Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Mestrado e Doutorado

Papel do *PTPN2* e *MDA5*, dois genes candidatos para diabete melito tipo 1, nas respostas das células beta pancreáticas a citocinas pró-inflamatórias e ao RNA de fita dupla intracelular.

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Orientador (Brasil): Prof Dr Luis Henrique dos Santos Canani

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Porto Alegre, 03 de setembro de 2010.

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Porto Alegre, 03 de setembro de 2010.

“Logo que, numa inovação, nos mostram alguma coisa de antigo, ficamos sossegados”

Friedrich Nietzsche

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Table of Contents

Tabela de Conteúdos

Lista de artigos que constituem essa tese

Esses artigos serão referidos em número romanos durante o texto:

I. Moore F, Colli ML, Cnop M, Esteve MI, Cardozo AK, Cunha DA, Bugliani M, Marchetti P, Eizirik DL. *PTPN2*, a candidate gene for type 1 diabetes, modulates interferon-γ-induced pancreatic beta cell apoptosis. *Diabetes* 58: 1283-91, 2009.
(IF: 8.50)

II. Colli ML, Moore F, Gurzov EN, Ortis F, Eizirik DL. *MDA5* and *PTPN2*, two candidate genes for type 1 diabetes, modify pancreatic beta cell responses to the viral by-product double-stranded RNA. *Hum Mol Genet* 19: 135-46, 2010.
(IF: 7.39)

Lista de abreviações

ADE	potencialização dependente de anticorpo	antibody-dependent enhancement
AP-1	proteína ativatora 1	activator protein 1
APC	célula apresentadora de antígeno	antigen-presenting cell
BB rat	rato Biobreeding	Biobreeding rat
BMI	índice de massa corporal	body index mass
CARD	domínio de recrutamento e ativação caspase	caspase activation and recruitment domain
CCL	ligante de quimiocina (grupo C-C)	chemokine (C-C motif) ligand
CCR	receptor de quimiocina (grupo C-C)	chemokine (C-C motif) chemokine receptor
Chga	cromogranina	chromogranin
CI	intervalo de confiança	confidence interval
CTLA4	proteína associada ao linfócito T citotóxico 4	cytotoxic T-lymphocyte-associated protein 4
CVB	Coxsackievírus	Coxsackievirus
CXCL	ligante de quimiocina (grupo C-X-C)	chemokine (C-X-C motif) ligand
CXCR	receptor de quimiocina (grupo C-X-C)	chemokine (C-X-C motif) receptor
DP5	proteína da morte 5 (também conhecida como Harakiri; *Hrk*)	death protein 5 (also known as Harakiri; *Hrk*)
dsRNA	RNA de fita dupla	double-stranded RNA
EGFR	receptor para o fator de crescimento epitelial	epidermal growth factor receptor
EMCV	vírus da encefalomiocardite	encephalomyocarditis virus
ER	retículo endoplasmático	endoplasmic reticulum
ERK	cinase relacionada ao sinal extracelular	extracellular signal–related kinase
EV	enterovírus	enterovirus
GAD	ácido glutâmico decarboxilase	glutamic acid decarboxylase
GFAP	proteína fibrilar glial ácida	glial fibrillary acidic protein
HLA	antígeno leucocitário humano	human leukocyte antigen
HSP	proteína *heat shock*	heat shock protein
IA2	proteína relacionada ao insulinoma 2	insulinoma-associated protein 2
IA2β	proteína relacionada ao insulinoma 2β	insulinoma-associated protein 2β

IAA	autoanticorpo contra insulina	insulin autoantibody
ICA	anticorpo contra células das ilhotas	islet cell antibody
IFN	interferon	interferon
IGRP	subunidade catalítica da proteína glicose-6-fosfatase ilhota específica	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL-1β	interleucina-1β	interleukin-1β
IL2RA	subunidade-α do receptor da interleucina-2	interleukin-2 receptor subunit-α
INS	insulina	insulin
IPS-1	estimulador do promotor do interferon-1	interferon promoter stimulator 1
IRAK	cinase associada ao receptor da interleucina-1	interleukin-1 receptor-associated kinase
IRF	fator regulador do interferon	interferon regulatory factor
ISGF3	fator estimulador dos genes do interferon 3	interferon-stimulated gene factor 3
JAK	cinase janus	janus kinase
JNK	cinase c-jun N-terminal	c-jun N-terminal kinase
KD	*knockdown*	knockdown
KO	*knockout*	knockout
LCMV	vírus da coriomeningite linfocítica	lymphocytic choriomeningitis virus
LGP2	laboratório de genética e fisiologia 2	laboratory of genetics and physiology 2
LPS	lipopolissacarídeo	lipopolysaccharide
MDA5	gene associado a diferenciação do melanoma 5 (também conhecido como helicase domínio C induzida por interferon 1; *IFIH1*)	melanoma differentiation associated gene-5 (also known as interferon induced with helicase C domain 1; *IFIH1*)
MHC	complexo de histocompatibilidade maior	major histocompatibility complex
mTEC	célula epitelial tímica medular	medullary thymic epithelial cell
MyD88	gene da resposta primária de diferenciação mielóide 88	myeloid differentiation primary response gene 88
NF-κB	fator nuclear κB	nuclear factor κB
NLR	receptor com domínio para ligação de nucleoídeo e repetições ricas em leucina	nucleotide-binding domain and leucine-rich repeat-containing receptor
NOD mice	camundongo diabético não-obeso	non-obese diabetic mice
PAMP	padrão molecular associado a patógenos	pathogen-associated molecular pattern
PIC	ácido poliinosínico-policitidílico	polyinosinic-polycitidilic acid
PKR	proteína cinase dependente do dsRNA	dsRNA-dependent protein kinase R

PPI	preproinsulina	preproinsulin
PRR	receptor para reconhecimento de padrões	pattern recognition receptor
PTPN2	proteína tirosina fosfatase N2	protein tyrosine phosphatase N2
PTPN22	proteína tirosina fosfatase N22	protein tyrosine phosphatase N22
PUMA	modulator da apoptosis induzido por p53	p53 upregulated modulator of apoptosis
RIG-I	gene induzido pelo ácido retinóico I	retinoic acid-inducible gene-I
RIP	promotor da insulina do rato	rat insulin promoter
RLR	receptor semelhante ao gene induzido pelo ácido retinóico I	retinoic acid-inducible gene-I-like receptor
SNP	polimorfimos em nucleotídeo único	single-nucleotide polymorphism
STAT	transdutor de sinal e ativador da transcrição 1	signal transducer and activator of transcription
T1D	diabete melito tipo 1	type 1 diabetes
TLR	receptor *toll-like*	toll-like receptor
TNF	fator de necrose tumoral	tumor necrosis factor
TRAF	fator associado ao receptor do TNF	TNF receptor associated factor
TRIF	adaptador e indutor de interferon-β contendo domínio TIR	TIR-domain-containing adapter-inducing interferon-β
VNTR	número variável de repetições tandem	variable number of tandem repeats
XCL	ligante de quimiocina (grupo C)	chemokine (C motif) ligand
ZnT8	transportador de zinco das ilhotas	islet zinc transporter

