controls.

Table 7.4

mRNA expression of Reg genes and Reg receptor in human tissues.

Human Tissue	EXTL3	Reg1α	Reg1β	Reg3α	Reg3γ
Salivary Glands (n=14)	+++	No	No	No	+
Synovia (n=12) RA	++	No	No	No	No
Chondrocytes (n=8)	++	No	+	+	No
Fibroblasts (n=12) RA	++	No	No	No	No

CHAPTER 8

DISCUSSION

8.1 Development of tertiary lymphoid structures in pancreatic islets and salivary glands of NOD mice

Development of TLS is a common feature of several chronic inflammatory diseases (300), and in particular those characterised by organ-specific autoimmunity such as Hashimoto's thyroiditis (307), rheumatoid arthritis (RA) (185, 308-310) and Sjogren's syndrome (SS) (109, 119, 121, 217, 218, 302). It has been known for some time that in these conditions ectopic lymphoid tissues acquire several features characteristic of SLO such as segregation of T and B cells in distinct areas, formation of HEVs, development of FDC networks and expression of lymphoid chemokines CXCL13, CCL19 and CCL21. More recently, it has been demonstrated that these GC-like structures not only recapitulate the cellular organization of SLOs, but are also capable of supporting activation of the molecular machinery responsible for the *in situ* production of high affinity class-switched autoantibodies as demonstrated by expression of AID, ongoing CSR and somatically hypermutated Ig V genes (218, 299, 311, 312). Thus, it is now evident that TLS represent preferential niches for the accumulation and functional activation of Ag-specific autoreactive B cells, contributing to the autoimmune process over and above SLOs.

T1D is an autoimmune disorder where T and B cells gradually infiltrate pancreatic islets (insulitis) eventually leading to the destruction of insulin-producing β cells. In comparison with the above mentioned autoimmune diseases, however, the complexity of obtaining human pancreatic tissue in vivo has hampered a systematic investigation of the prevalence, dynamic evolution, functionality and relevance of TLS in T1D insulitis. Studies of the cellular dynamics involved in the development of autoimmune insulitis have been largely derived from the NOD mouse strain, a robust model of T1D. Studies in this model have revealed that T and B lymphocytes invade the

islets simultaneously (46), progressing from an initial peri-insulitis towards a destructive intra-insulitis.

Furthermore, although T cells no doubt have a fundamental pathogenic role, there is compelling evidence that B lymphocytes play a non-redundant function in the autoimmune process and disease development. B cells are required for the initiation and progression of the insulitis in NOD mice as depletion of B cells completely abrogates the development of autoimmunity in this model (53). In addition, it has been shown that B cells must display autoreactive specificities towards islets associated Ags, i.e. IAA, in the context of appropriate MHC-II alleles in order to prime islets CD4+ T cells (52, 54, 156), suggesting that B cells exert a critical role in Ag presentation, a process which requires an organized lymphoid setting to promote T-B cell interactions (55, 313). The translational relevance of these observations has been recently confirmed in phase II clinical trials showing that B cell depletion achieved with a short course of the anti-CD20 MoAb Rituximab is capable of significantly preserve β cell function over a period of 1 year in patients with newly diagnosed T1D (314). Thus, although a key role for B cells has been identified, the cellular and molecular mechanisms leading to their activation within islet infiltrates are poorly understood.

In the work presented in this thesis I provided the first in depth demonstration that the development of inflammatory infiltrates within pancreatic islets of NOD mice is a highly organized process leading to the progressive acquisition of features of SLOs and the formation of functional lymphoid aggregates. First I used double IF on sequential sections for CD3/B220 and I clearly demonstrated that during the evolution from peri-insulitis to intra-insulitis T/B cell aggregates display progressive acquisition of T/B cell segregation (Fig. 4.1). This process further allows the development of FDC networks, which were exclusively present within the B cell area and required the presence of segregation of T and B cells in distinct areas (Fig. 4.2). Interestingly, despite a similar prevalence of insulitis, the highest incidence of both T/B cell segregation and FDC network formation was observed in NOD mice developing diabetes early (average 14 weeks) as opposed to late onset

diabetic mice, suggesting that evolution of TLS is associated with a more aggressive phenotype. This evidence is the confirmation in an animal model of recent data obtained from post-mortem evaluation of patients with recent-onset T1D in which it was shown that islets with a high degree of destruction and loss of β cell are characterised by a large accumulation of B lymphocytes (315). In addition, this is in keeping with the demonstration in other chronic autoimmune conditions, such as RA, that B cell infiltration and TLS formation are associated with a more severe clinical phenotype and are more likely to be resistant to anti-TNF therapy (316).

