UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

NATURAL IgM SECRETION IN HEALTH AND DISEASE: GENETIC CONTROL AND ROLE IN TYPE 1 DIABETES

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS
ESPECIALIDADE DE CIÊNCIAS BIOLÓGICAS E BIOMÉDICAS

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“Success is a journey, not a destination.
The doing is often more important than the outcome”.

Arthur Robert Ashe, Jr. (1943-1993)

This thesis is dedicated to my husband and children.

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Title

Natural IgM secretion in health and disease: genetic control and role in type 1 diabetes

Abstract

Germline-encoded autoreactive natural antibodies (NAbs) of the IgM isotype are secreted and circulate as a result of basal immune system stimulation, and constitute an important first line of defense against microorganism invasion, bridging the innate and the adaptive immune responses. NAbs are mostly secreted by positively selected B1a cells, and have been claimed to have a protective role against autoimmunity. Nevertheless, NAbs binding to cell surface self-antigens could have implications in the initiation of autoimmunity.

Article I focused on the genetic control of NAbs secretion in healthy mice. Importantly, interferon regulatory factor 4 (*Irf4)*, a transcription factor required for plasma cell differentiation and antibody secretion, was identified as the most probable candidate for the control of homeostatic serum IgM levels in the mouse.

Type 1 diabetes (T1D) is a complex autoimmune disease that develops spontaneously in humans and is pathogenically similar in the non-obese-diabetic (NOD) mouse model. B cells are necessary in the NOD diabetogenic process, and the presence of anti-pancreatic beta cell antibodies is the earliest manifestation of T1D.

Article II revealed that NOD peritoneal cavity B1a cells are more prone to spontaneously secrete NAbs that recognize pancreatic beta cell autoantigens, which could promote T1D either by enhancing professional antigen presentation of islet antigens, by activating the complement cascade or by directly promoting beta cell damage and self-antigens release. The studies reported in article III have explored these possibilities and have proven that NAbs of NOD B1a cells origin could bind and directly induce oxidative stress on pancreatic beta cells. Moreover, these studies have shown that NOD B1a cells have a lower threshold for innate-like stimulation and have established a link between NOD B1a cells properties, NAbs specificities and impact of IgM binding on beta cells physiology. Finally, article IV provides evidence that early treatment with antibodies that evoke NOD B1a cells proliferation and differentiation into IgM secreting cells correlates with T1D precipitation.

In conclusion, this thesis has shown that *Irf4* is a critical player in the genetic network that controls IgM secretion in healthy individuals, and that in the NOD mouse model of T1D, a lower threshold for innate like stimulation of peritoneal cavity B1a cells contributes to a naturally increased state of B1a cells activation and autoreactive IgM secretion, determining the initiation and/or contributing to the fueling of beta cells autoimmunity.

Keywords: Autoimmunity, autoreactive natural antibodies of the IgM isotype, B1a cells, genetic control, innate immune system, interferon regulatory factor 4, non-obese-diabetic mouse, oxidative stress, pancreatic beta cells, type 1 diabetes.

Título

Secreção de IgM natural na saúde e na doença: controlo genético e papel na diabetes tipo 1

Resumo

Os autoanticorpos naturais (NAbs) da classe IgM existem no organismo na ausência de imunização e constituem uma primeira linha de defesa fundamental contra infecções. Os NAbs são secretados maioritariamente por células B1a e a sua ligação a autoantigénios na superfície celular pode ter implicações para a iniciação de autoimunidade. O trabalho descrito no artigo I focou-se na compreensão do controlo genético da secreção de NAbs em murganhos saudáveis. Este estudo identificou o interferon regulatory factor 4 *(Irf4),* um factor de transcrição necessário para a diferenciação de plasmócitos e secreção de anticorpos, como o candidato mais provável para o controlo da homeostasia dos níveis de IgM circulante no murganho.

A diabetes tipo 1 (T1D) é uma doença autoimune complexa que se desenvolve espontaneamente nos humanos e que tem uma patogenia semelhante no murganho NOD (non-obese-diabetic). Os linfócitos B são necessários para o processo diabético do NOD, em que a presença de anticorpos anti-células beta pancreáticas é uma das manifestações mais precoces. O artigo II revelou que as células B1a da cavidade peritoneal do NOD têm uma elevada predisposição para secretarem NAbs que reconhecem autoantigénios de células beta pancreáticas e que podem promover o desenvolvimento de T1D quer pelo aumento da apresentação de autoantigénios, quer pela activação da cascata do sistema de complemento, quer pela indução directa de danos nas células beta pancreáticas. A investigação descrita no artigo III provou que os NAbs secretados por células B1a do NOD têm a capacidade de se ligarem e induzirem stress oxidativo nas células beta do pâncreas. Estes estudos revelaram ainda que as células B1a do NOD têm um limiar reduzido para activação inata e estabeleceram uma relação entre as propriedades das células B1a do NOD, as especificidades dos NAbs e o impacto da ligação de IgM na fisiologia das células beta. Finalmente, o artigo IV evidenciou que a indução de proliferação e diferenciação das células B1a em células secretoras de IgM contribui para o início da T1D.

Esta tese demonstrou que o *Irf4* é um factor de transcrição com um papel fundamental no controlo da secreção de IgM em animais saudáveis e que, no murganho NOD, as células B1a da cavidade peritoneal têm um menor limiar para estimulação inata, que contribui para o seu estado de activação e para a secreção de IgM autoreactiva, determinando a iniciação e/ou contribuindo para a progressão da diabetes tipo 1.

Palavras-chave: Autoanticorpos naturais da classe IgM, autoimunidade, células B1a, células beta pancreáticas, controlo genético, diabetes tipo 1, interferon regulatory factor 4, murganho non-obese-diabetic, sistema imune inato, stress oxidativo.

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List of abbreviations

AAb	Autoantibodies
AAg	Autoantigens
AICD	Activation induced cell death
AID	Autoimmune diseases
AP	Alkaline-phosphatase
APCs	Antigen-presenting cells
ATA	Anti-thymocyte autoantibody
BAFF	B cell-activating factor
BCL1	BALB/c-derived B lymphoma
BCL6	B-cell leukemia/lymphoma-6
BCR	B cell receptor
BLIMP1	B lymphocyte inducer of maturation program 1
BLYS	B cell-activating factor
BSA	Bovine Serum Albumin
Btk	Bruton's tyrosine kinase
CLP	Common lymphoid progenitors
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
ds-DNA	Double-stranded DNA
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FCγR	Fcγ-Receptor
FO B cell	Follicular B cell
GAD	Glutamate decarboxilase
GC	Germinal center
hCD20	Human CD20
HEL	Hen-egg lysozyme
HSP70	70 kilodalton heat shock protein
IA-2	Islet cell antigen 512
ICOS	Inducible T-cell costimulator
IDDM	Insulin dependent diabetes mellitus
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgH	Immunoglobulin heavy chain
IgJ	Immunoglobulin joining segments
IgL	Immunoglobulin light chain
IgM	Immunoglobulin M
IgMSC1	IgM secreting cells locus 1
IL10	Interleukin-10
INF	Interferon
iNOS	Inducible nitric oxide synthase
IP	Intra-peritoneal
Irf	Interferon regulatory factor
Irf4	Interferon regulatory factor 4
Irf5	Interferon regulatory factor 5
κB elements	kappa-light-chain-enhancer of activated B cells
LOD	Logarithm of odds
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAP kinases	Mitogen-activated protein kinases

MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MHC-II	Major Histocompatibility Complex of Class II
Mitf	Microphthalmia-associated transcription factor
MyD88	Myeloid differentiation factor 88
Mz B cell	Marginal zone B cell
NAbs	Natural antibodies
NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese-diabetic
NZB	New Zealand Black
NZB/W	New Zealand Black/ New Zealand White F1
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PAX5	Paired box gene 5
PCA	Principal component analysis
PKCβ	Protein kinase C β
PRRs	Pattern recognition receptors
QTL	Quantitative trait *loci*
RA	Rheumatoid arthritis
Rag	Recombination activating genes
SD	Standard deviation
SCID	Severe combined immunodeficient
SHP-1	Src homology phosphatase-1
Siglec	Sialic acid–binding, immunoglobulin-like lectin protein
SLE	Systemic lupus erythematosus
SPF	Specific pathogen free
ss-DNA	Single stranded-DNA
STAT3	Signal transducer and activator of transcription 3
T1D	Type 1 diabetes
TdT	Terminal deoxynucleotidyl transferase
TFh	Follicular helper T cells
Tg	Transgenic
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
Th17	Type 17 helper T cell
TIR	Toll/Interleukin-1 receptor
TLRs	Toll-like receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β
XBP1	X-box binding protein-1
VDJ	Variable, Diverse, and Joining gene sequences
VH genes	Variable genes of immunoglobulin heavy chain

List of publications

The work developed under the scope of this thesis resulted in the following publications and manuscripts, which will be referred by their roman numerals:

I. Côrte-Real, J., Rodo, J., Almeida, P., Garcia, J., Coutinho, A., Demengeot, J. & Penha-Gonçalves, C. *Irf4* is a positional and functional candidate gene for the control of serum IgM levels in the mouse. Genes Immun. 10, 93-9 (2009).

II. Côrte-Real, J., Duarte, N., Tavares, L. & Penha-Gonçalves, C. Autoimmunity triggers in the NOD mouse: a role for natural auto-antibody reactivities in type 1 diabetes. Ann. N. Y. Acad. Sci 1173, 442-448 (2009).

III. Côrte-Real, J.*, Duarte, N.*, Tavares, L. & Penha-Gonçalves, C. Innate stimulation of B1a cells enhances the autoreactive IgM repertoire in the NOD mouse: implications for type 1 diabetes (revised and accepted for publication in Diabetologia on the 17th of January 2012).

IV. Duarte, N.*, Côrte-Real, J.*, Tavares, L., Parkhouse, M. & Penha-Gonçalves, C. p58 surface glycoprotein is downregulated in NOD B1a cells and its targeting precipitates type 1 diabetes in the NOD mouse (manuscript).

* These authors contributed equally to the work.

Chapter I. General Introduction

1. Immune surveillance by natural IgM

The study of antibodies started in 1900, when Paul Ehrlich discovered that animals could be immunized against diphtheria and that the resulting animal antisera could be used to protect children against this disease (Ehrlich, 1900a; Ehrlich, 1900b). Ehrlich assumed that molecules in the sera of infected animals recognized and eliminated bacterial particles and he predicted that the immune system would not only eliminate microbial invaders, but would also inhibit the growth of tumors, by providing antibodies against malignant cells (Ehrlich, 1902). These theories were explored by Burnet, seven decades later, who first defined the immune surveillance concept as "*an evolutionary necessity"* (Burnet, 1970), and nowadays it is well established that nature has created an effective immune program in which antibodies are main players in mediating critical immune functions in vertebrates.

1.1. Innate and adaptive immune responses

An immune reaction is composed of an inherited, unspecific, immediate response and of a secondary one, which is adaptive and as so specific, that includes the generation of memory cells. The recognition system of the innate response works through a set of germ-line encoded pattern recognition receptors (PRRs), prominently toll-like receptors (TLRs) (Medzhitov, 2001). These receptors recognize specific patterns of antigens, termed pathogen-associated molecular patterns (PAMPs), like carbohydrates on glycoproteins and glycolipids, and repetitive structures as those found in bacterial lipopolysaccharide (LPS) and nucleic acids (Akira, Uematsu, & Takeuchi, 2006; Hemmi et al., 2000; Janeway & Medzhitov, 2002; Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003; Medzhitov & Janeway, 2000; Pasare & Medzhitov, 2005). Importantly, some of the factors released by damaged or dying cells, termed damage-associated molecular pattern molecules or DAMPs, also interact with TLRs, meaning that TLR activation may occur even in the absence of invasion by pathogens (Rubartelli & Lotze, 2007). As an example, TLR4 is part of the receptor for the bacterial product LPS, but also responds to the endogenous cellular molecule 70 kilodalton heat shock protein (Hsp70) and to the extracellular breakdown products of hyaluron, while TLR9 binds to DNA unmethylated CpG sequences that, although in different proportions, are found in all living creatures (Matzinger, 2002). This extremely important recognition system guarantees that a rapid immune response may be initiated upon the detection of structures that are most likely involved in primary cell stability and cell preservation mechanisms.

TLRs are expressed on various immune cells, including macrophages (Hausmann et al., 2002; Kim et al., 2009), dendritic cells (DCs) (Iwasaki & Medzhitov, 2004), B cells (Genestier et al., 2007), T cells (Kulkarni, Behboudi, & Sharif, 2011), and even on non-immune cells such as epithelial cells (Abreu, Fukata, & Arditi, 2005). The expression of TLRs is not static, but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stimuli. Furthermore, TLRs may be expressed extra- or intracellularly. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes, and their ligands require internalization to the endosome before signaling is possible (Akira et al., 2006). TLR stimulation recruits adaptor proteins containing a Toll/Interleukin-1 receptor (TIR) domain, such as myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Akira & Takeda, 2004), resulting in the triggering of downstream signaling cascades that rapidly induce the expression of genes involved in the inflammatory and immune responses. As an example, MyD88 signaling pathway modulates the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein (MAP) kinases and the transcription factor interferon regulatory factor 5 (Irf5), leading to the production of pro-inflammatory cytokines (Akira et al., 2006; Takaoka et al., 2005). While the activation of the TRIF signaling pathway induces type-I interferon (IFN) production and pro-inflammatory signals, leading to DCs maturation, expression of co-stimulatory molecules, and IFN-α/β secretion (Akira et al., 2006; Hoebe et al., 2003; Hoshino, Kaisho, Iwabe, Takeuchi, & Akira, 2002; Kaisho, Takeuchi, Kawai, Hoshino, & Akira, 2001; Yamamoto et al., 2003). Upon TLR activation the source of infection can be immediately eliminated by macrophage and DC mediated phagocytosis (Blander & Medzhitov, 2004) or neutralized by immunoglobulins of the IgM isotype, secreted mostly by B1a and marginal zone B cells (Genestier et al., 2007). Furthermore, activated antigen-presenting cells (APCs) contribute to the initiation of the adaptive immune response by migrating from the site of stimulation to the regional lymph nodes, where they express co-stimulatory molecules and present antigens to naïve $CD4^+$ T cells, inducing their activation (Akira, Takeda, & Kaisho, 2001; Medzhitov, Preston-Hurlburt, & Janeway, 1997). Consequently, the interaction of activated $CD4^+$ T cells with B cells in the germinal centers (GC) determines the increase of the B cell receptor (BCR) affinity for the cognate antigen by somatic hypermutation of their variable region (V) exons and change of their immunoglobulin (Ig) heavy chain (IgH) class by Ig class switch recombination from IgM to IgA, IgE or IgG. GC B cells with high-affinity and class-switched BCRs can then develop either into memory B cells or antibody-secreting plasma cells, fundamental components of protective adaptive immunity (LeBien & Tedder, 2008; Oracki, Walker, Hibbs, Corcoran, & Tarlinton, 2010; Radbruch et al., 2006). Therefore, the innate response triggered upon TLR

activation is a key element in inflammation as well as in the initiation of adaptive immunity against invading pathogens (Akira et al., 2006).

1.2. Natural IgM bridges the innate and the adaptive immune system

Antibodies of the IgM isotype perform a wide range of actions in the immune system having an important role in the primary defense mechanisms as suggested by the high concentration of IgM found in human cord blood, germ free mice and healthy individuals in the absence of immunization (Avrameas, 1991; Casali & Schettino, 1996; Coutinho, Kazatchkine, & Avrameas, 1995; Pereira et al., 1986; Tlaskalová-Hogenová et al., 1992). These so denominated "natural antibodies" (NAbs) are characterized by low antigen affinity, relatively high avidity and polyreactivity, providing the organism with a first line of defense against the invasion by foreign microorganisms (Boes, Prodeus, Schmidt, Carroll, & Chen, 1998). NAbs are mainly encoded by germline V gene segments and have a low percentage of nontemplated nucleotide (N)-additions. This lack of somatic mutations results in a restricted repertoire of reactivities and consequently NAb recognize phylogenetically conserved structures, such as nucleic acids, heat shock proteins, carbohydrates, and phospholipids (Carmack, Shinton, Hayakawa, & Hardy, 1990; Gu, Förster, & Rajewsky, 1990; Kantor & Herzenberg, 1993; Hardy & Hayakawa, 1994).

Natural IgM has been claimed to bridge the innate and the adaptive immune system, since the binding of IgM to previously unfamiliar antigens may mediate their direct neutralization and also contribute to the mounting of affinity-matured IgG responses to T cell-dependent antigens (Ehrenstein, O'Keefe, Davies, & Neuberger, 1998). Importantly, antigen-IgM complexes potently induce the activation of the complement pathway (Brown et al., 2002), an event that is central to the initiation of the adaptive immune response (Fearon & Locksley, 1996; Klaus, Humphrey, Kunkl, & Dongworth, 1980; Pepys, 1972). In fact, a single IgM pentamer can trigger the classical pathway of complement activation as efficiently as a thousand molecules of IgG (Cooper, 1985). Therefore, naturally secreted IgM is able to primarily defend the organism against pathogens to which it is unspecific, while sustaining the first steps of an antigen directed specific immune response.

1.3. Natural IgM regulates the homeostasis of the B cell compartment

Natural IgM has also an important role in the regulation of B cell homeostasis, by controlling their longevity and turnover, as elegantly demonstrated in studies performed on the $S\mu^-$ mouse, which lacks circulating but not B cell membrane bound IgM (Notley, Baker, &

Ehrenstein, 2010). It is well established that BCR signaling is an essential component for B cell survival (Fuentes-Pananá, Bannish, & Monroe, 2004), however while the reduction on basal BCR signaling observed in $S\mu^-$ mice results in impaired mature B cell survival and increased turnover in the spleen, enhanced B cell subsistence is observed in the peritoneal cavity (Notley et al., 2010). As a consequence of these and possibly other regulatory features of natural IgM, follicular B cell (FO) numbers are reduced in the $S\mu^-$ mouse (Baker & Ehrenstein, 2002), while peritoneal B1 cells (Boes, Esau, et al., 1998; Ehrenstein et al., 1998) and splenic marginal zone B cell (Mz) numbers are increased (Baker & Ehrenstein, 2002). These observations allow presuming that different B cell subpopulations have distinct thresholds for BCR responsiveness and survival.

1.4. Natural IgM and autoimmunity

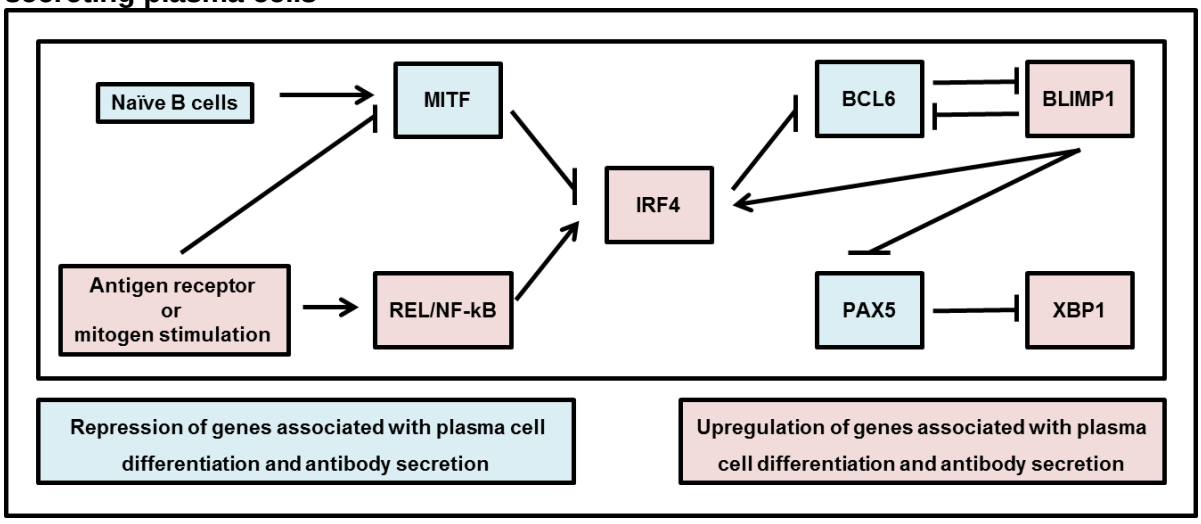
Natural IgM has been shown to bind apoptotic cells/debris and contribute to the clearance of autoantigens from the organism through C1q binding mechanisms (Chen, Park, Patel, & Silverman, 2009; Ogden, Kowalewski, Peng, Montenegro, & Elkon, 2005; Quartier, Potter, Ehrenstein, Walport, & Botto, 2005). Since apoptotic cells provide a potential source of immunogens for the development of autoreactivity, this property has been associated with a protective role for natural IgM against autoimmunity in several models of systemic lupus erythematosus (SLE) (Manson, Mauri, & Ehrenstein, 2005). Nevertheless, it is still controversial whether IgM would always provide the organism with this shielding effect. Given that a high proportion of natural IgM is self-reactive (Casali & Notkins, 1989; Hardy, Carmack, Li, & Hayakawa, 1994; Kantor & Herzenberg, 1993), it is reasonable to expect that the binding of IgM to a surface self-antigen could directly mediate apoptosis of the target cell (Brändlein et al., 2003; Varambally et al., 2004) or result in the activation of the complement cascade, which in turn would contribute to a pathogenic autoantibody response. Furthermore, it has been claimed that highly abundant IgM autoantibodies can rescue the development of autoreactive follicular and marginal zone B cells, by masking tolerogenic self-antigens (Ferry, Crockford, Leung, & Cornall, 2006). Hence, it is plausible that, in certain instances, natural autoreactive IgM would have a role in the predisposition to autoimmunity.

2. Genetic control of natural IgM secretion

The capacity for immunoglobulin secretion is acquired in the process of terminal B-cell differentiation to plasma cells (Calame, Lin, & Tunyaplin, 2003) and requires the expression of the transcription factor interferon regulatory factor 4 *(Irf4)* (Klein et al., 2006), a member of

the interferon-regulatory factor family (Mamane et al., 1999) that is required for lymphocyte proliferation (Mittrücker et al., 1997) and has an inhibitory role on interferon-induced transcription (Yamagata et al., 1996). *Irf4* expression is restricted to cells of the immune system (Marecki & Fenton, 2002; Pernis, 2002), playing a critical role in both early (Mittrücker et al., 1997) and late B-cell differentiation (Klein et al., 2006). Moreover, the role that *Irf4* plays in the differentiation of plasma cells is complex, highly regulated, and interdependent on the sequential expression of several transcription factors that are also crucial for the generation of antibody secreting cells (Figure I.1).

Figure I.1 IRF4 upregulation initiates the differentiation of mature B cells into antibody secreting plasma cells



The co-expression of MITF, BCL6 and PAX5 maintains naïve B cells in a resting state while, upon B cell stimulation, the activation of the REL/NF-κB pathway initiates the transcription of IRF4 and consequently induces the expression of BLIMP1 and XBP1, leading resting B cells to a plasma cell fate. Induction: (→); Inhibition: (⊣).

2.1. IRF4 plays a central role in the differentiation of plasma cells and in antibody secretion

Irf4 transcription is induced by antigen receptor or mitogen stimulation (Sciammas et al., 2006), is dependent on the binding of complexes of the transcription factors *Rel/NF-κB* to two kappa-light-chain-enhancer of activated B cells (κB) elements located in the *Irf4* promoter (Grumont & Gerondakis, 2000) and is repressed by the microphthalmia-associated transcription factor (*Mitf*), which is highly expressed on naive B cells, antagonizing the process of terminal B cell differentiation (L. Lin, Gerth, & Peng, 2004). When expressed, the IRF4 transcription factor binds to a motif in the Ig light (IgL) chain gene enhancers $E_{\kappa 3'}$, $E_{\lambda 2\text{-}4}$, and $E_{\lambda 3\text{-}1}$, playing an essential role in immunoglobulin production (Eisenbeis, Singh, & Storb,

1995). Also, IRF4 directly downregulates the expression of *Bcl6 (*B-cell leukemia/lymphoma-6), a transcription factor that is necessary for the germinal center events to occur (Saito et al., 2007), inhibiting its repression effect on *Prdm1* (the gene encoding B lymphocyte inducer of maturation program 1 or BLIMP1). BLIMP1 is a master regulator of terminal B cell differentiation whose upregulation represses the expression of paired box gene 5 (*Pax5*), allowing the transcription of genes in the immunoglobulin joining segments (IgJ) as well as in the IgH chain and IgL chain *loci* (K.-I. Lin, Angelin-Duclos, Kuo, & Calame, 2002; Shaffer, Peng, & Schlissel, 1997; Singh & Birshtein, 1996; Rinkenberger, Wallin, Johnson, & Koshland, 1996). *Pax5* expression is required to maintain B-cell identity (Cobaleda, Schebesta, Delogu, & Busslinger, 2007), it directly represses the transcription of components of the antibody-secreting-cell pathway such as genes encoding for IgJ (Rinkenberger et al., 1996) and *Xbp1* (X-box binding protein-1) (Reimold et al., 1996), which are crucial for immunoglobulin secretion and plasma cell development (Reimold et al., 2001; Shaffer et al., 2004). Once upregulated, *Prdm1* also inhibits *Bcl6* (Tunyaplin et al., 2004), and induces the expression of *Irf4* (Kallies et al., 2004*;* Sciammas & Davis, 2004) and *OBF1*, a B lymphocyte-specific transcription factor (Shaffer et al., 2002; Shaffer et al., 2004) that regulates the transcription and recombination of IgL chain κ genes (Casellas et al., 2002). Furthermore, the switch to expression of mRNA that encodes the secreted form of the μ IgH chain requires BLIMP1 (Shapiro-Shelef et al., 2003). Thus, *Irf4* upregulation initiates a cascade of events that culminate in the differentiation of plasma cells and in antibody secretion (Figure I.1). Importantly, in the context of this thesis (article I), we have identified *Irf4* as the most probable candidate for the control of homeostatic serum IgM levels in the mouse. In this work we have demonstrated that, in the absence of deliberate stimulation, BALB/c mice have significantly higher levels of circulating IgM when compared to C57BL/6. Further, by performing a genome-wide screen and quantitative trait *loci* (QTL) analysis on a C57BL/6xBALB/c F2 generation, we have identified a region on mouse chromosome 13 that contained a *locus* (*loci*) controlling IgM serum levels. Importantly, this genomic region encompassed the *Irf4* locus, and we were able to correlate higher levels of serum IgM with increased differentiation of IgM secreting B cells and *Irf4* expression.

3. B1a cells, the main natural IgM secretors

Different B cell subsets have been characterized in the mouse coelomic cavities and spleen, based on multiple surface markers, origin and functions (Table I.1).

B1 cells are enriched in the peritoneal and pleural cavities, express low surface levels of B220 and CD11b and are classically subdivided into $CD5^+$ B1a and $CD5^-$ B1b subsets (Herzenberg, 2000). Although commonly overlooked, a subset of $CD11b^-CD5^+$ peritoneal

B1c cells has also been recently described (Hastings, Gurdak, Tumang, & Rothstein, 2006). B1a cells derive from fetal liver precursors and spontaneously secrete natural autoantibodies of the IgM isotype (NAbs) (Hayakawa, Hardy, Herzenberg, & Herzenberg, 1985; Herzenberg, 2000), while B1b cells are generated from neonatal bone marrow progenitor cells (Stall, Wells, & Lam, 1996) and can rapidly respond to exogenous antigens (Martin, Oliver, & Kearney, 2001; Ochsenbein et al., 1999). The less studied B1c population appears earlier in ontogeny and is believed to contain both B1a and B1b progenitors (Ghosn, Yang, Tung, Herzenberg, & Herzenberg, 2008) and cells that have not yet fully differentiated into the typical $CD11b^{+}$ B1 phenotype (Hastings et al., 2006). Importantly, $CD19^{+}CD11b^{-}CD5^{-}$ B2 cells with an intermediate functional phenotype between peritoneal B1b and splenic B2 cells are also present in the peritoneal cavity (Hastings, Tumang, Behrens, & Rothstein, 2006).
B1 cells characterized by the $CD11b^{-}IgM^{high}B220^{low}CD5^{+}$ surface phenotype that spontaneously secrete natural antibodies (Holodick, Tumang, & Rothstein, 2010) and that respond quickly to innate stimulation (Fischer et al., 2001) are also found in the spleen, although in lower frequencies and with extensive differences when compared to their peritoneal counterparts (Tumang, Hastings, Bai, & Rothstein, 2004; Wells, Kantor, & Stall, 1994). However, the spleen is mostly enriched for conventional or B2 cell sub-populations that include FO B cells and the less abundant Mz B cells. Splenic B2 cells arise predominantly during adult life (Carey, Moffatt-Blue, Watson, Gavin, & Feeney, 2008) and while $CD23^{+}CD21^{-}$ FO B cells are the main players in the adaptive humoral immune response, the $IgM^{high}CD23^{-/low}CD21^{high}$ Mz B cells, along with B1 cells, are part of the natural immune system response, acting as a first and rapid line of defense against bacterial pathogens (Martin & Kearney, 2002).

3.1. B1a cells are functionally different from conventional B cells

Conventional B cells are predominantly generated in adult life, from bone marrow precursors, through a process of negative selection which ensures that only non-autoreactive B cells exit to the periphery (D. Nemazee, 2000; Seagal & Melamed, 2003). So, during development, B cells that react with self-antigens are removed in the bone marrow by mechanisms that include anergy (Brink et al., 1992; Goodnow, Crosbie, Jorgensen, Brink, & Basten, 1989; Hartley et al., 1991), receptor-editing, particularly on the Ig light chain (Casellas et al., 2001; D. Nemazee, 2000; Yachimovich, Mostoslavsky, Yarkoni, Verbovetski, & Eilat, 2002), and clonal deletion (D. A. Nemazee & Bürki, 1989; D. Nemazee & Buerki, 1989). In contrast, B1a cells develop mostly early in ontogeny and are positively selected through their BCR (Ferry et al., 2006; Hayakawa et al., 1999). The BCRs of B1a cells are encoded by a relatively restricted set of light and heavy chain rearrangements, including heavy chains that lack N

region insertions at the V (Variable)-D (Diverse) and D (Diverse)-J (Joining) gene segment junctions. These phenomena contribute to the maintenance of a limited and germ line programmed repertoire (Kantor, Merrill, Herzenberg, & Hillson, 1997; Rowley, Tang, Shinton, Hayakawa, & Hardy, 2007) throughout adult life, when B1a cells are predominantly maintained by their "self-renewing" properties. In fact, it has been shown that adult B1a cells are able to repopulate the peritoneal cavity of allotype-congenic mice and irradiated mice as efficiently as fetal liver progenitors, while the same was not observed when B cell progenitors from the bone marrow were transferred to the same recipients (Förster & Rajewsky, 1987; Hayakawa, Hardy, Stall, Herzenberg, & Herzenberg, 1986).

Table I.1 Principal features of the predominant B cell subsets in the peritoneal cavity and spleen of the mouse

B CELL SUBSET	LOCATION	ORIGIN	TYPE OF SELECTION	CELL SURFACE PHENOTYPE	FUNCTION
B1a	PERITONEAL CAVITY	Fetal liver	+	$CD19^+$ $CD11b^+CD5^+$	Natural IgM spontaneous secretion
B1b		Bone marrow	+	$CD19^+$ $CD11b^+CD5^-$	Rapid response to exogenous antigens
B1c		Fetal Liver/ Bone Marrow	+	$CD19^+$ $CD11b^-CD5^+$	B1a and B1b progenitors and cells that have not yet fully differentiated into the typical $CD11b^+$ B1 phenotype
B2		Bone marrow	-	$CD19^+$ $CD11b^-CD5^-$	Intermediate between B1b and splenic B2 cells
B1	SPLEEN	Fetal liver	+	$CD19^+CD11b^-$ $IgM^{high}B220^{low}CD5^+$	Spontaneous secretion of natural IgM
Marginal Zone		Bone marrow	+	$CD19^+CD11b^-$ $IgM^{high}CD23^{-/low}CD21^{high}$	Rapid response to exogenous antigens
Follicular		Bone marrow	-	$CD19^+CD11b^-$ $CD23^+CD21^-$	Main players in the adaptive humoral immune response

The location, origin, type of selection, cell surface phenotype and function of peritoneal cavity B1a, B1b, B1c and B2, and of splenic B1, marginal zone (Mz) and follicular (FO) B cells are summarized.

B1a cells differ functionally from conventional B cells in several ways. A distinctive property of B1a cells is the ability to spontaneously secrete IgM in the absence of specific immunization or T cell help (Casali & Schettino, 1996). Therefore, the maintenance of a restricted repertoire of B1a cells throughout life warrants a stable reactivity pattern of circulating natural IgM. Additionally, "resting" B1a cells present antigen to T cells more efficiently than conventional B cells, strongly promoting T cell proliferation, cytokine secretion and the differentiation of naive ($Foxp3^-CD4^+$) T cells into type 1 helper T cells (Th1) and type

17 helper T cell (Th17) pro-inflammatory cells, in marked contrast to conventional antigen-presenting B cells which favor the differentiation of naive ($Foxp3^{-}CD4^{+}$) T cells into $Foxp3^{+}$ regulatory T cells (Zhong et al., 2007). Furthermore, B1a cells display evidence of tonic signaling in a "steady" state, namely due to the constitutive activation of signal transducer and activator of transcription 3 (STAT3) (Karras et al., 1997), extracellular signal-regulated kinase (ERK) and nuclear factor of activated T-cells (NF-AT) (S.-C. Wong et al., 2002). The activation of these molecules occurs normally after BCR stimulation (Healy et al., 1997; Sutherland, Heath, Pelech, Young, & Gold, 1996; Wang, Kurosaki, & Corey, 2007), and has been shown to have implications for cell growth, proliferation and differentiation (Marshall, 1995; Peng, Gerth, Ranger, & Glimcher, 2001; Tarakhovsky, 1997). Thus, it can be hypothesized that their steady state level of activation on B1a cells has implications on the activity of this cell subset in the absence of external immunization.

3.2. BCR signaling on B1a cells physiology

BCR signaling has a double-edged sword role in B1a cell physiology. While robust BCR stimulation induces proliferation in conventional B cells, the same stimulus induces weak proliferative responses in mature B1a cells (Martin & Kearney, 2001). Conversely, during development, B1a cells positive selection requires strong BCR engagement by self-antigen and a certain degree of BCR signaling is also critical for B1a cells survival (Ahearn et al., 1996; Casola et al., 2004; Hayakawa et al., 1999; Leitges et al., 1996; Rickert, Rajewsky, & Roes, 1995; Tsukada, Rawlings, & Witte, 1994). Thus, positively selected B1a cell subpopulation has a naturally high autoreactive potential that is heavily compensated by the incapacity to respond to BCR stimulation.