Resumo

Na patogênese do diabete melito tipo 1 (DM1) vários genes e fatores ambientais, como as infecções virais, interagem para iniciar um ataque autoimune contra as células beta pancreáticas. Durante a fase inicial desse processo, as células beta desempenham um papel importante através da promoção de um "diálogo" com o sistema imune. Recentemente, o uso de técnicas de genotipagem em larga escala proporcionou um aumento significativo no número de genes conhecidos potencialmente associados ao desenvolvimento do DM1. Para compreender como esses novos genes candidatos modificam as respostas das células beta pancreáticas a mediadores inflamatórios e aos vírus, nós analisamos os dados de estudos prévios de *array* e um banco de dados online (www.t1dbase.org) para identificar os genes expressos nas células beta e modificados por citocinas ou pelo subproduto da replicação viral, RNA de fita dupla (RNAfd). Dois genes foram selecionados para serem estudados nesta tese, *PTPN2* e *MDA5*. PTPN2 é uma proteína tirosina fosfatase que tem entre os seus alvos o STAT1, um fator de transcrição chave no processo de morte das células beta. Inicialmente, confirmamos a presença de *PTPN2* pela quantificação do seu RNAm e produto protéico em uma linhagem de células beta (INS-1E), células beta primárias de rato purificadas por FACS e ilhotas humanas. Tratamento com citocinas ou RNAfd intracelular significativamente aumentou a sua expressão. O *knockdown* específico deste gene pela técnica de RNA de interferência aumentou significativamente a apoptose das células beta expostas a uma combinação de citocinas (interleucina-1β (IL-1β) + interferon-γ (IFN-γ)) ou RNAfd intracelular, e converteu IFN-γ isoladamente em um estímulo pró-apoptótico. O silenciamento do *PTPN2* amplificou a fosforilação do STAT1. O duplo *knockdown*, *PTPN2* + *STAT1*, protegeu as células beta contra a apoptose induzida por citocinas, sugerindo que *PTPN2* age como um regulador negativo dos efeitos pró-apoptóticos do STAT1. Contudo, o silenciamento do *PTPN2* não produziu nenhuma alteração maior na expressão de citocinas e

quimiocinas. O segundo gene candidato, *MDA5*, é uma helicase associada com o reconhecimento de ácidos nucléicos virais intracelulares. A principal função do *MDA5* é a detecção de infecções virais; sendo assim, esse gene foi avaliado apenas no contexto do mimético viral RNAfd. A transfecção de células INS-1E e células beta primárias de rato purificadas por FACS com RNAfd induziu um aumento significativo no RNAm codificando *MDA5*. O silenciamento do *MDA5* e do *RIG-I* (outra helicase envolvida no reconhecimento do RNAfd intracelular) não modificou a frequência da apoptose induziu por RNAfd. Por outro lado, o *knockdown* do *MDA5*, mas não do *RIG-I*, significativamente reduziu a expressão de várias citocinas/quimiocinas produzidas pelas células beta expostas ao RNAfd intracelular. Concluindo, os dados apresentados sugerem que esses dois genes candidatos, através de suas funções nas células beta, podem ter importantes papéis no desenvolvimento do DM1. *PTPN2* aparentemente previne a apoptose das células beta controlando a ativação do STAT1, enquanto *MDA5* pode regular o ataque imune local através da diminuição no recrutamento e ativação das células do sistema imune.

Abstract

In type 1 diabetes (T1D) several genes and environmental factors, such as viral infections, interact to trigger a chronic autoimmune assault against the insulin-producing pancreatic beta cells. During this process beta cells have an important role in maintenance/amplification of this autoimmune response via a cross-talk with the immune system. In recent years the development of high-throughput techniques for searching new genetic variants significantly increased the number of known genes potentially contributing for T1D. To clarify how these new candidate genes modify pancreatic beta responses to proinflammatory mediators and viruses, we used data from our previous array studies and an online beta cell database (www.t1dbase.org) to select candidate genes that are expressed in beta cells and modified by cytokines or the viral by-product double-stranded RNA (dsRNA). Two genes were identified, *PTPN2* and *MDA5*, and further studied in this thesis. PTPN2 is a protein tyrosine phosphatase with several targets including STAT1, a key transcription factor involved in beta cell death. We confirmed at mRNA and protein levels the expression of *PTPN2* in a beta cell linage (INS-1E), primary FACS-purified rat beta cells and human islets. Exposure to cytokines or to intracellular dsRNA increased *PTPN2* expression. Knockdown of *PTPN2*, by using specific small interference (si)RNAs, exacerbated beta cell apoptosis after treatment with a combination of cytokines (interleukin-1β (IL-1β) + interferon-γ (IFN-γ)) or intracellular dsRNA, and converted IFN-γ alone in a pro-apoptotic stimulus. Importantly, *PTPN2* silencing amplified STAT1 phosphorylation. The double knockdown of *PTPN2* and STAT1 protected beta cells against cytokine-induced apoptosis, suggesting that *PTPN2* acts as a negative regulator of the pro-apoptotic transcription factor STAT1. Knocking-down *PTPN2,* however, did not affect to any major extent the expression of cytokines and chemokines. The second candidate gene, *MDA5*, is an helicase involved in recognition of intracellular viral nucleic acids. Since *MDA5* works as a receptor for detection of viral infection, this gene was only

evaluated in the context of the viral mimetic dsRNA. Transfection of INS-1E cells and FACS-purified rat beta cells with dsRNA significantly upregulated the mRNA expression of *MDA5*. The silencing of *MDA5* and *RIG-I* (another helicase involved in recognition of intracellular dsRNA) did not modify dsRNA-induced apoptosis. On the other hand, the knockdown of *MDA5*, but not of *RIG-I*, decreased the expression of several cytokines/chemokines in beta cells exposed to intracellular dsRNA. In conclusion, our data suggest that these two candidate genes may have important roles in the development of T1D via their actions at the beta cell level. *PTPN2* seems to prevent beta cells apoptosis by controlling STAT1 activation, while *MDA5* might regulate the local autoimmune assault via decreasing recruitment and activation of immune cells.

1. Introduction

The American Diabetes Association (ADA) classifies type 1 diabetes (T1D) in two different groups: type 1 diabetes A, which is an immune-mediated disease, and type 1 diabetes B, which includes idiopathic forms of insulinopenic diabetes [1].

Type 1 diabetes A accounts for 80-85% of T1D cases, and it represents 5-10% of all cases of diabetes. T1DA is characterized by a severe deficiency of insulin secretion due to an autoimmune attack against the insulin-producing pancreatic beta cells. It usually affects children and adolescent, but it can be diagnosed in adults independently of their age. As in other autoimmune diseases, these patients can develop immune-mediated assault against other organs, and present increased prevalence of diseases such as Grave's disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, and pernicious anemia [1]. This is the form of type 1 discussed in this thesis, and from now it will be just called type 1 diabetes.

Type 1 diabetes B has an idiopathic etiology, and no markers of autoimmunity were identified in these patients. It occurs mainly in people from Asiatic or African ancestry, and is characterized by recurrent episodes of ketoacidosis with variable degree of insulinopenia between these episodes. Interestingly, a recent study identified a significant increase in genomic RNA and antibodies against human herpesvirus 8 in patients from Sub-Saharan Africa with a ketosis-prone form of diabetes [2]. It was also demonstrated the virus ability to infect pancreatic beta cells [2]. Moreover, a form of diabetes called fulminate diabetes was described in Japanese, and immunohistochemical analysis revealed the presence of the enterovirus capsid protein VP1 in pancreas of three patients that died after diagnosis [3]. These data suggest that this "idiopathic" form o type 1 diabetes may be caused by a direct cytopathic effect of viruses, with destruction of pancreatic beta cells by non-autoimmune mechanisms as observed in some animal models [4].

2. Epidemiology of Type 1 diabetes

Type 1 diabetes is one of the most frequent autoimmune diseases in children and young adults, with an annual estimative of 76,000 new cases worldwide and a prevalence of approximately 480,000 patients [5]. The fact that it affects mainly children, requires a long-life treatment and causes several chronic complications, makes a better understanding of the pathogenesis of T1D a priority.

To study the epidemiology of T1D, two important consortiums were established in the 80's: the EURODIAB, which includes 44 European centers and has data from more than 30 million children [6], and the DIAMOND network, that covers 112 centers from 57 countries around the world and 84 million children [7].