Importantly, I also provide original demonstration that the development of TLS in autoimmune insulitis in NOD mice is paralleled by the *in situ* overexpression of a critical set of genes which are known to be master regulators of physiological as well as ectopic lymphoid organogenesis (317). In particular, I clearly showed that the ectopic expression of lymphoid chemokines CXCL13 and CCL19 appeared already at 4 weeks of age and preceded the pancreatic infiltration of immune cells bearing the specific receptors CXCR5 and CCR7 (Fig. 4.4). In addition, the peak of expression of these factors was in keeping with the histological detection of fully formed TLS within the islets. The expression of lymphoid chemokines in embryonic lymphoid organogenesis by subsets of VCAM-1+ICAM-1+LTβR+ mesenchymal cells is critically dependent on the signalling through the LTβR upon binding by the membrane-bound heterotrimeric member of TNF family LTβ (LTα1β2), which, in embryonic development, is provided by CD3-CD4+CD45+IL-7Ra+RANK+ lymphoid inducer cells which also express CXCR5 (203, 318). In keeping with this evidence, I showed that the TNF family members LTα and LTβ are significantly upregulated in NOD mice in parallel with the induction of CXCL13/CCL19, suggesting that the LT/lymphoid chemokine positive feedback loop is active and functional and precedes the development of TLS during the autoimmune insulitis in NOD mice (Fig. 4.4). These data is supported by my observation, which confirms and expands previous evidence in the literature (319), that blockade of LTβ with a LTβR-Ig fusion protein completely prevents diabetes in NOD mice.

The importance of this functional pathway was further strengthen by evidence that the vast majority of B cells infiltrating pancreatic islets express CXCR5 and belong to the subpopulation of follicular B cells as shown by a B220+/IgD+/CD23+/CD21- phenotype (Figures 4.6 and 4.7). This follicular pattern is also consistent with the B cell subpopulation that I could characterize in submandibular gland infiltrates in NOD mice. Conversely, only in occasional large infiltrates it was possible to identify a subset of B220+/IgD-/CD1d+ B cells localizing at the periphery of the B cell area of the insulitis, possibly consistent with a transitional 2/MZ phenotype (320).

It has been recently demonstrated that the Ig V light chain gene repertoire of islet infiltrating B cells is highly diversified and differs from that observed in regional lymph nodes (49), suggesting clonal diversification within islet infiltrates. However, previous work could not rule out the possibility that B cells are pre-diversified elsewhere and simply accumulate during the course of the insulitis. Moreover, formal demonstration that B cells can activate *in situ* the molecular machinery to support SHM/CSR of the Ig genes has been missing. Here, I provide clear evidence, both at mRNA and protein level, that in the pancreas of NOD mice immune cell infiltrates characterised by T/B cell segregation, FDC networks and differentiation of GL7+ GC B cells invariably express AID, the enzyme responsible for SHM and CSR of the Ig genes (230) (Fig. 4.3). AID initiates SHM by introducing single point mutations in WRC (W = A⁄T, R = A⁄G) motifs hot spots within the Ig variable genes, which encode for the antigen-binding region of the antibody (321, 322). In addition, AID regulates CSR via its carboxyterminal region (323) by inducing double-strand breaks and recombination of Ig switch regions of DNA followed by excision of switch circles within the constant heavy-chain (CH) region. As AID is only transiently and exclusively expressed by B cells undergoing SHM and/or class-switching its detection has allowed me to directly demonstrate the capability of TLS to support activation and differentiation of B lymphocytes to antibody producing cells within the pancreatic tissue, a possibility further supported by evidence that TLS are surrounded by CD138+ plasma cells.