Surface expressed CD5 controls B1a cell proliferation upon BCR engagement (Bikah, Carey, Ciallella, Tarakhovsky, & Bondada, 1996) and it has been speculated that this molecule does so by recruiting the tyrosine phosphatase Src homology phosphatase-1 (SHP-1), an intracellular negative regulator, to the BCR complex (Tarakhovsky, 1997). CD22 and Siglec-G are two members of the sialic acid–binding immunoglobulin-like lectin protein family (Siglec) that are expressed in a B cell–restricted way and are able to inhibit BCR signaling through mechanisms which are also thought to be SHP-1 dependent (Crocker, Paulson, & Varki, 2007; Jellusova, Wellmann, Amann, Winkler, & Nitschke, 2010; Nitschke, 2009). In fact it has been shown that B1a cells and serum autoreactive IgM accumulate in mice that carry mutations on the *Shp-1* (O'Keefe, Williams, Davies, & Neuberger, 1996; Shultz et al., 1993; Sidman, Shultz, Hardy, Hayakawa, & Herzenberg, 1986), *Cd22* or *Siglec-G* genes (Hoffmann et al., 2007). On the other hand, several studies have clearly shown that BCR signaling is essential for B1a cells survival. Actually, mice deficient on CD19, CD21, Bruton's

tyrosine kinase (Btk) or protein kinase C β (PKCβ) have virtually no B1a cells in their peritoneal cavities (Tsukada, Rawlings, & Witte, 1994). These mice models share a deficient BCR activation signaling pathway since CD19 and CD21 proteins amplify IgM-mediated signaling (Carter & Fearon, 1992; Fearon & Carter, 1995), while Btk and PKCβ are activated upon BCR cross-linking (Leitges et al., 1996; Rawlings & Witte, 1995). Thus, it could be that CD5, SHP-1, CD22 and/or Siglec-G keep the threshold of the antigen receptor mediated activation of B1a cells at a level insufficient to induce their proliferation, but sufficient to provide signals that promote the survival of B1a cells. On the other hand, the expression of CD19 and CD21 might be essential for the amplification of survival signals mediated by the Btk/PKCβ signal transducing pathway (Tarakhovsky, 1997).

The expression of CD5 on B1a cells has a dual regulatory role. While down-modulating the B1a cell responsiveness to BCR mediated signaling (Bikah et al., 1996), and by this way controlling the production of a potentially autoreactive antibody repertoire, CD5 favors the secretion of interleukin-10 (IL10) by this cell subset (Gary-Gouy et al., 2002). In fact, B1a cells are major secretors of IL10 (O'Garra et al., 1992), a cytokine that contributes to the balance of type 1 and type 2 helper T cell (Th1/Th2) responses, that inhibits pro-inflammatory cytokine production by monocytes and macrophages, and that controls the proliferation of antigen-specific $CD4^+$ T cells by suppressing the antigen presenting capacity and decreasing co-stimulatory molecule expression by professional antigen-presenting-cells (DiLillo, Matsushita, & Tedder, 2010). Thus, through the secretion of IL10, B1a cells contribute to the orchestration of cellular immune responses involved in inflammation and autoimmunity.

3.3. B1a cells response to innate-like stimulation

CD5 expressing B1a cells respond poorly to BCR stimulation, but their activation status, proliferation, antibody secretion capacity and migration is strongly influenced by the gut microflora modifications and by innate-like stimulation through TLR (Alam et al., 2011; Alam, Valkonen, Palagani, et al., 2010; Genestier et al., 2007; Gururajan, Jacob, & Pulendran, 2007; Ha et al., 2006). B1a cells express several TLRs, which play a central role as sensors of infection and inducers of innate and adaptive immune responses (Kiyoshi Takeda, Kaisho, & Akira, 2003). Additionally, peritoneal B1 cells express CD9 (Won & Kearney, 2002) a membrane protein of the tetraspanin superfamily that is known to regulate cell motility (Hemler, 2005), and high levels of α4, α6, and β1 integrins that are involved in cell adhesion. Importantly, it has been shown that TLR stimulation of peritoneal B1 cells results in proliferation, differentiation to antibody secreting cells and the coordinated downregulation of integrin-CD9, resulting in a rapid and efficient mobilization of these cells from the peritoneal

cavity to other lymphoid organs (Ha et al., 2006). B1 cell migration from the peritoneal cavity was also shown to be reduced in germ-free mice, when compared with mice kept under specific pathogen free conditions that harbor a diverse gut microbiota (Ha et al., 2006). So, it is feasible that TLR signals control B1 cell recruitment and participation not only in acute responses, but also in steady-state conditions for the maintenance of immune system homeostasis.

Further, B1a cells are involved in the production of gut IgA, that is generated through a primitive T independent and follicular-independent pathway, and play an important role in the regulation of bacterial communities in the intestine (Fagarasan et al., 2002; Fagarasan, Kinoshita, Muramatsu, Ikuta, & Honjo, 2001; Macpherson et al., 2000; Suzuki et al., 2004). It is thus tempting to speculate that B1 cells surveying the abdominal cavity may be sensing the gut microbiota through TLRs, which are constantly contributing to their functional status. Importantly, TLRs can recognize both exogenous (PAMPs) and endogenous (DAMPs) molecules. Therefore, it can be speculated that peritoneal cavity B1a cells TLRs stimulation could occur either by gut derived microbial signals or ligands resulting from damaged or dying cells, and be instrumental in their secretory functions.

3.4. B1a cells across animal species

Notably, human B1a cells, with the capacity for spontaneous IgM secretion, efficient T cell stimulation, and tonic intracellular signaling, have recently been identified in both the umbilical cord and the adult peripheral blood. These cells are largely $CD5^+$ and were characterized according to their cell surface phenotype as $CD20^+CD27^+CD43^+CD70^-$ cells (Griffin, Holodick, & Rothstein, 2011). Thus, studies of B1a cells in mice may provide insight on human B1a cells properties and immunological roles, since they widely share cell surface markers and functional properties.

$CD5^+$ B cells have also been described in cattle, sheep, pigs, rabbits and chickens, where important species-related differences were found. In contrast with the relative low numbers (15–30%) of CD5 expressing B cells existing within the human (Griffin et al., 2011), mouse (chapter VI, article III) and pig B cell populations (Wilson & Wilkie, 2007), CD5 is expressed at low levels on all B-cells in the rabbit (Raman & Knight, 1992), chicken (Koskinen, Göbel, Tregaskes, Young, & Vainio, 1998) and cattle (Naessens, 1997), while in sheep the percentages of $CD5^+$ B cells may vary tremendously between animals and within the same individual in time (16-82%) (Chevallier et al., 1998).

In cattle and sheep $CD5^+$ B cells share important characteristics with murine B1a cells. Namely, they express low levels of IgD (Naessens, 1997), have a similar tissue distribution and express CD11b on their surface (Naessens & Williams, 1992). Also, rabbit $CD5^+$ B cells

express a restricted repertoire of variable genes of Ig heavy chain (V_H) and have been suggested to be functionally homologous to murine B1a cells (Becker, Suter, & Knight, 1990; Knight & Becker, 1990). On the other hand, functional analysis of CD5 expressing cells has shown that this cell surface marker cannot be used to discriminate B1 and B2 cell subpopulations in pigs (Wilson & Wilkie, 2007). Finally, chicken B cells largely resemble mouse B1a cells, in that they have a generalized expression of CD5, are generated during a limited period of time (in the bursa of Fabricius) and are maintained by self-renewal in adult life (Pink, 1986). Although these reports clearly show that B1a cells are present across animal species, further studies are needed to better understand the properties of this B cell subpopulation in these and other species.

4. Mechanisms of immune tolerance

The immune system has a remarkable capacity to maintain a state of equilibrium. The discrimination of self and non-self-antigens is critical for the mounting of immune responses against invading microorganisms while preventing autoimmunity. In healthy individuals, the absence of immune response against self-antigens is known as immune tolerance or self-tolerance (Daniel, Nolting, & von Boehmer, 2009; Waldmann, 2010). During development, lymphocytes with receptors capable of recognizing self-antigens are constantly being generated. Before immunization, the maintenance of the pool of naïve B cells (Fuentes-Pananá et al., 2004; Lam, Kühn, & Rajewsky, 1997) and T cells (Brocker, 1997; Kirberg, Berns, & von Boehmer, 1997; Rooke, Waltzinger, Benoist, & Mathis, 1997; Takeda, Rodewald, Arakawa, Bluethmann, & Shimizu, 1996) is dependent on antigen receptor–mediated signals. Thus, avoiding autoimmune reactions to continuously presented self-antigens requires effective mechanisms for preventing and terminating lymphocyte responses (Daniel et al., 2009; Waldmann, 2010) (Table I.2).

4.1. Immune tolerance in the B cell compartment

4.1.1 Conventional B cells

During adult life, B lymphocyte lymphopoiesis occurs mainly in the bone marrow where the development of self-reactive B cells has been shown to be controlled by the mechanisms of clonal deletion (D. A. Nemazee & Bürki, 1989; D. Nemazee & Buerki, 1989), anergy induction (Brink et al., 1992; Goodnow et al., 1989; Hartley et al., 1991) and receptor-editing (D. Nemazee, 2000; Casellas et al., 2001; Yachimovich et al., 2002).

In transgenic mice systems, antigen specific B cells can develop in the absence of their corresponding antigen, accumulate in peripheral lymphoid organs and produce antigen specific immunoglobulins. On the other hand, when the antigen to which B cells react to is present in the system, specific B cells and antibodies are absent from the periphery from the same transgenic mice. Further, B cells bearing a low level of membrane immunoglobulin with the referred antigen specificity are found in the bone marrow of these transgenic mice, suggesting that clonal deletion of autoreactive cells occurs in the pre-B-cell to B-cell transitional stage of B-cell development (Brink et al., 1992; D. A. Nemazee & Bürki, 1989; D. Nemazee & Buerki, 1989).

In the presence of soluble antigens, BCR down-regulation may be induced in developing B cells, which are rendered anergic and may exit to the periphery (Goodnow et al., 1989). This was verified in Ig-transgenic mice expressing hen-egg lysozyme (HEL)-specific BCRs, where conventional B cells matured and populated the usual microenvironments in peripheral lymphoid tissues in the absence of the antigen, and could be stimulated by HEL to produce antibody or to enhance proliferation responses to LPS stimulation *in vitro*. Conversely, when soluble HEL was encountered *in vivo* as a self-antigen, B cells expressing HEL-specific BCRs were rendered tolerant. Importantly, clonal deletion of Ig-transgenic B cells expressing HEL-specific BCRs occurred when HEL was recognized as a membrane-bound self-antigen (Brink et al., 1992).

In response to self-antigens, developing B lymphocytes can also modify their receptors through secondary Ig gene rearrangements in a process called receptor editing. This mechanism was observed in mice transgenic for the 3–83 antibody where, in the absence of its specific antigen, B cells in the periphery were numerous and expressed almost exclusively transgene-encoded BCRs (Tiegs, Russell, & Nemazee, 1993). However, when cognate antigen was introduced in the system, peripheral B cells were reduced in number, and lacked self-reactivity. These peripheral B cells retained the expression of the 3–83 Ig heavy chain, but not the 3–83 Ig light-chain. Furthermore, transgenic B cells in the bone marrows of antigen-expressing mice, expressed high levels of recombination activating genes (*Rag-1* and *Rag-2)* mRNA, and manifested rearrangements at their endogenous Ig light-chain *loci*. These findings suggest that autoantigen binding by immature bone marrow B cells could re-induce or prolong Ig light-chain gene rearrangements, allowing a cell to alter its autoreactive specificity and escape death (Tiegs et al., 1993). Independent evidence for receptor editing was obtained from transgenic mice carrying IgH+L chain genes specific for double-stranded DNA (ds-DNA) (Gay, Saunders, Camper, & Weigert, 1993).

Immature B cells, the end products of central B cell maturation, exit the bone marrow, migrate to the spleen and develop through transitional stages into mature B cells. Different selective pressures that contribute to the maintenance of immune tolerance in the B cell compartment occur in this stage of differentiation. While BCR simulation of early transitional

B cells leads to their arrest in an unresponsive state (Goodnow et al., 1988) or cell death (Norvell, Mandik, & Monroe, 1995), as transitional B cells progress towards the mature stage they gain sensitivity to positive selection pressures, being rescued from BCR induced apoptosis by T-cell help signals (Chung, Sater, Fields, Erikson, & Monroe, 2002). Thus, late transitional B cells that were not eliminated by negative selection pressures, resemble the mature B cells that they give rise to, which are activated and proliferate upon BCR stimulation (Niiro & Clark, 2002; Harwood & Batista, 2008).

Furthermore, studies using the monoclonal B cell receptor 3369 mouse line, specific for the thymocyte Thy glycoprotein, have demonstrated that the strength of BCR crosslinking induced by low-dose self-antigen in the spleen may influence the progression of naive immature B cells. In fact, the default maturation pathway of transitional B cells into follicular B cells may be altered by an increase in the strength of BCR signaling which preferentially directs B cells to mature into the marginal-zone B cell subset (Wen et al., 2005).

4.1.2. B1a cells

The use of transgenic mice systems has shown that during fetal and neonatal life the presence of self-antigen influences positively the fate of B1 cells (Hayakawa et al., 1999; Ferry et al., 2006). When the VH3609 μa heavy chain transgene, specific for the thymocyte Thy glycoprotein, was introduzed into the C.B17 (μb) background, the mice generated had high IgM anti-thymocyte autoantibody (ATA) serum titers detectable as early as at 1 week of age. Transgenic peritoneal cavity $CD5^+$ B1a cells secreted high levels of ATA, while transgene positive B2 cells in the spleen did not. $CD5^+$ transgenic B cells were also present in the spleen, as a minor fraction, and contributed with some ATA secretion. In the same study, the sequencing of the mRNA obtained from hybridomas of sorted B cell sub-populations has shown that while $CD5^-$ B2 cells had a diverse light chain usage, $CD5^+$ cells with ATA specificity used preferentially canonical Vκ21C light chains that lacked nucleotide additions. Furthermore, mice resulting from the cross of VH3609 μa transgenic mice with antigen-deficient Thy-1^- mice were deprived from ATA secreting B1a cells, proving that the presence of self-antigen is essential in the development of this B cell subset (Hayakawa et al., 1999). The antigen dependent positive selection of peritoneal cavity B1a cells was also suggested in an anti-erythrocyte BCR transgenic model of autoimmune hemolytic anemia (Okamoto et al., 1992), and was later validated by studies using transgenic mice carrying HEL specific B cell receptors (Ferry et al., 2006).

At present there are two alternative hypotheses to explain the origin of $CD5^+$ B1a cells. The lineage hypothesis, first proposed by the Herzenberg laboratory, holds that B1a pre-committed precursors exist in fetal tissues, such as the liver and the omentum (Hayakawa et

al., 1985; Kantor & Herzenberg, 1993; Kantor, Stall, Adams, Herzenberg, & Herzenberg, 1992). On the other hand, the specificity hypothesis (Cong, Rabin, & Wortis, 1991; Haughton, Arnold, Whitmore, & Clarke, 1993) proposes that any naïve B cell may be induced to become CD5 positive by antigen-induced surface Ig cross-linking. The later hypothesis is supported by the fact that, under some circumstances, B2 cells can assume a B1a-like phenotype, that includes the expression of CD5 (Wortis, Teutsch, Higer, Zheng, & Parker, 1995). However, this phenotype switch rarely occurs under normal circumstances (Hamilton & Kearney, 1994; Kantor & Herzenberg, 1993) and several studies have demonstrated the existence of different progenitors for the B1 and the B2 cell lineages (de Andrés et al., 2002; Fossati, Kumar, & Snoeck, 2010; Tung, Mrazek, Yang, Herzenberg, & Herzenberg, 2006). Consequently, the physiological relevance of the specificity hypothesis is currently questioned, while the lineage hypothesis is generally accepted.

B1a cells are generated mostly during fetal development and neonatal life (Hayakawa et al., 1999), and their maintenance in adult life is mainly assured by their self-replenishment capacity (Förster & Rajewsky, 1987; Hayakawa et al., 1986). Although widely accepted, this assumption has some inherent contradictions, since the decline of the ability to generate B1a cells during ontogeny is relative rather than absolute. Early studies have shown that adult bone marrow cells can, to a limited extent, give rise to B1a cells in the peritoneal cavity and spleen of severe combined immunodeficient (SCID) mice or of lethally irradiated allotype-congenic mice recipients (Huang, Henry, Iacomini, Imanishi-Kari, & Wortis, 1996; Iacomini & Imanishi-Kari, 1992; Kantor et al., 1992; Thomas-Vaslin, Coutinho, & Huetz, 1992). Moreover, the early precursors of B1a and B1b cells have recently been identified as Lin^- $CD45R^{low\text{-}neg}CD19^+$ cells in the mouse fetal liver and fetal bone marrow, and clearly distinguished from the $CD45R^+CD19^-$ B2 cell progenitors in the adult bone marrow (de Andrés et al., 2002; Tung et al., 2006). Although less abundant, the $CD45R^{low\text{-}neg}CD19^+$ B1 cell precursors have also been shown to persist in the bone marrow after birth (Montecino-Rodriguez, Leathers, & Dorshkind, 2006), and have been suggested to be mostly originated from kit^- common lymphoid progenitors (kit^-CLP) (Fossati, Kumar, & Snoeck, 2010). Interestingly, kit^-CLP have lower expression levels of *Rag* genes and terminal deoxynucleotidyl transferase (TdT), in comparison with CLP (Harman, Northrup, & Allman, 2008; Kumar, Fossati, Israel, & Snoeck, 2008), which could favor the usage of germline encoded IgH Variable, Diverse, and Joining (VDJ) gene sequences on bone marrow originated B1 cells.

Although common B1 precursors exist in the adult bone marrow, a more efficient reconstitution was observed in the B1b than in the B1a peritoneal cavity compartment at this stage. This observation suggested that differences in the fetal and adult microenvironment could influence the fate of B1 cell progenitors (Herzenberg, 2000; Tung et al., 2006). Later studies, from the Herzenberg laboratory, have suggested that CD138 and Major

Histocompatibility Complex of Class II (MHC-II) expression would help to better characterize the early-stage bone marrow B lineage progenitors previously defined. This suggestion came from the observation that the B2 progenitor subset is enriched in $CD138^{high}$ cells, while $CD138^{-}$ cells constitute around 90% of the neonatal subset that gives rise to B1a cells and $CD138^{int}$ cells constitute roughly 50% of the adult precursor subset that predominantly gives rise to B1b cells. Moreover, MHC-II is expressed in the B2 cell lineage progenitors and mature cells but only in B1a and B1b mature B cells. The functional correlates of this pattern of CD138 and MHC-II expression on B cell precursors requires further investigation (Tung et al., 2006).

More recently the Carsetti laboratory has claimed that, before birth, fetal liver B1a precursors may migrate to the spleen, giving origin to peritoneal cavity B1a cells during adult life (Rosado et al., 2009). This finding is sustained by studies from the same laboratory showing that adult asplenic and splenectomized mice have a reduced number of peritoneal cavity B1a cells (Wardemann, Boehm, Dear, & Carsetti, 2002).

Independently of their origin, B1a cells persist from early life and continuously give rise to IgM autoantibodies which, if in abundance, may directly bind antigens on the cell surface and mediate cellular damage (Brändlein et al., 2003; Varambally et al., 2004). Additionally, by masking tolerogenic self-antigens, natural IgM of B1a cell origin may rescue the development of autoreactive conventional B2 cells and predispose to autoimmunity in the later cell compartment (Ferry et al., 2006).

Importantly, the maintenance of peripheral immune tolerance in the positively selected B1a cell subset is guaranteed by the expression of regulatory molecules, such as CD5, SHP-1, CD22 and Siglec-G, which keep the BCR mediated activation of B1a cells below the levels necessary to induce their extensive proliferation and autoantibody secretion, thus preventing autoimmunity (Bikah et al., 1996; Hoffmann et al., 2007; O'Keefe, Williams, Davies, & Neuberger, 1996; Tarakhovsky, 1997).

4.2. Immune tolerance in the T cell compartment

In normal physiological conditions, tolerance mechanisms which avoid autoreactivity in the T cell compartment are present both centrally, in the thymus, and in the periphery. During thymic development, the affinity/avidity of the recognition of self-antigens presented in the context of Major Histocompatibility Complex (MHC) molecules is essential for T cell maturation. If the self-peptide-MHC molecule complex expressed on cortical epithelial cells is recognized with low/moderate affinity/avidity the T cells are positively selected and further mature (Ashton-Rickardt & Tonegawa, 1994; Kisielow & Miazek, 1995; Surh & Sprent, 1994). Yet, if the affinity/avidity to self-peptide-MHC molecule complex is high, the self-reactive T

cells are negatively selected and apoptosis is induced (Ashton-Rickardt & Tonegawa, 1994). The negative selection of thymocytes is mostly mediated by self-peptide presenting bone marrow-derived dendritic cells in the thymic medulla (Surh & Sprent, 1994; Viret, Barlow, & Janeway, 1999). Finally, the absence of recognition of the self-peptide-MHC molecule complex leads to T cells death by neglect (Janeway & Bottomly, 1994; Kisielow & von Boehmer, 1995; Surh & Sprent, 1994).

The presence of peripheral autoreactive T cells in healthy individuals has suggested that central tolerance is not the only barrier against autoreactivity (Lohmann, Leslie, & Londei, 1996; Semana, Gausling, Jackson, & Hafler, 1999). In fact, peripheral tolerance mechanisms that deal with autoreactive T cells that have escaped negative selection in the thymus have been described. They include T cell-intrinsic mechanisms that act directly on the responding cell, such as ignorance, immune deviation and apoptosis, and T cell extrinsic mechanisms that evoke additional cell subsets such as tolerogenic dendritic cells and regulatory T cells (Walker & Abbas, 2002). Clonal ignorance occurs when T cells are irresponsive to self-antigens either because the self-antigens are anatomically sequestered or presented in the absence of co-stimulatory signals needed to trigger the immune response (Zinkernagel, 1996). Immune deviation has been suggested to avoid T cell autoimmunity, by balancing the $CD4^+$ T cell responses between Th1, which have been associated with many cases of autoimmunity (Bradley et al., 1999), and Th2 which have been linked with downregulation of autoimmunity in experimental autoimmune encephalomyelitis (EAE) and type 1 diabetes (T1D) experimental models (Bradley et al., 1999; Young et al., 2000). Activation induced cell death (AICD) of autoreactive T cell clones in the periphery may also occur upon repetitive encounter with self-antigen, and both the ligation of the Fas death receptor to its ligand (Watanabe-Fukunaga, Brannan, Copeland, Jenkins, & Nagata, 1992) and the cytotoxic T-lymphocyte antigen 4 (CTLA-4) protein, a negative regulator of T cell activation, have been suggested to have an important role in this mechanism (Colucci, Bergman, Penha-Gonçalves, Cilio, & Holmberg, 1997; Tivol et al., 1995). Specialized subsets of dendritic cells, named tolerogenic DCs, may also cross-present self-antigens and mediate the suppression of antigen-specific T cell immune responses through the deletion of self-reactive T cell clones (Bonifaz et al., 2002; Mukhopadhaya et al., 2008) or via the induction of regulatory T cells (Gregori, 2011; Maldonado & von Andrian, 2010). Regulatory T cells are characterized by the expression of $CD4^+CD25^+FoxP3^+$ (Sakaguchi, 2005) and, being engaged in the dominant control of self-reactive T cells, give an important contribute to the maintenance of immunologic self-tolerance (Sakaguchi, 2004).

Table I.2 Major immune tolerance mechanisms taking place in the B and T cell compartments of the mouse

LOCATION	B CELL COMPARTMENT	IMMUNE TOLERANCE MECHANISM
Bone marrow	Developing B cells	Clonal deletion Receptor editing Anergy induction
Spleen	Early transitional B cells	Apoptosis induction Anergy induction
Coelomic cavities and spleen	B1a cells	Expression of regulatory molecules
LOCATION	**T CELL COMPARTMENT**	**IMMUNE TOLERANCE MECHANISM**
Thymus	Developing thymocytes	Apoptosis induction Anergy induction
Periphery	Autoreactive T cells	<u>T cell intrinsic:</u> Ignorance Immune deviation Apoptosis
		<u>T cell extrinsic:</u> Tolerogenic dendritic cells Regulatory T cells

The location and the main immune tolerance mechanisms taking place in the mouse B and T lymphocyte compartments are represented.

5. Immune tolerance failure and autoimmunity

The primary function of the immune system is to confer protection of the organism against infection. However, the maintenance of normal immune function is a very intricate process, and the failure to establish and maintain tolerance to self-antigens results in autoimmunity.

The phenomenon of autoimmunity was first described by Paul Ehrlich in 1899, who introduced the term "*horror autotoxicus" to* describe the reluctance of the organism to produce toxic autoantibodies (Himmelweit, 1957). Importantly, autoimmunity and autoimmune diseases are distinct phenomena. A certain degree of autoimmunity is a built-in feature of the immune system and can be shown in healthy individuals (Ermann & Fathman, 2001), while autoimmune diseases are the result of aberrant genetic, environmental, and pathogenic factors that, over variable periods of time, lead to a dysfunction of the immune system characterized by inflammation and attack on the body's own organs, tissues, and cells (Ermann & Fathman, 2001; Marrack, Kappler, & Kotzin, 2001). Among the genetic markers of predisposition to autoimmune disease are specific sets of genes for the MHC

molecules that both shape and regulate the specificity of the adaptive immune response. In addition, genetic variability in a number of other genes that are important in the regulation of immune responses has been associated with the development of autoimmune diseases. The genetic makeup of humans and mice determines not only how the immune system deals with antigenic challenges from the environment, but also how the immune system is regulated to remain tolerant towards self. Certain environmental conditions, such as an infection, failure of regulatory mechanisms and/or an inappropriate immune response to crossreactive self-antigens, may condition the onset of autoimmune disease, leading to self-antigen attack, organ damage and dysfunction (Ermann & Fathman, 2001).

Autoimmune diseases (AID) exhibit a spectrum of clinical features that range from local or organ-specific to systemic diseases. According to their principal clinical-pathologic features, each AID can be classified in two main groups: (1) systemic autoimmune diseases, in which the autoimmune response is directed to antigens which are not organ-specific and thus is a more generalized condition, such as the case of systemic lupus erythematosus (Lipsky, 2001) and rheumatoid arthritis (RA) (Feldmann, 2001); and (2) organ specific autoimmune diseases, as is the case of autoimmune thyroiditis (Weetman, 2001) and type 1 diabetes (Gershwin & Shoenfeld, 2011; Wucherpfennig & Eisenbarth, 2001).

Several of the mechanisms involved in the removal, inactivation or regulation of self-reactive lymphocytes, conferring the property of immune tolerance or self-tolerance, are now understood as described above. However, the conditions under which, and the mechanisms by which, this self-tolerance can break down, giving rise to autoimmunity manifestations and autoimmune diseases remain less clear.

6. Type 1 diabetes in Portugal and in the Western World

The urgency to identify the subtle factors contributing to type 1 diabetes (T1D) onset and progression is compelled by recent statistics indicating that the number of T1D new cases is rising tremendously in European Societies. According to the 2010 Annual Report of the Portuguese National Observatory of Diabetes (Observatório Nacional da Diabetes, 2010), the prevalence of diabetes in Portugal for the year of 2009 was of 12.3% ($983*10^3$ patients) for people between 20 and 79 years of age, corresponding to 7,3% of the total population. Furthermore, incidence studies over the last 7 years have allowed to estimate that around 500 new cases per 100 000 inhabitants arise in our country each year. Of these, roughly 90% are patients with type 2 diabetes and a bit less than 10% are cases of type 1 diabetes. However, T1D incidence is rising, due to yet unknown causes, and since 2000 the number of new cases per year doubled in children and young people till the age of 19, being its current prevalence in this age group of 0.12%. Importantly, the Portuguese picture of type 1 diabetes

mimics its general evolution in Europe, where the number of T1D new cases in children under the age of five is increasing by more than 5% each year and where is predicted that, by 2020, the T1D incidence in this age group has doubled (Patterson, Dahlquist, Gyürüs, Green, & Soltész, 2009).

Type 1 diabetes is a complex autoimmune disease, contributed by multiple genetic and environmental factors that results in the selective destruction of insulin secreting beta cells from the pancreatic islets of Langerhans (Yang & Santamaria, 2003). The absence of insulin secretion in this disease leads to persistent high blood glucose levels which results in general tissue damage and severe complications affecting mostly the kidneys, the eyes, the peripheral nerves and the vascular system. In almost all the so-called "developed countries" diabetes is the major cause of blindness, renal failure and lower limb amputation and one of the leading causes of death due to its implications in coronary disease and cardiac failure (Gordon, 2004; Putaala et al., 2011; Tong et al., 2008).

Beyond the suffering that diabetes provokes in patients and relatives, there are also serious economic implications to diabetic people (salary loss, high expenses on medicines, prolonged sick leave) and the society in general (high costs with health care services, lower productivity of the working population). As an example, the use of drugs for diabetes therapy (dosage/1000 inhabitants/day) in Portugal experienced a 49% raise between 2000 and 2009 and the predicted increase on the sales for these medicines will quadruple by 2020, reaching $858*10^6$ euros. The total costs associated with diabetes in 2009 were equivalent to 0.9% of the Portuguese Gross Domestic Product, and represented 9% of the money spent on Health in Portugal reflecting well the tremendous impact that this disease has on our economy (Observatório Nacional da Diabetes, 2010).

6.1. Type 1 diabetes in veterinary species

In veterinary medicine, diabetes is one of the most frequently diagnosed endocrine disorders in cats and dogs that, although rarely, can also be detected in the equine veterinary practice. While in cats (Rand, 1999) and horses (Durham, Hughes, Cottle, Rendle, & Boston, 2009) the most commonly diagnosed diabetes cases are of the non-insulin-dependent type 2, type 1 diabetes marked by the presence of pancreatic beta cell reactive autoantibodies (Davison, Herrtage, Steiner, Williams, & Catchpole, 2003; Elie & Hoenig, 1995; Hoenig & Dawe, 1992), is the most frequent form of disease in dogs. Interestingly, in both humans, dogs and mice, the major genetic determinants of type 1 diabetes are the major histocompatibility complex alleles on the leukocyte antigen genes (Hattori et al., 1986; Kennedy et al., 2006; She, 1996). Thus, the research effort put on the understanding of mouse type 1 diabetes pathogenesis may benefit both humans and veterinary animal species, particularly the dog.

7. The NOD mouse model of type 1 diabetes

Type 1 diabetes develops spontaneously in humans and other animal species and the pathogenic events leading to its development are largely represented in the non-obese-diabetic (NOD) mouse, an insightful experimental model for the study of this autoimmune disease (Castaño & Eisenbarth, 1990). The genetic architecture of T1D in the NOD mouse and human patients reveals a combination of apparently normal alleles at numerous *loci* that in isolation afford only a small degree of disease susceptibility, but together set in motion a series of immunological events that lead to islet inflammation and overt diabetes. Although strongly contributed by genetic elements, environmental factors, such as the composition of the gut microbiota (Alam et al., 2011) or the exposition to certain viral infections (Horwitz et al., 1998; Hyöty & Taylor, 2002; Jun & Yoon, 2001; See & Tilles, 1995; Serreze, Ottendorfer, Ellis, Gauntt, & Atkinson, 2000; Yang & Santamaria, 2003) may also help to trigger the type 1 diabetes disease process.

7.1. Type 1 diabetes pathogenic events in the NOD mouse

T1D pathogenesis in the NOD mouse is influenced by a variety of defects that interfere with the establishment or maintenance of T cell tolerance (Choisy-Rossi, Holl, Pierce, Chapman, & Serreze, 2004; Kreuwel et al., 2001; Lesage et al., 2002), and can be tracked back to around 3 to 5 weeks of age, when antigen-presenting-cell mediated priming of autoreactive T cells in the pancreatic lymph nodes and the recruitment of mononuclear cells into the pancreatic islets of Langerhans lead to the inflammation phenomena termed as insulitis. The clinical signs of diabetes, due to massive beta cell loss, arise only from 12 weeks onwards on this mouse model and are determined by the selective destruction of insulin secreting beta cells by autoreactive cytotoxic T cells (Yang & Santamaria, 2003). In fact, it has been proposed that diabetogenesis in the NOD requires two checkpoints temporally distinct: (1) initial targeting of islet beta cells and (2) later conversion of nondestructive insulitis into a diabetogenic inflammatory process (André et al., 1996). This two-step model is supported by the gender comparison of type 1 diabetes development in the NOD where, although all mice develop insulitis, the occurrence of diabetes is much higher in females (60-80%) than males (20-30%) (Lampeter, Signore, Gale, & Pozzilli, 1989).

Cytotoxic T cells are the effectors of pancreatic beta cell destruction (Yang & Santamaria, 2003). Nevertheless, several factors have been shown to be involved in T1D pathogenesis. In particular, studies on NOD.Igμ.null mice, completely deficient in B cells, have clearly demonstrated the requirement of this lymphocyte sub-population for the diabetic autoimmune process. In fact, NOD.Igμ.null mice have normal numbers of T cells but have a delay in the

onset and a reduction in the severity of insulitis and are strongly protected from type 1 diabetes development (Akashi et al., 1997; Serreze et al., 1996). Further, the reconstitution of NOD.Igμ.null mice with NOD B cells restores T1D susceptibility (Serreze et al., 1998). It has been previously demonstrated that T lymphocytes from diabetic NOD donors can efficiently transfer disease to neonatal recipients depleted of B lymphocytes (Bendelac et al., 1988). Thus, once generated, diabetogenic T lymphocytes can mediate destruction of pancreatic beta cells in the absence of B lymphocytes. Together these reports suggest that B lymphocytes play an essential role in the initial development and/or activation of beta cell autoreactive T cells in the NOD mouse.

7.2. B cells are important players on type 1 diabetes pathogenesis

Intense investigation on the role that B cells play in NOD type 1 diabetes has shown that B cells capacity to present antigen to T cells is a determinant factor in disease progression. NOD mice with functionally competent macrophages, dendritic cells and T cells, but with a MHC-II deficient B cell compartment were shown to be resistant to the development of autoimmune diabetes. Importantly, the pancreas of these mice presented rare foci of peri-insulitis, suggesting that MHC-II mediated antigen presentation by NOD B cells serves to overcome a checkpoint in T cell tolerance to islet beta cells, after their initial targeting has occurred (Noorchashm et al., 1999). In addition, the introduction of a mutant IgM heavy chain transgene allowing the restoration of B cells on NOD.Igμ.null mice, but not the recovery of circulating antibodies, was only partially able to restore T1D. Importantly, this transgenic construct had a fixed heavy chain reactive to the hapten (4-hydroxy-3-nitrophenyl) acetyl but maintained the potential for selection of a diversity of light chains. Therefore, in theory, albeit at lower levels, these transgenic B cells could still present the relevant diabetes related autoantigens to the autoreactive T-cells (F. S. Wong et al., 2004). Furthermore, NOD BCR specificity has been proven as a determinant factor of B cells pathogenicity in T1D. In fact, NOD mice carrying transgenic (Tg) V_H genes with different potential for insulin binding have different disease outcomes. While VH125.Tg.NOD that produce a repertoire in which 1-3% of mature B cells are insulin specific develop accelerated diabetes, VH281.Tg.NOD mice, with limited insulin binding capacity, are partially protected from T1D (Hulbert, Riseili, Rojas, & Thomas, 2001). Consistently, the presence of a fixed BCR due to the introduction of transgenic Ig molecules specific for the disease irrelevant antigen hen egg lysozyme (HEL) retards the onset of diabetes in NOD mice (Silveira et al., 2002). One of the important aspects of the function of B cells as APCs is the ability to concentrate antigen many fold by virtue of the antigen specificity of their BCR (Rock, Benacerraf, & Abbas, 1984). Thus, these studies prove that the antigen presenting function and BCR specificity of B cells have an

important role in T1D pathogenesis. However, they also leave open the possibility that beta cell reactive autoantibodies may be a part of autoimmune diabetes initiation.