The results obtained by these groups indicate the wide variation of T1D incidence (Figure 1) [8]. Thus, the differences between countries range from 0.1/100,000 per year in the Zunyi region of China to more than 40/100,000 per year in Finland [7]. Other important epidemiological aspects observed were the age and sex-specific variations of incidence, temporal trends, seasonality of onset and geographic variation of clinical presentation [8].

2.1. Geographic Variation

Europe has the best databanks on T1D incidence, with many countries having information covering different regions or the whole nation. Based on these information, a progressive increase in T1D incidence from south to north was described in Europe [9]. Sardinia, in the south of Italy, is an exception, with high values close to Finland.

The data available from sub-Saharan African populations are incomplete and a good picture about its T1D incidence is not possible at the moment. However, in regions bordering the Mediterranean Sea and in the Middle East a relative low incidence was observed (1/100,000 per year (Pakistan) and 8/100,000 per year (Egypt)) [8].

In North America, Canada (21.7/100,000 per year) and United States (16.1/100,000 per year) have incidence rates similar to populations from North of Europe. On the other hand, Mexico presents a low incidence (1.5/100,000 per year) [8]. Although the incidence of T1D in Central and South America is usually low, there are large differences between countries. This seems related to an inverse ecological correlation between the proportion of the Amerindians population and the incidence of T1D [10]. The Brazilian cases are estimated to accounts for 70% of the region's total numbers of T1D patients (7.7 to 18.5/100,000 per year) [7, 11].

The Southeast Asia region includes India, the country with the largest childhood population, but that unfortunately does not have accurate data regarding T1D incidence. Two studies with significant variation in the incidence rates and covering only cases from urban area were published [5], indicating values in the range of 4.2/100,000 to 8.0/100,000 per year [5, 12]. The other country evaluated, Mauritius, has a low incidence (1.4/100,000 per year) [5].

Finally, the West Pacific region, with the exception of Australia and New Zealand, has usually low incidence [5]. China, the most populous country in the world, presents a very low incidence of T1D [7]. For that reasons, only 6% of the T1D cases come from the West Pacific region.

Several studies have tried to explain these disparities in T1D incidences between regions. Two important factors are usually considered: the genetic background of the individuals and the environmental determinants (see below).

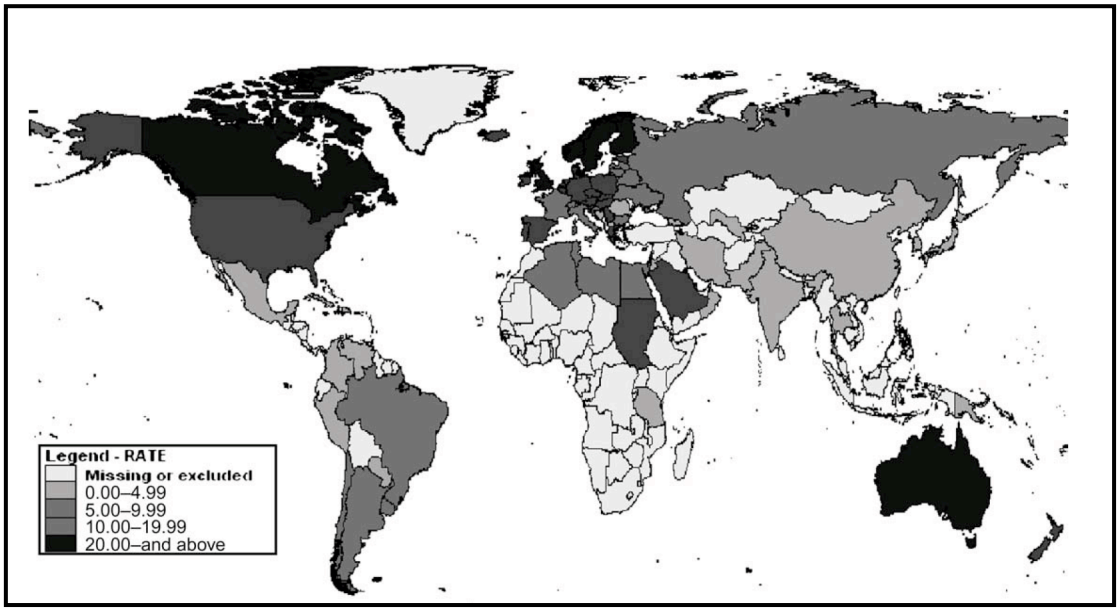

Figure 1. Map of published incidence rates (per 100,000) of type 1 diabetes in children (adapted from [5]).

2.2. Migration Studies

Migration studies provide valuable information on the role of environmental components as etiological factors. For instance, by comparing the T1D incidence in a population that migrate between regions with different incidence (low to high, or the other way around) to the one that still lives in the original regions it is possible to evaluate the effect of the new environment.

T1D incidence in the first generation of immigrants coming from the Asiatic continent, a region with a very low incidence of T1D, to Bradford, U.K. during the period from 1978 to 1990 was compared [13]. In the first 4 years of the study the incidence among Asians was 3.1/100,000 per year versus 11.8/100,000 per year in non-Asians, but in the subsequent 3 years the incidence increased to 11.7/100,000 per year, similar to values in the local population (12.0/100,000 per year) [13]. A similar effect was observed in Asian children that moved to another region of the U.K. (Leicestershire) [14]. These data, together with other studies, show that T1D risk increases in relation to both geography, and early growth, as

defined by height, weight and BMI [15, 16]. This suggests that both modifications in the environment and the lifestyle have an important role in the development of T1D.

Several studies indicate an important role for genetic factors in the pathogenesis of T1D. It was reported that from 1980 to 1990 the Japanese children living in Hawaii presented a similar incidence of T1D as those living in Japan (3/100,000 per year versus 2/100,000 per year, respectively), which is much lower than the Hawaiian children (15.4/100,000 per year) [17]. Italy presents one of the most significant within-country variations in the T1D incidence, ranging from 7.9 in the Lazio region to 38.8/100,000 per year in Sardinia [18, 19]. After World War II, a significant community of descendents from Sardinia was established in Lazio [20]. Of note, children of Sardinian-heritage born in Lazio had the same high T1D incidence as the population of origin. Moreover, children with one Sardinian parent had a diabetic rate half of that of Sardinians and double of that observed in the local population, suggesting that in this high risk population the genetic factors are crucial, overruling eventual environmental contributions [21]. In contrast, an elegant study evaluated the incidence of T1D among second-generation immigrants and adoptees from other countries that moved to Sweden, a country with high incidence of T1D [22]. Since families with compatriot marriages normally keep their original cultural and social conditions, there is a tendency to slow integration with the host population, which can reduce the effect of the environment as a cofactor for diseases. By analyzing second-generation immigrants and children adopted by Swedish couples, the authors could assume that environmental, cultural, and lifestyle exposures were rather similar to the native Swedes, with a difference mostly in the ethnic/genetic background. In comparison to Swedish children, the incidence of T1D was lower in all second-generation immigrants and all adoptees from abroad, with the exception of those having parents from Finland, a country with a higher incidence of T1D than Sweden [22]. Of note, adoptees,

especially those adopted at an older age, could have been exposed to environmental risk factors from their home country, which may affect data interpretation.

In summary, the available data suggest that both environmental and genetic factors are important for T1D development, but their relative weight may vary [13, 14, 21, 22]. Most probably there is an interaction between different environmental factors acting in different genetic backgrounds, producing the previously published diversity of results.