Interestingly, by comparing the characteristics of TLS in pancreas and submandibular glands of NOD mice, I could demonstrate several consistencies and differences. It is evident that in salivary glands the expression of AID increases with disease severity and mice age, together with T/B cell segregation, FDC network presence, lymphoid chemokines, B cells survival and proliferating factors expression. On the contrary, the expression of AID at mRNA level that I report in NOD pancreas shows a peak at disease onset and declines together with the loss of antigens and disappearance of infiltrates. Overall, this evidence strongly suggest that *in situ* chronic antigen stimulation is a critical factor which is required to sustain the immune activation within the target tissue which in turn leads to the activation of the lymphoneogenetic programme and the formation of functional niches of autoreactive B cells.

Importantly, I have shown that TLS in the pancreas are surrounded by CD138+ plasma cells, a proportion of which displays immunoreactivity against insulin, strongly indicating that TLS in NOD insulitis provide niches capable of supporting the differentiation of islet-autoreactive B cells and contribute to autoimmunity over and above SLOs. These data are in agreement with similar evidence showing reactivity of isolated B cells towards the same antigen in fluid phase (49).

Overall, my results in NOD insulitis provide strong evidence that supports the following concepts i) TLS progressively evolve in pancreatic insulitis in NOD mice following an extremely tight cellular and molecular organizational programme; ii) this process is associated with a more aggressive phenotype, and iii) once formed, these structures display the capacity to activate the molecular machinery to sustain in situ Ig V repertoire diversification, class-switching and differentiation of B cells into autoantibody-producing cells, iv) upon cessation of antigenic stimulation, due to destruction of the pancreatic islets, overt inflammation subsides and TLS progressively recede.

In parallel with the development of TLS in the pancreas of NOD mice, I also characterized TLS formation in the submandibular glands.

My group recently showed that ectopic lymphoid structures in SS salivary glands acquire functional properties typical of SLOs and are capable of supporting autoreactive B cell activation and autoantibody production as demonstrated by expression of AID and Ig class switching (218). Similarly to T1D, the dissecting of the dynamics of TLS formation in patients with SS is technically and ethically challenging. Thus, I used the NOD mouse model of spontaneous autoimmune insulitis in order to characterize the cellular and molecular basis of autoreactive B cell activation and evolution of functional TLS in the chronically inflamed NOD salivary glands.

In this work I provided the first in depth demonstration that the development of inflammatory infiltrates within sumbandibular glands of NOD mice is a highly organized process leading to the progressive acquisition of features of SLOs and the formation of functional lymphoid aggregates. Similarly to my work on pancreas, I first used double IF on sequential sections for CD3/B220 and I was able to demonstrate that during the development of inflammatory infiltrates T/B cell aggregates display progressive acquisition of T/B cell segregation (Fig. 5.1). NOD infiltrates in submandibular glands displayed progressive features of TLS from week 8, with over 80% of periductal aggregates displaying partial or complete T/B cell segregation, and over 75% of mice developing FDC networks with GL7+ ectopic GCs from week 20. The development of FDC networks was exclusively present within the B cell area and required the presence of segregation of T and B cells in distinct areas, equally to pancreatic insulitis.

The evolution of TLS in the submandibular glands was preceded by the mRNA upregulation of genes regulating ectopic lymphoid tissue organization (317) and function such as lymphoid CKs CXCL13/CCL19 and their specific receptors CXCR5/CCR7, lymphotoxins and B cell survival factors BAFF and APRIL (Figures 5.3 and 5.4).

In particular, ectopic LTα and LTβ mRNA expression appears early in the inflammatory process and CXCL13 is the first lymphoid CK to be upregulated in the salivary glands. This data, consistent with my results in pancreas, suggests that the LT/lymphoid CK positive feedback loop is active and functional and precedes the development of TLS also during the autoimmune sialoadenitis in NOD

mice. In agreement with CXCL13/CXCR5 mRNA expression, B cells infiltrating NOD submandibular glands displayed strong CXCR5 expression and were mostly characterised by a follicular phenotype (B220+/IgD+/IgMlow/CD23+/CD21low) as demonstrated by both IHC and FACS analysis on isolated cells (Fig. 5.6 and 5.7). Finally, functionality of TLS was demonstrated by the abundant expression of AID mRNA and protein within the FDC networks, which paralleled the detection of circulating SS-related autoantibodies.