7.3. Natural IgM and B1a cells role on type 1 diabetes development

The earliest manifestation of diabetogenesis is the presence of islet-related autoantibodies, both in pre-diabetic patients and in the NOD mouse (Leslie, Lipsky, & Notkins, 2001). These autoreactive immunoglobulins, of the IgG isotype, recognize beta cell antigens (insulin, glutamate decarboxilase (GAD), islet cell antigen 512 (IA-2)) (Bingley et al., 1997; Kaufman et al., 1992; L. Yu et al., 2000), and are believed to be bystander products of the disease, resulting from the ongoing autoimmune process (Leslie et al., 2001). Thus, it is generally accepted that in the stage of cytotoxic beta cell destruction, the exposure of conventional B cells to high loads of beta cell related autoantigens would lead to the preferential activation of B cells with autoreactive potential. The long-sustained interactions between autoreactive T and B cells would then condition the repertoire of autoantibodies typical of pre-diabetic patients, where the beta cell destruction is already in progress, though clinical signs are not yet present.

The work presented in the articles II, III and IV of this thesis focuses on the role that autoreactive antibodies play in T1D pathogenesis in the NOD mouse. Our studies were encouraged by previous research showing that, before the development of insulitis, antibodies of the IgM isotype bound to pancreatic beta cells surface may be found in the NOD mouse while such antibodies were not detected in non-diabetic C57BL/6 controls (Shieh, Cornelius, Winter, & Peck, 1993). Also, analysis of spleen hybridomas, produced in the absence of immunization, has revealed that the NOD has more spontaneously active B cells when compared to non-diabetes-prone strains. Additionally, this study has shown that the NOD mouse has more cells naturally secreting insulin reactive antibodies with natural IgM properties. This reactivity pattern was detectable already at 4 weeks of age and was maintained throughout life (Thomas, Kendall, & Mitchell, 2002). Consistently, *in situ* hybridization studies by Holmberg and colleagues have clearly shown that NOD mice maintain in adult life the neonatal bias for expression of D-proximal variable genes of Ig heavy chain (V_H), and thus exhibit an antibody repertoire biased to germline-encoded specificities (Andersson, Ekstrand-Hammarström, Eriksson, Overmo, & Holmberg, 1994; Leijon, Freitas, & Holmberg, 1993).

Together these findings suggest that the genetic background of NOD mice determines developmental abnormalities that may favor the generation of B cells that secrete anti-insulin natural antibodies. They also supported our work hypothesis that natural antibodies with pancreatic autoantigen specificity are therefore produced in the NOD from early life and bind

to beta cells, possibly having a triggering effect in the diabetogenic cascade of events that lead to specific beta cell destruction.

Most of the antibodies of the IgM isotype arise from spontaneously secreting B1a cells, independently of germinal center reactions, exogenous stimuli or T cell help (Casali & Schettino, 1996). Importantly, B1a cells have a high potential for autoreactivity and have been shown to be critical for autoantibody development and autoimmune symptoms in the New Zealand Black (NZB) and New Zealand Black/ New Zealand White F1 (NZB/W) mouse models of systemic lupus erythematosus (Steinberg, Smathers, Frederiksen, & Steinberg, 1982; Murakami, Yoshioka, Shirai, Tsubata, & Honjo, 1995). Moreover, B1a cells have been recently proven to be necessary for the insulitis onset in the DO11 x RIP-mOVA transgenic mouse model of T1D, where diabetes is mediated by cytotoxic T cells recognizing pancreatic beta cells antigens (Ryan et al., 2010). Also, in the 125Tg mouse, B1a cells prevail as anti-insulin IgM secretors while B2 cells with the same specificity are anergized (Rojas, Hulbert, & Thomas, 2001). Evidence that B1a cells are augmented in the peripheral blood of T1D patients (Gyarmati, Szekeres-Barthó, Fischer, & Soltész, 1999) and that B1a cells are naturally increasingly activated in the NOD strain (Alam, Valkonen, Ohls, Törnqvist, & Hänninen, 2010), further suggest that B1a cells may be involved in the diabetogenic autoimmune process. These data justified our effort to investigate whether natural antibodies with the ability to bind to pancreatic beta cells are produced by NOD B1a cells and have an impact on beta cell physiology upon binding.

In the article II, published in the context of this PhD thesis, we were able to determine that NOD peritoneal cavity B1a cells are more prone to spontaneously secrete IgM that recognizes pancreatic autoantigens and we set out the hypothesis that soluble, islet-specific, natural antibodies could promote disease either by enhancing professional antigen presentation of islet antigens, by activating the complement cascade or by directly causing the initial tissue damage and islet cell antigens release.

In the article III of this thesis we have investigated these hypothesis and we have established that IgM of NOD B1a cells origin could bind and directly induce stress responses on pancreatic beta cells. Moreover, we have determined that NOD B1a cells have a lower threshold for innate-like stimulation and we have established a link between NOD B1a cells properties, natural autoreactive specificities and impact of IgM binding on beta-cells physiology. Finally, the article IV of this thesis provides evidence that NOD B1a cells proliferation and differentiation into IgM secreting cells contributes to T1D onset.

In summary, B1a cells carrying germ-line-encoded specificities recognizing self-antigens are an inherited component of the immune system that is selected during fetal life and secretes natural IgM, serving as an early line of defense against invading microorganisms. B1a cells are highly regulated by inhibitory molecules that, in healthy individuals, keep their autoreactive potential under control. Nevertheless, in certain autoimmune conditions, as type

1 diabetes, a lower threshold for innate like stimulation of B1a cells contributes to a naturally increased state of B1a cell activation and autoantibody secretion, determining the initiation and/or contributing to the fueling of autoimmunity.

In this PhD thesis the genetic factors controlling IgM concentration in the serum of non-manipulated healthy mice were investigated, as well as the role that B1a cells and natural antibodies play in autoimmunity, by studying their properties in the NOD mouse model of type 1 diabetes.

Chapter II. Objectives

Spontaneous, constitutive secretion of IgM is a key distinct characteristic of B1a cells that accounts, in large extent, for the circulating protective pool of natural antibodies in both mice and humans. However, B1a cells and the immunoglobulins that they produce are potentially autoreactive, so that regulation of natural antibody secretion is a crucial control mechanism in the immune system.

This thesis main objective was to understand the role of natural IgM in health and disease. Thus, the studies were focused on:

(1) Determining the genetic factors controlling natural IgM secretion in healthy mice;

(2) Dissecting the natural antibody secretion profile and the properties of B1a cells in the non-obese-diabetic (NOD) mouse model of type 1 diabetes;

(3) Investigating the impact of autoreactive IgM of B1a cell origin in pancreatic beta cells physiology and type 1 diabetes initiation.

Chapter III. Materials and methods

1. Mice

All mice used were females between 1 and 32 weeks of age, bred and maintained in either conventional or specific pathogen free (SPF) housing facilities at the Instituto Gulbenkian de Ciência. This study included C57BL/6, BALB/c, NOD, NOD.SCID, C57BL/6.H2g7 and NOD.H2b strains as well as mice from the genetic crosses F1 (C57BL/6 X BALB/c) and F2 (C57BL/6 X BALB/c). Blood was collected from the facial vein and urinary glucose levels were measured weekly from 12 to 32 weeks of age with Diabur 5000 test stripes (Roche). Mice were considered to be diabetic after two consecutive positive measures of glycosuria. Euthanasia was performed by CO_2 inhalation or cervical dislocation. The experimental protocols were approved by the competent Portuguese authority (Direcção Geral de Veterinária) and are in accordance with international regulations (U.S. Department of Health and Human Service, Office of Extramural Research, National Institutes of Health, Office of Laboratory Animal Welfare, 1985).

2. Cell lines

BCL1 cells (BALB/c-derived B lymphoma) were kindly provided by Dr. John F. Kearney, University of Alabama at Birmingham, and maintained in RPMI 1640 complete medium (supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 50 mM 2-mercaptoethanol, 100 U penicillin and 100 mg/ml streptomycin, all from Life Technologies).

3. Intra-peritoneal injection

For the assessment of NIM-R7 impact on lymphocyte distribution we have performed 4 weekly intra-peritoneal (IP) injections of 50 μg of antibody diluted in 100 ml of RPMI 1640 (Life Technologies) on NOD female mice starting at 5 weeks of age. Mice were euthanized one week after the last injection and the organs were taken for flow cytometry analysis. For the verification of the NIM-R7 effect on T1D development the same IP injections protocol was started at 3 weeks of age and was followed by two IP injections with 2 weeks of interval.

4. Genetic methods

4.1. Genotyping

Genomic DNA was extracted from mouse tails with standard digestion/precipitation methods. Genotyping was performed using microsatellite DNA markers, polymorphic for the parental strains. Markers were chosen according to their chromosomal position, as given by the Broad Institute for Genome Research (USA), to cover uniformly the whole mouse genome at an average density of 20 cM. The genotypes for each locus were determined by DNA marker amplification using conventional PCR techniques. Amplification products were analyzed in 4% agarose gels (Cambrex BioScience) with ethidium bromide (Sigma) and the allele size was determined using the Eagle Eye II video system (Stratagene).

4.2. Genetic analysis

Quantitative trait locus analysis was performed by using the normal model in the R/QTL software (Broman, Wu, Sen, & Churchill, 2003). This program calculates logarithm of odds (LOD scores) over intervals between linked markers, generating likelihood plots of genetic association with the phenotype across the genotyped chromosomal regions. The genome-wide statistical significance of the results was empirically determined by performing permutation tests (Lander & Kruglyak, 1995) and the QTL confidence interval was calculated using the Bayesian method of R/QTL (Broman & Sen, 2009).

4.3. RNA isolation and real-time PCR

Total RNA from either sorted or cultured cells was obtained using the RNeasy Mini Kit (Qiagen) or the High Pure RNA Isolation Kit (Roche) following the manufacturer protocol for animal cells. RNA was converted to cDNA with a Transcriptor First Strand cDNA Synthesis Kit or a Transcriptor High Fidelity cDNA Synthesis Kit (both from Roche). The following TaqMan Gene Expression Assays with FAM-reporter (Applied Biosystems) were used: *Irf4* (Mm0051634-m1), *Tlr2* (Mm00442346_m1), *Tlr4 (*Mm00445274_m1*), Tlr6 (*Mm02529782_s1), *Tlr7 (*Mm00446590_m1), *Tlr9 (Mm00446193_m1), Fas (*Mm00433237_m1*), Nos2 (*Mm01309901_m1*), Caspase 3 (*Mm01195084_m1*) and Ccl2 (*Mm00441242_m1*).* Gene expression quantification was performed in ABI Prism 7900HT (Applied Byosistems, Foster City, CA, USA). Relative quantification in each real-time PCR

reaction was obtained after normalization for mouse GAPDH (VIC/MGB probe) expression using the $2^{-\Delta\Delta CT}$ analysis method (Livak & Schmittgen, 2001).

5. Immunological methods

5.1. ELISA

Standard indirect ELISA was used to quantify total IgM and anti-insulin IgM in the serum. Total IgM, IgM with reactivity to a pool of T1D related autoantigens (AAg) and IL10 levels were measured in cell culture supernatants after TLR stimulation. Briefly, ELISA plates (Nunc) were coated with IgM, insulin only (Sigma) or a T1D AAg pool of insulin, IA-2 (GenWay Biotech), GAD65 and GAD67 (United States Biologicals). Bound seric IgM was detected by goat anti-mouse IgM-HRP human adsorbed antibody or goat-anti-mouse-IgM-AP conjugated antibody (both from Southern Biotech.) and revealed respectively with the substrate o-phenylenediamine (Calbiochem, Gibbstown, NJ, USA) and H_2O_2 or the sigma phosphatase substrate. Optical densities were read in a spectrophotometer at 490 nm or 405 nm. IgM concentrations were calculated against standard curves obtained with either purified monoclonal IgM (clone 11E10, Southern Biotech.) or anti-insulin IgM kindly provided by Dr. JW Thomas, Vanderbilt University Medical Center (Thomas, Kralick, & Ewulonu, 1997). IL10 concentration was determined with a mouse IL-10 ELISA MAX™ Deluxe kit (Biolegend) according to the manufacturer's instructions.

5.2. ELISPOT ASSAY

The number of IgM-secreting B cells or B cells secreting IgM with reactivity to a T1D related AAg pool of insulin (Sigma) and GAD67 (United States Biologicals) or insulin, IA-2 (GenWay Biotech), GAD65 and GAD67 (United States Biologicals), to thyroiditis associated AAg (thyroglobulin and thyroperoxidase), to the sialitis related AAg muscarinic-3-receptor, to SLE associated AAg (ds-DNA, single stranded-DNA (ss-DNA) and histone) or to AAg typically recognized by B1a cells (phosphatidylcholine, phosphorylcholine and dextran) (Baumgarth, Tung, & Herzenberg, 2005) were determined by ELISPOT ASSAY.

The plates were coated overnight at 4 ºC with goat anti-mouse IgM-human adsorbed (Southern Biotech.) or with the referred antigens diluted in coating buffer (0.05M Na_2CO_3). Cell dilutions were performed in RPMI 1640 complete medium and incubated overnight at 37ºC. When specific IgM reactivity was being measured 1% Bovine Serum Albumin (BSA) was added to the RPMI 1640 complete medium. Non-adherent cells were thereafter removed

by flicking the plate followed by lysis with 0.1% Tween 20 in water. After extensive washing with PBS containing 0.05% Tween 20 the plates were incubated with goat anti-mouse IgM-AP-conjugated antibody (Southern Biotech.), washed again and revealed with BCIP-AMP substrate (2.3 mM of 5-bromo-4-chloro-3-indolyl phosphate diluted in 2-amino-2-methyl-1-propanol buffer, Sigma). After incubation overnight at 4 ºC and washing three times with distilled water, the traces of IgM-secreting cells appeared as blue spots that were counted under the microscope.

5.3. Flow cytometry analysis

Lymphocytes were isolated from peritoneal cavity by peritoneal lavages performed with PBS supplemented with 2% FCS or from spleen by straining through a nylon mesh. Single cell suspensions were then stained according to standard procedures. Fc-receptors unspecific binding was prevented by incubating the cells with unlabeled anti-mouse-Fc-block/CD16/32 (clone 2.4G2, BD Pharmingen). The following antibodies were used for staining: FITC or PE-Cy7-anti-CD19 (clone 1D3), FITC or A647-anti-IgM (clone R33.24.12), PE-anti-CD23 (clone B3B4), Percp or PE-anti-CD5 (clone 53-7.3), Pacific-Blue, Cy5, or APC-anti-CD45R/B220 (clone RA3-6B2), biotinylated or FITC-anti-CD11b/Mac-1 (clone M1/70), FITC-anti-CD21 (clone 7G6), PE-anti-CD43 (clone S7), biotinylated-anti-CD86 (clone GL1), FITC-anti-CD62L (clone Mel14) and biotinylated or PE-anti-Syndecan-1 (clone 281-2). Monoclonal NIM-R7 (anti-p58) antibody (Parkhouse, Santos-Argumedo, Teixeiral, Henry, & Wawrzynczak, 1992) was biotinylated and used for staining. Biotinylated antibodies were revealed by FITC, PercP or APC-Cy7-streptavidin. Traceable latex beads (Beckman coulter) were added for counting cells. Stained cell suspensions were analyzed by flow cytometry either with a FACSCalibur™ (Becton Dickinson) or a FACS CYAN ADP (Dako Cytomation) and the data was respectively acquired with CELLQuest or *Summit* software. Analysis was performed with FlowJo software (TreeStar Inc.).

5.4. Cell purification

Peritoneal or splenic B cell subpopulations were purified using the high speed cell sorter MoFlo (Dako-Cytomation, Berkeley, CA, USA) or the BD FACSAria III cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Cell purity was always higher than 90%.

5.5. “Panama” Blot

Global serum IgM repertoire was evaluated by the “Panama” Blot semi-quantitative immunoblotting technique that allows evaluation of antibody reactivities against large panels of antigens and has been described in detail elsewhere (Haury, Grandien, Sundblad, Coutinho, & Nobrega, 1994; Nobrega et al., 1993). Briefly, protein extracts were prepared and, following electrophoresis, the separated proteins were transferred onto nitrocellulose membranes that where then incubated with serum in miniblot incubation units (Immunetics). Goat anti-mouse IgM-AP conjugated secondary antibody (Southern Biotech.) was used and immunoreactivities were revealed with nitroblue tetrazolium (NBT) and bromo-chloro-indolylphosphate (BCIP) substrates (Promega Corp.). Quantitation of immunoreactivities was performed by densitometry in high resolution reflective mode. A second densitometry was performed after colloidal gold staining (Protogold, Biocell). The irregularities of the electrophoretic migration were corrected by the superimposition of the two stainings and the intensity of reactivity development was adjusted to a standard serum. For all these procedures, specially developed macros using the software IGOR (Wavemetrics) were used. The adjusted reactivity profiles were divided into sections defined empirically for each protein extract and the intensity of the respective serum immunoreactivity was quantified as the mean optical density within these limits. A sample profile thus consisted of a list of reactivity measures, one for each section, and was analyzed by Principal Component Analysis.

6. Cell biology

6.1. Toll-like receptor stimulation

Purified peritoneal B1a cells were cultured in RPMI 1640 complete medium with or without 1-5 μg/ml of purified LPS and with or without 0.5 μM CpG (ODN 1826, Invivogen). On the second day of culture the cells were collected, counted and plated for ELISPOT ASSAY of anti-T1D AAg IgM secreting cells. Supernatants were taken for antibody and IL10 quantification by ELISA at day 3 of culture. For proliferation analysis cells were pulsed in the last 6 hours of culture, harvested and ^{3}H-thymidine (Perkin Elmer) incorporation was measured. Expression of cell activation markers after one day of stimulation was assessed by staining using biotinylated-anti-CD69 (clone H1.2F3), biotinylated-anti-CD86 (clone GL1), Alexa488-anti-CD25 (clone PC 61) and FITC or Percp-Streptavidin antibodies followed by flow cytometry analysis.

6.2. *In vitro* transwell cultures

Islets of Langerhans were isolated from the pancreas by collagenase type V digestion (1.4 mg/ml, Sigma) followed by hand picking under a stereomicroscope. Islets were subsequently dissociated into single cell suspensions by simultaneous mechanical and dispase enzymatic treatment (5 mg/ml, Roche). Freshly dissociated cells were cultured in Ham's F-10-glutamax medium (Gibco) supplemented with 10 mm glucose (Sigma), 0.5% BSA, 50 μM isobutylmethylxanthine (Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco). 24 hours after, sorted B cells were added in transwells (0.4 μM pore size, Millipore). After 24 hours of co-culture beta cells were either collected for flow cytometry analysis or processed for RNA isolation. Alexa647-anti-IgM (clone R33.24.12) was used for staining of bound IgM on beta cells. Negative controls consisted of beta cells with lymphocyte medium only in the transwell.

7. Statistical analysis

Statistical significant differences for the analyzed phenotypes were estimated by either Kruskal-Wallis or unpaired student's t-test. Two-tailed tests with 95% of confidence interval where used and differences with $p<0.05$ were considered significant.

Chapter IV. Article I: *Irf4* is a positional and functional candidate gene for the control of serum IgM levels in the mouse

Irf4 IS A POSITIONAL AND FUNCTIONAL CANDIDATE GENE FOR THE CONTROL OF SERUM IgM LEVELS IN THE MOUSE

by

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ABSTRACT

Natural IgM are involved in numerous immunological functions but the genetic factors that control the homeostasis of its secretion and upholding remain unknown. Prompted by the finding that C57BL/6 mice had significantly lower serum levels of IgM when compared to BALB/c mice, we performed a genome-wide screen and found that the level of serum IgM was controlled by a QTL on chromosome 13 reaching the highest level of association at marker D13Mit266 (LOD score=3.54). This *locus* was named *IgMSC1* and covered a region encompassing the interferon regulatory factor 4 gene (*Irf4*). The number of splenic mature B cells in C57BL/6 did not differ from BALB/c mice but we found that low serum levels of IgM in C57BL/6 mice correlated with lower frequency of IgM secreting cells in the spleen and in the peritoneal cavity. These results suggested that C57BL/6 mice have lower efficiency in late B cell maturation, a process that is highly impaired in *Irf4* knockout mice. In fact, we also found reduced *Irf4* gene expression in B cells of C57BL/6 mice. Thus, we propose *Irf4* as a candidate for the *IgMSC1 locus*, which controls IgM homeostatic levels at the level of B cell terminal differentiation.

Keywords: serum IgM, homeostasis, *Irf4*

INTRODUCTION

Natural IgM play a significant role in the immunology of health and disease. IgM antibodies are involved in numerous immunological functions including protection against infection (Boes, Prodeus, Schmidt, Carroll, & Chen, 1998; Brown et al., 2002; Ochsenbein et al., 1999), prevention of autoimmunity (Boes et al., 2000; Ehrenstein, Cook, & Neuberger, 2000), B cell homeostasis (Baker & Ehrenstein, 2002; Boes et al., 1998; Ehrenstein, O'Keefe, Davies, & Neuberger, 1998) and immunosurveillance against tumors (Brandlein et al., 2003; Vollmers & Brandlein, 2005). IgM biological roles also include opsonization of apoptotic cells, resulting in accelerated clearance by phagocytic cells (Peng, Kowalewski, Kim, & Elkon, 2005). Many of the circulating IgM correspond to natural antibodies that exist prior to infection or immunization (Bos et al., 1987; Haury et al., 1997; Lacroix-Desmazes et al., 1998). Natural IgM are polyreactive to evolutionary conserved structures (Boes, 2000) and bind efficiently to previously un-encountered antigen (Lydyard et al., 1990). These antibodies compensate their low antigen affinity with relatively high avidity and furthermore the effectiveness of the antigen-antibody interaction is enhanced by the high efficiency of IgM in engaging the complement pathway (Manson, Mauri, & Ehrenstein, 2005; Peng, et al., 2005).

The preimmune Ig repertoire is thought to be composed by mature B cells either recirculating through follicles of secondary lymphoid organs (B2 cells), or joining compartments in specific locations as the marginal zone in the spleen (MZ B cells) and the pleural or peritoneal cavities (B1 cells) (Lacroix-Desmazes, et al., 1998). B2 and marginal zone B cell development is initiated in the bone marrow and completed in the periphery (Shapiro-Shelef & Calame, 2005; Srivastava, Quinn, Hazard, Erikson, & Allman, 2005) throughout life. B cells exported from the bone marrow maturate in the spleen (Chung, Silverman, & Monroe, 2003) into subsets that differ in their surface phenotype, anatomic localization, and immunologic function (Martin & Kearney, 1999; Martin & Kearney, 2002; Oliver, Martin, Gartland, Carter, & Kearney, 1997) and that ultimately are able to differentiate into antibody-producing plasma cells, upon antigenic stimulation (Shapiro-Shelef & Calame, 2005). Most frequently primary antigen contact of mature naïve B cells leads to IgM production detectable in the serum.

On the other hand, studies on germ-free or axenic mice suggest that in the absence of pathogens such stimulation is conveyed by the action of self-antigens, leading to production of natural antibodies (Holmberg et al., 1986; Pereira et al., 1986). The B1 cell population, is derived almost exclusively from foetal liver (Herzenberg, 2000), is established early in ontogeny and is predominantly maintained by self-replenishment (Hardy & Hayakawa, 1994). These cells locate preferentially in the pleural and peritoneal cavities (Herzenberg, 2000) and spontaneously secrete natural Ig (Tumang, Frances, Yeo, & Rothstein, 2005) participating in relatively few antigen-stimulated antibody responses (Herzenberg, 2000).

The capacity for Ig secretion is acquired in the process of B cell terminal differentiation (Calame, Lin, & Tunyaplin, 2003), at the plasma cell stage and requires the expression of the transcription factor *Irf4* (Klein et al., 2006), a member of the interferon regulatory factor (IRF) family (Mamane et al., 1999)**.** *Irf4* expression is induced by antigen receptor or mitogen stimulation (Sciammas et al., 2006) and is restricted to cells of the immune system (Marecki & Fenton, 2002; Pernis, 2002), playing a critical role in both early (Mittrucker et al., 1997) and late B cell differentiation (Klein, et al., 2006). Interestingly, *Irf4* upregulation results in spontaneous differentiation of B cells into plasma cells and production of IgM in the absence of external stimuli (Lin, Gerth, & Peng, 2004).

Several familiarity and genetic studies have indicated that heritable components control the serum levels of IgE, IgA, and IgG (Barbosa, Rao, & Morton, 1981; Borecki, McGue, Gerrard, Lebowitz, & Rao, 1994; Grundbacher, 1974) and identified a number of *loci* influencing total serum levels of these antibodies in humans (Barnes et al., 1996; Bleecker, Amelung, Levitt, Postma, & Meyers, 1995; Mansur, Bishop, Holgate, Markham, & Morrison, 2004). There is also convincing evidence for parent-offspring effects and genetic heritability in human serum IgM levels (Barbosa, et al., 1981; Borecki, et al., 1994; Grundbacher, 1974), as well as evidence for genetic control of natural IgM titres in mammals (Gobet, Cerny, Ruedi, Hengartner, & Zinkernagel, 1988), birds (Parmentier et al., 2004) and fish (Kachamakova et al., 2006). However, no linkage evidence for quantitative trait *loci* influencing serum IgM has been reported and the genetics of the homeostatic regulation of serum IgM remains largely unknown. Here, we characterized a total IgM serum phenotype in mouse inbred strains and performed a genome-wide screen that identified a region on mouse chromosome 13 that contained a *locus* (*loci*) controlling IgM serum levels. Searching for the cellular and genetic basis of serum IgM phenotype we correlated the level of serum IgM with the frequency of IgM producing cells and the expression of the *Irf4* gene.

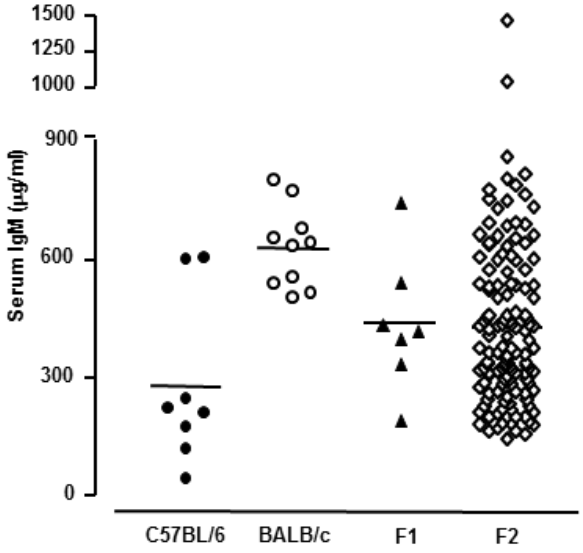
RESULTS

Serum IgM phenotype

To investigate the genetic control of serum IgM we analyzed the IgM concentration in the serum of C57BL/6 and BALB/c inbred strains. We found that the serum IgM level was lower in C57BL/6 than in BALB/c mice, albeit the phenotype showed reduced penetrance (Figure IV.1). The F1 [C57BL/6 X BALB/c] generation showed an intermediate phenotype and a F2 [C57BL/6 X BALB/c] progeny of 136 mice showed a phenotype spectrum, from low to high values, indicating that alleles controlling the phenotype were segregating in the cross (Figure IV.1). The serum IgM trait, measured as the logarithm of IgM concentration, was normally

distributed in the F2 [C57BL/6 X BALB/c] progeny required for quantitative trait *locus* (QTL) analysis (data not shown).

Figure IV.1 Serum IgM levels in naive mice

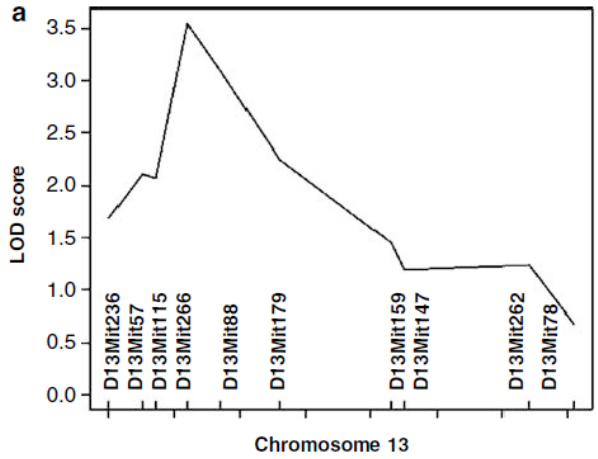


IgM serum concentration was measured in 8 C57BL/6 (black circles), 10 BALB/c (white circles), 7 F1 [C57BL/6 X BALB/c] (black triangles) and 136 F2 [C57BL/6 X BALB/c] (white diamonds) mice by Indirect ELISA. Average concentrations are shown as horizontal bars.

Genetic mapping of serum IgM phenotype

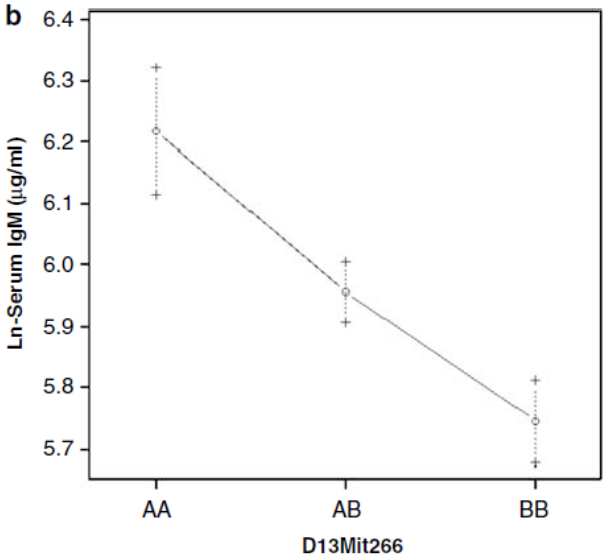
We performed a QTL analysis for this trait scanning the genome with 100 microsatellite markers in the 136 F2 progeny. We found that a region in proximal mouse chromosome 13 was linked to the serum IgM trait, with a 95% confidence interval between the markers D13MIT57 and D13MIT179, where the highest associated marker D13Mit266 reached a LOD score of 3.54 corresponding to an empirically estimated genome-wide *p*-value of 0.04. The chromosome 13 *locus* was named *IgMSC1* (*IgM-secreting cells locus 1*) (Figure IV.2a) and covered a region encompassing the *Irf4* gene. Although no other chromosomal region reached the level of genomic significance, suggestive LOD scores were detected in chromosome 7 at marker D7MIT318 (LOD 2.05) and in chromosome 9 at marker D9MIT53 (LOD 2.02) that could represent additional *loci* controlling the serum IgM phenotype, albeit with a smaller genetic effect as compared to *IgMSC1*. To evaluate the *IgMSC1* genetic effect in the serum IgM phenotype F2 mice were classified according to their genotype at the highest linked marker in the *IgMSC1 locus* (D13Mit266). This analysis shows that the *IgMSC1 locus* has an additive mode of action (Figure IV.2b) and controls a significant fraction of the phenotypic variance (15.5%).

Figure IV.2a Mapping the *IgMSC1* locus



LOD score curve for mouse chromosome 13 represents the likelihood for linkage of the serum IgM trait with the represented markers. Regions of significant linkage show LOD score above 3.0. The x-axis ticks represent the relative position of microsatellite markers along this chromosome, from left to right: D13Mit236, 4cM; D13Mit57, 9cM; D13Mit115, 11cM; D13Mit266, 16cM; D13Mit88, 21cM; D13Mit179, 30cM; D13Mit159, 47cM; D13Mit147, 49cM; D13Mit262, 68cM; and D13Mit78, 75cM.

Figure IV.2b IgM serum trait control by the *IgMSC1* locus



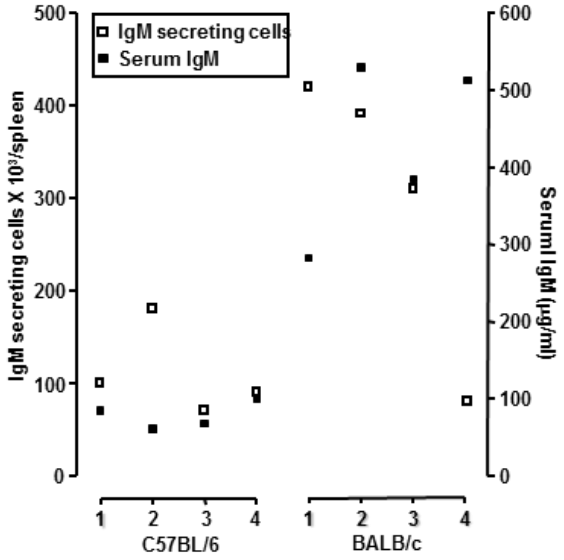
The F2 (C57BL/6 X BALB/c) mice were classified according to their genotype as AA (BALB/c) AB (heterozygous) or BB (C57BL/6) at D13Mit266 and the mean trait value and the standard error are represented for each group.

IgM secreting cells and serum IgM

With the aim of determining the cellular basis of the IgM serum trait we started by counting the total number of B cells, sIgM$^+$ B cells and IgM secreting cells in the spleen of C57BL/6 and BALB/c adult mice. We found no significant differences in the total number of B cells or in the total number of mature sIgM$^+$ B cells (data not shown), suggesting that regulation of B

cell spleen cellularity and B cell maturation in the spleen is similar in these strains. However, we also found that C57BL/6 have significantly lower number of IgM secreting cells in the spleen, offering an explanation for the observed lower serum IgM phenotype (Figure IV.3).

Figure IV.3 Number of IgM secreting cells in the spleen correlates with serum IgM concentration

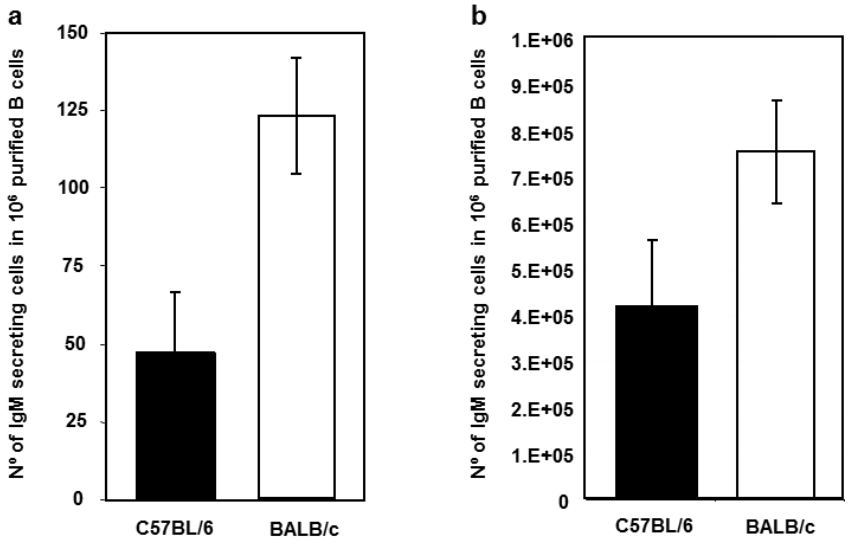


The values represent results that were obtained by ELISA-Spot-Assay and indirect ELISA in individual C57BL/6 and BALB/c mice.

Moreover, we purified B cell populations to show that the number of IgM secreting cells within splenic B cells was lower in C57BL/6, suggesting that these mice have less efficient plasma cell differentiation as compared to BALB/c mice (Figure IV.4a). These findings suggested that the cellular basis for the serum IgM trait resides in final steps of the peripheral B cell maturation and terminal differentiation in the spleen.