2.3. Temporal Tendencies

Studies from different regions around the world have been describing an increase in T1D incidence starting in the 1950s [23-28]; the EURODIAB and DIAMOND consortiums confirmed these temporal tendencies in their collective countries database [7, 29]. The DIAMOND group identified an average of 2.8% annual increase in incidence during 1990-1999 for all but two regions (Central America and West Indies) [7]. Recently, the EURODIAB published a new analysis of incidence trends for childhood T1D in Europe during 1989-2003 [29]. This informative study, based on accurate databases, corroborates the increase in diabetes incidence with an overall annual increase for all centers of 3.9% (95% CI 3.6-4.2). Furthermore, it shows that this rise in incidence varies between age-groups: in general, the peak of incidence is at puberty [30], but the increase in the youngest groups is more pronounced: 0-4 years: 5.4% (4.8-6.1); 5-9 years: 4.3% (3.8-4.8) and 10-14 years: 2.9% (2.5-3.3). These increased rates are not homogeneous, and Central/Eastern European countries, typically regions of low T1D incidence, present the most pronounced increase in diabetes incidence [29]. These rapid changes over time can not be explained by modification in the genetic factors, and indicate a heightened environmental pressure [31]. The Western-related lifestyle such as increased weight and height development [32], obesity [33, 34], caesarean section deliveries [35] and reduced early infection [36] could be causes of this

differential increase in T1D incidence [37], suggesting a convergence of incidences all over Europe due to risk factors harmonization [29].

The data about temporal trends in T1D incidence in Brazil provide some divergent information. The first study describing the annual incidence in two urban regions (Sao Paulo and Passo Fundo) demonstrated a slight decrease in the incidence over 2-4 years [7]. Another study from a different urban region (Bauru, in the state of Sao Paulo) identified a significant increase in the incidence from 1987 (2.82/100,000) to 2002 (18.5/100,000) [11]. Data covering a large number of regions in Brazil are needed to unveil the real temporal trends in our country.

2.4. Seasonality of Onset

The seasonality of onset or diagnosis of T1D has been reported in many Northern Hemisphere countries [38], but it has been less studied in the Southern Hemisphere [39]. The incidence data collected by the DIAMOND Project over the period 1990–1999 in 53 countries worldwide was recently analyzed [40], and a significant seasonality identified, with peaks in Winter and decreases in incidence in the Summer months for countries in both Hemispheres [40]. Regions further away from the equator were on average more likely to exhibit seasonality. Among potential environmental causes, observational studies suggest that seasonal viral infections can change the incidence levels of T1D [41] or modify the pattern of seasonality [42]. Since the seasonal variability is more evident in higher latitude, another explanation for the phenomenon could be the decreased levels of vitamin D, previously described in patients with T1D [43, 44].

3. Pathogenesis of T1D

Type 1 diabetes was first described as a possible autoimmune disease more then 40 years ago, based on the observation of T1D development in patients with other well known autoimmune disease [45-47] and the histological findings of inflammatory pancreatic infiltrate (insulitis) [48, 49]. In 1974, two independent groups used the indirect immunofluorescence technique to identify circulating islet cell antibodies (ICA) in the sera of patients with T1D and coexistent autoimmunity [50, 51]. Despite this first enthusiasm with the role of autoantibodies, further studies indicated that ICAs do not have a major role in T1D pathogenesis [52]. However, the fact that their appearance often precedes diabetes development by several years [53] have justified the use of ICA and other autoantibodies to predict T1D [54].

3.1. Autoimmunity

The progressive autoimmune attack against beta cells observed in T1D leaves characteristic "signal autoantibodies" that, associated with genetic susceptibility traits, can be used in predictive models of the disease [54]. As discussed above, ICAs were the first markers of autoimmunity identified in patients with T1D. They may have oscillatory patterns and are directed against different islet antigens during disease progression. Since T1D is mainly a T cell-mediated disease [55], the characterization of T cell responses to autoantigens have also been evaluated in human and animal models of T1D. This approach demonstrated that in mice and humans the target antigens for B and T cells are overlapping, but not always identical [56]. For example, insulin was identified as an autoantigen for B and T cells in both species. In humans, ZnT8 (islet zinc transporter) was demonstrated to be a B cell target, but it remains to be proven as a T cell target [57]. On the other hand, chromogranin A (Chga) is a T cell target in mice, but there are no known ICAs against this protein [58]. The different islets autoantigens already described are listed below (Table 1) and described in 3.1.1.

Table 1. Immune responses to autoantigens in T1D. (modified from [59], with additional information from [57, 58]).

Antigen	Expression	Subcellular location	Antibodies	CD4+ T Cells	CD8+ T Cells
Chga	NE	Secretory granule		NOD	
CPE (CPH)	NE	Secretory granule membrane	H[a, c], NOD	NOD	
DNA TopII	WE	Nucleus	H[d]		
GAD65	NE	Synaptic-like microvesicle	H[b, c]	NOD, H[b, c]	NOD, H[b, c]
GAD67	NE	Cytosol	H[b]	NOD	
GFAP	NE	Intermediate filament		NOD	NOD, H[b,c]
Glima38	NE	Secretory granule membrane	H[a, b]		
GLUT2	WE	Cell membrane	H[b]		
GM2–1e	NE	Secretory granule membrane	H[a, b, c]		
GT3e	NE	Cell membrane	H[b]		
HIP/PAP	Pancreas	Secretory granule		NOD	
HSP-60	WE	Mitochondrial matrix	NOD, H[b]	NOD, H[b]	
HSP-70	WE	Mitochondrial matrix, cytosol, ER	H[b]	H[b]	
HSP-90	WE	Cytosol	H[c]		
IA-2 (ICA512)	NE	Secretory granule membrane	H[b, c]	NOD, H[b, c]	H[c]
IA-2b (phogrin)	NE	Secretory granule membrane	H[b, c]	NOD	
IAPP (amylin)	IS	Secretory granule			H[b]
ICA69	NE	Trans-Golgi network	H[a, b, c]	NOD, H[b]	
IGRP	IS	ER membrane			NOD, H[b,c]
Imogen38	WE	Mitochondrial matrix		H[b]	
Insulin	IS	Secretory granule	H[b, c], NOD	NOD, H[b, c]	NOD, H[b]
Jun – B	WE	Nucleus	H[b]	H[b, c]	
Peripherin	NE	Cytosol	NOD	NOD	
S100b	NE	Cytosol		NOD, H[b, c]	
SOX13 (ICA12)	WE	Nucleus	H[d]		
Sulfatide	NE	Secretory granule	H[b]		
ZnT8	IS	Secretory granule	H[b]		

H: human; IS: islet specific; NE: neuroendocrine pattern; WE: widely expressed; NOD: NOD mice

[a]Prediabetic subjects (later developed T1D).
[b]Recent-onset T1D patients.
[c]At-risk first-degree relatives of T1D patients.
[d]Long-standing T1D patients.
[e]Lipid antigens.

3.1.1. Autoantigens

3.1.1.1. Proinsulin and insulin

Insulin was the first autoantigen to be recognized in patients with T1D [60] and it is a major target for both B and T cells in mice and humans. Insulin represents one third of the

total protein content in pancreatic beta cells. Since there are also humoral responses to exogenous insulin, patients should be examined for these antibodies before insulin therapy. Taking this variable into consideration, improved assays demonstrated that around 70% of new-onset patients have circulating antibodies to insulin [61]. Antibodies against insulin are among the first to appear in the 1-2 year age period, and patients presenting these antibodies usually have a high prevalence of *HLA DRB1*04; DQB1*0302* [62]. The insulin autoantibodies (IAAs) are of high-affinity IgG1 class already at the start, suggesting rapid maturation [63]. After this early appearance of IAAs, there is spreading of the immune response to other islet antigens, suggesting that the progressive destruction of pancreatic beta cells leads to exposure of several autoantigens. Some of these patients develop high-affinity responses to GAD65 (Glutamic acid decarboxylase 65 kDa) prior to IAAs, but not to other autoantigens that usually follow IAAs appearance [64]. This pattern suggests that in patients who develop T1D in childhood the initial immune response is targeted to antigens specifically expressed in beta cells (e.g insulin), while other and ubiquitous cell proteins (e.g. IA-2) are probably secondary targets, recognized due to massive release of proteins from dying beta cells. In addition, both the genetic background (HLA groups) and the inflammatory cytokine milieu present seem to be important to determine these autoantibodies responses [54].