It is relevant to notice that several important differences distinguish TLS formation during autoimmune insulitis and sialoadenitis in NOD mice. The development of TLS in the pancreas begins earlier, peaks at disease onset and recedes after 25 weeks of age. Conversely, lymphoid neogenesis in the salivary glands starts later and progressively develops with the ageing of the mouse. This suggests that the autoimmune process leading to the formation of TLS progress completely independently in the two compartments and are critically dependent upon the local expression of TLS-related genes for induction and maintenance. Most likely, chronic antigenic stimulation in the salivary glands, fuelled by ductal epithelial cells, which, differently from β cells in the pancreas, display high regenerating and proliferating capacity, is responsible for the long-term over-expression of the LT/lymphoid CK axis and the persistence of functional TLS. In addition, the prevalence, size, degree of lymphoid organization of the lymphocytic aggregates in the salivary glands of late stage NOD mice seems to be significantly greater in comparison with the pancreas. In keeping with this observation, quantitative analysis of LTs, lymphoid CKs and AID demonstrated that indeed salivary glands in the late stage of the disease express significantly higher levels of these factors. Most likely, this phenomenon is strictly dependent on the chronic nature of the sialoadenitis, with the NOD mouse closely mimicking the human disease, which is characterised by late onset and prolonged B cell immune activation compared to T1D.

Regardless of these differences, overall my results contribute to the understanding of the cellular and molecular mechanisms underlying the evolution of functional TLS within the target organs of

T1D and SS. These data strongly support the hypothesis that B cells are activated within TLS and promote an in situ autoantibody response over and above SLO.

A critical question is whether TLS in chronic autoimmune diseases are amenable for targeting by blocking critical pathways regulating the lymphoneogenetic programme. In this regard, my work in the NOD mouse model of T1D and SS has identified this model as a suitable model to test therapeutic strategies aimed at modulating the development and maintenance of TLS and B cell functionality.

In this regard, in this PhD project, I investigated whether inhibition of the LT-β dependent pathway was sufficient to prevent/delay the formation of TLS and the generation of local B cell autoimmunity both in pancreas and submandibular glands of NOD mice by using a LTβR-Ig fusion protein.

The lymphotoxin system is part of the tumor necrosis factor family and is required for lymph node development. Manipulation of this system has provided an invaluable tool for the dissection of processes which are critical not only for lymphoid organ development but also in the maintenance of SLO homeostasis and the formation of ectopic organized lymphoid tissues in chronically inflamed sites. A soluble LTβR-Ig fusion protein has been shown to efficiently block this pathway and is currently being tested in the treatment of several autoimmune diseases (324).

Previous work in male NOD mice, which differently from female NODs are protected from sialoadenitis but susceptible to develop lacrimal gland infiltrates, showed that this treatment was capable of ablating the development of HEVs and the formation of TLS (325)

In this study I carried out a trial in NOD mice in order to inhibit the LT-β pathway as a therapy for both autoimmune insulitis and sialoadenitis. For this purpose, mice were treated relatively late (weeks 12) in order to investigate the capacity of LTβR-Ig to revert rather than prevent the development of TLS. In T1D, after 5 weeks none of the mice treated with LTβR-Ig developed

hyperglycaemia. Importantly, pancreas from these mice displayed mostly normal islets and no inflammatory infiltrates. On the contrary, all the mice treated with MOPC-21 isotype control eventually developed diabetes and most had to be culled or died before the termination of the study. This result confirms previous work demonstrating the efficacy of LTβ blockade in the evolution towards destructive insulitis and prevention of diabetic onset. Because most of the work in NOD mice focused on the modulation of insulitis, I mainly concentrated in the effect of LTβ blockade in the autoimmune sialoadenitis. Strikingly, submandibular glands of NOD mice treated with LTβ-Ig displayed strongly reduced number and size of lymphoid aggregates which were characterised by the virtual absence of lymphoid organization in terms of T/B cell segregation, FDC network formation and expression of AIDcompared to mice treated with the isotype control.