It has been described that the B cell population (B1) localizing in the pleural and peritoneal cavities is an important source of natural IgM in the mouse (Herzenberg et al., 1986). To assess the contribution of peritoneal B cells to the observed phenotype we decided to characterize the population of B cells and IgM secreting cells in the peritoneal cavity of C57BL/6 and BALB/c mice. We found that C57BL/6 mice have a decreased number of IgM secreting cells in the peritoneal cavity when compared to the BALB/c strain (Figure IV.4b). Thus, the number of IgM secreting B cells in the peritoneal cavity may also contribute to the cellular basis of the serum IgM trait in these mouse strains. These data indicate that the reduced number of IgM secreting cells in the C57BL/6 mice is not specific of the spleen environment and appears to be an intrinsic genetic property of the B cell compartment.

Figure IV.4 Decreased number of IgM secreting cell counting in purified splenic B cells and purified peritoneal cavity B cells in C57BL/6 mice

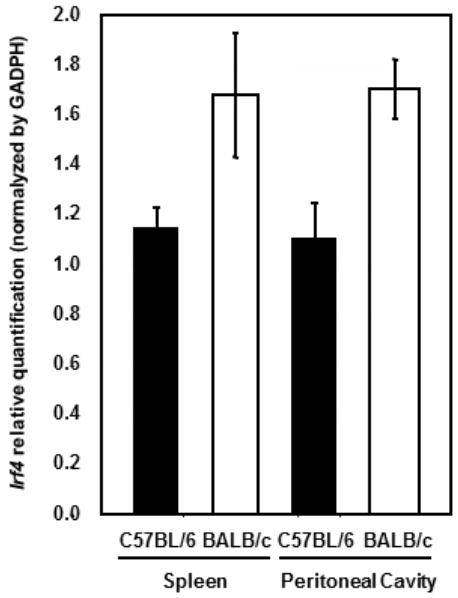


(a) Number of IgM secreting cells in splenocyte B cells pooled from groups of 8 C57BL/6 (black bars) and 8 BALB/c (white bars) mice. The results represent the mean and standard deviation of triplicate samples assayed simultaneously. (b) Number of IgM secreting cells in peritoneal cavity B cells pooled from groups of 5 mice and representing the mean and standard deviation of two separate experiments assayed in triplicates.

Irf4 gene expression

The *Irf4* gene is a key player in several stages of B cell development, namely in controlling plasma cell differentiation (Klein, et al., 2006; Mittrucker, et al., 1997) and co-localizes with *IgMSC1* on chromosome 13. In principle, several genes in the region flanked by D13MIT115 and D13MIT88 that encompass the highest linked marker in the *IgMSC1 locus* could mediate the observed IgM serum phenotype. Nevertheless, the *Irf4* gene is the most plausible functional candidate in this interval as it was previously related to antibody secretion (Klein, et al., 2006; Mittrucker, et al., 1997). To ascertain whether the *Irf4* gene could be involved in controlling the serum IgM trait, we quantified *Irf4* RNA by real-time PCR in purified B cells from the spleen and the peritoneal cavity. We found that *Irf4* expression was significantly reduced in splenic B cells and in peritoneal cavity B cells from the C57BL/6 (Figure IV.5). These data strongly suggest that *Irf4* is a candidate gene to *IgMSC1* and contributes to the serum IgM phenotype by controlling the number of IgM secreting cells.

Figure IV.5 *Irf4* gene expression is decreased in purified B cells from the spleen and peritoneal cavity of C57BL/6 mice



RNA of the *Irf4* gene was quantified in purified B cells from spleens of 5 C57BL/6 (black bars) and 5 BALB/c (white bars) adult mice or in purified B cells from pooled peritoneal cavity lavages of 5 C57BL/6 (black column) or 5 BALB/c (white column) adult mice. The results represent the mean and standard deviation of the average of samples assayed simultaneously.

DISCUSSION

In this work we used a mouse genome screen approach to search for genetic factors that control the IgM levels in the serum. We identified a major *locus* on mouse chromosome 13 controlling the IgM serum concentration that we called *IgM-secreting cells locus 1* (*IgMSC1*). Our results support a correlation of this phenotype with the number of IgM producing cells in the spleen and in the peritoneal cavity, leading us to hypothesize that the *IgMSC1* controls IgM serum concentration by regulating the differentiation and/or maintenance of plasma cells both in the spleen and in the peritoneal cavity. It has been described that the serum anti-RBC IgM concentration is linked to the 40 to 49.5 cM region on chromosome 13 (Lee et al., 2004) and we show that gene(s) controlling natural IgM secretion are also localized in this chromosome. However, the anti-RBC *locus* (40-49.5 cM) and the *IgMSC1 locus* (9-30 cM) are somewhat apart on chromosome 13 raising the possibility that they would not correspond to the same genetic factor. We noted that the IgG serum concentration in C57BL/6 mice was significantly higher as compared to BALB/c and that *IgMSC1* was not controlling levels of serum IgG in the genetic cross we analyzed (data not shown). This indicates that the genetic mechanisms that control IgM and IgG serum steady state levels have distinct and independent components.

The *IgMSC1 locus* is tightly linked to the interferon regulatory factor 4 (*Irf4*) gene region on

mouse chromosome 13 and we showed that the relative expression of this transcription factor is reduced in C57BL/6 B cells isolated from the spleen and from the peritoneal cavity. Thus, we propose the interferon regulatory factor 4 as a candidate gene to mediate the *IgMSC1* phenotypic effect.

A series of recent studies have demonstrated critical functions for IRF-4 at several stages of B cell development, where it seems to preferentially limit clonal expansion and promote differentiation processes (Lu, Medina, Lancki, & Singh, 2003; Sciammas, et al., 2006). *Irf4* is a member of the interferon-regulatory factor family of transcription factors characterized by a specific DNA-binding domain and the ability to either activating or repressing the transcription of interferon-inducible genes (Mamane, et al., 1999; Shapiro-Shelef & Calame, 2005). Expression of *Irf4* is restricted to cells of the immune system, including lymphocytes, dendritic cells and macrophages, in which it has been linked to a variety of functions, such as proliferation, apoptosis and differentiation (Fanzo, Hu, Jang, & Pernis, 2003; Marecki & Fenton, 2002; Pernis, 2002). *Irf4* knockout mice have normal early B cell development but profound reduction in serum immunoglobulin concentrations and show incapability to mount antibody responses due to a late stage blockage in peripheral B cell maturation that leads to the absence of plasma cells, plasmablasts and class-switched B cells (Klein, et al., 2006; Mittrucker, et al., 1997). Recently, the *Irf4* gene has been demonstrated to have a biphasic expression pattern: it is expressed in immature B cells in the bone marrow (Lu, et al., 2003), absent from proliferating germinal center centroblasts and then re-expressed in a subpopulation of centrocytes in the germinal center and in plasma cells (Falini et al., 2000). Extending these studies, the evidences provided here introduce the notion that natural genetic variation at the *Irf4* gene impacts in its expression levels, being a major factor in the quantitative control of terminal B cell differentiation and, consequently, of natural IgM secretion.

It is plausible that a differential effect of *Irf4* alleles could explain, at least in part, the observed differences in frequency of IgM secreting cells and IgM serum concentration when we compared the C57BL/6 and BALB/c mouse strains. It is reported that the coding region of the *Irf4* gene in C57BL/6 and BALB/c mice shows no sequence differences except for a synonymous point mutation in the exon 6 (NCBI entries with accession numbers AAA75309.1 and AAA75283.1). On the other hand, regulatory elements have been described in the human *Irf4* gene encompassing the 5 kb upstream the *Irf4* coding region (Nishiya, Yamamoto, Imaizumi, Kohno, & Matsuyama, 2004). However, we have sequenced 4763 bp upstream the translational starting site of the *Irf4* gene and did not find sequence differences between C57BL/6 and BALB/c mice. Thus, it is possible that the allelic variation controlling the observed differences in RNA expression lies in yet unidentified *Irf4* gene regulatory regions. In fact, the functional conformation of the *Irf4* promoter region suggests that its expression is strictly regulated in the chromatin context (Nishiya, et al., 2004).

Detailed functional studies of the control region of the mouse *Irf4* gene would be needed to enable a systematic analysis of the differential expression of the C57BL/6 and BALB/c *Irf4* alleles.

This work highlighted a region on mouse chromosome 13 that contains genetic factor(s) involved in the circuitry of the serum immunoglobulin homeostasis and led us to hypothesize that allelic variation in *Irf4* gene may control the homeostasis of serum IgM in the mouse at the level of the generation of antibody-producing cells. These findings may motivate human studies on the genetic control of IgM and natural antibody levels in normal individuals and in the context of Ig deficiencies and inflammatory autoimmune diseases, particularly focused on genes mapping in the region ortholog to *IgMSC1*, including the *Irf4* gene.

MATERIALS AND METHODS

Mice

The mouse strains used in this study included the C57BL/6 and BALB/c as well as mice from the genetic crosses F1 (C57BL/6 X BALB/c) and F2 (C57BL/6 X BALB/c). All mice were females between 6 and 8 months of age, bred and maintained in conventional housing facilities at the Instituto Gulbenkian de Ciência. F1 (C57BL/6 X BALB/c) mice were used to generate an F2 (C57BL/6 X BALB/c) progeny of 136 animals.

Genotyping

Genomic DNA was extracted from mouse tails with standard digestion/ precipitation methods. The F2 generation was genotyped using 100 microsatellite DNA markers, polymorphic for the parental strains. Markers were chosen according to their chromosomal position as given by the Broad Institute for genome research (USA), to cover uniformly the whole mouse genome at an average density of 20 cM. The genotypes for each *locus* were determined by DNA marker amplification using conventional PCR techniques. Amplification products were analyzed in 4% agarose gels (Cambrex BioScience, East Rutherford, NJ, USA) with ethidium bromide (Sigma-Aldrich, St Louis, MO, USA) and the allele size was determined using the Eagle Eye® II video system (Stratagene, San Diego, CA, USA). Each individual of the F2 progeny was typed for each marker as homozygous for one or the other parental strain or as heterozygous when presenting both the parental strain alleles.

IgM serum concentration

Standard indirect ELISA was used to quantify total IgM concentrations in mouse sera. Briefly, flat-bottomed 96-well ELISA plates were coated overnight at 4ºC, with goat anti-mouse-IgM-UNLB human adsorbed antibody (Southern Biotech. Birmingham, AL, USA) diluted in coating buffer (0.05M Na_2CO_3). The plates were washed with PBS, blocked with PBS-gelatin 1% and incubated at 37ºC for 60 minutes. After washing, eight serial dilutions of sera to be tested (starting at 1:90 and using 1:3 dilution steps) were incubated for 60 minutes at 37ºC and washed. Bound seric IgM was detected by the peroxidase-conjugated goat anti-mouse IgM-HRP human adsorbed antibody (Southern Biotech.) diluted in PBS-Gelatin 1%-Tween 0.075% (1:4000), revealed with the substrate o-phenylenediamine (Calbiochem, Gibbstown, NJ, USA) and H_2O_2 and the OD read at 490 nm. The concentrations of IgM antibody in the samples were calculated against standard curves obtained with purified monoclonal IgM (mouse-IgM UNLB, clone 11E10, Southern Biotech.) tested in the same microtitre plate.

Genetic analysis

Quantitative trait *locus* analysis was performed by using the normal model in the R/QTL software (Broman, Wu, Sen, & Churchill, 2003). This program calculates logarithm of odds (LOD scores) over intervals between linked markers, generating likelihood plots of genetic association with the phenotype across the genotyped chromosomal regions. The genome-wide statistical significance of the results was empirically determined by performing permutation tests (Lander & Kruglyak, 1995) and the QTL confidence interval was calculated using the Bayesian method of R/QTL (Broman & Sen, 2009).

IgM-secreting cells

The number of IgM-secreting cells was determined by ELISA-Spot-Assay (ESA). The plates were coated overnight at 4ºC with goat anti-mouse IgM-human adsorbed (Southern Biotech.), diluted in coating buffer (0.05M Na_2CO_3) and the plates saturated with PBS containing 1% gelatin. Serial three-fold dilutions of total splenocytes (starting with 10^6 cells), spleen purified B-cells (starting with $3x10^6$ cells) or sorted peritoneal cavity B cells (starting with $7{,}5*10^3$ cells) were performed in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 50 mM 2-mercaptoethanol, 100 U penicillin and 100 mg/ml streptomycin (all from Life Technologies, N.Y. USA). Cell dilutions were added and the plates were sealed and incubated overnight at 37ºC in a humidified 5% CO_2 atmosphere. Non-adherent cells were thereafter removed by flicking the plate followed by lysis with 0.1% Tween-20 in water. After extensive washing with PBS containing 0.05% Tween-20 the plates were incubated with goat anti-mouse IgM-AP conjugated antibody (Southern Biotech.), washed again and revealed with BCIP-AMP

substrate (2.3 mM of 5-bromo-4-chloro-3-indolyl phosphate diluted in 2-amino-2-methyl-1-propanol buffer, Sigma-Aldrich). After incubation overnight at 4ºC and washing three times with distilled water, the traces of IgM-secreting cells were revealed as blue spots and counted.

B cell purification

Splenocyte suspensions were prepared by straining of the spleens through a nylon mesh and peritoneal cavity cell suspensions were obtained from peritoneal lavage with 7 ml of PBS supplemented with 2% FCS. Purified B cells, with an average purity of 98%, were obtained from splenocyte and peritoneal cavity cell suspensions through high speed cell sorting (MoFlo, Cytomation Inc., Fort Collins, CO, USA), after labeling with FITC-anti-mouse CD19 (clone 1D3, BD Biosciences, California, USA).

RNA isolation and Real-Time PCR

Total RNA from sorted splenic and peritoneal B cells was obtained using an RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer´s protocol for animal cells. RNA was converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche, Mannheim, Germany) using random hexamer primers. *Irf4* (Mm0051634-m1) expression was quantified using TaqMan Gene Expression Assays from ABI with TaqMan Universal PCR master mix. The gene expression quantification reactions were performed in an ABI Prism 7900HT system. Relative quantification of *Irf4* (Mm0051634-m1) in each real-time PCR reaction was obtained after normalization for GAPDH expression measured in the same PCR reaction and used the $2^{-\Delta\Delta CT}$ analysis method (Livak & Schmittgen, 2001).

Statistical analysis

Statistical significant differences in serum IgM concentration, B cell number, IgM secreting cell number and *Irf4* relative expression were estimated by student's t-test. Differences with $p<0.05$ were considered significant.

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Chapter V. Article II: Autoimmunity triggers in the NOD mouse: a role for natural auto-antibody reactivities in type 1 diabetes

AUTOIMMUNITY TRIGGERS IN THE NOD MOUSE: A ROLE FOR NATURAL AUTO-ANTIBODY REACTIVITIES IN TYPE 1 DIABETES

by

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ABSTRACT

The non-obese diabetic mouse (NOD) is widely used as a model to study human type 1 diabetes (T1D). In the NOD mouse T1D is a T cell mediated autoimmune disease of complex etiology in which B cells play an essential role. One of the major unresolved issues in T1D is the genetic and/or environmental factors that trigger the autoimmune reaction. In the NOD mouse, as in humans, autoantibodies to pancreatic islets are present at early ages and are highly correlated to diabetes progression, but their etiological role has long been disputed. NOD autoantibodies have the characteristics of a natural repertoire and B1 cells, the main natural antibody producers, exhibit functional differences in this strain that could have consequences for disease determination. Using a genetic approach, we propose to test if the NOD natural autoantibody repertoire includes innate reactivities that participate in diabetes pathogenesis by promoting insulitis initiation.

Keywords: Type 1 diabetes, NOD, natural autoantibodies

TEXT

Type 1 diabetes (T1D) is well established as a multifactorial disease with an initial insulitis phase that involves dendritic cells, macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells (Yang & Santamaria, 2003) followed by a diabetic phase where onset of disease is caused by selective and progressive autoimmune destruction of the insulin-secreting β-cells in the pancreas (Bach, 1994). However, the mechanisms underlying the loss of tolerance to specific self-antigens are unknown. In diabetic patients and in non-obese-diabetic (NOD) mice T1D develops spontaneously, is pathogenically similar (Castano & Eisenbarth, 1990) and is under complex genetic control (Ghosh et al., 1993). Environmental factors have also been demonstrated to have a modulatory role in T1D progression (Bach, 1994) and could act as disease triggers in humans while in NOD laboratory mice, bred and maintained in controlled housing conditions, genetically determined factors should assume a major role as initiators of the autoimmune process.

It has been shown that absence of B cells prevents diabetes in the NOD mouse (Akashi et al., 1997) but the roles that B cells may play in the NOD autoimmune reaction cascade are not fully understood. B cell receptor specificity (Hulbert, Riseili, Rojas, & Thomas, 2001) and B cell antigen-presenting function have been demonstrated to play a part in disease development (Wong et al., 2004), but other functional aberrations or distortions in the development of B cell repertoires could have implications for pathogenesis in this strain.

Autoantibodies have served historically as the *sine qua non* evidence to indicate the involvement of B cells in autoimmune diseases. They provide diagnostic and prognostic criteria, play a requisite role in disease pathogenesis and serve as surrogate markers for disease activity (Martin & Chan, 2004). For most autoimmune diseases, however, the mechanistic relationship between pathogenic B cells, their products and clinical manifestations remains elusive.

NOD mice produce various islet β-cell specific autoantibodies although it is still a subject of debate whether they contribute to T1D development (Inoue et al., 2007). Currently, autoantibodies are looked at as being T1D biomarkers, secondary to the occurring pathogenic process, but their presence largely precedes disease and is highly correlated with its progression (Bingley et al., 1997; Bonifacio et al., 2001). In addition, maternal antibodies have been proven to be important for T1D development in the NOD progeny (Greeley et al., 2002), thus it is likely that their role is being underestimated.

It has been demonstrated that the NOD B cell compartment is highly active and that early detected autoantibodies have the characteristics of a natural repertoire (Thomas, Kendall, & Mitchell, 2002). Natural antibodies are spontaneously secreted by B1 cells, characterized by the expression of the CD5 molecule (Herzenberg, 2000), by the capacity for self-renewal and by the preferential localization in the peritoneal and pleural cavities (Dorshkind & Montecino-

Rodriguez, 2007). These antibodies belong mainly to the IgM isotype, are encoded by germline genes and are polyreactive, being implicated in a variety of functions including immunoregulation, homeostasis and repertoire selection (Coutinho, Kazatchkine, & Avrameas, 1995), and also in autoimmunity (Thomas, et al., 2002).

Previous studies showed that in a transgenic mouse model some insulin specific B cells evade the mechanisms of tolerance induction by entering a B1-like compartment in the peritoneal cavity (Rojas, Hulbert, & Thomas, 2001), and that B cells secreting anti-insulin with structural characteristics of natural autoantibodies exist in human T1D, evoking the contribution that B1 cells may have to the autoimmune process (Ichiyoshi, Zhou, & Casali, 1995). The suggestion of a role for B1 cells in T1D pathogenesis arose once more in a recent report showing their presence in the pancreatic infiltrates of NOD mice and a delayed onset of disease upon its specific elimination from the peritoneal cavity. In this study, the protection from diabetes correlated with the maintenance of B-cell tolerance, measured as the concentration of insulin-specific IgG in the sera (Kendall, Woodward, Hulbert, & Thomas, 2004).

It has been argued that the large pool of natural autoantibodies in the NOD mouse may fuel the autoimmune process if appropriate T-cell help is available (Thomas, et al., 2002) and the idea that natural IgM of certain specificities, most probably genetically determined, may have a role as pathogenic initiators of insulitis and T1D autoimmune pathogenesis through increased antigen presentation, direct islet injury or complement activation, is sustained by several lines of evidence described in the following paragraphs.

Natural autoantibodies as initiators of autoantigen presentation to T cells

1) The majority of B lymphocytes and macrophages constitutively express Fcα/μ receptors that allow phagocytosis of IgM-antigen complexes and facilitate subsequent antigen processing and presentation (Shibuya et al., 2000). In agreement, mice deficient in the secretory form of IgM exhibit delayed development of T cell dependent humoral responses (Ehrenstein, O'Keefe, Davies, & Neuberger, 1998), suggesting that the uptake of IgM-antigen complexes through Fcα/μ receptors plays an essential role in the priming of helper T lymphocytes (Shibuya, et al., 2000), which is a critical step in the autoimmune process evolving in the NOD mouse.

2) Fc-γ receptor deficient NOD mice have decreased incidence of insulitis and diabetes, independently of autoantibody levels. In this scenario, it is inferred that activating Fc-γ receptors augment autoantigen presentation leading to the activation of autoreactive T cells (Inoue, et al., 2007). Most likely Fc-γ receptors interaction with IgG containing immune complexes occurs only after the initiation of the autoimmune reaction and could serve as an autoimmunity amplification mechanism. This leaves open the possibility that a similar process of antigen presentation to T cells could take place at the initial steps of disease process,

before IgG presence in the system, through IgM-antigen interaction with Fcα/μ receptors on antigen presenting cells.

3) B cell surface Ig can also mediate an increased loading onto MHC molecules as a result of an augment in antigen uptake (Takai, 2002). It has been shown that GAD autoantibodies are implicated in GAD presentation to T-cells, thus antibody specificities are likely modulators of the T cell epitopes made available by antigen-presenting cells (Reijonen, Daniels, Lernmark, & Nepom, 2000). This may represent a mechanism of revealing otherwise cryptic T cell determinants to which tolerance has not been established and may be relevant in the drive toward high affinity antibodies. Autoantibody specificity and titers may therefore alter the focus of the T cell response over time, contributing to the initiation and perpetuation of autoimmunity in T1D (Takai, 2002).

Natural autoantibodies as pancreatic islet injurers

1) Prior to the first appearance of insulitis NOD mice are sensitized to islet-cell associated antigens and a small percentage of β-cells have autoantibody bound to their surface. Additionally, sera collected from these animals contain autoantibodies that bind to β-cells of both T1D-prone and T1D-resistant mice. Autoantibodies binding to pancreatic β-cells in this strain are predominantly of the IgM isotype (Jaume, Parry, Madec, Sonderstrup, & Baekkeskov, 2002), therefore an early humoral response may be involved in β-cell targeting, the initiation of insulitis and subsequent disease development.

2) Natural autoantibodies bound to pancreatic islet antigens can trigger the complement system mediated inflammation (Shieh, Cornelius, Winter, & Peck, 1993), which may contribute to the observed deregulation in the NOD mouse immune system and modulate T1D development.

Moreover Fc-receptor activation can regulate immune-cell responses such as B cell proliferation (Shieh, et al., 1993). Thus, at early times, IgM-antigen interaction with Fcα/μ receptors could have an autocrine effect on B cell populations leading to expansion of B cells bearing autoreactive specificities and facilitating the autoimmunity process.

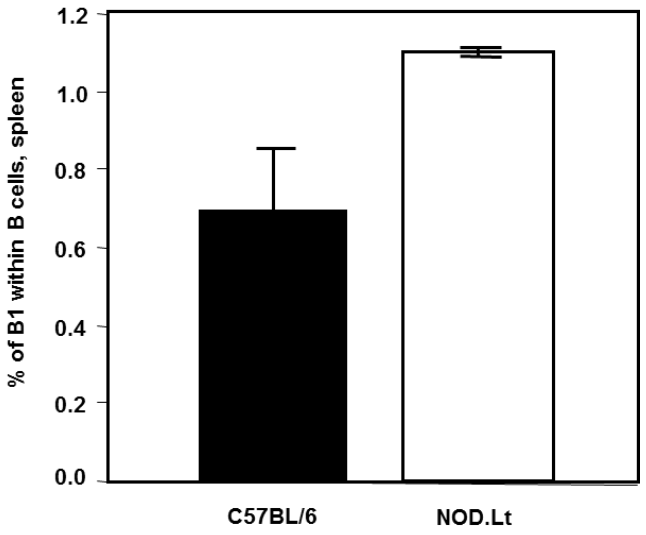
NOD mice display defects at the level of the B1 cell compartment that may contribute to IDDM pathogeny

Early-detected autoantibodies in the NOD mouse show characteristics of a natural repertoire (Thomas, et al., 2002), suggesting a genetic determination of the specificities of the major contributors for natural autoantibody production in this strain, namely non-anergic B cells and autoreactive B1 cells. Despite the multitude of factors that have been suggested to participate in the T1D pathogenesis it has been difficult to identify those that are involved in the very first steps of the autoimmune reaction. Here, we propose that soluble islet-specific natural antibodies promote disease either by enhancing professional antigen presentation of islets

antigens or by directly causing the initial tissue damage and islet cell antigens release. In this context, the genetically specified pre-insulitic IgM antibody repertoire would have a role in the initiation of T1D pathogenesis.

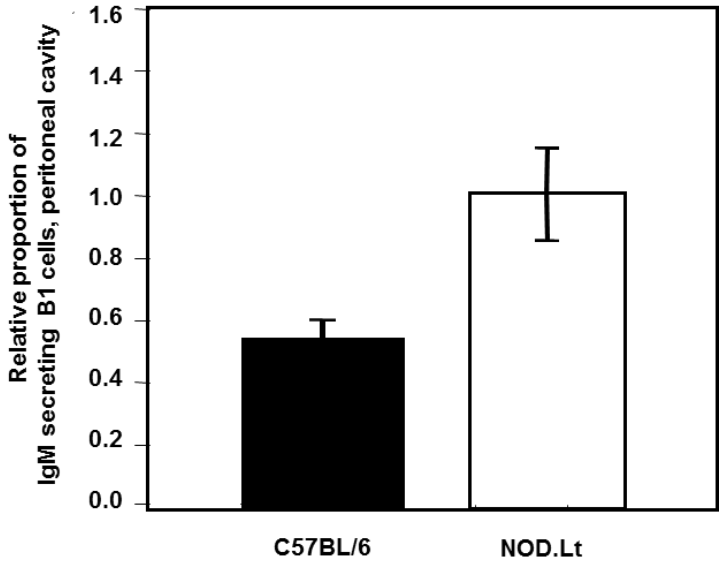
To address this hypothesis we initiated the characterization of the NOD and the C57BL/6 strains B cell compartment at the populational and functional levels at early ages, when insulitis is still incipient. We did not find in the NOD mouse B2 cell population abnormalities that could be easily correlated to the described pattern of autoreactivity, but NOD mice show alterations at the level of the B1 cell compartment that can be contributing to its increased natural autoreactivity and involved in T1D pathogenic process. Flow cytometry analysis of stained splenocyte and peritoneal cell suspensions revealed that B1 cells are overrepresented in the NOD spleen (Figure V.1) and ELISPOT ASSAY (ESA) characterization of purified B1 cells in these locations showed that peritoneal cavity NOD B1 cells have an increased capacity for natural IgM secretion (Fig V.2). Importantly no such alterations were observed in the B1 cell population in the spleen (data not shown).

Figure V.1 Percentage of B1 cells within B cells in the spleen at 4 weeks of age



The bars represent the mean and standard deviation of groups of five animals from a representative experiment.

Figure V.2 Proportion of IgM secreting B1 cells in the peritoneal cavity relative to the NOD strain, at 4 weeks of age

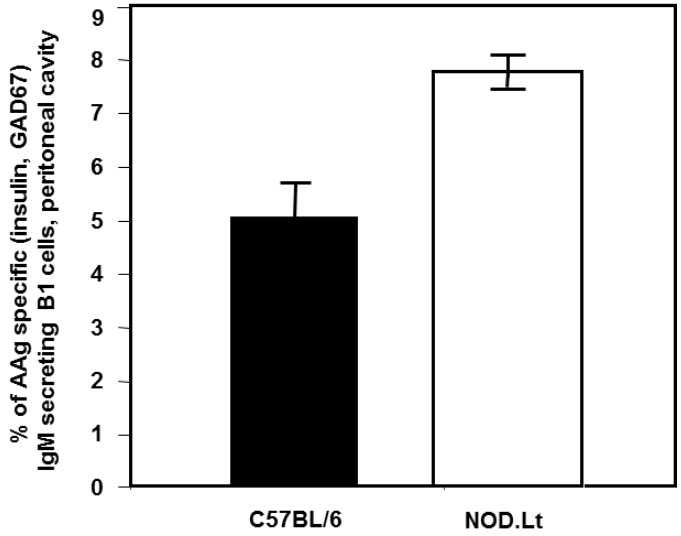


The bars represent the mean and standard deviation of three separate experiments of groups of ten animals, assayed in triplicates.

Insulin and Glutamate Decarboxylase (GAD) have been validated as main autoantigens associated with T1D in humans and in the NOD mouse (Bonifacio, et al., 2001), being the GAD67 isoform highly expressed in the NOD pancreas (Pleau, Esling, Van Acker, & Dardenne, 1996) and its overexpression connected to increased insulitis and diabetes development (Kim et al., 2002). Interestingly, when looking for B1 secretion of autoantigen-binding natural IgM by ESA, we found that peritoneal cavity NOD B1 cells are more prone to spontaneously secrete IgM that recognizes insulin and GAD67 than C57BL/6 B1 cells (Figure V.3). This property was exclusive of B1 cells in this location, since the homologous population in the spleen was unable to recognize such antigens (data not shown). These results are consistent with a study that suggests that unique features of the peritoneal cavity microenvironment may foster natural autoantibody production by B1 cells in transgenic systems (Rojas, et al., 2001). Therefore, we propose that before disease onset the NOD B1 cell compartment is overrepresented in the spleen and is over-activated in the peritoneal cavity where it generates a repertoire of antibody specificities biased to the recognition of T1D related antigens. As previously discussed, such alterations could have implications to disease process initiation and to the autoimmune reaction amplification.

To further test the hypothesis that the NOD repertoire of natural antibodies has particularities that may be involved in the progression to T1D, we measured the NOD and C57BL/6 early reactivity patterns of serum IgM against a large antigen collection. Using peptide chip arrays and principal component analysis, we found that particular specificities discriminate these strains reactivity profile (data not shown). Although these findings require further investigation they are consistent with our hypothesis.

Figure V.3 Percentage of IgM secreting B1 cells able to recognize type 1 diabetes related autoantigens (Insulin, GAD67) in the peritoneal cavity, at 4 weeks of age



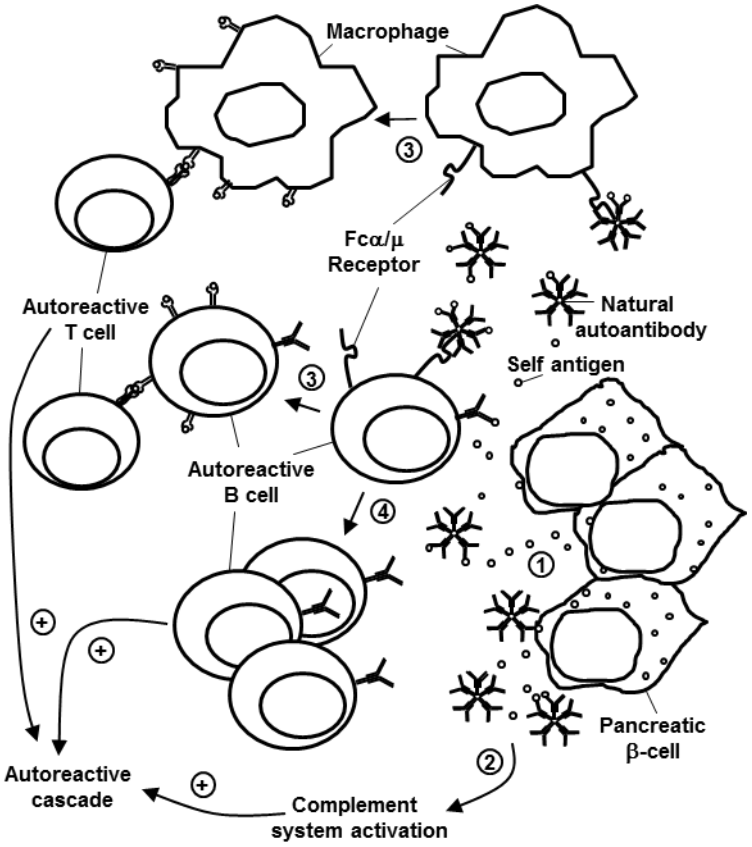
The bars represent the mean and standard deviation of one representative experiment of groups of ten animals, assayed in triplicates.

Genetic control of B1 cell autoreactivity in the NOD mouse

The pathogenic role of autoantibodies in T1D is controversial and many abnormalities observed in the NOD mouse B cell compartment are hard to link to T1D pathogenesis on the basis of correlations with other immunological parameters. We are particularly interested in understanding the role that B1 cells, as natural autoantibody producers, may have in T1D progression. We are addressing this question by using a genetic approach that will allow us to establish the connection between abnormalities in the B1 cell compartment, generation of autoantibodies and diabetogenesis in the NOD mouse. One possibility is to test whether already described insulin-dependent-diabetic *loci* (*Idd*) control the development and functional activation of autoreactive B1 cells, whose natural autoantibodies would have a role in initiating the pancreatic β-cell destruction, ultimately leading to overt T1D (Figure V.4). Following this approach we are currently characterizing the NOD natural IgM autoreactive pattern and analyzing several NOD congenic strains, aiming to map these phenotypes within established congenic regions.

This approach will help clarify the role of autoantibodies in the murine T1D and in particular to distinguish germline encoded pathogenic specificities from the effects of epitope spreading induced by the disease progression. The demonstration of a role for B1 cells and natural autoantibodies in the development of T1D, through the dissection of its genetic control, may contribute to develop strategies that block disease progression in the NOD mouse and possibly in susceptible individuals.

Figure V.4 The presence of autoreactive natural antibodies, due to the genetically determined development and functional activation of autoreactive B1 cells, may initiate the pancreatic β-cell autoimmune reaction (1) and fuel the autoimmune process through complement system activation (2), increased antigen presentation (3) and autoreactive B cell proliferation (4) in the pancreatic lymph nodes



Due to the length of this paper, we are unable to summarize all of the cutting edge issues that surround this research. For this reason, we refer to the following recent literature on this subject (Bresson & von Herrath, 2007; Knip & Siljander, 2008; Marino & Grey, 2008; Poletaev, Stepanyuk, & Gershwin, 2008).

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Chapter VI. Article III: Innate stimulation of B1a cells enhances the autoreactive IgM repertoire in the NOD mouse: implications for type 1 diabetes

INNATE STIMULATION OF B1a CELLS ENHANCES THE AUTOREACTIVE IgM REPERTOIRE IN THE NOD MOUSE: IMPLICATIONS FOR TYPE 1 DIABETES

by

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ABSTRACT

Aims/Hypothesis: We sought to determine whether the presence of natural autoreactive antibodies of B1a cell origin would play a role in type 1 diabetes (T1D) initiation.

Methods: We have compared IgM repertoires and B1a cell compartments in NOD and C57BL/6 mice. Serum IgM autoreactivity profiles were determined by ELISA and the secretory properties and activation status of B1a cells were characterized by ELISPOT Assay and flow cytometry. B1a cells response to innate activation was analyzed by gene expression assays, ELISA and ^{3}H-thymidine incorporation. The effect of IgM produced by NOD B1a cells on NOD.SCID beta cells was examined in co-cultures: IgM binding was measured by flow cytometry and Real-Time PCR was used to study oxidative stress responses.

Results: NOD displayed increased levels of serum anti-insulin-IgM that were independent of the H2 locus, that were maintained up to pre-diabetic stages, and that correlated with the NOD B1a cells secretion profile. NOD B1a cells were naturally more activated, expressed higher levels of Toll-Like-Receptors (TLR) and responded to TLR stimulation *in vitro* with higher proliferation and increased capacity to secrete anti-T1D related IgM, but produced lower amounts of IL10. Spontaneously secreted IgM of NOD B1a cells origin was able to bind to pancreatic beta cells *in vitro* and induced *iNOS* expression in islet cultures.