Different from the group of patients that present T1D during childhood, individuals affected by the second peak of T1D incidence that occurs around puberty present heterogeneous profiles of autoantibodies. Thus, they often have antibodies directed against single autoantigens, typically insulin or GAD65, and spreading to other beta cell antigens is less frequent (Figure 2). The correlation with HLA class II is also not so strong as in childhood T1D [54, 65]. Those differences could be related to particular etiologies and immunization occurring in older individuals. Alternatively, the immune threshold for immune activation may differ with age, and patients who develop islets autoimmunity during

childhood might have been “programmed” during fetal life, instead of by environmental factors putatively present in puberty T1D. Supporting this hypothesis, exposure to elevated blood glucose levels and maternal islets autoantibodies during pregnancy are associated with reduced risk of islets autoantibodies in the child [66].

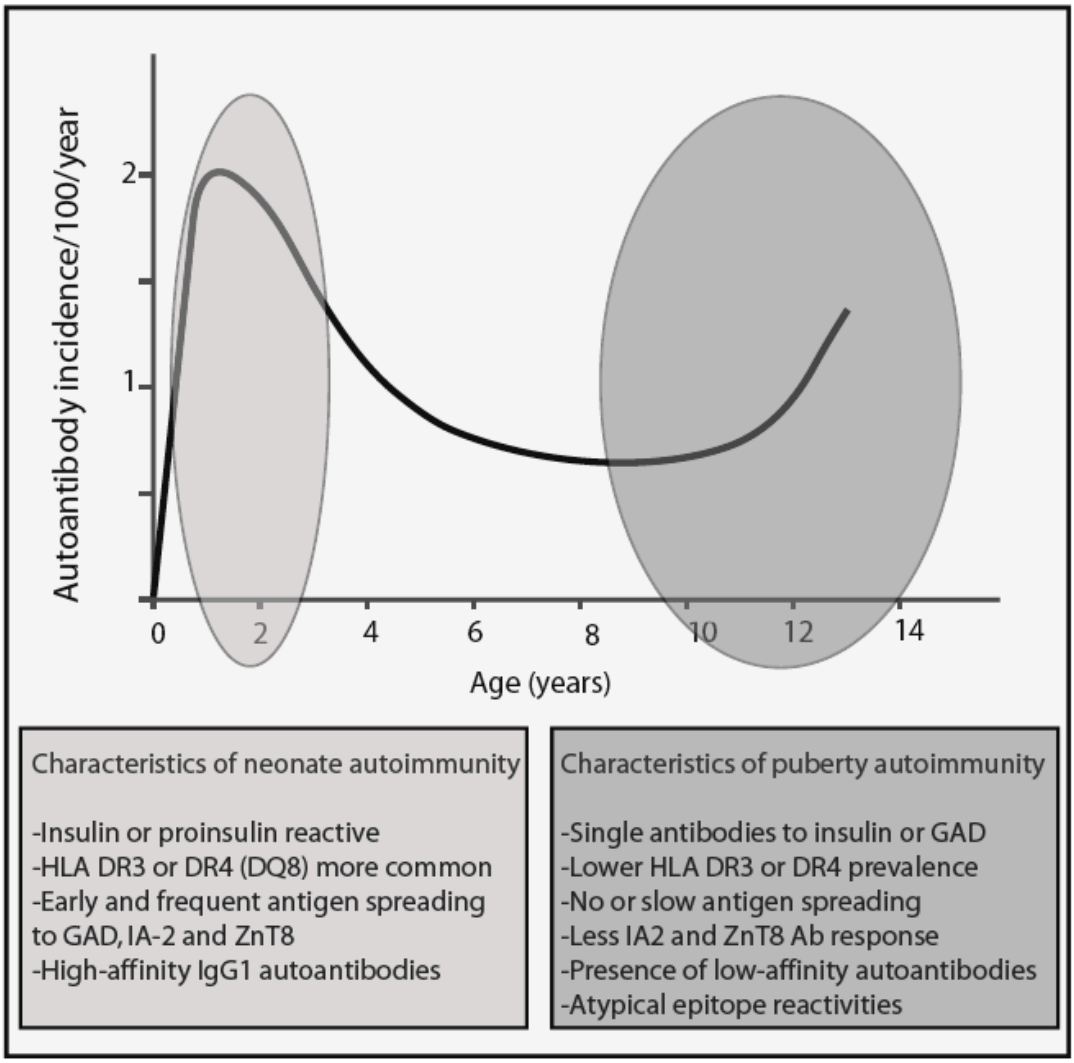

Figure 2. Incidence of islets autoantibodies in different ages. (modified from [54])

Unlike humans, mice and rats express two preproinsulin genes, insulin 1 and insulin 2. Insulin 1 is expressed in the pancreas and insulin 2 in both the pancreas and certain epithelial cells of the thymus (medullary thymic epithelial cells [mTECs]) that coexpress the transcription factor Aire, related to Autoimmune Polyendocrine Syndrome 1 (APS1).

Systemic insulin 2-deficient NOD mice develop an accelerated form of diabetes [67], whereas insulin 1–deficient NOD mice develop IAAs but are free from insulitis and diabetes [68]. Interestingly, selective deletion of *Ins2* in the mTECs of *Ins1*$^{-/-}$ mice triggers diabetes [69]. These findings suggest that thymus expression of insulin has an important role in controlling immune tolerance to beta cell antigens. T cell reactivity against mature insulin has been mainly localized to the B9-23 epitope of *Ins2* [70]. This epitope differs only at one amino acid in insulin 1, and the clones of CD4+ T cells isolated from prediabetic NOD mice also recognize it [59]. The transferring of those B9-23 clones to young NOD mice accelerate diabetes development [70] and NOD mice KO (knockout) for *Ins1* and *Ins2,* but expressing functional insulin transgene encoding a mutant B9-23 epitope, are diabetes resistant [71]. In humans, 83% of recent-onset diabetes patients show a significant proliferative response of the peripheral lymphocytes to B9-23, as compared with none of the age and HLA paired controls [72].

The effector CD8+ T cells also react against epitopes from insulin. Thus, a population of CD8+ T cells obtained from mice recognizes an epitope from the insulin B-chain (B15-23) [73]. These cells were already present in the islets of 4-week-old NOD mice, suggesting that insulin is one of the early targets for the beta cell cytotoxic CD8^{+} T cell response [74]. This peptide is present in both insulin 1 and 2, and it is entirely contained within B9-23. Studies in humans confirmed these results and additionally identified several other epitopes, not only in insulin but also in pre- and proinsulin, as targets of T cell responses in patients with T1D [75-77].

3.1.1.2. Glutamic acid decarboxylase (GAD)

GAD is the rate-limiting enzyme in the conversion of glutamic acid to γ-aminobutyric acid (GABA), a well-known inhibitory neurotransmitter, which also functions as a signaling molecule in the pancreatic islets [78]. GAD, differently from insulin, is expressed in several

tissues such as central and peripheral nervous system, epithelial cells of the fallopian tube, and spermatozoa. There are two isoforms of GAD [59]. The first, GAD65 (65 kDa) is the only one expressed in human beta cells, and is present mainly in synaptic-like microvesicles together with GABA. Seventy percent of new-onset T1D patients have antibodies against GAD65, compared to 4% in the control population [79]. The second, GAD67 (67 kDa) presents a significant homology (65%) with GAD65, and 10% of new-onset diabetic patients have antibodies to GAD67; their crossreativity with GAD65, and the absence of GAD67 in human islets, suggests that GAD65 is the primary antigen [79]. The production of antibodies against GAD by NOD mice is variable and not reproducible between colonies [80, 81].