In addition, the ectopic expression of TLS-related genes was significantly dampened following LTβR-Ig, confirming that the lymphoneogenetic programme is critically dependent on the continuous production of LTβ and that its blockade critically impairs the functionality of ectopic GCs. It remains to be established whether inhibition of the LT-β pathway will be effective in the human setting, as it would be expected by the capacity to prevent the formation of TLS and the local activation of autoreactive B cells.

8.2 Tissue modulation of Reg genes characterizes autoimmune insulitis and sialoadenitis in NOD mice and Reg proteins are potential biomarkers of β-cell regeneration in the human disease

A continuous process of tissue remodelling and regeneration is a fundamental feature of the homeostatic response of the target organ of several autoimmune diseases. In type 1 diabetes (T1D) the β-cell mass is in a constant process of death and renewal in order to regenerate the islets damaged by the autoimmune process. The relationship linking inflammation and regeneration during autoimmunity remains elusive. Reg genes, a multigene family discovered using cDNA libraries derived from rat regenerating islets, have been suggested to play an important role in epithelial regeneration not only in the pancreas but also in the salivary glands (SG) of Sjogren's Syndrome (SS) during autoimmune sialoadenitis.

There is evidence that the β-cell mass, affected by autoimmune destruction in T1D, is not static. Strategies for influencing the replication and growth of β cells are therefore important for prevention and the possibility of advances towards therapeutic solutions. The family of Reg genes has been shown to be associated with regeneration of pancreatic islets in several studies (245, 251, 276, 326-328). Reg genes promote β cell growth signal during islet regeneration upon binding to a unique receptor (266). Interestingly, the expression of the Reg receptor EXTL3 is not increased in regenerating islets as compared to normal islets, suggesting that regeneration is primarily regulated by modulation of Reg expression. In this regard, inflammation exerts a pivotal role in regulating Reg gene expression as demonstrated by evidence that Reg genes transcription can be directly mediated by IL-6 upon binding to an IL-6 responsive element in the Reg promoter (272, 329). Despite the large body of evidence in support of the role of Reg in islets regeneration and homeostasis, an in depth characterization of the expression levels of all Reg genes during the

different stages of the autoimmune insulitis is lacking. Likewise, whether pancreatic Reg expression is modulated in physiological conditions with aging is unclear.

In my thesis, by performing a comprehensive and quantitative analysis of the dynamic of Reg genes expression during autoimmune insulitis, I unravelled a novel pattern of regulation for Reg genes both in physiological and pathological conditions. I firstly showed that all Reg genes, except Reg4, are highly expressed in the pancreas of both NOD and Balb/c mice at 4 weeks of age but their expression dramatically decreases to become virtually undetectable by week 8-10 (Fig. 7.2). This strongly suggest that Reg genes exert a pivotal role in pancreas homeostasis at early stages of pancreas development (326), but would normally cease their function in adult life as demonstrated by their lack of expression in Balb/c mice at up to 25 weeks of age.

Conversely, in the autoimmune environment of the adult NOD mouse pancreas, Reg genes display a bimodal pattern of expression, with re-induction in parallel with disease onset, most likely as an attempt to regenerate the β-cells mass following the autoimmune destructive process. In female NOD mice Reg mRNA expression peaked at around 12-14 weeks, in parallel with disease onset, and showed a sustained expression followed by a novel sharp decrease at week 20 and onwards. The reliability of the transcript analysis was strengthened by the histological evaluation of Reg3β and Reg3δ which was coherent with the gene expression results (Fig. 7.2 and 7.3).

In keeping with a pivotal role of inflammation in this *de novo* expression, I could observe a rapid increase in local IL-6 mRNA that preceded Reg gene overexpression and peaked in correspondence of the peak in Reg followed by a progressive and rapid decline, in line with complete disappearance of islets and inflammatory infiltrates and return to normal IL-6 levels (Fig.7.2). Thus, the absence of local inflammation and IL-6 stimuli upon islet destruction could explain the decrease in Reg gene expression in older NOD mice. Another possible explanation for the disappearane of Reg genes, would be that Reg at this point become target of the autoimmune process, consistently with previous evidence both in NOD mice and patients with T1D (273, 278).