Conclusions/interpretation: NOD B1a cells revealed lower innate activation threshold for secretion of autoreactive IgM capable to trigger oxidative stress responses upon binding to pancreatic beta cells and providing an early pathogenesis mechanism in murine T1D.

Keywords: NOD, type 1 diabetes, autoreactive IgM, B1a cells, Toll-like-receptor, pancreatic beta cell, oxidative stress.

Abbreviations: AAb: autoantibodies, AAg: autoantigens, BCR: B cell receptor, IA-2: islet cell antigen 512, iNOS: inducible nitric oxide synthase, LPS: Lipopolysaccharide, NAbs: natural antibodies, PCA: Principal Component Analysis, SLE: systemic lupus erythematosus, SPF: specific pathogen free, TLR: Toll-Like receptor, T1D: type 1 diabetes.

INTRODUCTION

The urgency to identify the subtle factors contributing to Type 1 Diabetes (T1D) onset and progression is compelled by recent European statistics indicating that the number of new cases of T1D in children under the age of five is increasing by more than 5% each year and predicting the doubling of T1D incidence in this age group, by 2020 (Patterson, Dahlquist, Gyurus, Green, & Soltesz, 2009). Diabetic patients and non-obese-diabetic (NOD) mice share the spontaneous pathogenic events of T1D development (Castano & Eisenbarth, 1990) in which autoreactive cytotoxic T cells selectively destroy insulin secreting beta cells within the pancreatic islets of Langerhans (Bach, 1994). Nevertheless, studies on NOD mice devoid of B cells have shown that this lymphocyte population is necessary in the diabetic autoimmune process (Serreze et al., 1996) and it is widely accepted that islet-related autoantibodies (AAb) represent the earliest manifestations of diabetogenesis both in pre-diabetic patients and in NOD mice (Leslie, Lipsky, & Notkins, 2001). These autoreactive immunoglobulins, of the IgG isotype, recognize beta cell antigens and are believed to be bystander products of the disease resulting from an ongoing autoimmune process (Leslie, et al., 2001). Thus, in the stage of cytotoxic beta cell destruction, B cells would be highly exposed to beta cell related autoantigens (AAg) leading to preferential activation of B cells with autoreactive potential. In fact, NOD BCR specificity and B cell antigen presentation capacity to T cells are determinant factors in T1D progression (Hulbert, Riseili, Rojas, & Thomas, 2001; Noorchashm et al., 1999). It is believed that long-sustained autoreactive T-B cell interactions condition the repertoire of AAb typical of pre-diabetic patients, where the beta cell destruction is already in progress, though clinical signs are not yet present. Importantly, B-cell–null NOD mice that express a transgene encoding a mutant heavy chain immunoglobulin on the cell surface but cannot secrete antibodies only partially restored T1D suggesting that circulating antibodies could play a role in the disease (Wong et al., 2004).

Here, we revisited the role of autoantibodies in T1D pathogenesis under the contention that the early presence of AAb against beta cell antigens can have implications for T1D pathogenesis. Insulitis consists of the infiltration of pancreatic islets of Langerhans by mononuclear cells and is the hallmark of T1D initiation. In NOD mice the first insulitic events start around 3-5 weeks of age but clinical signs of diabetes, due to massive beta cell loss, arise only from 12 weeks onwards (Yang & Santamaria, 2003). It has been previously shown that NOD, but not C57BL/6 non-diabetic controls, have antibodies bound to beta cells before development of insulitis. Interestingly, these antibodies are mainly of the IgM isotype (Shieh, Cornelius, Winter, & Peck, 1993) and thus unlikely to originate in germinal center reactions. Consistently, young NOD mice have a pronounced repertoire of IgM AAb (Thomas, Kendall, & Mitchell, 2002). Most of the antibodies of the IgM isotype are spontaneously secreted by B1a cells in the absence of exogenous stimuli or T cell help (Casali & Schettino, 1996). The

B1a cells are part of the B1 cell subset of lymphocytes that are mainly found in the peritoneal and pleural cavities and at lower levels in the spleen. These B1 cells express low levels of B220 and are subdivided into B1a and B1b subsets based on the presence or absence of the CD5 molecule on their surface, respectively. B1a cells are mostly of fetal origin, do not undergo class switching and affinity maturation and spontaneously secrete natural AAb (Herzenberg, 2000). On the other hand, B1b cells are generated mainly during neonatal life in the bone marrow (Stall, Wells, & Lam, 1996) and are involved in the earliest specific reaction against pathogens (Martin, Oliver, & Kearney, 2001; Ochsenbein et al., 1999). The CD5 expression on B1a cells is known to downmodulate responses to BCR mediated signaling while favoring IL10 secretion (Bikah, Carey, Ciallella, Tarakhovsky, & Bondada, 1996; Gary-Gouy et al., 2002). Interestingly, the B1 cells activation status is influenced by gut microflora modifications (Alam et al., 2011; Alam et al., 2010) and innate stimulation through toll-like receptors (TLRs) can induce their proliferative and antibody secretion capacities (Genestier et al., 2007). Thus, B1 can be considered as bridging cells between innate and adaptive immunity.

Importantly, B1a cells have recently been proven to be necessary for insulitis onset in a transgenic model in which disease is mediated by T cells recognizing pancreatic beta cell antigens (Ryan et al., 2010). Also, in the 125Tg mouse, B1a cells prevailed as anti-insulin IgM secretors while B2 cells with the same specificity were anergized (Rojas et al., 2001). Further, B1a cells are increased in the peripheral blood of T1D patients (Gyarmati, Szekeres-Bartho, Fischer, & Soltesz, 1999) and have been suggested to play a role in the NOD autoimmune process (Kendall, Woodward, Hulbert, & Thomas, 2004).

We have previously hypothesized that NAbs of NOD B1a cell origin could promote the initiation of insulitis (Corte-Real, Duarte, Tavares, & Penha-Goncalves, 2009). However, the link between B1a cell properties, autoreactive IgM profile and autoimmune process triggering has never been established for T1D. Here, we dissect this intricate chain of events by analyzing the autoreactive repertoire of NOD IgM, by uncovering the B cell sub-populations contributing to its secretion and by examining the impact of these immunoglobulins on NOD beta cell physiology.

METHODS

Mice

C57BL/6, NOD, NOD.SCID, C57BL/6.H2g7 and NOD.H2b mice were bred and maintained in SPF facilities at the Instituto Gulbenkian de Ciência. All mice used were females with 1 to 12 weeks of age. NOD female insulitis was unapparent at 3 weeks. Experimental protocols were

approved by the competent Portuguese authority (Direcção Geral de Veterinária) and are in accordance with international regulations (U.S. Department of Health and Human Service, Office of Extramural Research, National Institutes of Health, Office of Laboratory Animal Welfare, 1985).

ELISA and ELISPOT assays

ELISA was used to quantify total IgM and anti-insulin IgM concentrations in mouse sera. Total IgM, IgM with reactivity to a pool of T1D related AAg and IL10 levels were measured in cell culture supernatants after TLR stimulation. Briefly, ELISA plates (Nunc) were coated with IgM, insulin only (Sigma) or a T1D AAg pool of insulin, IA-2 (GenWay Biotech), GAD65 and GAD67 (United States Biologicals). Bound seric IgM was detected by goat-anti-mouse-IgM-Alkaline-Phosphatase (AP) conjugated antibody (Southern Biotech.) revealed with sigma phosphatase substrate and optical densities read in a spectrophotometer at 405 nm. IgM concentrations were calculated against standard curves obtained with either purified monoclonal IgM (clone 11E10, Southern Biotech.) or anti-insulin IgM kindly provided by JW Thomas (Thomas, Kralick, & Ewulonu, 1997). IL10 concentration was determined with a mouse IL-10 ELISA MAX™ Deluxe kit (Biolegend) according to manufacturer's instructions.

The number of B cells secreting IgM with reactivity to the previously described T1D AAg pool, to thyroiditis associated AAg (thyroglobulin and thyroperoxidase), to the sialitis related AAg muscarinic-3-receptor, to Systemic Lupus Erythematosus (SLE) associated AAg (ds-DNA, ss-DNA and histone) or to AAg typically recognized by B1a cells (phosphatidylcholine, phosphorylcholine and dextran) (Baumgarth, Tung, & Herzenberg, 2005) were determined as described elsewhere (Corte-Real et al., 2009). Briefly, cell dilutions were performed in RPMI 1640 complete medium with 1% BSA and incubated overnight at 37ºC. Cells were removed by washing with 0.1% Tween-20 and plates were incubated with goat anti-mouse IgM-AP conjugated antibody and revealed with BCIP-AMP substrate (2.3 mM of 5-bromo-4-chloro-3-indolyl phosphate diluted in 2-amino-2-methyl-1-propanol buffer, Sigma).

Cell analysis and purification

Single cell suspensions were prepared from spleen and from peritoneal cavity lavage and incubated with anti-mouse-Fc-block/CD16/32 (clone 2.4G2, BD Pharmingen). Peritoneal B1a cells were identified as $B220^{+}CD5^{+}$ cells and B1b+B2 cells as $B220^{+}CD5^{-}$ cells. Splenic B1a cells were identified as $CD23^{-}IgM^{high}B220^{low}CD5^{+}$ cells, follicular B lymphocytes as $CD23^{+}IgM^{low}$ and marginal zone B cells as $CD23^{-}IgM^{high}CD21^{+}CD5^{-}$. FITC-anti-CD19 (clone 1D3), FITC-anti-IgM (clone R33.24.12), PE-anti-CD23 (clone B3B4), Percp or PE-anti-CD5 (clone 53-7.3), APC-anti-CD45R/B220 (clone RA3-6B2), biotinylated or FITC-anti-CD11b/Mac-1 (clone M1/70), FITC-anti-CD21 (clone 7G6), PE-anti-CD43 (clone S7), biotinylated-anti-CD86 (clone GL1), FITC-anti-CD62L (clone Mel14), biotinylated or PE-anti-

Syndecan-1 (clone 281-2) and FITC or Percp-Streptavidin antibodies were used for staining. Traceable latex beads (Beckman coulter) were added for counting cells. Flow cytometry (FACSCalibur^{TM}) with CELLQuest software (Becton Dickinson, Franklin Lakes, NJ, USA) was used and data analysis performed with FlowJo software (TreeStar). Peritoneal and splenic B cell populations were purified by high speed cell sorting MoFlo (Dako-Cytomation, Berkeley, CA, USA) or BD FACSAria III cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Cell purity was higher than 90%.

RNA isolation and Real-Time PCR

Total RNA from sorted peritoneal cavity B1a cells or from cultured beta cells was obtained using a High Pure RNA Isolation Kit (Roche) following the manufacturer's protocol. RNA was converted to cDNA with Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The following TaqMan Gene Expression Assays with FAM-reporter (Applied biosystems) were used: *Tlr2* (Mm00442346_m1), *Tlr4 (*Mm00445274_m1*), Tlr6 (*Mm02529782_s1), *Tlr7 (*Mm00446590_m1), *Tlr9 (*Mm00446193_m1*), Fas (*Mm00433237_m1*), Nos2 (*Mm01309901_m1*), Caspase 3 (*Mm01195084_m1*) and Ccl2 (*Mm00441242_m1*).* Gene expression quantification was performed in ABI Prism 7900HT (Applied Byosistems, Foster City, CA, USA). Relative quantification in each real-time PCR reaction was obtained after normalization for mouse GAPDH (VIC/MGB probe) expression using the $2^{-\Delta\Delta CT}$ analysis method (Livak & Schmittgen, 2001).

Lipopolysaccharide stimulation

Purified peritoneal B1a cells were cultured in RPMI 1640 complete medium with or without 1-5 μg/ml of purified LPS and supernatants were taken for antibody and IL10 quantification by ELISA at day 3 of culture. For proliferation analysis cells were pulsed in the last 6 h of culture, harvested and ^{3}H-thymidine (Perkin Elmer) incorporation was measured. Expression of activation markers after one day of stimulation was assessed on pooled duplicates using biotinylated-anti-CD69 (clone H1.2F3), biotinylated-anti-CD86 (clone GL1), Alexa488-anti-CD25 (clone PC 61) and Percp-Streptavidin.

***In vitro* transwell cultures**

Islets of Langerhans were isolated from the pancreas of 9 weeks old NOD.SCID females by collagenase type V digestion (1.4 mg/ml, Sigma) followed by hand picking under a stereomicroscope. Islets were subsequently dissociated into single cell suspensions by simultaneous mechanical and dispase enzymatic treatment (5 mg/ml, Roche). Freshly dissociated cells were cultured at $1*10^5$ cells per well in Ham's F-10-glutamax medium (Gibco) supplemented with 10 mM glucose (Sigma), 0,5% BSA, 50 μM

isobutylmethylxanthine (Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco). 24 hours after, sorted peritoneal cavity B1a cells ($5*10^5$) or follicular splenic B cells ($5*10^5$) were added in transwells (0,4 μm pore size, Millipore). After 24h of co-culture beta cells were either collected for flow cytometry analysis or processed for RNA isolation. Alexa647-anti-IgM (clone R33.24.12) was used for staining of bound IgM on beta cells. Negative controls consisted of beta cells with lymphocyte medium only in the transwell.

Statistical analysis

Statistics were estimated by either Kruskal-Wallis or unpaired student's t-test as specified in each figure legend. Two-tailed tests with 95% of confidence interval where used and differences with $p<0.05$ were considered significant.

RESULTS

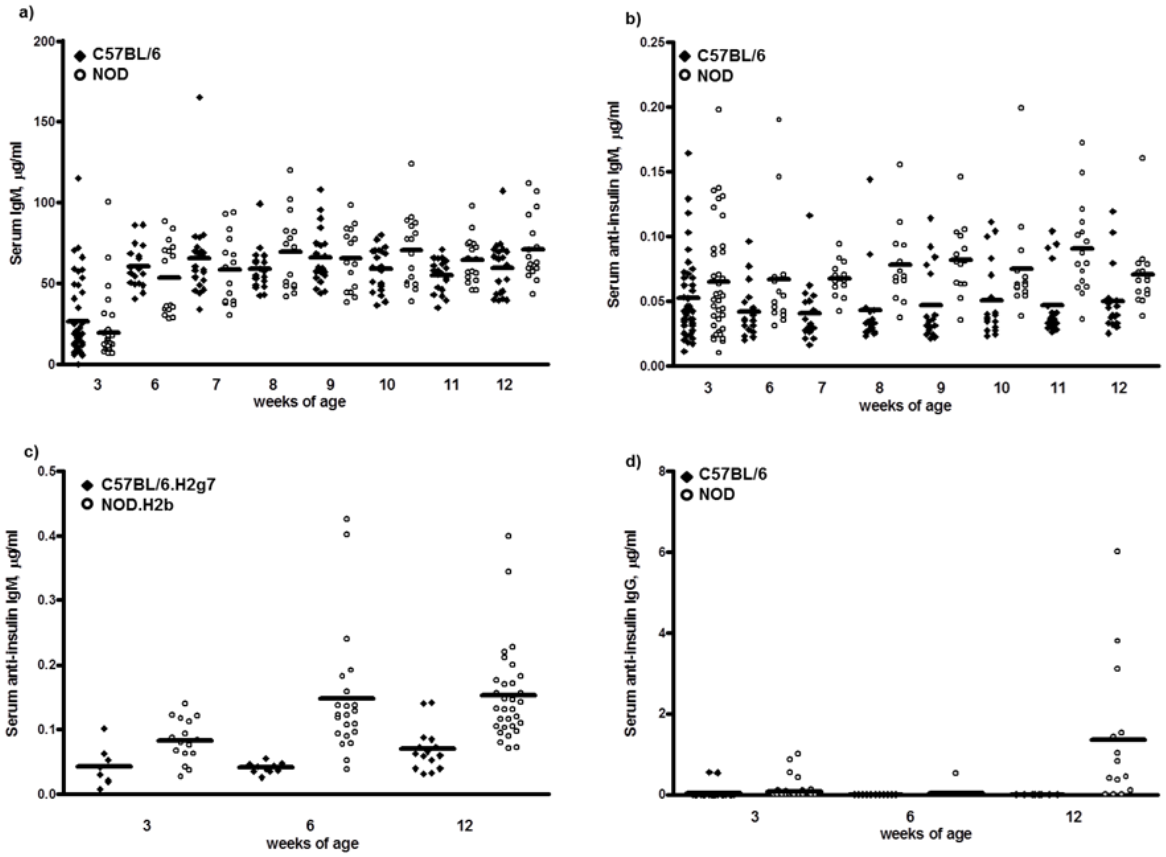
High anti-insulin IgM level in the NOD mouse is independent of T cell autoreactivity

To ascertain whether the NOD natural AAb repertoire was specifically directed to islet-related antigens, we quantified the serum concentration of total IgM and of anti-insulin IgM in NOD and C57BL/6 mice from 3 to 12 weeks of age. Although no differences were found in total IgM levels (Figure VI.1a), significantly increased levels of IgM with reactivity towards insulin were found in NOD mice (Figure VI.1b). Importantly, these values were stable between 6-12 weeks indicating that they were not influenced by the progression of the disease in the NOD mice. This suggests that the insulin-IgM autoreactivity is a T-cell independent, intrinsic characteristic of the NOD natural IgM repertoire. In support, we have analysed the profile of anti-insulin IgM secretion in C57BL/6.H2g7 mice, whose genetic background is similar to that of the C57BL/6 parental strain but contains a MHC locus of NOD origin, and in its reciprocal NOD.H2b congenic strain that is protected from T1D. The levels of anti-insulin IgM were significantly increased in the NOD.H2b compared to C57BL/6.H2g7 controls (Figure VI.1c). No differences were found in total IgM concentration (data not shown).

Additionally, we have analyzed the global pattern of natural IgM reactivity by comparing the capacity of NOD and C57BL/6 serum IgM to recognize AAg present in NOD protein extracts and inferred that not only were there different patterns of IgM autoreactivity but also that global IgM reactivity was higher in NOD compared to C57BL/6 mice (Supplementary Figure VI.1). We have also measured the serum levels of anti-insulin IgG in NOD and C57BL/6 mice. As expected, the reactivity profile was quite different from that observed for anti-insulin IgM. Although very low to undetectable levels were present in the serum of both strains up to

6 weeks, NOD revealed a progressive increase in antibody concentration that was most relevant at 12 weeks (Figure VI.1d).

Figure VI.1 Antibody IgM reactivity in the NOD is biased towards T1D antigens from pre-insulitic ages



Serum antibody concentration was measured individually by ELISA. Total IgM (a) and anti-insulin IgM (b) was assessed in groups of 13 to 47 C57BL/6 or NOD mice at 3 weeks of age and over the interval of 6 to 12 weeks of age. Anti-insulin IgM was determined in groups of 8 to 31 C57BL/6.H2g7 or NOD.H2b mice (c) and anti-insulin IgG was analyzed in groups of 14 to 46 C57BL/6 or NOD mice (d) at 3, 6 and 12 weeks of age. Average concentrations are shown as horizontal bars. Anti-insulin IgM (b and c) and IgG (d) were different between strains over the 3 to 12 weeks of age interval ($p<0.0001$) in Kruskal-Wallis test. NOD anti-insulin IgG concentration significantly increased with time ($p<0.0001$) while NOD anti-insulin IgM did not vary in the 6-12 weeks of age in Kruskal-Wallis test.

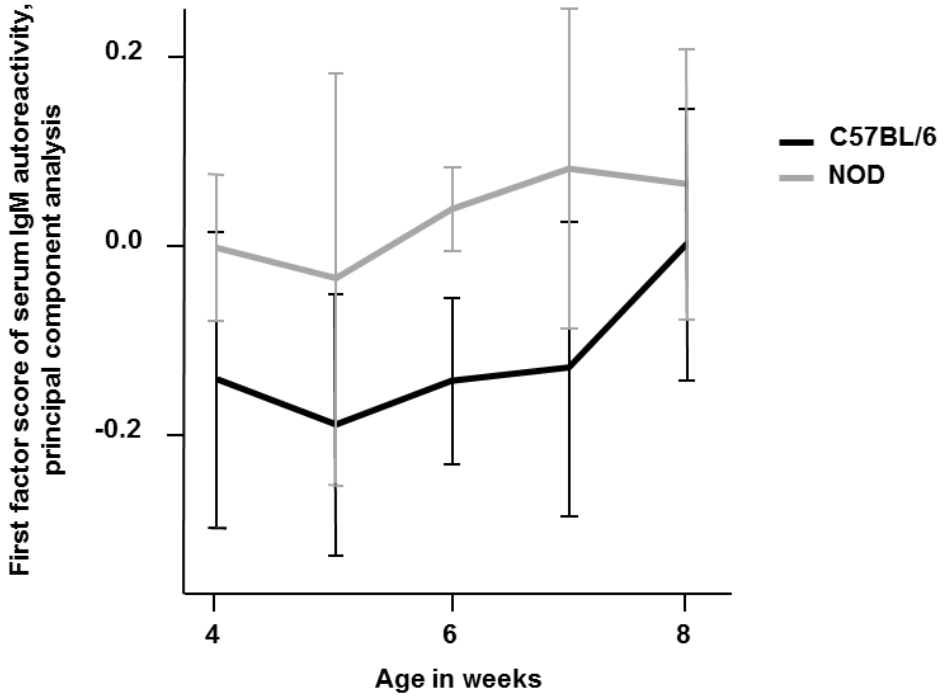
High proportion of NOD peritoneal cavity B1a cells secretes IgM anti-T1D autoantigens in absence of patent insulitis

In search of the cellular source for the altered natural antibodies repertoire in the NOD mouse, we quantified the number of B cells secreting IgM that were able to recognize a pool of T1D related AAg. We have analysed the peritoneal B1a and B1b+B2 cells and the splenic follicular B cells, marginal zone B cells and B1a cells (Figure VI.2a). We observed that already at 5 weeks of age the B1a cell peritoneal compartment contained the highest population of cells spontaneously secreting IgM with anti-T1D AAg reactivity. Importantly,

there were significantly higher numbers of B1a cells secreting IgM with these reactivities in the NOD when compared to the C57BL/6 control, which is consistent with the pattern that we have found in the serum. We have detected a low proportion of antibody-secreting cells with anti-T1D AAg reactivity in peritoneal B1b+B2 cells, though no differences were observed between strains (Figure VI.2a). In the splenic compartment the percentage of cells with these properties was either zero or extremely low (Figure VI.2a).

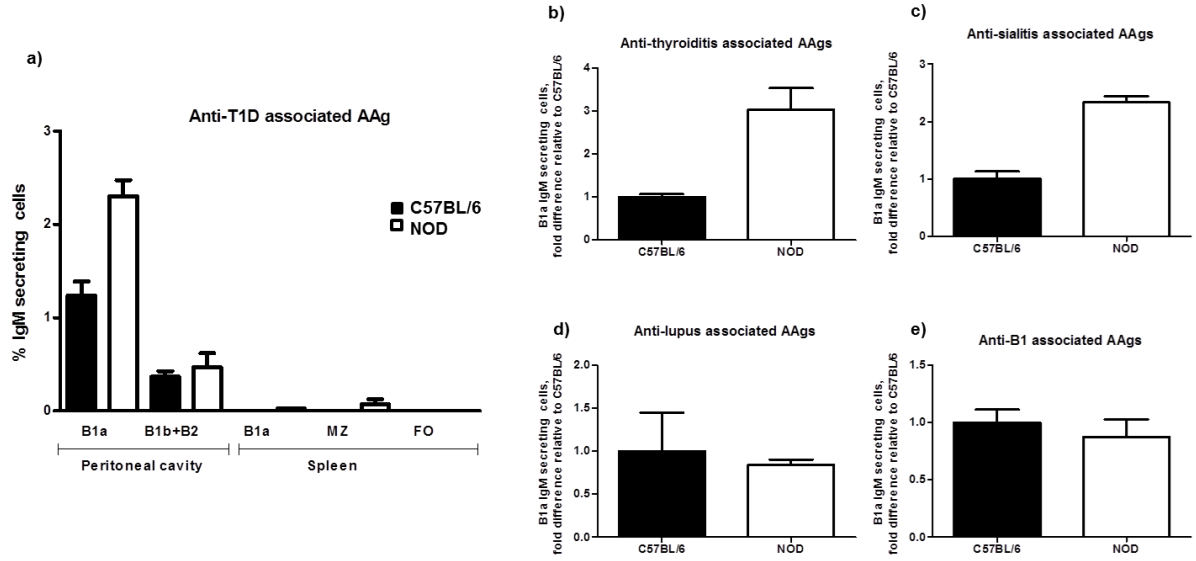
We next wanted to ascertain whether the observed difference in autoreactivity was restricted to T1D associated AAg or whether it could represent a general feature of NOD B1a cells. Thus, we have quantified the proportion of B1a cells secreting IgM recognizing either a pool of AAg related to autoimmune thyroiditis (Figure VI.2b), the main self-antigen of immune mediated sialitis (Figure VI.2c), a pool of antigens that are targets of SLE (Figure VI.2d), or a pool of antigens that B1a cells generally recognize (Figure VI.2e). Consistent with the NOD polyendocrine phenotype we detected more peritoneal B1a cells secreting IgM that recognized thyroiditis and sialitis AAg (Figure VI.2b and VI.2c), although no differences were found for the other AAgs tested (Figure VI.2d and VI.2e).

Supplementary Figure VI.1 NOD and C57BL/6 mice exhibit distinct profiles of IgM autoreactivity



Serum IgM reactivity of 5 C57BL/6 or NOD mice were tested on liver and kidney extracts, over the 4 to 8 weeks of age interval. The numerical values obtained from the quantification of the immunoreactivity profiles were submitted to PCA. First principal component mean scores per strain and time point are represented with the respective SD. Group differences were found for the first PCA factor averaged over the 4 to 6 weeks of age interval ($p<0.05$) in unpaired t-student test.

Figure VI.2 Higher proportions of autoreactive IgM secreting B1a cells are present in the NOD peritoneal cavity

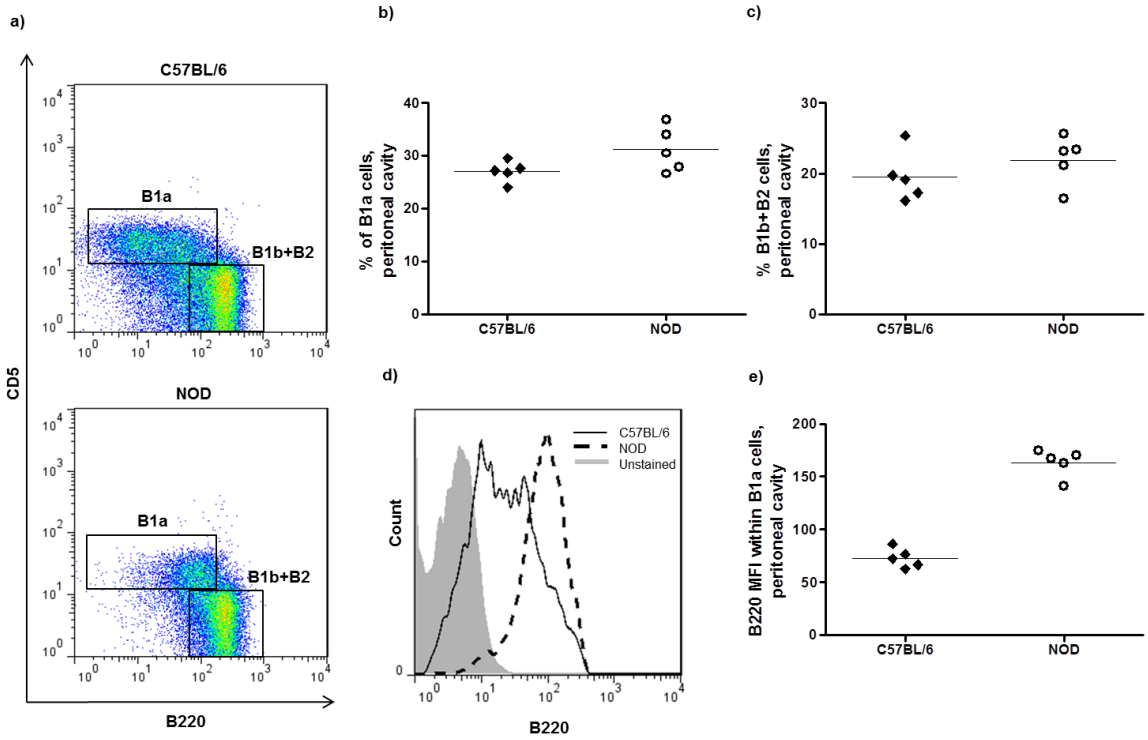


The percentage of cells spontaneously secreting IgM against: a pool of insulin+GAD65+GAD67+IA-2 (a), thyroglobulin and thyroperoxidase (b), muscarinic-3-receptor (c), ds-DNA, ss-DNA and histone (d), or phosphatidylcholine, phosphorylcholine and dextran (e) was determined by ELISPOT Assay. Peritoneal cavity B1a (a-e) and B1b+B2 cells, splenic follicular B cells (FO), marginal zone B cells (Mz) and splenic B1a cells (a) were purified from pooled samples of groups of 8 to 12 mice between 3 and 5 weeks of age. The bars represent the average results of triplicates with the respective SD. Significant differences between strains were found in (a) ($p<0.01$), in (b) ($p<0.05$) and in (c) ($p<0.001$) with unpaired t-student test. Data are representative of three independent experiments.

NOD B1a cells are B220^{bright} at 1 week of age

Subsequently, we have analysed whether the differences in AAb secretion patterns between NOD and C57BL/6 could be attributed to disturbances in the peritoneal cavity B1a cell compartment. We observed that although the proportion and numbers of peritoneal B cells did not differ between mouse strains, striking differences in the B1a surface phenotype could be observed (Figure VI.3a-VI.3c) with NOD cells expressing higher levels of B220 already at 3 weeks (Figure VI.3d and VI.3e).

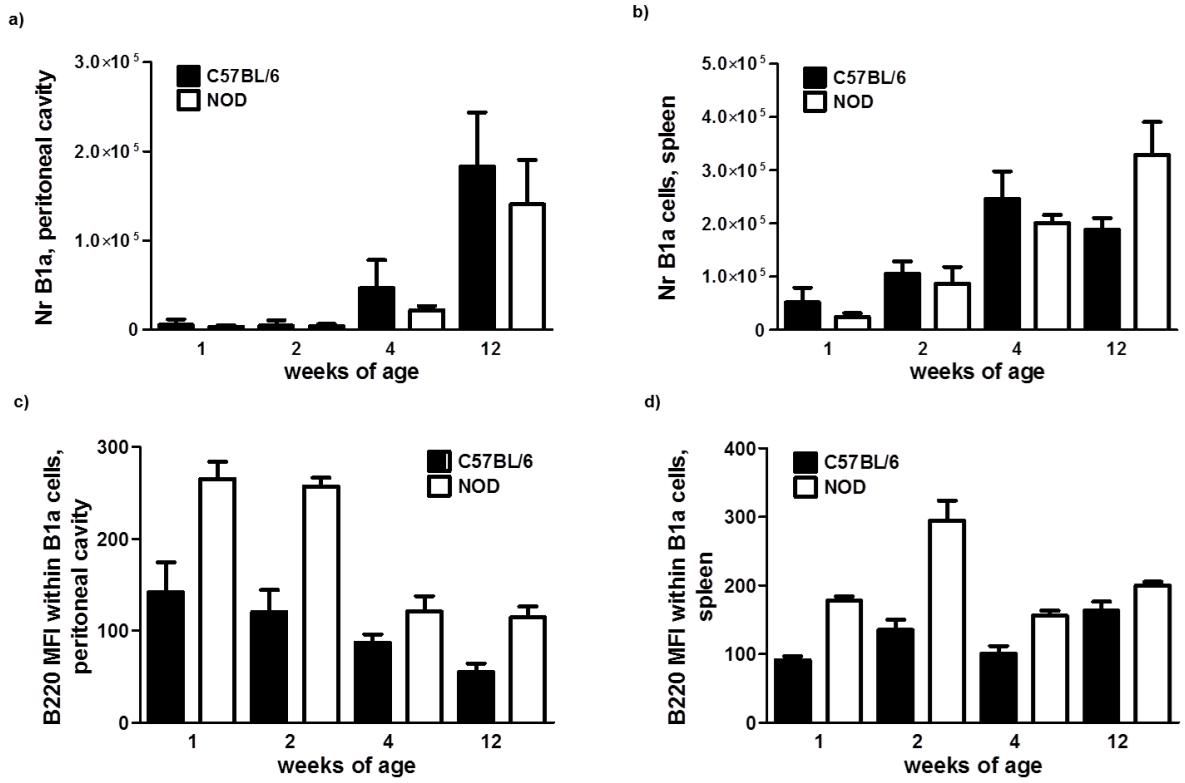
Figure VI.3 NOD peritoneal cavity B1a cells are B220 high



Peritoneal cavity B1a and B1b+B2 cell subpopulations were analyzed by flow cytometry in NOD and C57BL/6 mice. B220 and CD5 expression was evaluated within $CD19^+$ B cells and representative dot plots are shown (a) as well as a representative histogram of typical B220 expression intensity (d). The individual percentages of B1a and B1b+B2 cells with respective mean values for 5 C57BL/6 or NOD mice with 3 weeks of age are represented (b-c) as well as the B220 mean fluorescent intensity (MFI) individual values from the same samples (e). Strain differences were found for B220 MFI levels (e) ($p<0.0001$) in unpaired t-student test. Data are representative of more than three independent experiments.

To understand the origin of this NOD distinctive feature, we have performed a time course analysis of B1a cells both in the peritoneal cavity and in the spleen in comparison to C57BL/6. We found identical kinetics and no differences in the number of B1a cells in the peritoneal cavity (Figure VI.4a). In the spleen, the B1a cell numbers were relatively similar, except at 1 and at 12 weeks where NOD had respectively slightly less or more B1a cells in comparison to C57BL/6 (Figure VI.4b). Interestingly, the NOD B1a cells both in peritoneal cavity and in the spleen consistently presented increased levels of B220 molecules on their surface, detected as early as at 1 week of age (Figure VI.4c and VI.4d). In the peritoneal cavity, B220 intensity decreased with aging, though the strain difference persisted (Figure VI.4c). Intriguingly, the B220 levels were higher in NOD mouse spleen around 2 weeks (Figure VI.4d).