GAD is also a target of autoimmune attack by T cells. GAD65-specific $CD4^+$ and $CD8^+$ T cells are present in the peripheral blood of at risk individuals and recent-onset T1D patients [76, 82]. Treatment of patients with GAD-alum injections promoted a less pronounced decrease of C-peptide without changing the insulin requirement [83]. Studies of GAD65 in animal models have, however, produced contradictory results. In NOD mice GAD65-specific T cell responses have been associated with both pathogenesis and regulation of diabetes [84-86]. These conflicting results, in comparison to human data, could be due to the limited GAD expression in mouse islets whereas GAD is well expressed in human islets [87].

3.1.1.3. Insulinoma-associated protein 2 (IA2) and Insulinoma-associated protein 2β (IA2β)

IA2 (also known as ICA512) and IA2β (also known as phogrin) were identified after the digestion with trypsin of immunoprecitates from the sera of T1D patients [88, 89]. These proteins are localized in the dense secretory vesicles of neuroendocrine tissues, including the brain, pituitary and pancreatic islets [88, 89]. IA2 and IA2β are members of the receptor-type protein tyrosine phosphatase family, but multiple substitutions within the tyrosine

phosphatase domain appear to have induced loss of their catalytic activity [90]. Studies in knockout animals suggest that these proteins have a role in the regulation of insulin release and glucose tolerance [91]. Around 46% and 66% of the patients with T1D present antibodies against IA2 and IA2β, respectively, at the time of diagnosis [88, 89]. These antibodies usually appear later than antibodies against insulin and GAD, and are highly associated with the expression of other autoantibodies. The presence of two or more autoantibodies (anti-insulin, GAD, and IA2) is one of the best predictors of progression to T1D [92]. IA2β reactivity can be completely blocked by preincubation with IA2, while reactivity to IA2 is only partially prevented by preincubation with IA2β (89). These findings suggests that IA2 is the primary target of the humoral immune response [59].

CD4+ T cell proliferative responses against IA2 were identified in 59% of newly diagnosed T1D patients, compared to 8% in controls matched by HLA of risk [93]. Moreover, IA2 peptides naturally processed and presented by HLA-DR4 were shown to elicit T cell responses in HLA-DR4+ patients but not HLA-DR4+ controls [94]. Similar CD4+ T cell reactivity to IA2 and IA2β was observed in NOD mice [95, 96]. IA2-specific CD8+ T cell responses have been documented in recent-onset T1D patients [97] and antibody-positive first-degree relatives [98]. However, neither knockout of IA2 [99] nor IA2β [100] prevented the development of diabetes in NOD mice, suggesting that these antigens are not essential targets for the progression of diabetes.

3.1.1.4. Zinc transporter ZnT8

The ZnT8 transporter was recently identified as a candidate diabetes autoantigen based on a bioinformatic screening [57]. ZnT8 is a multispanning transmembrane protein mainly localized in the pancreatic beta cell secretory granules. It is responsible for transporting zinc into the granules where it forms a complex with insulin [101]. Polymorphisms in ZnT8 have been associated with risk of T2D, but apparently do not have a

role in T1D development [102]. Sixty to eighty percent of the patients with recent-onset T1D have ZnT8 antibodies. Based on data from the DAISY cohort, they usually appear after insulin and GAD antibodies [57]. NOD mice do not produce antibodies against ZnT8, and this antigen has not yet been characterized as a T cell target [56].

3.1.1.5. Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)

IGRP is an ER-resident, nine-span transmembrane protein that is expressed predominantly in beta cells and to a lesser extent in alpha cells [103]. IGRP is a homolog of the hepatic glucose-6-phosphatase; one study suggests that IGRP has enzymatic activity and is the authentic islet-specific glucose-6-phosphatase catalytic subunit [104]. In NOD mice, IGRP-specific CD8+ T cells correspond to a significant proportion of the islet-infiltrating population [105]. Humans also have CD8+ T cell responses to IGRP. The peptides $IGRP_{152-160}$, $IGRP_{215-223}$, and $IGRP_{293-301}$, which bind to *HLA-A2*0201*, induce both interferon-γ and granzyme B responses in peripheral blood mononuclear cells of recent-onset diabetic as well as in antibody-positive individuals [106, 107]. The fact that the diabetogenic role of IGRP-specific CD8+ T cells requires a previous autoimmune response to proinsulin [108] suggests that IGRP responses are not primary events in T1D development.

3.1.1.6. Heat shock protein-60 (HSP-60)

Antibodies against HSP-60 were demonstrated in NOD mice [109] and in children with newly diagnosed T1D [110]. The proliferative response of T cells in NOD mice [111] and T1D patients (<14 weeks since the diagnosis) [112] was also increased by exposure to different peptides originated from HSP-60, especially the amino acids sequence 437-460 (designated p277). These findings motivated a small clinical trial in recent-onset T1D patients (< 6 months). In this study, patients treated with p277 (DiaPep277) seemed to preserve some beta cell function, as C-peptide concentrations were maintained and the need for exogenous

insulin was lower, despite a similar metabolic control (similar HbA1c) [113]. Since HSP-60 is an ubiquitous protein, some authors raise questions about its role in the highly beta cell-specific destruction observed in T1D [59].

3.1.1.7. Additional auto-antigens

Several other potential auto-antigens have been identified in animal models of diabetes and in T1D patients. Usually autoantibodies from serum are the first to be characterized. The antibodies identified against widely expressed proteins include DNA topoisomerase II [114], HSP-70 [115], HSP-90 [116], ICA69 [117], glucose transporter 2 (GLUT2) [118], and the transcription factors JunB [119] and SOX13 (also known as ICA12) [120]. The antigens with a neuroendocrine pattern include carboxypeptidase H (CPE) [121], glycosylated islet membrane antigen of 38kDa (glima38) [122], and the lipid antigens sulfatide [123] and the gangliosides GM2-1 [124] and GT3 [125].

T cell responses to additional beta cell antigens were also identified, usually following discovery of autoantibodies. Studies in NOD mice demonstrated CD4+ T cell responses to CPE [84], ICA69 [126], peripherin [84], and the calcium-biding protein S100β [127]. Recently, chromogranin A (Chga) was identified as the antigen recognized by the pathogenic BDC 2.5 T cell clone of NOD mice [58], but no antibodies specific for Chga have been described in either humans or NOD mice. In humans, CD4+ T cell responses against junB [119], HSP-70 [115], islet mitochondrial antigen of 38 kDa (Imogen38) [128] and hepatocarcinoma-pancreas/pancreatic associated protein (HIP/PAP) [129] were detected in T1D patients and/or antibodies-positive first-degree relatives. Finally, CD8+ T cell responses to amylin, or islet amyloid polypeptide (IAPP) [130], and the glial fibrillary acidic protein (GFAP) [107, 127], expressed on the peri-islet Schwann cells, were identified in both NOD mice and humans. This finding is particularly interesting, because it suggests that responses to non-beta cell antigens can be involved in insulitis and in the pathogenesis of T1D.

3.1.2. Molecular mimicry

Molecular mimicry due to similarity between beta cell antigens, such as GAD65 and IA2, and molecules expressed by some virus, such as Coxsackie B virus [131] and Rotavirus [132], could be another initiating factor for T1D. The results supporting this hypothesis are contradictory and will be further discussed below, during the description of the role of viral infection in the pathogenesis of T1D (section 5.4).