Overall, here I provide novel observations on the tight regulation of Reg genes expression in physiological and pathological conditions, further confirming a large body of evidence which strongly supports a pivotal role for Reg genes in regulating the homeostasis of the pancreas and its capacity to respond to insults, either induced by inflammation and/or metabolic demand following β cell destruction. Thus, the degree of expression of Reg genes might constitute a new tool in the evaluation and monitoring of β cell regeneration in T1D.

In this thesis, not only I characterized Reg genes expression in pancreas of NOD mice, also I demonstrated that Reg genes are expressed in other target inflamed tissues. I verified in fact that, not only in pancreas but also in NOD mice salivary glands Reg1 and Reg3γ are highly expressed. Interestingly, Reg3γ has an age- and inflammation-related increase and is also expressed in salivary glands of control mice at a significant lower degree. On the contrary Reg1 peaks at disease onset and is not expressed in salivary glands of control mice. These data provide a strong, although indirect evidence of a regenerating environment in tissues that are being damaged by the autoimmune process. In salivary glands this is the first report demonstrating the expression of a restricted number of Reg genes and their likely implication in the attempt of the gland to regenerate. The evidence that Reg3γ expression increases gradually during the disease evolution, in parallel with the progressive development of inflammatory infiltrates in the salivary glands, reflects the close relationship between inflammation and Reg expression. Overall, the dynamic of Reg expression pattern, showing a close association between disease onset/evolution and *de novo* Reg induction strongly suggests that the target cells are attempting to re-establish their original mass/function following the immune-mediated damage.

These results provide a strong observational base on which it will be possible to build functional studies to investigate the role of individual Regs in regulating the interaction between chronic inflammation and regeneration.

In the next part of my project, following characterization of Reg genes expression in the NOD mouse model of T1D and SS, I evaluated the potential role of Reg1α protein as biomarker of regeneration and damage in patients affected by T1D and SS.

In T1D auto-reactive inflammatory cells recognize antigens on β cells and destroy them via T- and B- cells driven mechanisms (41). Autoantibodies are generated and the autoimmune process, with cell damage and antigen exposure, enhances inflammation within the islets. This damaging effect, together with the inflammatory environment, can stimulate cells to replicate in order to replace destroyed β cells (330).

In my study, I demonstrated that circulating levels of Reg1α are significantly increased in both type 1 and type 2 diabetes compared to age and sex-matched controls. In addition, I showed that circulating Reg1α is not increased in a population of SLE patients included as an autoimmune/inflammatory disease control population. Interestingly, increase in serum Reg1α was observed not only in newly diagnosed type 1 patients, but also, although to a lesser extent, in type 1 patients with long standing disease. Of particular interest, T1D patients with longer disease duration displayed reduced levels of Reg1α and a negative correlation between circulating Reg and disease duration was observed.

The increased expression of Reg1α both in type 1 and type 2 diabetes provides the first confirmation in human subjects of previous extensive work in experimental models of diabetes and pancreas regeneration. In animal models of T1D (i.e. NOD mice) it has been clearly shown that loss of β cells, due to local infiltration of immune cells during the autoimmune process, is responsible for up-regulation of Reg genes expression (245). This effect has been shown to be due to a homeostatic mechanism in the attempt to restore the β cells mass and function, as well as a direct consequence of local inflammation. Thus, my demonstration of increased levels of circulating Reg1α protein in patients with T1D at disease onset is in keeping with the hypothesis that the Reg system is activated in humans upon islets inflammation and autoimmunity. Importantly, progressive

reduction of Reg in patients with long standing T1D would suggest that the reservoir for regeneration of β cells might be lost over time.

Similarly, demonstration of increased levels of Reg1α in patients with T2D, who are characterised initially by high insulin levels and an increased demand for insulin followed by a stress-induced reduction in the β-cell mass (331, 332), is in keeping with the hypothesis that a local up-regulation of Reg genes results from an increased metabolic demand as well as triggered by a depletion of the β-cell mass. In T2D β-cell function declines as disease duration increases (333) so that in patients with long duration at least 30% of them are on insulin therapy.

It is of interest that circulating levels of Reg1α are not raised in patients with SLE, suggesting that inflammation *per se* is not sufficient (i.e. high levels of circulating IL-6) to induce Reg genes overexpression.