Figure VI.4 Ontogenic distribution of B1a cells in the spleen and peritoneal cavity

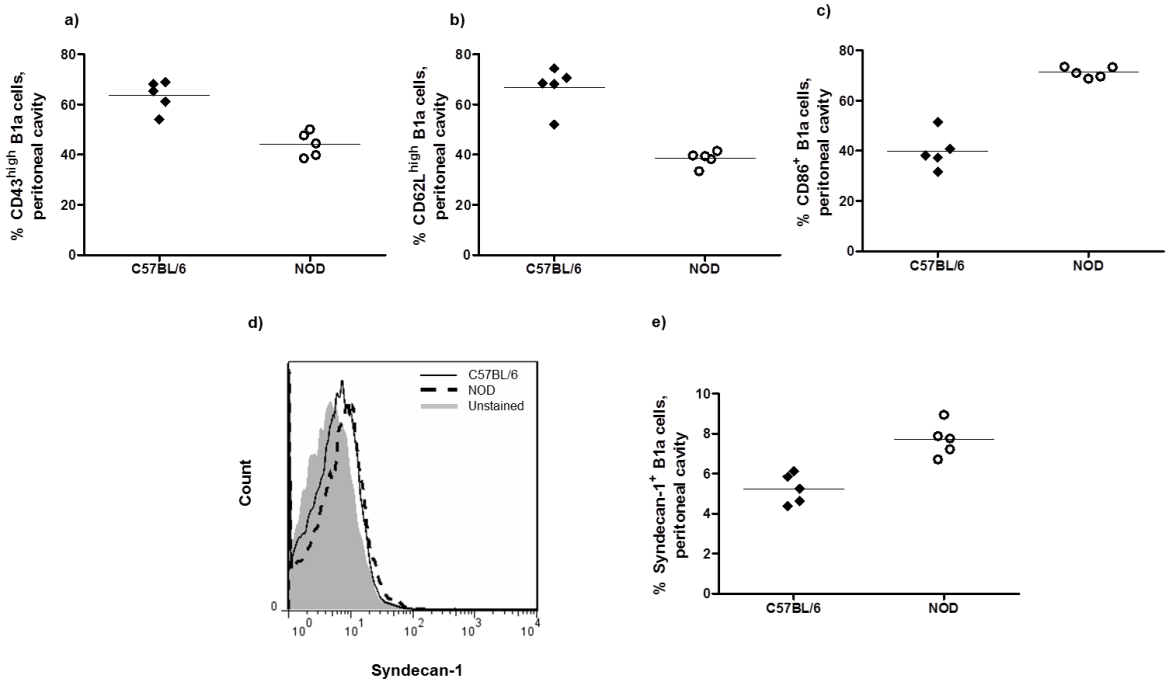


The number of peritoneal cavity (a) and splenic (b) B1a cells and the B220 MFI within the same populations (c and d respectively) were determined by flow cytometry at 1, 2, 4 and 12 weeks of age. The average results for 4 to 9 C57BL/6 or NOD mice are represented with the respective SD. Differences were found for NOD and C57BL/6 B1a cells B220 MFI for all analyzed ages ($p<0.05$) in unpaired t-student test. B1a numbers were identical between strains except at 1 ($p<0.05$) and 12 weeks ($p<0.01$) in the spleen in unpaired t-student test. Data are representative of 2 to 4 independent experiments for each time point.

Peritoneal cavity NOD B1a cells display increased expression of surface activation molecules

We hypothesized that the greatest expression of the B220 molecule on NOD B1a cells could be linked to increased basal activation. Indeed, we observed that at 3 weeks NOD B1a cells showed decreased proportion of $CD43^{high}$ expression as compared to C57BL/6 (Figure VI.5a). Also, NOD B1a cells expressed lower amounts of CD62L and showed enhanced expression of the co-stimulatory molecule CD86 (Figure VI.5b and VI.5c). Thus, the surface phenotype of NOD B1a cells corroborates with the observed increased activation state at pre-insulitic stages. In addition, 4 to 9% of B1a cells expressed Syndecan-1, indicating that some of these cells share the antibody secretion phenotype of plasma cells (Figure VI.5d). Interestingly, the proportion of Syndecan-1^{+} B1a cells was consistently larger in the NOD mouse (Figure VI.5e).

Figure VI.5 NOD peritoneal cavity B1a cells are naturally more activated



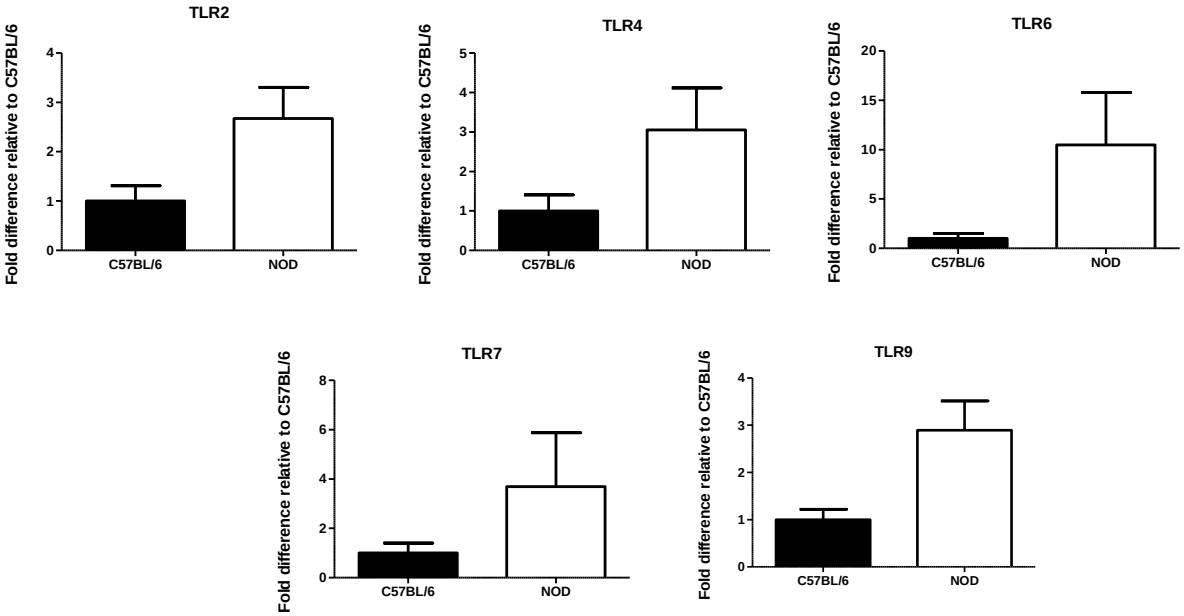
Peritoneal cavity B1a cells were analyzed in 5 C57BL/6 or NOD mice, with 3 weeks of age. The expression of CD43 (a), CD62L (b), CD86 (c) and Syndecan-1 (d-e) was assessed by flow cytometry. Individual percentages and the respective means are represented (a-c, e). A typical Syndecan-1 expression histogram (d) is shown for each strain and unstained controls. Differences were found in the expression of all the referred cell surface activation markers ($p<0.01$) in unpaired t-student test. Data are representative of 3 independent experiments.

NOD B1a cells secrete increased levels of autoantibodies upon TLR stimulation

The innate-like properties of B1a cell stimulation prompted us to test whether alterations in TLR expression or response to TLR stimuli could condition the secretion profiles observed in the NOD B1a compartment. We have quantified TLR 2, 4, 6, 7 and 9 mRNA levels in purified peritoneal NOD B1a cells in comparison to C57BL/6 and observed that all these genes were expressed more in the NOD mice (Figure VI.6). To identify physiological correlates of these gene expression differences we have stimulated sorted B1a cells with purified LPS, a known ligand to TLR4, and found no consistent strain differences in CD69, CD86 or CD25 cell surface expression, indicating that similar activation levels were obtained (Figure VI.7a-VI.7c). Despite seeing no differences in total IgM levels within the stimulated B1a cell supernatants between NOD and C57BL/6, the concentration of NOD IgM with reactivity to islet-related AAg was consistently higher (Figure VI.7d and VI.7e). Accordingly, an increased proportion of TLR4 stimulated NOD B1a cells secreted IgM with these specificities (Supplementary Figure VI.2a), mimicking the secretion behaviour that we observed *ex vivo*. TLR4 stimulation induced potent secretion of the immunoregulatory cytokine IL10 by B1a cells and, in contrast with the observed AAb secretion profile, lower amounts were detected in the NOD B1a cell cultures (Figure VI.7f). In addition, after 3 days of stimulation NOD B1a cells proliferation was substantially higher than in C57BL/6 (Figure VI.7g). Similar

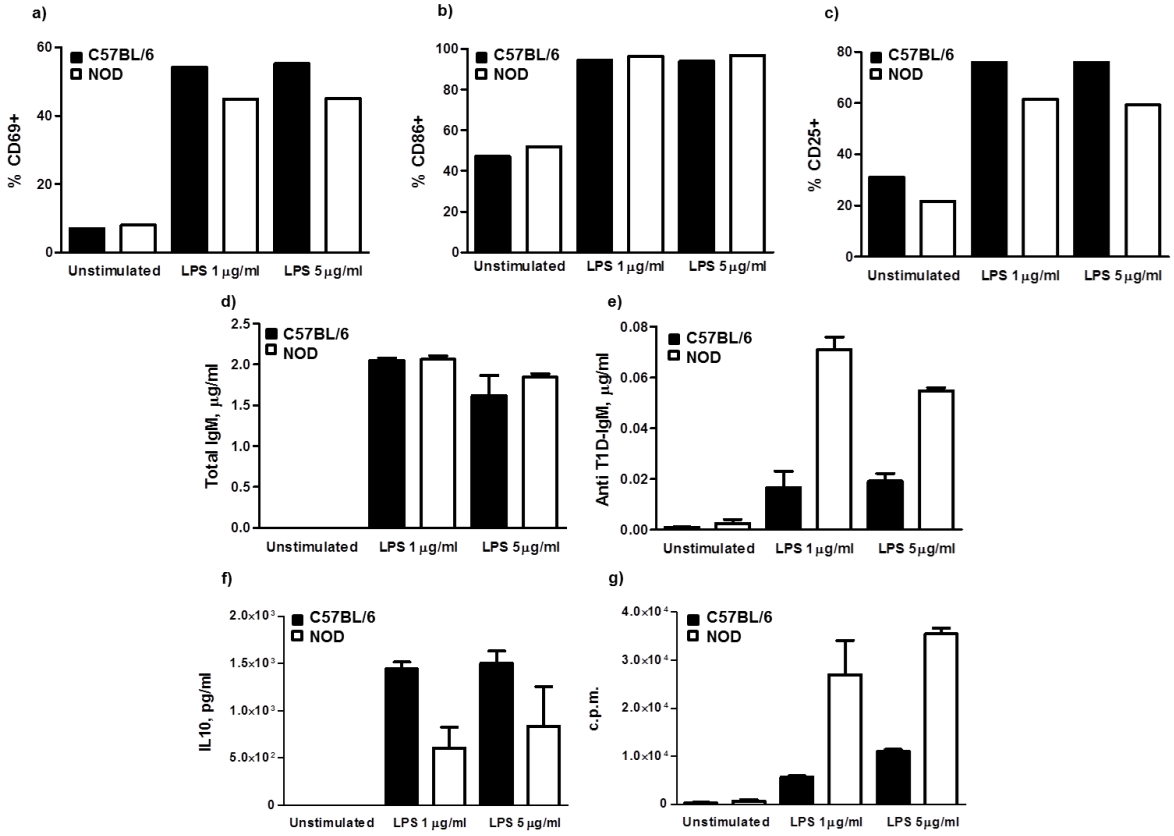
observations were obtained upon TLR9 stimulation (Supplementary Figure VI.2b and VI.2c).

Figure VI.6 TLR gene expression is increased in young NOD peritoneal cavity B1a cells



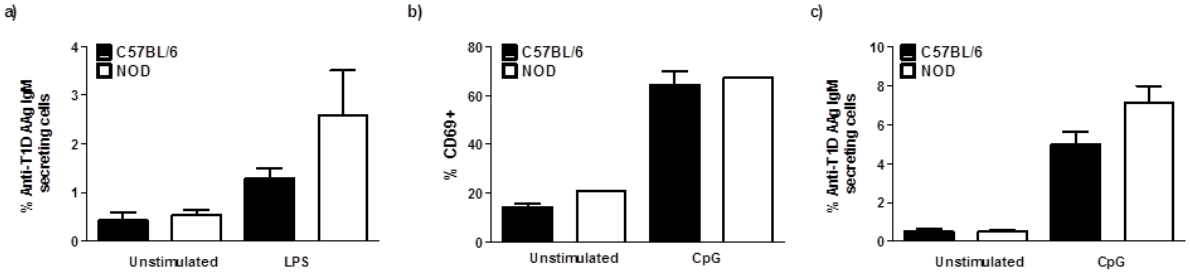
Purified peritoneal B1a cells were obtained from 15 females with 3 weeks of age, pooled for RNA extraction, and TaqMan Gene Expression assays were used to quantify the expression of the indicated genes. Relative quantification of these genes was obtained in triplicates, after normalization to GAPDH expression. Mean fold-differences to C57BL/6 and the respective SD from a pool of two identical experiments are represented. Differences were found for all the studied genes ($p<0.05$) in unpaired t-student test.

Figure VI.7 NOD peritoneal cavity B1a cells respond to LPS stimulation *in vitro* with increased proliferation and secretion of autoantibodies



Purified peritoneal cavity B1a cells were obtained from 16 C57BL/6 or NOD mice with 6 to 10 weeks of age and were stimulated with 1 or 5 μg/ml of purified LPS. Unstimulated cells were used as negative controls. After one day the cells were assayed for activation phenotypes by flow cytometry, namely CD69, CD86 and CD25 cell surface expression (a-c). On the third day of culture, supernatants were assessed for total IgM (d), anti-T1D AAg IgM (e) and IL10 concentration (f) by ELISA. Proliferation was measured by ^{3}H-thymidine incorporation (e). The average of duplicate samples is represented with the respective SD, except for activation markers analysis where pooled duplicates were used for flow cytometry acquisition. Strain differences were found for anti-T1D AAg IgM concentration in supernatants of B1a cells stimulated with both 1 and 5 μg/ml of LPS (p<0.05) for IL10 levels in supernatants of B1a cells stimulated with 1 μg/ml of LPS (p<0.05) and for proliferation after 3 days of 5 μg/ml LPS stimulation (p<0.01) in unpaired t-student test. Data are representative of 2 independent experiments.

Supplementary Figure VI.2 NOD peritoneal cavity B1a cells innate-like stimulation *in vitro* is consistent with a lower threshold for autoreactive IgM secretion



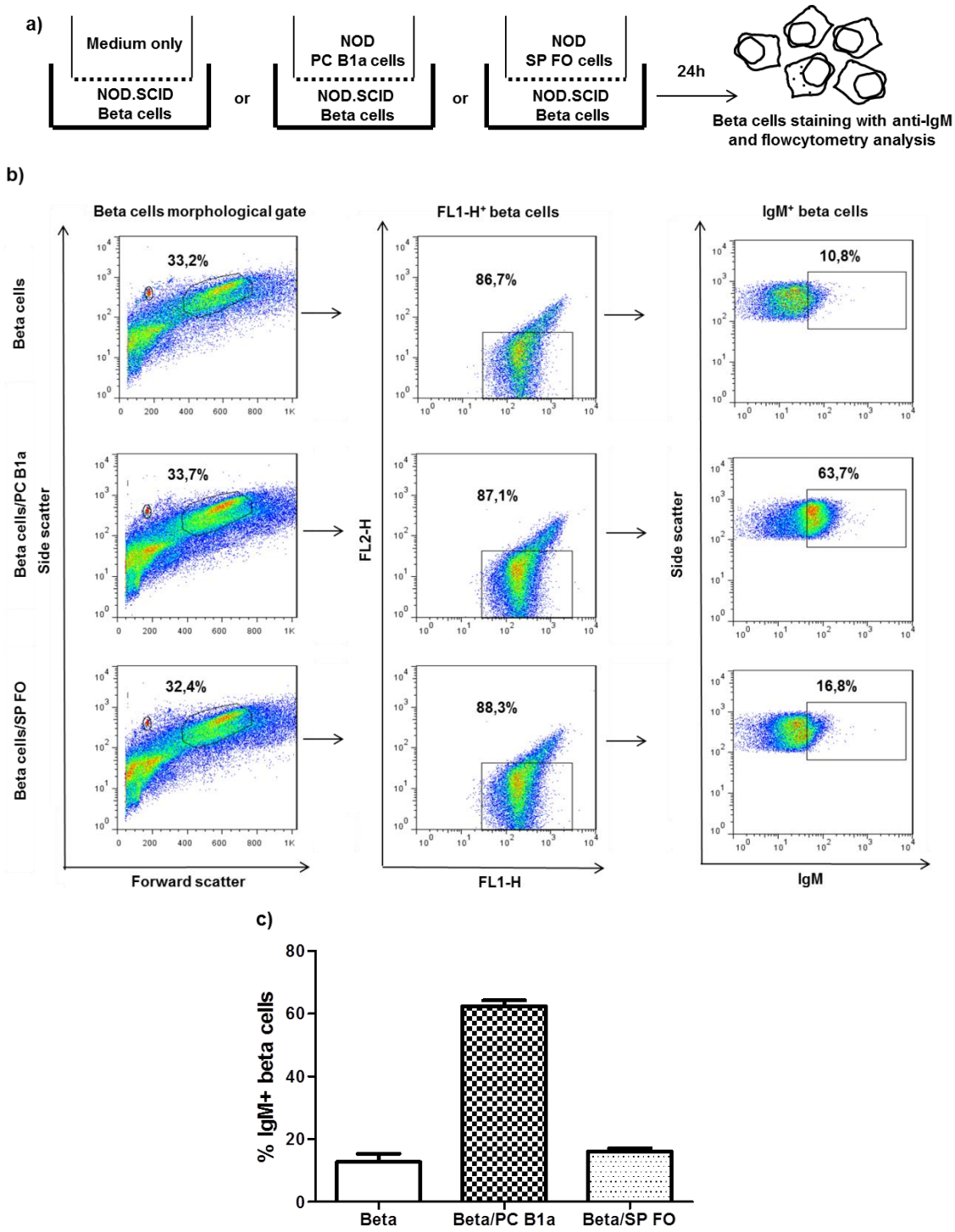
Purified peritoneal B1a cells were obtained from 16 C57BL/6 or NOD mice with 6-10 weeks of age and cultured with 5μg/ml of LPS (a) or 0.5 μM of CpG (b-c). Unstimulated cells were used as negative controls. Anti-T1D AAg IgM secreting cells were quantified by ESA after 2 days of culture (a, c). The

expression of CD69 on CPG stimulated B1a cells was assessed by flow cytometry on the 1st day of culture, the bars represent the average of duplicates (C57BL/6) or a single well acquisition (NOD) (b). For ESA results the average of triplicate samples is represented. Error bars represent SD. Strain differences were found in (a) ($p<0.05$) and (c) ($p<0.05$) in unpaired t-student test.

IgM secreted by NOD B1a cells can bind to pancreatic beta cells *in vitro* and trigger *iNOS* expression

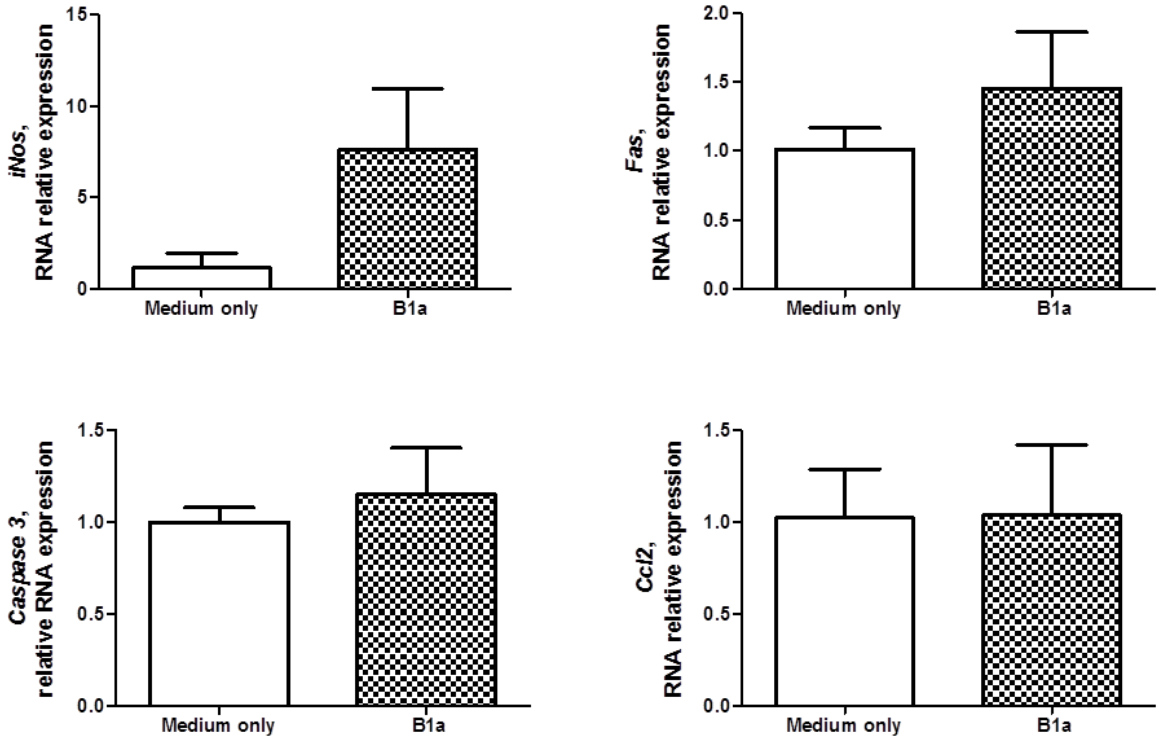
To analyze the mechanisms by which IgM of NOD B1a cell origin may contribute to disease, we have established *in vitro* cultures where secreted antibody but not B cells are in contact with beta cells. We have purified NOD peritoneal B1a cells and splenic follicular B cells and cultured them in transwell with dispersed pancreatic islets of Langerhans from immunodeficient NOD.SCID mice (Figure VI.8a). After 24 hours of culture, beta cells were analysed for the presence of bound IgM (Figure VI.8b). Beta cells could be distinguished by their size, granulosity and autofluorescence in the FL1-H channel (Figure VI.8a and VI.8b). In agreement with our analysis of IgM reactivity, beta cells that were co-cultured with sorted B1a cells presented IgM bound on their surface. On the other hand, beta cells co-cultured with follicular B cells showed only background levels of IgM staining (Figure VI.8b and VI.8c). Using the same system we next analyzed the expression levels of genes associated with pancreatic beta cell responses to stress. Gene expression analysis revealed upregulation of *iNOS* in islet cells co-cultured with NOD B1a cells when compared to beta cells cultured with medium alone (Figure VI.9). No difference was observed in the expression of *Fas*, *Caspase 3* and *Ccl2* (Figure VI.9).

Figure VI.8 IgM secreted by NOD B1a cells binds to NOD.SCID pancreatic beta cells *in vitro*



The enriched beta cell population collected from 9 NOD.SCID females, with 9 weeks of age, was co-cultured in a transwell system with purified peritoneal cavity B1a cells (PC B1a) or splenic follicular B cells (SP FO) obtained from 15 NOD females with 7-10 weeks of age. After 24 h, beta cells were stained and the presence of bound IgM was analysed by flow cytometry (a). Beta cells cultured in transwell with lymphocyte culture medium only were used as negative controls. Representative dot plots of the beta cells morphological gate, auto-fluorescence in FL1-H channel and IgM staining are represented for each experimental condition (b). The mean and SD of duplicates is shown in (c). Beta cells co-cultured with sorted peritoneal B1a cells presented increased levels of IgM bound on their surface, in comparison to negative controls or beta cells co-cultured with follicular B cells from the spleen ($p<0.01$) in unpaired t-student test. Data are representative of 3 independent experiments.

Figure VI.9 *In vitro* autoreactive natural IgM binding in pancreatic beta cells triggers *iNOS* expression



Dispersed pancreatic islets of Langerhans obtained from 9 NOD.SCID females, with 9 weeks of age, were co-cultured in duplicates in a transwell system with purified peritoneal cavity B1a cells collected from 12-14 NOD females with 9-10 weeks of age. After 24 h, beta cells RNA was extracted and TaqMan Gene Expression assays were used to quantify the expression of *iNOS*, *Fas*, *Caspase 3* and *Ccl2*. Relative quantification of these genes was obtained in triplicates of each sample, after normalization to GAPDH expression. Average fold-differences with SD from a pool of two identical experiments are represented in relation to negative controls, where beta cells were co-cultured with lymphocyte medium only. Differences were detected for *iNOS* expression ($p<0.05$) in unpaired t-student test.

DISCUSSION

This study demonstrates that NOD B1a cells have an increased responsiveness to innate activation and secrete natural antibodies with higher reactivity to T1D associated AAg. Importantly, NOD B1a cell-derived IgM is able to bind pancreatic beta cells and trigger *iNOS* expression, a starting point in the beta cell oxidative stress response. Together, these results provide supporting evidence to propose that the NOD natural AAb repertoire is biased from an early age towards endocrine autoreactivity, thus having the potential to fuel the autoimmune beta cell attack prior to the T cell mediated destruction.

In our time course analysis NOD mice presented increased but stable serum levels of IgM with insulin reactivity when compared to C57BL/6. This pattern of natural antibody autoreactivity did not correlate with the IgG serum levels that clearly followed the progression of the disease in the NOD mouse (Figure VI.1d; Koczwara et al., 2003). The remarkably

different trajectories of serum anti-insulin IgG or IgM isotypes highlights their different roles in the NOD immune response. While the detection of anti-T1D AAg IgG is widely accepted as an early disease marker in clinical practice and is generally considered to be a by-product of the T cell mediated destruction of beta cells (Serreze et al., 1998; Yu et al., 2000), autoreactive IgM comprises the pool of NAbs, the first antibodies to arise during ontogeny produced in the absence of exogenous stimuli (Meffre & Salmon, 2007). Analysis of MHC congenic strains (Wicker et al., 1992) showed that, unlike IgG, IgM reactivity observed in the NOD is independent of T-cell help and disease progression, and is consistent with the hypothesis that an increased autoreactivity in the NOD NAb compartment would contribute to the initiation of autoimmunity (Corte-Real, Duarte, et al., 2009). This suggested the possibility that IgM reactivities would also be altered in human T1D and we have preliminary evidence that common genetic variants (SNPs) in the IgM locus control the levels of anti-GAD serum IgM and are associated with disease in a collection of T1D patients (unpublished results). These findings strengthen the link between NAbs and T1D pathogenesis and give importance to further investigations into the origin of autoreactive IgM.

We found that B220 expression strikingly distinguished NOD B1a cells in peritoneal cavity from as early as 1 week of age, as compared to C57BL/6 and BALB/c (not shown). Interestingly, peritoneal B1a cells that develop from adult bone marrow progenitors showed increased levels of B220 expression (Esplin, Welner, Zhang, Borghesi, & Kincade, 2009; Wardemann, Boehm, Dear, & Carsetti, 2002). Thus, it is possible that bone marrow progenitors play a larger role in NOD B1a cells ontogeny, conditioning a higher B220 expression. Additionally, increased B220 expression is associated with a lower threshold of activation (Casola et al., 2004; Jellusova, Wellmann, Amann, Winkler, & Nitschke, 2010) and possibly underlies the surface expression profiles of CD43, CD62L and CD86 in NOD peritoneal B1a cells. Some of these phenotypes have been previously described on NOD B1a cells and associated with increased activation, migration and capacity for T cell co-stimulation (Alam, Valkonen, Ohls, Tornqvist, & Hanninen, 2010). Interestingly, alterations in the NOD microflora have been shown to condition some of the surface B1 cell traits (Alam, et al., 2011; Alam, Valkonen, Palagani, et al., 2010). Thus, peritoneal cavity B1a cells are able to rapidly respond to external stimuli, namely microbial alterations in the intestine. Our observations of higher B220 and TLR expression at an early age and increased proliferation upon TLR stimulation, concur to strongly suggest that B1a B220^{high} cells may represent a B1 cell population in the NOD mouse that colonizes the peritoneal cavity in early ontogeny and is exquisitely sensitive to innate stimuli.

In accordance, NOD B1a TLR stimulation resulted in increased levels of IgM secretion with reactivity against T1D AAg while the secretion of IL10 remained lower when compared to C57BL/6 B1a cells. Stimulation through TLR has been shown to strongly promote plasma cell differentiation in the B1 cell compartment (Genestier, et al., 2007). Interestingly, NOD

B1a cells produced similar amounts of total IgM in comparison to C57BL/6 in the presence of TLR ligands. This indicates that the NOD B1a cell population harbours an increased population of autoreactive cells with increased responsiveness and propensity for plasma cell differentiation upon innate stimulation. This is in agreement with increased numbers of NOD B1a cells secreting IgM with reactivity towards endocrine AAg and with the pattern of serum anti-insulin IgM.

Importantly, we did not detect strain differences in the numbers of B1a cells in the pancreatic lymph nodes, nor their presence in the pancreas of pre-diabetic NOD mice (data not shown), strengthening the hypothesis that the role of NOD B1a cells on T1D onset may be mediated by autoreactive IgM (Corte-Real, Duarte, et al., 2009). Accordingly, binding of IgM to beta cells has been described *in vivo* in very young NOD mice (Shieh, et al., 1993). We showed here that NOD B1a-derived IgM binds to beta cells *in vitro* and that the expression of *iNOS* is upregulated in islet cells in contact with NOD B1a derived supernatant, suggesting that IgM binding is involved in triggering the beta cell oxidative stress response. On the other hand, *Fas*, *Caspase 3* and *Ccl2* gene expression were not altered indicating that the pattern of gene induction differed from what has been described for cytokine induced beta cell damage (Cardozo, Kruhoffer, Leeman, Orntoft, & Eizirik, 2001). This reinforces the notion that the effects observed in *iNOS* expression are due to the binding of autoreactive IgM, a hypothesis that requires further confirmation. Whether other immunological components, such as complement in the serum, could potentiate the IgM effect and determine the fate of beta cells in T1D remains also to be addressed.

In conclusion, in this study we have linked alterations in the B1a cell population to serum IgM autoreactivities and beta cell oxidative stress, strengthening the hypothesis that NAbs are an early factor in T1D pathogenesis evolution in the NOD mouse.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

"Panama" Blot

To evaluate NOD and C57BL/6 mice global serum IgM repertoire we have used a semi-quantitative immunoblotting technique that allows evaluation of antibody reactivities against large panels of antigens and has been described in detail elsewhere (Haury, Grandien, Sundblad, Coutinho, & Nobrega, 1994; Nobrega et al., 1993). Briefly, protein extracts were prepared from the livers and kidneys of 25 NOD females with average age of 6 weeks. Following electrophoresis the separated proteins were transferred onto nitrocellulose membranes that where then incubated with serum from 5 C57BL/6 and 5 NOD females from 4 to 12 weeks of age in miniblot incubation units (Immunetics). Goat anti-mouse IgM-AP conjugated secondary antibody (Southern Biotech.) was used and immunoreactivities were revealed with nitroblue tetrazolium (NBT) and bromo-chloro-indolylphosphate (BCIP) substrates (Promega Corp.). Quantitation of immunoreactivities was performed by densitometry in high resolution reflective mode. A second densitometry was performed after colloidal gold staining (Protogold, Biocell). The irregularities of the electrophoretic migration were corrected by the superimposition of the two stainings and the intensity of reactivity development was adjusted to a NOD standard serum, obtained from a pool of 19 females with 15 to 21 weeks of age. For all these procedures, specially developed macros using the software IGOR (Wavemetrics) were used. The adjusted reactivity profiles were divided into sections defined empirically for each protein extract and the intensity of the respective serum immunoreactivity was quantified as the mean optical density within these limits. A sample profile thus consisted of a list of reactivity measures, one for each section, and was analyzed by Principal Component Analysis (PCA).

Innate stimulation

Purified peritoneal B1a cells were cultured in RPMI 1640 complete medium with or without 0.5 μM CpG (ODN 1826, Invivogen). After one day, cells from duplicate wells were pooled, stained with biotinylated-anti-CD69 (clone H1.2F3) followed by FITC-streptavidin and were analyzed by flow cytometry. On the second day of culture the cells were collected, counted and plated for ESA quantification of anti-T1D AAg IgM secreting cells. The percentage of cells secreting antibodies with these reactivities was also assessed on purified peritoneal B1a cells cultured in RPMI 1640 complete medium with or without purified LPS (5 μg/ml).

SUPPLEMENTARY REFERENCES

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Chapter VII. Article IV: p58 surface glycoprotein is downregulated in NOD B1a cells and its targeting precipitates type 1 diabetes in the NOD mouse

p58 SURFACE GLYCOPROTEIN IS DOWNREGULATED IN NOD B1a CELLS AND ITS TARGETING PRECIPITATES TYPE 1 DIABETES IN THE NOD MOUSE

by

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ABSTRACT

Peritoneal cavity B1a cells have been implicated in the type 1 diabetes (T1D) autoimmune process in several mouse models and also in humans, though a direct link between alterations in the peritoneal cavity compartment and diabetes progression has been hard to establish. Recently, we have described that peritoneal B1a cells of the non-obese-diabetic (NOD) mouse are naturally more activated and have a lower threshold for innate-like stimulation. In line with these findings we here show that p58, a surface glycoprotein that is constitutively found on B1a cells and has been correlated to the natural activated state of this subpopulation, is expressed at lower levels in the NOD peritoneal B1a cell compartment, when compared to non-diabetic prone strains. After p58 specific targeting in NOD mice *in vivo* we have observed: (1) down-modulation of IgM expression and increased proliferation of peritoneal cavity B1a cells; (2) increased IgM serum levels and; (3) an earlier onset of diabetes. We here propose that low p58 expression levels may contribute to a less efficient regulation on NOD B1a cells and that p58 targeting could abrogate the existing inhibition of proliferation signals, accelerating T1D onset in the NOD. These results reinforce the notion that B1a cells may have a role in this autoimmune diabetogenesis process.

INTRODUCTION

Type 1 diabetes (T1D) is a complex genetic autoimmune disease, contributed by various immunological alterations, which culminates in the destruction of pancreatic beta cells by specific T cell cytotoxicity (Bach, 1994). Although a pathogenic role for beta cell specific autoantibodies has never been identified in T1D, their early presence is highly correlated with progression to disease. Therefore, we have hypothesized that a genetically determined alteration in immunoglobulin reactivity could have implications in the diabetogenesis process (Côrte-Real, Duarte, Tavares, & Penha-Gonçalves, 2009). In fact, a germline encoded repertoire of autoreactive antibodies is naturally present in the body and is mainly contributed by spontaneous IgM secreting peritoneal and splenic B1a cells (Feeney, 1990; Gu, Förster, & Rajewsky, 1990; Tlaskalová-Hogenová et al., 1992).

Extensive studies on B1 cell phenotypes and functions have revealed that they could be involved in the development of autoimmune diseases (Duan & Morel, 2006). In particular, we have been studying the non-obese-diabetic (NOD) mouse model (Castaño & Eisenbarth, 1990) to understand the role of peritoneal cavity B1a cells in the development of type 1 diabetes. Recently, we have correlated alterations in the NOD B1a cell compartment to serum IgM autoreactivities and to induction of pancreatic beta cell oxidative stress, which led us to propose that NOD B1a cells contribute to diabetes progression through the secretion of beta cell autoreactive natural antibodies (Côrte-Real et al., 2009; chapter VI, article III). In this later study we have also demonstrated that peritoneal cavity NOD B1a cells display increased expression of surface activation molecules and have a lower threshold for innate-like stimulation (chapter VI, article III).

The expression of the 58 kD surface glycoprotein designated by p58 (Parkhouse, Santos-Argumedo, Teixeiral, Henry, & Wawrzynczak, 1992) has been previously associated with the naturally activated status of peritoneal B1 cells and has been shown to be constitutively expressed in BALB/c B1 cells from the peritoneal cavity and spleen, as well as in splenic marginal zone (Mz) B cells (Manjarrez-Orduño, Parkhouse, & Santos-Argumedo, 2003). Interestingly, p58 expression levels were only significant in follicular (FO) B cells and T cells upon stimulation (Manjarrez-Orduño et al., 2003). Despite that p58 identity and function are still unknown, a relevant tool for its study is the anti-p58 monoclonal antibody (NIM-R7). NIM-R7 has been generated against the cell membranes of the p58 expressing BALB/c-derived B cell lymphoma (BCL1) (Parkhouse et al., 1992), a typical B1 cell-derived lymphoma that arose spontaneously in the BALB/c strain (Slavin & Strober, 1978) and that is characterized by the expression of IgM, CD5 and low levels of B220 on its surface, as well as by the spontaneous secretion of IgM (Koganei, Ito, Yamamoto, & Matsumoto, 2005). Importantly, when conjugated to ricin-A, NIM-R7 was able to induce BCL1 cell death *in vitro* while no

effect was observed in resting FO B cells, where lower levels of expression of p58 were observed (Manjarrez-Orduño et al., 2003).