3.2. Innate Immunity

Vertebrates make use of two different "immune systems" to maintain host-microbial homeostasis: innate and adaptive. The innate immune system represents the first line of defense against infections. This system utilizes a limited number of receptors encoded in the germline, called pattern recognition receptors (PRRs), which detect invariant molecular patterns common to certain class of microbes [133]. These microbial components are denominated pathogen-associated molecular patterns (PAMPs). They are structures unique to microorganisms and often essential for their survival, such as double-stranded viral RNA, important for the replication of certain viruses, and lipopolysaccharide (LPS), a structural component of gram-negative bacteria cell walls.

3.2.1. Pattern Recognition Receptors (PRRs)

The pattern recognition receptors can be classified in three different classes: secreted, transmembrane and cytosolic [133].

Secreted PRRs include collectins, ficolins, and pentraxins, which act via binding to microbial cell surface and activation of the complement system and/or opsonization of pathogens for phagocytosis [134].

The transmembrane PRRs include the Toll-like receptors (TLRs) and the C-type lectins. The TLRs are a group of 13 paralogs expressed on the plasma membrane, endoplasmic reticulum and endosomal compartments of mammalian cells (Figure 3). TLRs 1, 2, 4, 5, 6 and

11 are located on the cell surface and detect peptidoglycans, lipoproteins and lipid from bacteria, protozoa and fungi, while TLRs 3, 7, 8 and 9 are mostly intracellular and detect viral and bacterial nucleic acids [135]. The expression of TLRs is cell-type specific, allowing different roles for cells involved in the immune response. The second class of transmembrane PRRs, C-type lectin, has the ability to recognize specific pathogen-associated carbohydrate structures, as β-glucans and mannan present on the fungal cell walls [136].

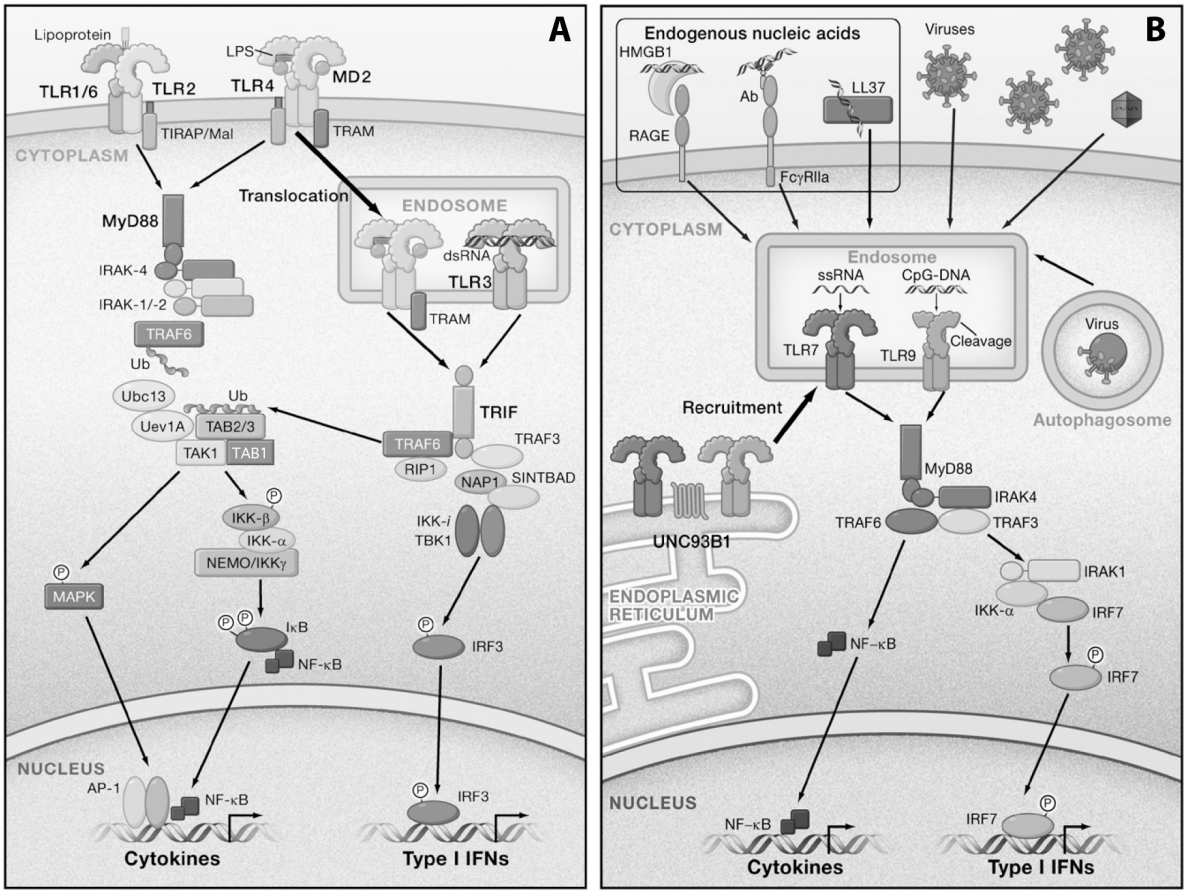

Figure 3. TLRs and signaling pathways. A: TLR1/6, 2, and 4 are localized in the plasma membrane; upon biding to their respective ligands the adaptors MyD88 and TIRAP are recruited and make a complex with TRAF6. The E3 ubiquitin ligase activity of TRAF6 promotes the assemblage of the complex with TAK1, TAB1, and TAB2/3, activating the transcription factors NF-κB, and AP-1. These induce the expression of several downstream genes, such as CCL2, CCL5, CXCL10, and NOS2. TLR3 recognizes different PAMPs and uses TRIF instead of the adaptor MyD88. TLR4, via the molecule TRAM, can also recruit TRIF-dependent pathways. TRIF, by activating TRAF6/RIP and TRAF3, induces several

cytokines/chemokines and type 1 interferons, via NF-κB and IRFs, respectively. B: TLR7 and 9 utilize a complex of MyD88, IRAK-1/4, TRAF6, TRAF3, IKKs, and IRF7 to promote the expression of cytokines/chemokines and type 1 interferons. (modified from [137])

The cytosolic PRRs include the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs). The RLR family has three members: retinoic acid-inducible gene-I (RIG-I), melanoma differentiation associated gene-5 (*MDA5*) and laboratory of genetics and physiology 2 (LGP2) (Figure 4). These proteins share a central DExD/H-box RNA helicase domain that senses viral RNA within the infected cell [138]. RIG-I and MDA5 also have two N-terminal caspase activation and recruitment domains (CARD). CARDs allow for the interaction with the adaptor protein mitochondrial antiviral signaling (MAVS, or IPS-1) and promote activation of several transcription factors, including interferon regulatory factor 3 (IRF3) and 7 (IRF7), nuclear factor κB (NF-κB) and components of the activator protein 1 (AP-1) family. LGP2 lacks the CARDs domain and may have a primary regulatory role on RIG-I and MDA5 activity [139, 140]. Animal models of *RIG-I* and *MDA5* knockout demonstrated that they have distinct virus specificities. RIG-I is important for mounting an immune response to many single-stranded RNA viruses (influenza A, measles, mumps, and Sendai virus) and positive-stranded viruses (hepatitis C and Japanese encephalitis virus) [141], while MDA5 is crucial for protection against other set of viruses, including poliovirus, encephalomyocarditis, and vaccinia virus [141, 142]. Interestingly, these helicases can also respond to viral DNA that was transcribed into uncapped RNA by the cytosolic RNA polymerase III [143, 144]. A new cytosolic antiviral pattern has been recently described: known as IFN-stimulatory B-DNA response it also senses intracellular foreign nucleic acids, but in this case independently from RIG-I [145].