Furthermore, I evaluated Reg1α circulating levels in patients affected by SS. Consistently with what I could find in T1D and in the salivary glands of NOD mice, SS patients show raised levels of the protein. This is in agreement with the hypothesis that Reg is induced in tissues target of the autoimmune process and that systemic autoimmunity (i.e. SLE patients) is not sufficient to induce Reg expression.

I was also able to confirm this finding by evaluating the mRNA expression of Reg genes at tissue level in the labial salivary glands of patients affected by SS and detecting Reg3γ mRNA expression in the affected tissue, together with high levels of the Reg receptor EXTL3.

Interestingly, circulating levels of Reg1α were also increased in patients with rheumatoid arthritis and, in preliminary experiments I could show that synovial tissues ad synovial fibroblasts from RA patients express EXTL3, although I could not detect local expression of any Reg gene transcript, possibly suggesting that soluble Regs might exert their role systemically, or alternatively, that additional ligands to EXTL3 exist.

In the final part of my project, I aimed at characterizing the presence of autoantibodies against Reg1α in patients with T1D and T2D. Anti-Reg1α antibodies were recently described in Japanese patients with T1D, with an overall prevalence of 24.9% (278). Here I provide the first demonstration that anti-Reg1α antibodies are present in almost 50% of caucasian patients with T1D, suggesting that the presence of such antibodies is a relatively common feature during autoimmune insulitis. Importantly, T2D patients were consistently negative for anti-Reg antibodies, suggesting that the autoimmune background, rather than the increased expression of Reg genes (also observed in T2D) is responsible for the breach of tolerance against this (auto)antigen.

Consistent with the hypothesis that T1D is characterised by an anti-Reg immune response, previous data in NOD mice demonstrated spontaneous T-cell responses to HIP/PAP (member of the Reg family). These Ag-specific T-cells can home to the pancreatic islets and can transfer disease when co-injected with CD8+ T-cells from diabetic NOD mice (273). In turn, autoreactive T cells can further drive a humoral anti-Reg response as demonstrated by the presence of anti-Reg antibodies in NOD mice (273) as well as in human subjects, as confirmed by my and previous data.

Overall, my data strengthen the hypothesis that a regenerating process, characterised by up-regulation of Reg genes expression with consequent Reg protein secretion, is active in the pancreas and SG of T1D and SS patients, respectively.

The work carried out in this thesis suggests that the processes of destruction and regeneration occurring in chronic autoimmune/inflammatory diseases are strongly interdependent whereby regeneration is induced by the chronic inflammatory process but that, in turn, autoimmunity can be further triggered by the attempt to regenerate and by the potential exposure of novel antigens. In this regard, it is fascinating to speculate that Reg proteins expressed in the attempt to regenerate β cells would become autoantigens and contribute to the autoimmune response in predisposed individuals, which are characterised by circulating anti-Reg1α antibodies. Thus, this vicious cycle may prevent Reg genes from restoring the islet homeostasis and fuel the autoimmune response in a

subset of patients. In this regard, it would be extremely interesting to perform larger prospective studies in order to identify whether subjects with high circulating levels of Reg1α protein at disease onset or absence of Reg1α autoantibodies during the course of the disease may represent a phenotype with higher regenerating capacity. This would potentially allow the study of Reg1α protein and specific autoantibodies as new tools in the evaluation and monitoring of β cells regeneration and autoimmunity.

APPENDICES

Appendix I

Immunohistochemistry

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

0.5M Tris Base, 9% NaCl, pH 7.6:

Trizma base ---------- 61 g

NaCl ----------90 g

Distilled water ----------1000 m

Mix to dissolve and adjust pH to 7.6 using concentrated HCl. Storage at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

Acid Alcohol

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

Tris HCL

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

Store the solution at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary. Levamisole hydrochloride 0.1% is added at the Tris HCL buffer before use to inhibit enadogenous alkaline phosphatase.

AP or HRP Avidin-Biotin Complex (DAKO)

10 μl of Solution 1 were added to 1 ml of TBS pH 7.6, and mixed well. Another 10 μl of solution 2 were added and mixed well. The ABC complex was prepared at least 45' before use.