Here, we have characterized the expression of p58 in the NOD mouse and we have found that peritoneal cavity B1a cells display p58 downregulation, therefore we have further explored the hypothesis that B1a cells activation status is altered in this diabetic prone strain. Furthermore, targeting of p58 on NOD peritoneal cavity B1a cells had an impact on B1a cell numbers and B1a surface IgM expression at this location, upon total IgM serum levels and T1D onset, strengthening the notion that B1a cells may play a role in the autoimmune diabetic process.

MATERIALS AND METHODS

Mice

C57BL/6, NOD and BALB/c mice were bred and maintained in specific pathogen free housing facilities at the Instituto Gulbenkian de Ciência. All mice used were females varying between 3 and 32 weeks of age. NOD female insulitis was unapparent at 3 weeks. Experimental protocols were approved by the Direcção Geral de Veterinária of the Portuguese Ministry of Agriculture, Rural Development and Fisheries and are in accordance with national and international regulations (U.S. Department of Health and Human Service, Office of Extramural Research, National Institutes of Health, Office of Laboratory Animal Welfare, 1985).

Cell culture and analysis

BCL1 cells were kindly provided by Dr. John F. Kearney, University of Alabama at Birmingham, and maintained in RPMI 1640 complete medium (supplemented with 2% FCS, 2 mM L-glutamine, 10 mM HEPES, 50 mM 2-mercaptoethanol, 100 U penicillin, 100 mg/ml streptomycin, all from Life Technologies, 50 μg/ml gentamycin from Sigma and 0.25 μg/ml fungizone from Invitrogen). Lymphocytes were isolated from peritoneal cavity by peritoneal lavage performed with PBS supplemented with 2% FCS or from spleen by straining through a nylon mesh. Single cell suspensions were then stained according to standard procedures. Fc receptors unspecific binding was prevented by incubating the cells with unlabeled anti-mouse-Fc-block/CD16/32 (clone 2.4G2, BD Pharmingen). Peritoneal cavity and splenic B cells were identified as $CD19^+$ cells. Peritoneal cavity B cells were divided in sub-populations according to the expression of CD11b and CD5: B1a as $CD11b^+CD5^+$, B1b as $CD11b^+CD5^-$, B1c as $CD11b^-CD5^+$ and B2 cells as $CD11b^-CD5^-$ cells. In the spleen, follicular B cells were defined as $CD23^+IgM^{low/+}$, marginal zone B cells as $CD23^-IgM^{high}CD21^+$ and B1a cells were

identified as $CD23^{-}IgM^{high}B220^{low}CD5^{+}$. T cells were defined as $CD19^{-}CD5^{+}$ in both peritoneal cavity and spleen. The antibodies used were Pacific-Blue or Cy5-anti-mouse CD45R/B220 (clone RA3-6B2), FITC or biotin-anti-mouse-CD11b/Mac-1 (clone M1/70), FITC-anti-mouse-CD21 (clone 7G6), FITC or A647-anti-mouse-IgM (clone R33.24.12), PE-anti-mouse-CD23/IgEFcR (clone B3B4), PE or Percp-anti-mouse-CD5/Ly-1 (clone 53-7.3) and PE-Cy7-anti-mouse-CD19 (clone 1D3). Monoclonal NIM-R7 (anti-p58) antibody (Parkhouse et al., 1992) was biotinylated and used for staining. Biotinylated antibodies were revealed by either FITC, Percp or APC-Cy7-streptavidin. Traceable latex beads (Beckman coulter Inc.) were added for counting cells. Stained cell suspensions were analyzed by flow cytometry either with a FACSCalibur™ (Becton Dickinson) or a FACS CYAN ADP (Dako Cytomation) and the data was respectively acquired with CELLQuest or Summit software. Analysis was performed with FlowJo software (TreeStar Inc.).

Intra-peritoneal injection, blood collection and glycosuria measurement

For the assessment of NIM-R7 impact on lymphocytes physiology we have performed 4 intra-peritoneal (IP) injections of 50 μg of this antibody diluted in 100 ml of RPMI 1640 (Life Technologies) into NOD female mice, starting at 5 weeks of age and with one week intervals. Mice were euthanized one week after the last injection and the organs were taken for flow cytometry analysis. For the verification of the effect of NIM-R7 on IgM serum levels and on T1D development, the same IP injections protocol was started at 3 weeks of age and was followed by a further two IP injections with 2 weeks interval. Blood was collected from the facial vein at 5, 7, 10 and 13 weeks of age and urinary glucose levels were measured weekly from 12 to 32 weeks of age with Diabur 5000 test stripes (Roche). Mice were considered to be diabetic after two consecutive positive measures of glycosuria.

ELISA

Briefly, 96-well ELISA plates (Nunc) were coated overnight at 4ºC with anti-mouse-IgM (clone R33.24.12, 2 μg/ml) diluted in coating buffer (0.05 M Na_2CO_3), and were subsequently blocked with PBS-gelatin (1%, powder food grade, Merck) at 37ºC for 1 hour. Then, 1:100 diluted serum samples were incubated for 1 hour at 37ºC and washed. Bound serum IgM was detected by goat anti-mouse-IgM-AP conjugated antibody (Southern Biotech.), the plates were developed with sigma phosphatase substrate and optical densities were read in a spectrophotometer at 405 nm. The concentrations of IgM antibody in the samples were calculated against standard curves obtained with purified monoclonal IgM (clone 11E10, Southern Biotech.) tested in the same microtitre plate.

Statistical analysis

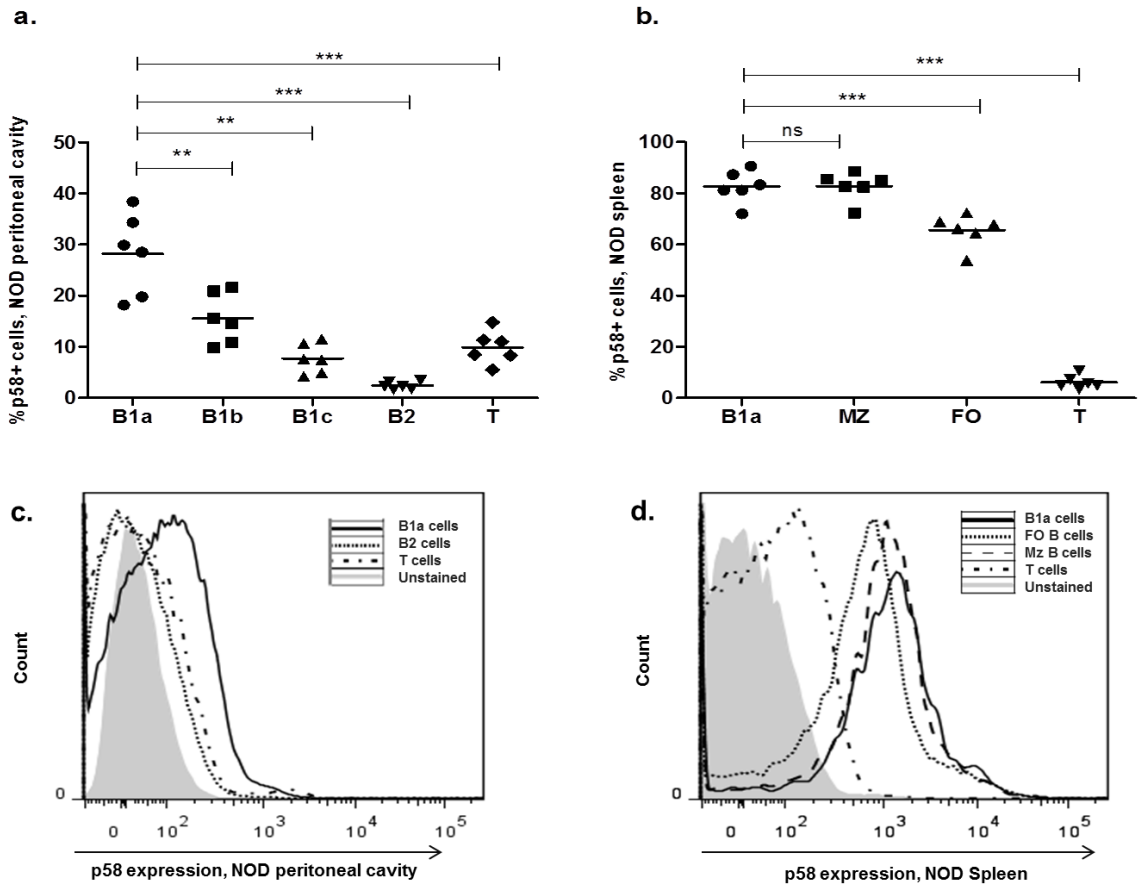
Statistical significant differences for the analyzed phenotypes were estimated by unpaired student's t-test. Two-tailed tests with 95% of confidence interval where used and differences with $p<0.05$ were considered significant. Differences with $p<0.05$ (*), $p<0.01$(**) or $p<0.001$(***) are represented in the respective figures.

RESULTS

p58 is expressed at high levels on NOD peritoneal cavity B1a cells and splenic B1a and marginal zone B cells

Previous studies have described that, in undisturbed conditions, p58 expression is restricted to BALB/c peritoneal cavity B1 cells and splenic B1 and marginal zone B cells, and have suggested that p58 may have a role in the lymphocyte activation processes (Manjarrez-Orduño et al., 2003). In general, the expression of p58 on NOD peritoneal cavity and spleen lymphocyte subpopulations was similar to that of BALB/c mice. The expression of p58 in NOD peritoneal cavity B1a cells was significantly increased when compared to other peritoneal lymphocyte sub-populations (Figure VII.1a and VII.1c). Similarly, in the NOD spleen, higher expression levels of p58 were detected in both B1a and marginal zone B cell sub-populations when compared to follicular B cells and T cells (Figure VII.1b and VII.1d).

Figure VII.1 p58 is constitutively more expressed on NOD B1a cells both in peritoneal cavity and spleen



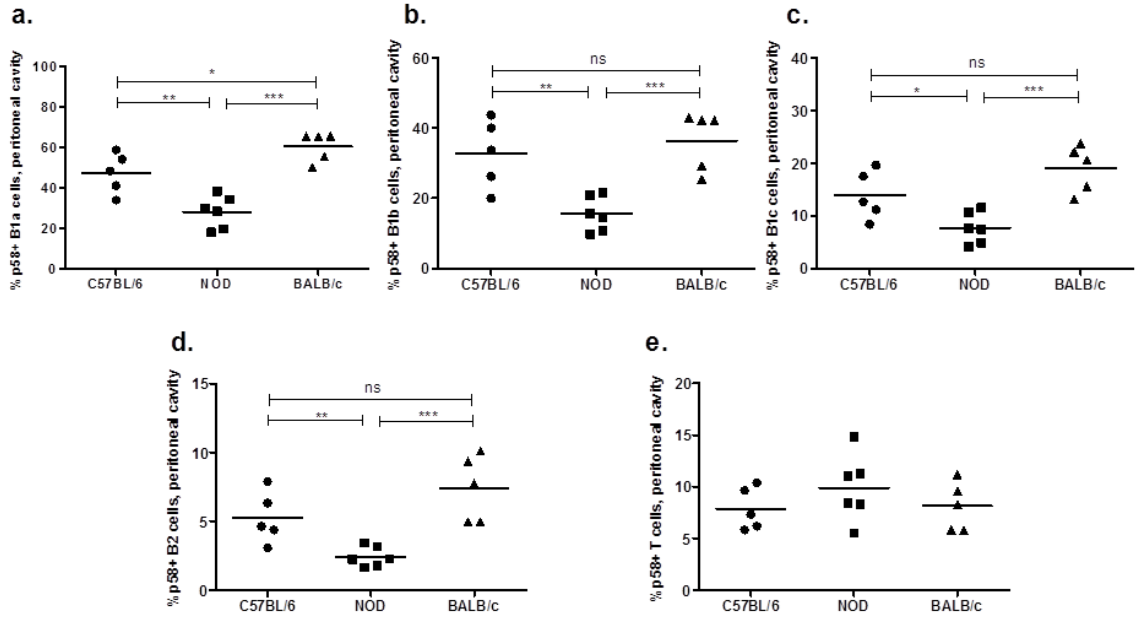
p58 expression within NOD peritoneal cavity B1a, B1b, B1c, B2 and T cell subpopulations (a) or NOD spleen B1a, marginal zone B (Mz), follicular B (FO) and T cell subpopulations (b) was analyzed by flow cytometry in 6 NOD females with 11 to 15 weeks of age. The individual percentages with respective mean values are represented for peritoneal cavity (a) and spleen (b) as well as the representative histograms of typical p58 expression intensity (c, d). Differences in p58 expression between sub-populations were found as denoted. Data are representative of two independent experiments.

p58 is naturally downmodulated in the NOD peritoneal B cell compartment

Given that p58 could have physiological implications for "naturally activated cells" and that NOD B1a cells have a lower threshold for innate-like stimulation (chapter VI, article III), we performed a comparative flow cytometry analysis of p58 expression on peritoneal cavity B1a, B1b, B1c, B2 and T cells (Figure VII.2) and splenic B1a, Mz B cells, FO B cells and T cell sub-populations (Figure VII.3) of NOD and non-diabetic C57BL/6 and BALB/c mouse strains. We have verified that, in the peritoneal cavity, p58 is expressed in all the analyzed strains mainly in B1a and B1b cells (Figure VII.2a and b) and at very low levels in B1c, B2 and T cell sub-populations (Figure VII.2c-VII.2e). Interestingly, p58 expression was lower in all the NOD peritoneal cavity B cells subsets, when compared to both C57BL/6 and BALB/c strains (Figure VII.2a-VII.2d). No strain differences in p58 expression levels were found for the peritoneal cavity T cell population (Figure VII.2e) and curiously, p58 expression within B1a

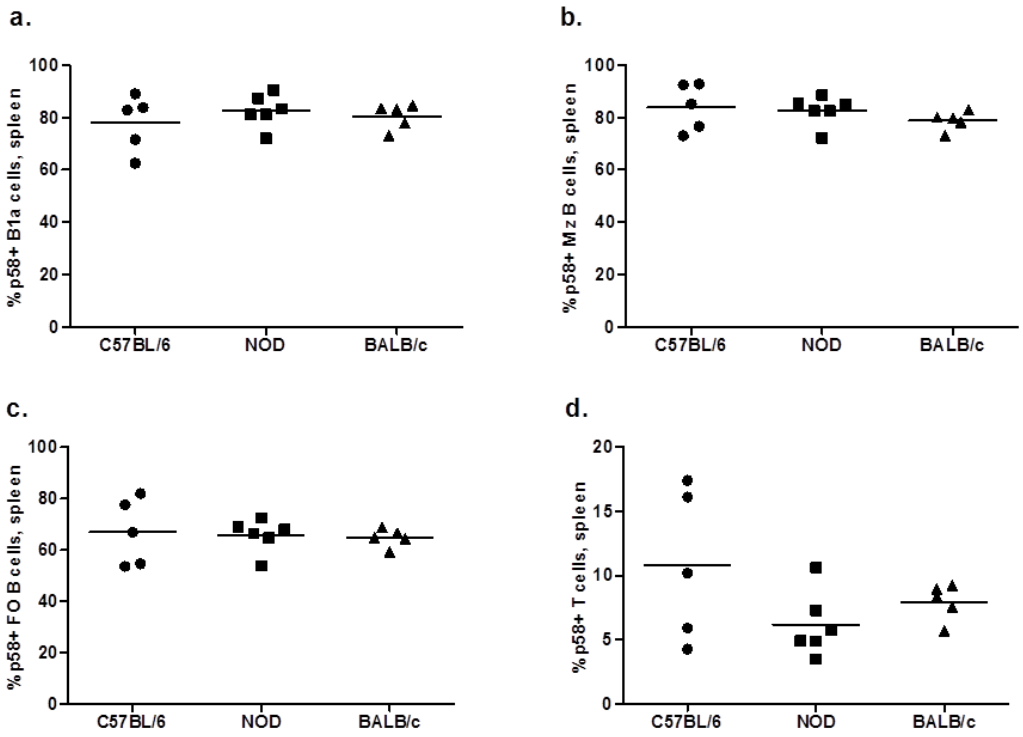
cells was higher in the BALB/c in comparison to the C57BL/6 (Figure VII.2a). In the spleen, we have observed that p58 is expressed at high levels in both B1a and marginal zone B cells in the three analyzed strains (Figure VII.3a and VII.3b) and, in contrast to what has been described (Manjarrez-Orduño et al., 2003), we have found that significant levels of p58 expression were also detectable in FO B cells for all the strains of mice that we have studied (Figure VII.3c). Very low levels of p58 were found in the splenic T cell subpopulation (Figure VII.3d). No differences in the p58 expression levels on splenic B1a, FO B cells, Mz B cells or T cells were found between NOD, C57BL/6 and BALB/c strains. In conclusion, our results show that p58 is generally expressed on mouse B1a and Mz B cells, and that NOD peritoneal B1a cells have lower levels of p58 than their non-diabetic counterparts.

Figure VII.2 p58 is expressed at low levels on NOD peritoneal cavity B cell populations



p58 expression within peritoneal cavity B1a (a), B1b (b), B1c (c), B2 (d) and T cell (e) subpopulations was analyzed by flow cytometry in 5 C57BL/6, 6 NOD and 5 BALB/c females with 11 to 15 weeks of age. The individual percentages with respective mean values are represented. Strain differences in p58 expression were found for B1a, B1b, B1c and B2 as depicted. Data are representative of two independent experiments.

Figure VII.3 p58 is constitutively more expressed on splenic B1a and marginal zone B cell subpopulations

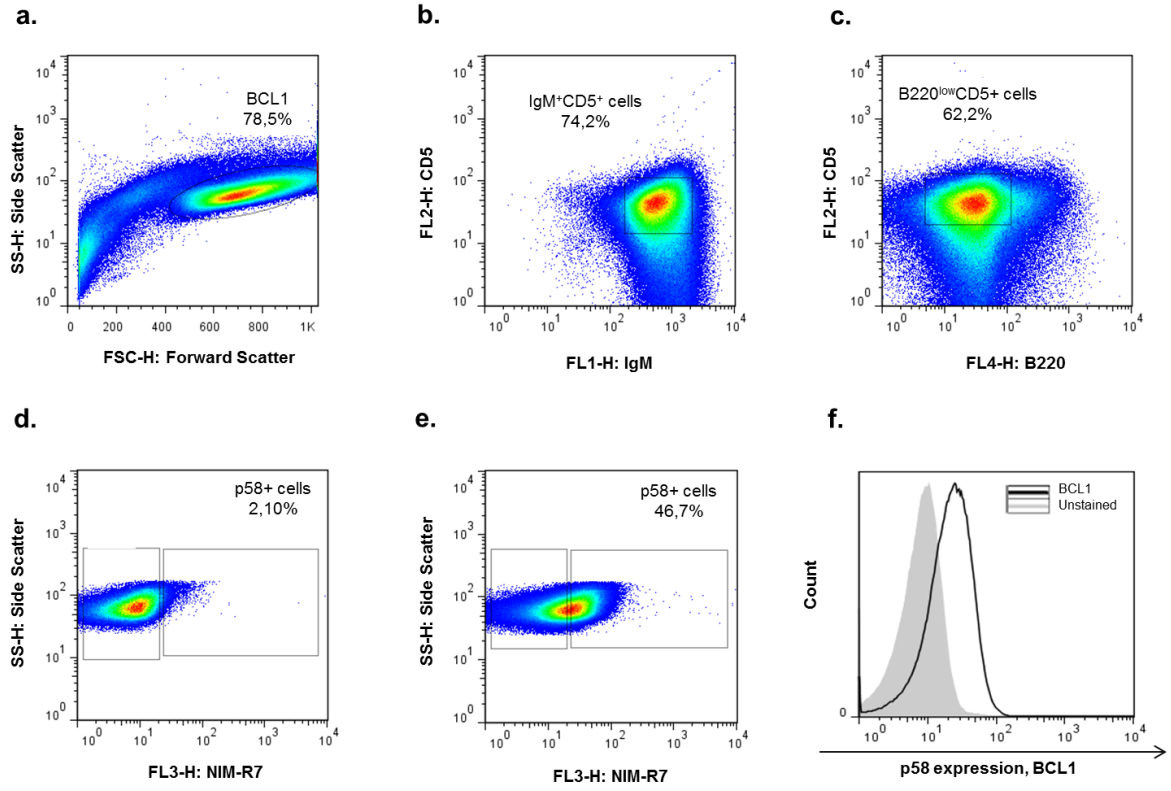


p58 expression within splenic B1a (a), marginal zone B (Mz) (b), follicular B (FO) (c), and T (d) cell subpopulations was analyzed by flow cytometry in 5 C57BL/6, 6 NOD and 5 BALB/c females with 11 to 15 weeks of age. The individual percentages with respective mean values are represented. No strain differences in p58 expression were found. Data are representative of two independent experiments.

p58 specific targeting affects B lymphocyte distribution and IgM expression in the peritoneal cavity

The anti-p58 specific NIM-R7 monoclonal antibody was generated against the membranes of BCL1 (Parkhouse et al., 1992) which are believed to have been originated on peritoneal cavity B1a cells (Koganei et al., 2005). In fact, we have confirmed by flow cytometry that the pattern of cell surface markers expression within the BCL1 cells morphological gate (Figure VII.4a) is very similar to that described for B1a cells. Most BCL1 cells were $IgM^{+}CD5^{+}$ (Figure VII.4b), $B220^{low}CD5^{+}$ (Figure VII.4c) and around 50% expressed p58 on their surface (Figure VII.4e).

Figure VII.4 BCL1 cells express p58 and have a similar phenotype to peritoneal cavity B1a cells

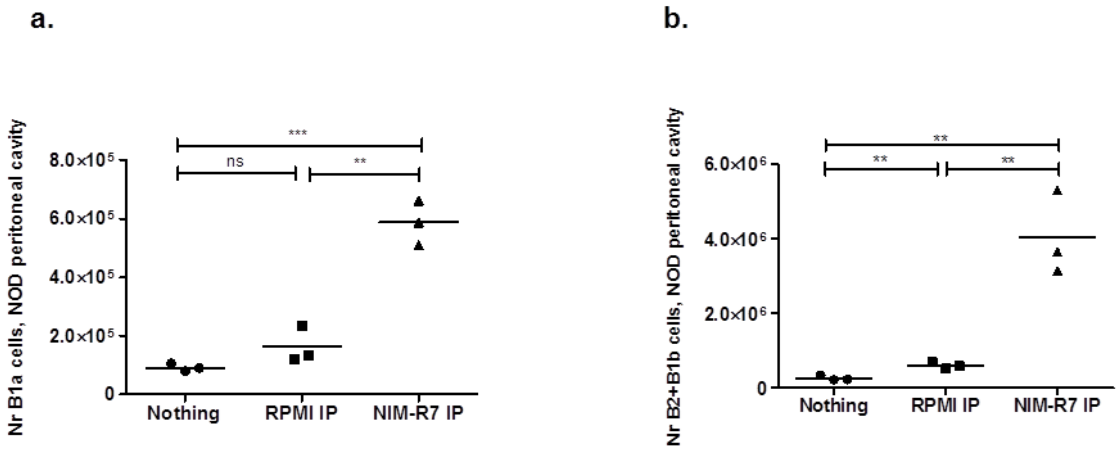


BCL1 cells were analyzed by flow cytometry and representative dot plots of BCL1 morphological gate (a), and $IgM^{+}CD5^{+}$ (b) and $B220^{low}CD5^{+}$ (c) staining profiles within the BCL1 gate are shown. p58 expression is represented for BCL1 unstained controls (d) and BCL1 stained cells (e). A histogram of typical p58 expression on the same cells is shown (f).

Further, it has been previously shown that BCL1 cells are effectively killed upon treatment with NIM-R7 conjugated to ricin-A, while no effect is observed in FO B cells or T cells (Manjarrez-Orduño et al., 2003). Given that BCL1 cells have a B1a like phenotype (Koganei et al., 2005; and Figure VII.4) and that p58 is constitutively expressed on NOD peritoneal B1a cells (Figure VII.1), we decided to test whether p58 targeting *in vivo* would have an impact on the numbers of B1a cells in NOD mice. With this aim, we performed a weekly IP injection of 50 μg of NIM-R7 for four consecutive weeks, in NOD female mice, starting at 5 weeks of age, and one week after treatment we assessed the flow cytometry profile and numbers of peritoneal cavity lymphocyte sub-populations in comparison with un-manipulated mice or RPMI injected controls. Contrary to our expectations, we have observed a dramatic increase in the numbers of p58 expressing B1a cells in the NIM-R7 antibody treated group in comparison to either un-manipulated mice (6 fold) or RPMI injected mice (3.5 fold) (Figure VII.5a). The numbers of B2+B1b cells in the peritoneal cavity have also increased enormously upon NIM-R7 treatment (15 fold when compared to un-manipulated mice, 6.5 fold when compared to RPMI injected controls) (Figure VII.5b). The simple injection of RPMI had a slight increasing effect on the number of peritoneal cavity B2+B1b cells in comparison

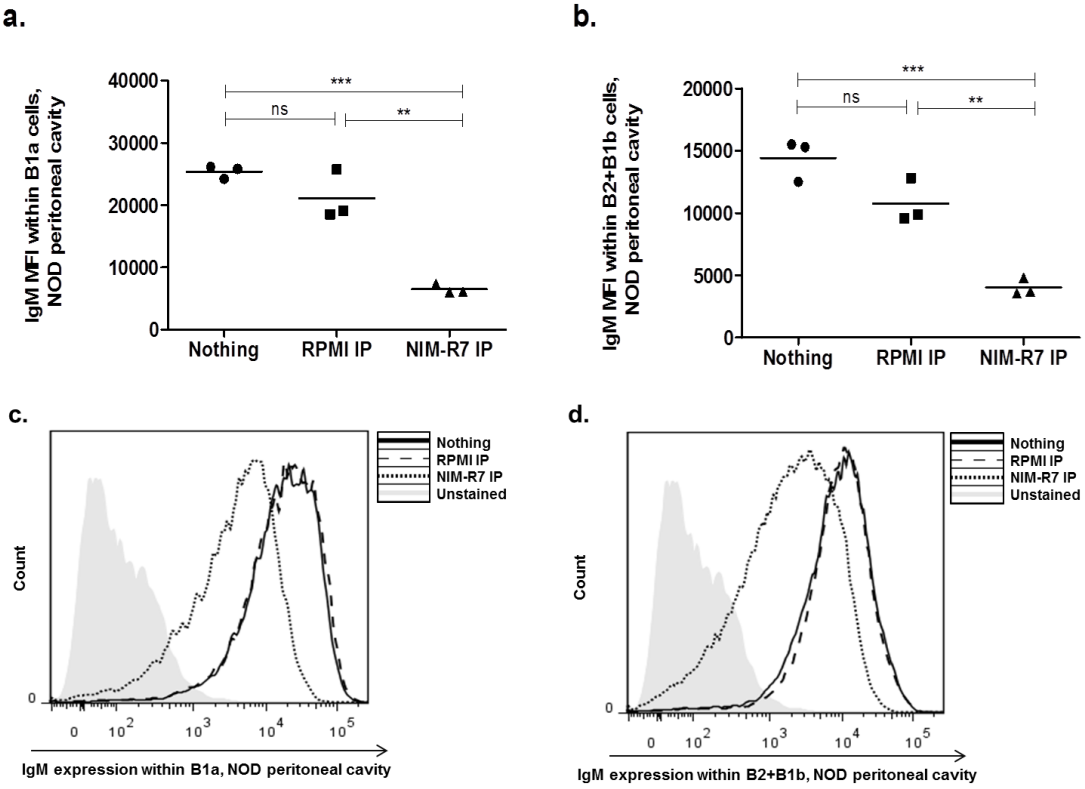
to un-manipulated mice, but this effect was not observed for B1a cells in the same location. T cell numbers were not significantly affected in the NIM-R7 antibody injected group and splenic lymphocyte subpopulations were unaltered by the NIM-R7 treatment (data not shown). Interestingly, there was a major decrease of surface IgM expression levels on both peritoneal B1a and B2 cells in the NIM-R7 treated NOD mice (Figure VII.6), although the levels of p58 remained unchanged (data not shown).

Figure VII.5 Specific targeting of p58 *in vivo* affects B lymphocyte distribution in the peritoneal cavity



Flow cytometry analysis of NOD peritoneal cavity after one week of intra-peritoneal treatment with purified NIM-R7 antibodies was performed. The individual numbers of B1a (a) and B2+B1b (b) cell subpopulations for 3 females with 9 weeks of age and the respective mean values are represented for non-manipulated mice (nothing), RPMI injected controls (RPMI IP) or the antibody treated group (NIM-R7 IP). Representative differences are shown and the data are representative of two independent experiments.

Figure VII.6 Specific targeting of p58 *in vivo* decreases surface expression of IgM on peritoneal cavity B cells



IgM mean fluorescence intensity (MFI) within NOD peritoneal cavity B1a (a) and B2+B1b (b) after one week of intra-peritoneal treatment. The individual values obtained for 3 females with 9 weeks of age and the respective mean values are represented for non-manipulated mice (nothing), RPMI injected controls (RPMI IP) or the antibody treated group (NIM-R7 IP). The representative histograms of typical IgM expression intensity (c, d) are presented. Differences in IgM expression levels between groups are denoted. Data are representative of two independent experiments.

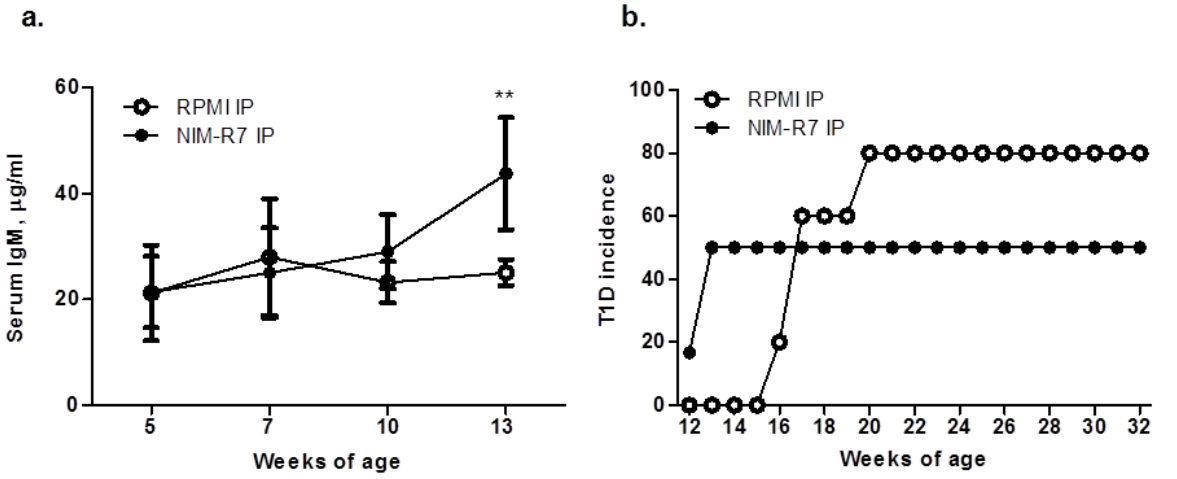
p58 specific targeting induces an increase in serum IgM titers and accelerates NOD T1D onset

B1a cells are the main natural IgM secretors (Casali & Schettino, 1996) and have been implicated in the development of several autoimmune syndromes (Duan & Morel, 2006), including type 1 diabetes (Alam, Valkonen, Ohls, Törnqvist, & Hänninen, 2010; Ryan et al., 2010). Our own results suggest that B1a cells could have a pathogenic role in type 1 diabetes progression in the NOD mouse model, by secreting pancreatic beta cell reactive autoantibodies that, upon binding, could initiate beta cell oxidative stress (article III). Therefore, we decided to determine if the proliferation of peritoneal cavity B1a cells, and the decrease of surface IgM expression levels, induced by the treatment of mice *in vivo* with NIM-R7 IP injection, would be accompanied by an impact on the total serum IgM levels, and induction of type 1 diabetes in the NOD mouse. Pre-insulitic NOD females with 3 weeks of age (Yang & Santamaria, 2003) were injected IP as described above and then given another two extra NIM-R7 IP administrations, 2 weeks apart. As a control, mice were injected IP with RPMI. Significant differences in the total levels of IgM between the two groups of mice were

observed at 13 weeks of age (Figure VII.7a) which correlated well with the increase in B1a cell number previously shown (Figure VII.5a). Contrary to our expectations, there was no difference in the levels of anti-insulin IgM between the two groups of mice over time (data not shown).

Strikingly, the onset of T1D was accelerated in the NIM-R7 IP injected group, where 50% of the NOD females developed diabetes by 13 weeks of age (Figure VII.7b). Equal incidence values were reached only by 17 weeks of age in the RPMI injected control group. The clear correlation of these results suggests a pathogenesis mechanism linking alterations in the NOD B1a cell compartment to increased serum IgM levels and early T1D onset.

Figure VII.7 Specific targeting of p58 *in vivo* induces an increased serum IgM titer and accelerates NOD T1D onset



Serum IgM concentration was measured individually at 5, 7, 10 and 13 weeks of age in 6 NOD females under intra-peritoneal NIM-R7 treatment (NIM-R7 IP) or in 6 NOD female controls where RPMI was injected IP (RPMI IP) (a). Urine glucose levels were followed weekly in the previously defined groups from 12 weeks of age, and type 1 diabetes incidence is shown (b). Significant differences in serum IgM levels are identified and the data are representative of two independent experiments.

DISCUSSION

The physiology of B1 cells, their role in normal immune responses and their implications in autoimmunity are not yet completely understood. We and others have described that peritoneal NOD B1a cells are altered in comparison to non-diabetic mice (Alam et al., 2010). In particular, we have demonstrated that this subset has a lower activation threshold for innate-like stimulation in this T1D mouse model (chapter VI, article III). By dissecting p58

expression levels in different B1 cell subsets in the NOD, we found that it is expressed mostly on B1a cells, while B1 precursors or immature B1 cells contained within the $CD11b^-$ B1c subset do not express p58. This observation is consistent with previous work which showed that p58 is expressed mainly on peritoneal B1a cells, and that this glycoprotein is not expressed at early stages of lymphocyte development, as well as it is associated with an increased lymphocyte activation status on the BALB/c mouse (Manjarrez-Orduño et al., 2003). In NOD mice, p58 expression is significantly decreased in the B1a cell compartment when compared to C57BL/6 and BALB/c mice. This difference was not observed for splenic B1a cells. This finding is puzzling and indicates that p58 function is more complex than initially thought, being that low p58 expression levels may contribute to a less efficient regulation and to the lower threshold for innate-like stimulation observed in NOD B1a cells.

Importantly, we have confirmed that p58 is mostly expressed on B1a and Mz B cells across mouse strains and constitutes a useful surface marker to define these subsets in cellular studies. Unlike the previous work (Manjarrez-Orduño et al., 2003), we have detected significant levels of p58 expression on follicular B cells in the spleen, a difference perhaps due to the fact that we have used older mice in our study (11-15 weeks *vs* newborn to 8 weeks old). Moreover, we did not morphologically discriminate non-activated from activated FO B cells, therefore we cannot exclude that an enriched population of splenic activated B cells in older mice is contributing to the observed p58 expression in our follicular B cell analysis.

Treatment with NIM-R7 has been shown to efficiently kill BCL1 cells (Manjarrez-Orduño et al., 2003). Since BCL1 cells express p58 and have a B1a-like-nature it was tempting to test whether targeting peritoneal cavity NOD B1a cells with NIM-R7 would have an impact on their phenotype and survival. Surprisingly, injection of NIM-R7 IP induced a major increase in NOD peritoneal B1a cell numbers and not in B1a cell death. This result points once again to the intricate function that p58 may have on the physiology of B1a cells. At the same time, IgM surface expression on peritoneal B1a cells was greatly decreased upon NIM-R7 treatment, an observation that suggests that p58 targeting induces maturation towards a plasma cell-like, secretory phenotype (Oracki, Walker, Hibbs, Corcoran, & Tarlinton, 2010). Further work, for example *in vitro*, is necessary to confirm if p58 treatment *in vivo* stimulates plasma cell differentiation. Still, these observations suggest that p58 expression could have an inhibitory function on NOD B1a cells proliferation and plasma cell differentiation, which would be abrogated upon its specific triggering with NIM-R7.

Phenotypic alterations in NOD B1a cells have been observed and correlated with T1D progression, and we have suggested that NOD B1a cells could be contributing to T1D pathogenesis through the secretion of beta cell reactive autoantibodies (Côrte-Real et al., 2009; article III). Here, despite the fact that we did not observe an increase in serum anti-insulin IgM levels, the total IgM titers in the NIM-R7 IP injected NOD group were augmented,

strengthening the hypothesis that targeting p58 *in vivo* favors the differentiation of B1a cells into antibody secreting cells. Furthermore, NIM-R7 administration to NOD females resulted in an earlier onset of T1D. Thus, alterations induced in B1a cells correlate with increased IgM levels in the serum and acceleration of diabetes, supporting the possibility of a causal relationship between B1a cells-originated IgM and diabetogenesis.

Unfortunately, this treatment *in vivo* impacts on the p58 cell surface marker whose function is still unknown, and so the observed effects may or may not be subsequent to the specific alterations observed in the B1a cell subset. Clarification of these aspects warrants further investigation on p58 identity and function.

Nevertheless, these results allow the proposal that p58 down-modulation observed in the NOD peritoneal B1a cells compartment could contribute to its described lower threshold of innate activation (article III) and could consequently have implications for T1D progression.

In summary, while further studies are needed to establish the cellular and molecular mechanisms underlying diabetes hastening by p58 targeting, we here show that p58 is a generalized mouse peritoneal cavity B1a cell surface marker which is down-modulated in the NOD B1a cell compartment and that alterations induced in the NOD peritoneal cavity compartment through p58 targeting accelerate type 1 diabetes.

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Chapter VIII. General Discussion

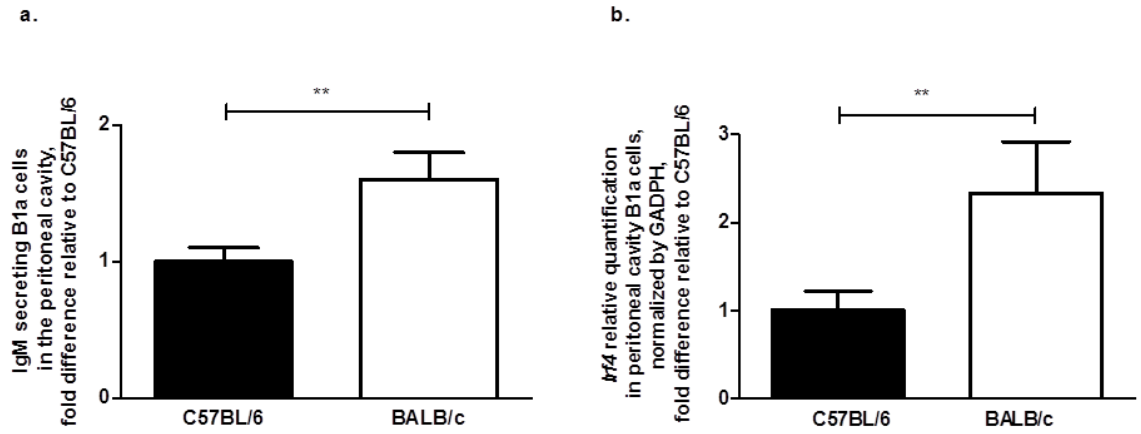
1. *Irf4* controls serum IgM concentration at the level of antibody secreting cells differentiation

In article I published in the context of this PhD thesis we were interested in identifying unknown genetic factors controlling the homeostatic levels of IgM secretion and upholding in the mouse. We have identified a region on mouse chromosome 13 that contained a *locus* (*loci*) controlling IgM serum levels. Interestingly, this genomic region, which was designated as *IgMSC1 (IgM secreting cells locus 1),* encompassed the *Irf4* gene, a known key player in the plasma cell differentiation pathway. Consistently, we detected higher levels of *Irf4* expression in the peritoneal cavity and splenic B cell populations in the BALB/c strain, which also showed greater serum levels of IgM and higher numbers of IgM secreting B cells in both locations, when compared to the C57BL/6 strain.

Our study was done in the absence of immunization, so we hypothesized that the serum IgM and IgM secreting cells that we were detecting originated mostly from the spontaneous secreting B1a cell subpopulation in both the peritoneal cavity and spleen. In fact, we have later confirmed that a higher proportion of peritoneal cavity B1a cells spontaneously secreted IgM in the BALB/c (Figure VIII.1a). The IgM secreting capacity of the purified peritoneal cavity B2+B1b cell pool was not different between C57BL/6 and BALB/c (data not shown). Notably, *Irf4* expression was also higher in purified peritoneal B1a cells of the BALB/c in comparison to the C57BL/6 (Figure VIII.1b). These data strongly suggest that a naturally higher expression of *Irf4* in BALB/c peritoneal cavity B1a cells contributes to an increased capacity to secrete IgM that is collected in the serum, determining the observed increase in the circulating levels of natural IgM in this strain.

Nevertheless, we cannot exclude that splenic marginal zone B cells, as well as activated follicular B cells, are also contributing to the observed phenotypes. To test whether the contribution of *Irf4* to natural antibody concentration in the serum comes mostly from the control of B1a cells spontaneous IgM secretion, we would have to assess and compare the spontaneous IgM secretory capacities of purified B cell subpopulations in the spleen of C57BL/6 and BALB/c, as well as their correlations to *Irf4* expression levels in the same cell subsets. Further, we would have to confirm if the pattern of IgM secretion and *Irf4* expression in splenic B1a cells is the same as the one observed in peritoneal cavity B1a cells. Genetic confirmation that *Irf4* is a main contributor to the control of serum IgM levels in naïve mice would require the construction of C57BL/6 and BALB/c congenic strains, reciprocal for the *Irf4 locus*, followed by knocking-in the BALB/c *Irf4* allele in the C57BL/6 genetic background.

Figure VIII.1 Higher proportions of IgM secreting B1a cells in the BALB/c peritoneal cavity correlate with greater expression of *Irf4*



The proportion of peritoneal cavity B1a cells spontaneously secreting IgM was determined by ELISPOT Assay (a) and the expression of *Irf4* was measured by Taqman Gene Expression Assay in the same cell subset (b). Peritoneal cavity B1a cells were purified from groups of 5 mice with 8 weeks of age. In (a) the bars represent the average results of triplicates with the respective standard deviation (SD). In (b) the relative quantification of *Irf4* was obtained in triplicates, after normalization to GAPDH expression, and the SD from a pool of two identical experiments is depicted. Mean fold-differences to the C57BL/6 are shown. Significant differences between strains were found ($p<0.01$) with unpaired t-student test. Data are representative of two independent experiments.

IRF4, BLIMP1 and XBP1 are accepted to be the main transcription factors responsible for plasma cell differentiation and antibody secretion upon B cell stimulation (Figure I.1). However, the control mechanism of natural IgM secretion by B1a cells, in the absence of immunization, is still contradictory and not completely understood. In the absence of external stimuli, *Irf4* upregulation in mice that lack *Mitf* activity results in the differentiation of B cells into plasma cells and in the production of IgM, but not IgG (L. Lin et al., 2004). Further, although having a normal development of peritoneal B1a cells, *Irf4*$^{-/-}$ mice have a 99% reduction in circulating IgM (Mittrücker et al., 1997) and a recent study has shown that *Irf4* controls the amount of IgM secreted by individual splenic B1a cells, while the individual low IgM secretion capacity of peritoneal cavity B1a cells remains unaltered in *Irf4*$^{-/-}$ mice (Holodick et al., 2010). These results indicate that accumulation of serum immunoglobulin M on wild type mice depends mostly on *Irf4* dependent high secretion by splenic B1a cells and less on the low secreting homologous population in the peritoneal cavity. However, and although we have purified B cells as a bulk in both the peritoneal cavity and spleen, our results in C57BL/6 and BALB/c wild type mice point to a much higher percentage of naturally secreting IgM secreting cells within the peritoneal cavity.

A few years ago, the studies from the Rothstein laboratory have reported undetectable expression levels of *Prdm1* (the gene encoding BLIMP1), as well as *Xbp1,* in spontaneous secreting peritoneal cavity B1a cells, suggesting at that time that this subpopulation could use a non-classical pathway to produce immunoglobulins (Tumang, Francés, Yeo, &

Rothstein, 2005). In contrast, more recent studies from both the Calame (Savitsky & Calame, 2006) and the Tarlinton groups (Fairfax et al., 2007) have demonstrated that, even if expressed at lower levels when compared with LPS stimulated conventional B2 cells, B1a cells require BLIMP1 in order to fully develop into antibody secreting plasma cells and to secrete IgM at normal levels. Furthermore, *Rag2*$^{-/-}$ blastocysts (which give rise to mice lacking endogenous B cells, serum Ig and T cells) when reconstituted with *Xbp1*$^{-/-}$ embryonic stem cells gave origin to mice with normal B (including peritoneal cavity B1a) and T cell subsets, yet with minimal immunoglobulin production of any isotype (Reimold et al., 2001). Thus, the prevailing hypothesis is that spontaneously IgM secreting B1a cells use the same regulatory mechanisms for immunoglobulin secretion as plasma cells resulting from the mitogen stimulation of B2 cells, but as B1a cells secrete less immunoglobulin per cell, they may require lower amounts of *Prmd1* and *Xbp1* mRNA and protein (Savitsky & Calame, 2006).

Importantly, the Nutt group has proven that when *Rag1*$^{-/-}$ mice (which are also deficient in endogenous B cells, T cells and serum Ig) were reconstituted with fetal liver cells mutant for the *Prdm1* gene, they lacked a defined plasma cell compartment, yet showed low but detectable amounts of all immunoglobulin isotypes (0.1%–10% compared to wild type controls), demonstrating the existence of a BLIMP1 independent initial stage of antibody secretion. Notably, upon stimulation, *Xbp1* was detected and *Pax5* downregulated, but *Irf4* expression levels did not increase on BLIMP1 mutant B cells, suggesting that the early steps of antibody secreting cell differentiation may occur independently of BLIMP1 and IRF4 (Kallies et al., 2007). These findings are consistent with the observation that low levels of IgM secretion by B1a cells in both the peritoneal cavity and spleen can be maintained in the absence of *Irf4* in the *Irf4*$^{-/-}$ mouse (Holodick et al., 2010). Thus, in wild type mice, the upregulation of *Irf4* can initiate the classical cascade of plasma cell differentiation (Figure I.1) and allow antibody secreting cells to use their full potential to produce immunoglobulins.

It is well established that by repressing *Bcl6* and *Pax5,* the expression of *Irf4* and *Prdm1* ensures that after plasma cell development is induced, B cells cannot return to an earlier developmental stage. However, the terminally differentiated state of plasma cells may be reversed under special conditions, as when BCL6 is expressed by plasmacytomas that consequently lose their immunoglobulin secretion capacities (Fujita et al., 2004). Although sharing the capacity of antibody secretion, B1a cells have particular properties, as the one of self-renewal (Förster & Rajewsky, 1987), that clearly distinguish them from classical plasma cells. Thus, it is possible that in B1a cells the combination of low expression levels of transcription factors, that are essential for immunoglobulin secretion, keep these cells in an intermediate stage of a continuum differentiation program that leads to terminal plasma cell differentiation in classically stimulated B2 cells. Additionally, it is possible that in B1a cells further differentiation to a terminal plasma cell state is blocked through yet unknown

mechanisms. Our results clearly suggest that, in natural conditions, higher expression levels of *Irf4* imbalances the B1a cell compartment to a more efficient secretory function.

2. NOD mice have increased levels of natural autoantibodies with the potential to induce pancreatic beta-cell damage

Natural antibodies are mostly produced by B1a cells, which link the innate and the adaptive immune system, and are also involved in several autoimmune processes, including type 1 diabetes (Gyarmati et al., 1999; Kendall, Woodward, Hulbert, & Thomas, 2004; Ryan et al., 2010). In fact, natural IgM plays a relevant role as immune protector, scavenger of apoptotic cells/debris and preventer of autoimmunity (Ehrenstein et al., 1998). Furthermore, germline encoded IgM of B1a cell origin has been shown to bind to human carcinoma cells and to directly control their proliferation through the induction of apoptosis, playing a crucial part in immunosurveillance against malignant cells (Brändlein et al., 2003). Thus, it is also reasonable to predict that the coupling of autoreactive IgM to self-antigens could provide a mechanism in the induction of target cell killing. Moreover, in an autoimmune context, IgM binding could result in the activation of the complement cascade contributing to an autoantigen-driven destructive response (Brown et al., 2002; Fearon & Locksley, 1996; Klaus et al., 1980; Pepys, 1972).

In article II of this thesis, we firstly set-out the hypothesis that in the framework of type 1 diabetes, natural IgM could serve as an autoimmunity trigger of beta cell injury. In fact, it has been reported that NOD mice pancreatic beta-cells have IgM bound to their surface prior to the appearance of insulitis (Shieh et al., 1993). Further, in article III, we have clearly demonstrated that young NOD mice have higher serum levels of IgM with insulin reactivity when compared to C57BL/6. Significantly, stable titres of anti-insulin-IgM are maintained in time, and do not increase even in pre-diabetic NOD mice, where the destructive anti-beta cell autoimmune process is already ongoing. In addition, the analysis of MHC congenic strains (Wicker et al., 1992) showed that IgM reactivity observed in the NOD is independent of T cell help and disease progression. These findings are consistent with the hypothesis proposed on article II that increased autoreactivity in the NOD natural antibody compartment is set prior to pancreatic autoimmunity onset, and could contribute to the initiation of autoimmunity.

An increased concentration of circulating serum IgM with the ability to recognize pancreatic islet antigens could determine their direct binding on beta cells. This could directly mediate cell damage or trigger the activation of the complement system and the establishment of a beta cell-directed adaptive immune response, contributing to enhanced immuno-deregulations in the NOD mouse and to the development of T1D. Furthermore, IgM–autoantigen interaction with Fcα/μ receptors on macrophages and B cells could determine a

more efficient autoantigen presentation capacity to autoreactive T cells or have an autocrine effect on beta cell reactive B cell populations, facilitating the autoimmunity process (Côrte-Real, Duarte, Tavares, & Penha-Gonçalves, 2009).

Importantly, in the work presented in article III of this thesis, we were able to demonstrate *in vitro* that the binding of natural IgM *per se* induces oxidative stress on pancreatic beta cells, as measured by the upregulation of *iNOS* expression. This indicates that autoreactive IgM has a role in promoting beta cell damage and is possibly contributing to T1D initiation. Further investigation is required to understand whether the early presence of beta cell reactive natural IgM has additional implications for the diabetogenic process occurring in the NOD.

Interestingly, as also shown in article III, the patterns observed for natural antibody and IgG autoreactivity in the NOD serum were not identical. While NOD anti-insulin IgM remained constant in time, the concentration of IgG with the same specificity clearly followed the progression of disease. Significantly, and contradicting the established notion that elevated beta cell reactive IgG is merely a by-product of the T cell mediated destruction of beta cells, a recent study from the Vinuesa laboratory has shown that anti-islet immunoglobulins of the IgG isotype can enhance the expansion of islet-reactive $CD4^+$ T cells in an Fcγ-Receptor (FcγR)-mediated manner, and cooperate with inherited defects in thymic and peripheral $CD4^+$ T cell tolerance, contributing to type 1 diabetes progression (Silva et al., 2011). These authors studied TCR^+HEL^+ transgenic mice carrying a point mutation in the *Roquin* gene ($Roquin^{san}$). The mutated *Roquin* gene exacerbates the expression of the inducible T-cell costimulator (*ICOS)* on follicular helper T (TFh) cells, with profound effects on cell function and immune tolerance. Consequently, $Roquin^{san/san}$ mice spontaneously develop germinal center reactions, IgG autoantibodies and systemic autoimmunity (D. Yu et al., 2007; Linterman et al. 2009; Vinuesa et al., 2005). TCR^+HEL^+ transgenic mice have an increased frequency of islet-reactive $CD4^+$ T cells. The HEL transgene encodes HEL under the insulin gene promoter, and mirrors the pattern of insulin expression, with high expression in islet beta cells and *Aire* dependent expression in thymic medullary epithelial cells (Akkaraju et al., 1997; Liston et al., 2004). The T cell receptor (TCR) transgene encodes a TCR which recognizes a dominant HEL peptide presented by MHC-II (Ho, Cooke, Goodnow, & Davis, 1994). Nevertheless, the presence of immune tolerance mechanisms in the T cell compartment prevents type 1 diabetes development in 80% of TCR^+HEL^+ transgenic animals in a non-diabetic background (Akkaraju et al., 1997; Lesage et al., 2002; Silva et al., 2011). On the other hand, the $Roquin^{san}$ mutation dramatically increases the progression to T1D in TCR^+HEL^+ mice, such that 100% develop diabetes by 8 weeks of age (Silva et al., 2011). These authors have clearly shown that the autoreactive B cells present in $Roquin^{san/san}TCR^+HEL^+$ mice have a role in T1D progression by secreting high titers of anti-HEL IgG autoantibodies. In this system, anti-HEL IgG enhances the antigen presenting

capacity of FcγR expressing cells, mediating the activation of islet-reactive $CD4^+$ T cells. Importantly, the presence of anti-HEL IgG antibodies in HEL^+ single transgenic mice was insufficient to precipitate diabetes, arguing against a direct antibody-mediated destruction of beta cells (Silva et al., 2011).

A role for autoreactive IgG in type 1 diabetes development has previously been proposed in studies of NOD mouse embryos implanted in non-diabetic surrogate mothers (Greeley et al., 2002; Kagohashi et al., 2005). Despite that the incidence of diabetes was reduced in NOD mice born from non-diabetic prone mothers, it was never clarified if the observed maternal effects reflected the absence of transmission of anti-islet autoantibodies or other factors, such as an altered colonization of the offspring by microbial commensals, which is known to influence T1D incidence in NOD mice (Wen et al., 2008). The Vinuesa laboratory has complemented these findings by demonstrating that spontaneously produced, maternally transmitted or passively transferred anti-islet IgG autoantibodies can overcome thymic and peripheral tolerance mechanisms in the TCR^+HEL^+ transgenic mouse model and trigger $CD4^+$ T cell mediated autoimmune diabetes in an FcγR-dependent manner (Silva et al., 2011). Further evidence supporting that this mechanism mediates a pathogenic role for autoantibodies comes from a report where NOD mice lacking FcγR were shown to be protected from diabetes (Inoue et al., 2007). Also, passive transfer of rabbit or mouse IgG against islet-expressed ovalbumin (OVA) was shown to enhance activation of transferred naïve OVA specific $CD8^+$ T cells, break tolerance and promote T1D in RIP-mOVA mice, which express OVA as a self-antigen in the thymus and in pancreatic beta cells. The transfer of anti-OVA IgG alone, to the same mouse recipients, had no effect on beta cells physiology (Harbers et al., 2007).

Although the presence of anti-islet autoantibodies is the best current predictor of human T1D, the role of B cells and autoantibodies in still poorly understood in diabetic people (Achenbach et al., 2004; L. Yu et al., 2000; Naserke, Bonifacio, & Ziegler, 2001; Reijonen, Daniels, Lernmark, & Nepom, 2000; Ziegler, Hummel, Schenker, & Bonifacio, 1999). Observational studies of subjects with either mothers or fathers with type 1 diabetes have revealed paradoxical protection associated with transmission of anti-islet autoantibodies (Harjutsalo, Reunanen, & Tuomilehto, 2006; Koczwara, Bonifacio, & Ziegler, 2004; Pociot, Nørgaard, Hobolth, Andersen, & Nerup, 1993; Warram, Krolewski, Gottlieb, & Kahn, 1984). These maternal effects in humans are still largely unexplored. Importantly, as well as the human population, the pathogenesis of human type 1 diabetes is likely to be heterogeneous, hence it is conceivable that the diabetes-promoting role of B cells and anti-islet antibodies may not be uniform nor easy to untangle.

The extraordinarily divergent paths of serum anti-insulin IgG and IgM isotypes that we have described for the NOD mouse (article III), an inbred mouse model of type 1 diabetes, highlight the different roles that these antibodies may play in the autoimmune response.

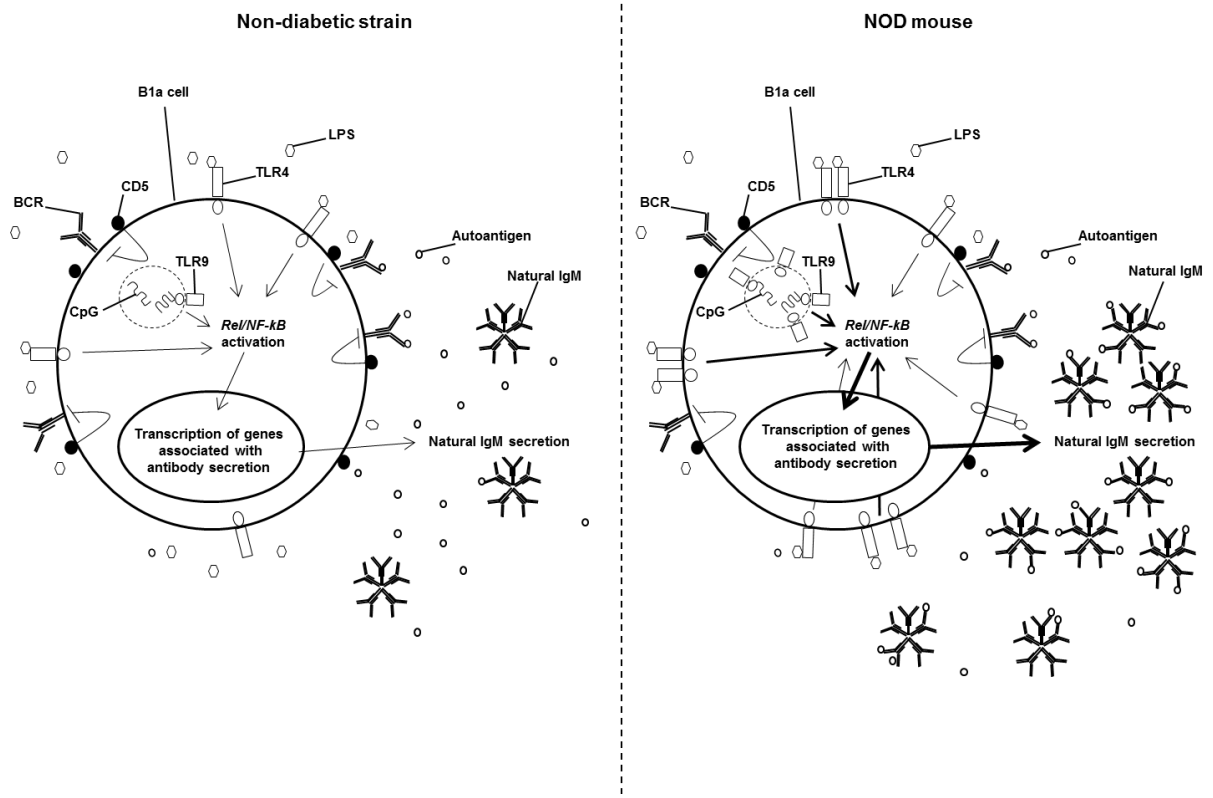
Therefore, diverging from what has been generally accepted, enough evidence exists that autoreactive antibodies of both the IgM and IgG isotypes have a part in the initiation and maintenance of the diabetic autoimmune process.

3. NOD B1a cells secrete beta cell reactive natural autoantibodies, fueling the autoimmune process in type 1 diabetes

B1a cells develop through a process of positive selection based on the BCR reactivity and are the predominant natural IgM secretors. Thus, membrane bound and secreted IgM of B1a cell origin are naturally autoreactive (Casali & Notkins, 1989; Hardy et al., 1994; Kantor & Herzenberg, 1993). In healthy individuals, strict regulation of B1a cells immunoglobulin secretion assures that this autoimmune predisposition is restrained, nevertheless B1a cells have been involved in autoimmune conditions like type 1 diabetes (Kendall, Woodward, Hulbert, & Thomas, 2004; Ryan et al., 2010).

Importantly, in the work published in articles II and III, we provide the first evidence that when insulitis is still incipient, NOD B1a cells spontaneously secrete IgM whose specificities contributes to the increased pattern of antibody autoreactivity observed in the serum of this strain, in comparison to non-autoimmune controls. Moreover, our early characterization of the B1a cell compartment in the NOD and C57BL/6 strains revealed that peritoneal cavity B1a cells in the NOD are naturally more activated and have a diminished threshold for innate-like stimulation, compared to its homolog population in the non-diabetic-prone strain. In fact, in resting conditions, NOD peritoneal cavity B1a cells have a profile of B220, CD43, CD62L and CD86 expression that is associated with an increased activation status (Casola et al., 2004; Jellusova et al., 2010). In addition, NOD peritoneal cavity B1a cells had naturally higher levels of *TLR2*, *TLR4*, *TLR6*, *TLR7* and *TLR9* expression and responded to TLR stimulation with increased proliferation, augmented secretion of IgM with reactivity against type 1 diabetes autoantigens and lower production of IL10, when compared to C57BL/6 B1a cells. Intriguingly, equivalent amounts of total IgM were secreted upon TLR stimulation of either C57BL/6 or NOD B1a cells. Therefore, the NOD B1a cell population must be enriched for autoreactive cells with increased responsiveness and propensity for plasma cell differentiation upon innate-like stimulation (Figure VIII.2). Accordingly, in resting conditions NOD mice have more B1a cells in the peritoneal cavity that secrete IgM towards T1D autoantigens and have higher levels of serum anti-insulin IgM, than the C57BL/6 controls.

Figure VIII.2 Autoreactive NOD B1a cells have a lower threshold for innate like stimulation



Representative image of peritoneal cavity B1a cells IgM secretion patttern in non-diabetic prone mice in comparison to the NOD strain. Autoreactive NOD B1a cells naturally express higher levels of TLR4 and TLR9 and respond to LPS and CpG stimulation with an increased capacity to secrete natural autoreactive IgM. Induction (→); Inhibition: (⊣).

Alterations in microflora have been shown to condition some of the surface B1a cell traits (Alam et al., 2011; Alam, Valkonen, Palagani, et al., 2010) and type 1 diabetes progression in the NOD mouse (Alam et al., 2011; Kriegel et al., 2011). Nevertheless, further investigation is required to understand whether endogenous TLR ligands or the external stimulation of TLR, provided for example by microbial alterations in the intestine, is essential for the establishment and maintenance of the secretory properties of NOD peritoneal cavity B1a cells and for the shaping of the serum IgM autoreactive repertoire.

It has also been established, in the article III of this thesis, that natural IgM derived from NOD B1a cell is able to bind to pancreatic beta cells and trigger the beta cell oxidative stress response *in vitro*. Together, the results discussed in articles II and III provide supporting evidence to propose that from early ages, the repertoire of natural antibodies spontaneously secreted by NOD peritoneal cavity B1a cells is skewed towards endocrine auto-reactivity and has the potential to fuel the autoimmune beta cell attack prior to T cell-mediated specific-cell destruction.

4. Peritoneal cavity NOD B1a cells triggering precipitates T1D in the NOD mouse

It is now clear that peritoneal cavity NOD B1a cells are altered in comparison to non-diabetic mice with potential implications for the diabetogenic process. Consistently, in article IV, we demonstrate that p58 surface glycoprotein expression, which has been correlated to the natural activated state of this subpopulation (Manjarrez-Orduño, Parkhouse, & Santos-Argumedo, 2003), is lower in the NOD peritoneal cavity B1a cell compartment when compared to C57BL/6 and BALB/c non-diabetic prone strains. Moreover, p58 specific targeting in NOD mice *in vivo* induces the down-modulation of IgM on peritoneal cavity B1a cells and the proliferation of this cell subset. Consistently with a more efficient differentiation to a plasma cell-like phenotype, p58 targeted NOD mice have an increased concentration of circulating IgM, and most relevantly, these features correlate with an earlier onset of diabetes in this strain.

The identity and function of p58 and its implications for B1a cells physiology in health and autoimmunity are still unknown and require further investigation. Nevertheless, the results of article IV suggest that lower expression levels of p58 on NOD B1a cells contribute to a less efficient regulation of this cell compartment, and that p58 targeting promotes the proliferation of NOD B1a cells and their differentiation to IgM secreting plasma cells, accelerating the onset of type 1 diabetes in the NOD. These results reinforce the initial hypothesis that peritoneal cavity B1a cells have an initial role on the autoimmune diabetogenesis process.

Anti-CD20 and anti-CD22 monoclonal antibody (mAb) therapy, which specifically deplete the B cell compartment in treated animals, have shown significant promise in disease prevention and reversal in young NOD mice (Fiorina et al., 2008; Hu et al., 2007; Xiu et al., 2008). Furthermore, the construction of transgenic NOD mice in which B cells express human CD20 (hCD20/NOD mice) has allowed the analysis of the therapeutic effects of B cell depletion using the murine anti-CD20 monoclonal antibody (2H7), which targets the same epitope as rituximab, the only B cell targeted drug approved for use in humans (Bour-Jordan & Bluestone, 2007; Hu et al., 2007). The first B cell depleting trials with rituximab in type 1 diabetes patients have reported responsiveness to therapy, broadly comparable with anti-CD3 monoclonal antibody treatment, highlighting an important role for B cells or antibodies in type 1 diabetes in humans (Pescovitz et al., 2009). Rituximab is highly effective in depleting recirculating and memory B cells and causes a rapid decrease in autoantibodies in a number of autoimmune diseases such as RA and SLE (Leandro & de la Torre, 2009). However, the effect of Rituximab on anti-islet antibodies has not yet been described in T1D patients (Pescovitz et al., 2009).

The B cell-activating factor (BAFF, BLyS) is a critical survival promoting factor for which peripheral B cells compete (Lesley et al., 2004; Thien et al., 2004). Consistently with the anti-

CD20 results, long-term anti-BLyS mAb therapy in NOD mice resulted in selective depletion of marginal zone and follicular B cells, correlating with a significant delay in autoimmune diabetes onset, and reduced anti-insulin IgG titers (Zekavat et al., 2008). Thus, anti-BLyS mAb therapy could be an alternative promising approach for the treatment of T1D in susceptible individuals.

It has been previously shown that BCL1 cells are effectively killed upon treatment with NIM-R7 conjugated to ricin-A, while no effect is observed in FO B cells or T cells (Manjarrez-Orduño et al., 2003). As shown in article IV BCL1 cells have a B1a like phenotype and p58 is constitutively expressed on NOD peritoneal B1a cells. Thus, our initial expectation was that *in vivo* NIM-R7 intra-peritoneal treatment would deplete NOD peritoneal cavity B1a cells. The induction of B1a cell proliferation, plasma cell differentiation and T1D precipitation has allowed us to infer the importance of peritoneal B1a cells in type 1 diabetes development. Nevertheless, future studies aiming to specifically deplete this cell subset would allow us to verify if T1D development in the NOD mouse would be prevented in the absence of B1a cells, and to better understand the role of NOD B1a cells in T1D pathogenesis.

Chapter IX. General Conclusion and Future Perspectives

The studies presented in this thesis provide evidence that:

- Natural IgM secretion in the mouse is regulated by the transcription factor *Irf4* which, in natural conditions, controls the number of spontaneous antibody secreting cells both in the peritoneal cavity and in the spleen;
- Peritoneal cavity B1a cells of the NOD mouse naturally secrete IgM with high reactivity towards pancreatic beta cells, which are able to bind to their target autoantigens and induce beta cell oxidative stress;
- The peritoneal cavity B1a cell compartment in the NOD mouse is enriched for autoreactive cells which are remarkably activated in resting conditions and respond vigorously to innate-like stimulation;
- The induction of proliferation and antibody secretion specifically on peritoneal cavity NOD B1a cells anticipates the onset of type 1 diabetes.

In conclusion, this thesis has contributed to the study of genetic factors controlling natural IgM secretion and to the elucidation of the early pathogenic steps of type 1 diabetes. Essentially, these studies have established that alterations in the NOD peritoneal cavity B1a cell subset are responsible for the existence of an autoreactive repertoire of serum IgM, which can induce beta cell oxidative stress, strongly suggesting that natural autoantibodies are an early factor in type 1 diabetes pathogenesis evolving in the NOD mouse.

The work here detailed has shed light on the mysterious field of B1a cells physiology in health and disease. Nevertheless, some questions remain unanswered and require further investigation: (1) The study of *Irf4* expression and spontaneous IgM secretion on the splenic B1a cell compartment, the construction of C57BL/6 and BALB/c congenic strains, reciprocal for the *Irf4* locus, as well as knocking-in the BALB/c *Irf4* allele into the C57BL/6 genetic background, will allow us to better understand the role of *Irf4* in the control of serum IgM levels in naïve mice. (2) The several pathways by which anti-pancreatic beta cell IgM could determine the initiation of T1D remain largely unexplored. It will be important to understand if the induction of oxidative stress responses on beta cells, upon IgM binding, has consequences on beta cell viability in the NOD mouse. The role of NOD natural IgM on the activation of the complement system and on the enhancement of the autoantigen presentation capacity to autoreactive T cells needs further clarification. Likewise, the potential autocrine effect of natural IgM autoantibodies on NOD beta cell reactive B cell populations remains to be established. (3) Further studies will help to understand whether endogenous or exogenous TLR ligands contribute to the establishment and maintenance of the secretory properties of NOD peritoneal cavity B1a cells, and for the shaping of the serum IgM autoreactive repertoire. (4) The identification of p58 as well as *in vitro* targeting of p58 on purified peritoneal cavity B1a cells will allow us to better comprehend the function of this

glycoprotein and its implication on B1a cells physiology. (5) Although complex, future work aiming to specifically reconstitute B1a cell populations in NOD.Igμ.null mice and to specifically deplete the B1a cell subset in NOD mice will allow us to verify the requirement of B1a cells and natural IgM in T1D development.

The demonstration of a role for B1a cells, and natural autoantibodies, in the development of type 1 diabetes may contribute to the development of strategies that block disease progression in the NOD mouse and possibly, in susceptible individuals.

Chapter X. General References

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