Appendix II

Molecular Biology Buffers and Reagents

Ethylenediaminetetraacetic acid (EDTA 0.5M pH 8.0)

18.6g EDTA were dissolved in 80ml H_2O. 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, 13.75g boric acid and 10 ml of EDTA 0.5M (pH 8.0) were dissolved in 400 ml of distilled water. Distilled ware was added to the final volume of 500 ml and the solution was stored at room temperature.

Tris Acetic acid EDTA (TAE buffer 5X)

12.1g of Tris base, 2.86 ml of acetic acid and 5 ml of EDTA 0.5M (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 room temperature.

Loading Dye (Bromophenol-blue dye 6X)

300 ml od 30% glycerol and 400 ml of EDTA 0.5M (pH8) were added to 300 ml of deionised H_2O and mixed well. 2mg of bromophenol-blue were added to the solution and dissolved by inversion. Solution was stored at -20C and diluted to working solution at the time of use.

Molecular weight markers (λ Hind III-cut)

The stock solution (1μg/μl) (New England Biolabs, UK Ltd, Hertfordshire, UK) was diluted 1:20 in molecular (nucleic acid, RNAse and DNAse free) water to obtain a working solution of 50ng/μl (New England Biolabs UK Ltd, Hertfordshire, UK). 1μl/lane was usually loaded in each agarose gel.

PUBLICATIONS

Original papers directly related to the work presented in this PhD dissertation:

Astorri E, Bombardieri M, Gabba S, Peakman M, Pozzilli P, Pitzalis C. Evolution of ectopic lymphoid neogenesis and in situ autoantibody production in autoimmune nonobese diabetic mice: cellular and molecular characterization of tertiary lymphoid structures in pancreatic islets. *Journal of Immunology* 2010 Sep 15;185(6):3359-68. Epub 2010 Aug 16.

Astorri E, Guglielmi C, Bombardieri M, Alessandri C, Buzzetti R, Maggi D, Valesini G, Pitzalis C, Pozzilli P. Circulating Reg1α Proteins and Autoantibodies to Reg1α Proteins as Biomarkers of β-Cell Regeneration and Damage in Type 1 Diabetes. *Horm Metab Res.* 2010 Oct 22. [Epub ahead of print]

Astorri E, Bombardieri M, Guglielmi C, Takasawa S, Okamoto H, Pitzalis C, Pozzilli P. A bimodal expression of Reg genes characterizes autoimmune insulitis in the NOD mouse model of type 1 diabetes. *Submitted to journal.*

This work has also been presented at several international meetings:

Astorri E, Guglielmi C, Bombardieri M, Pitzalis C, Pozzilli P. Cellular regeneration in type 1 diabetes: Reg genes family in the NOD mouse animal model. 2008 *SID Congress*, Turin.

Astorri E, Bombardieri M, Guglielmi C, Pitzalis C, Pozzilli P. Beta cell regeneration in Type 1 Diabetes: the role of Reg genes in NOD mice. 2008 *EASD Congress*, Rome.

Astorri E, Bombardieri M, Gabba S, Corsiero E, Barone F, Proctor G, Pitzalis C. Molecular and cellular evolution of functional tertiary lymphoid structures in salivary glands of NOD mice. 2010 *BSR Congress*, Birmingham.

Astorri E, Bombardieri M, Gabba S, Corsiero E, Barone F, Proctor G, Pitzalis C. Molecular and cellular evolution of functional tertiary lymphoid structures in salivary glands of NOD mice. 2010 *EULAR Congress*, Rome.

Astorri E, Bombardieri M, Gabba S, Corsiero E, Barone F, Proctor G, Pitzalis C. Molecular and cellular evolution of functional tertiary lymphoid structures in salivary glands of NOD mice: inhibition of the lymphotoxin-β pathway as a therapy for autoimmune diseases. 2010 *ACR/ARHP Congress*, Atlanta.

Astorri E, Bombardieri M, Gabba S, Peakman M, Pozzilli P, Pitzalis C. Evolution of Ectopic Lymphoid Neogenesis and in situ autoantibodies production in autoimmune diabetic NOD mice: cellular and molecular characterization of tertiary lymphoid structures in pancreatic islets. 2010 *Innochem Meeting*, Berlin.

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