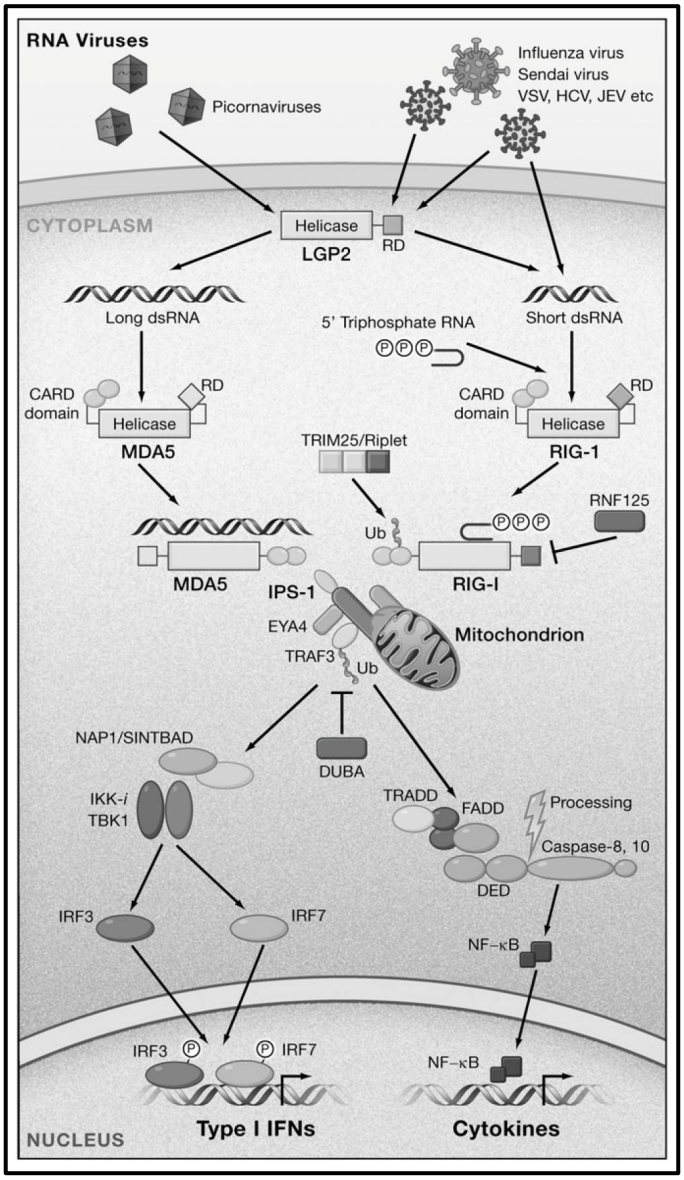

Figure 4. RNA viruses recognition by RLRs. The helicase LGP2 modifies viral RNA facilitating the recognition by MDA5 and RIG-I. These RLRs recognize RNA from different viruses and are negatively regulated by specific proteins, but use similar signaling pathways. Both MDA5 and RIG-I, interact with the mitochondrial protein IPS-1 activating a cascade of molecules that lead to production of proinflammatory cytokines/chemokines and type 1 interferons via activation of transcription factors IRF3, IRF7 and NF-κB. (modified from [137])

NLRs, the second group of cytoplasmic PRRs, mainly recognizes bacterial products present in the intracellular compartment, but can also be activated by some endogenous stress stimuli, namely uric acid crystals, reactive oxygen species and changes in intracellular ion

concentration [146] (Figure 5). These PRRs promote a proinflammatory environment by activation of the transcription factors NF-κB and AP-1, but also of caspase 1, involved in the processing of pro-IL-1β to IL-1β.

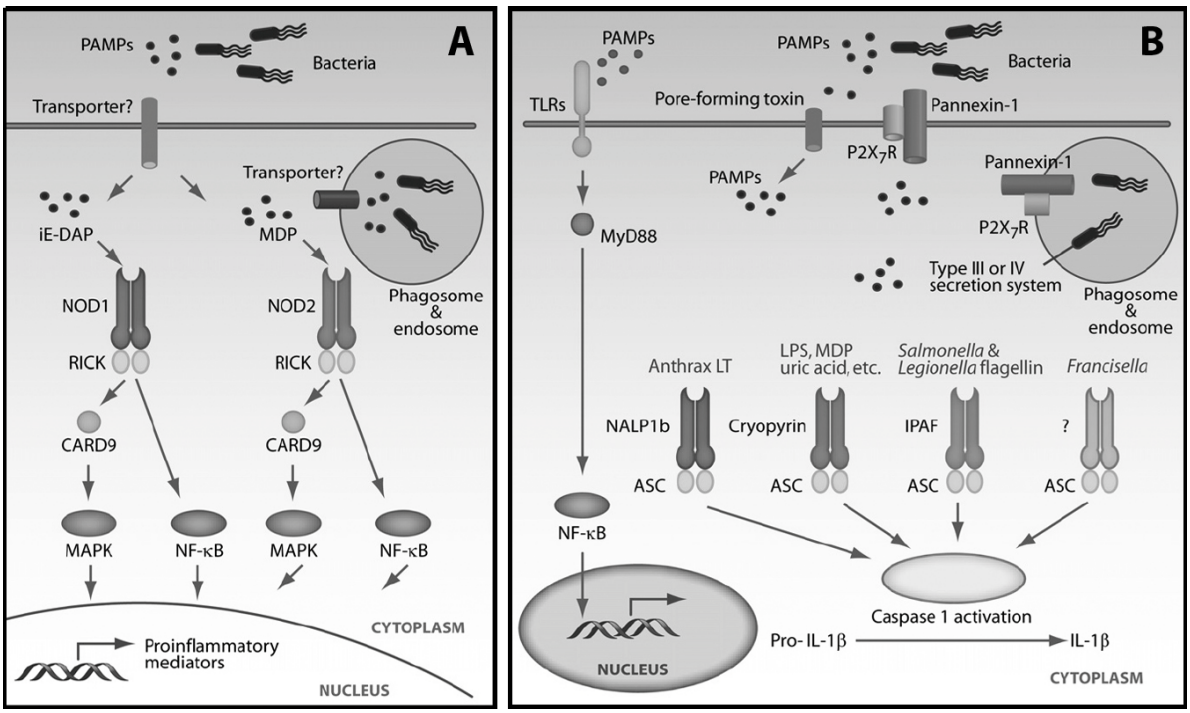

Figure 5. Recognition of PAMPs by NLRs and the signaling pathways activated. A: The NOD1 and NOD2 receptors can recognize the peptideoglicans-derived iE-DAP and MDP, respectively. These receptors, upon recruitment of the adaptor proteins RICK and CARD9, activate several proinflammatory mediators via NF-κB and AP-1. B: Other NLRs (NALP1b, cryopyrin/Nalp3, IPAF, etc) are activated by different bacterial PAMPs, and also endogenous stimulus (cryopyrin/Nalp3). These receptors activate the inflammatory caspase 1 by a complex with the protein ASC (apoptosis-associated speck-like protein containing a CARD). After TLR-dependent production of pro-IL-1β, the active caspase 1 process the immature pro-IL-1β in active IL-1β that is then secreted and trigger the local inflammatory response. (modified from [146]).

Discrimination between viral and self-nucleic acids is sometimes imperfect, which may lead to autoimmunity [147]. Activation of innate immune signaling pathways, including the PRRs, contributes for the triggering of several autoimmune diseases, such as lupus, rheumatoid arthritis and autoimmune hepatitis [147, 148]. There is also evidence that TLRs

and other components of the innate immunity response contribute for triggering insulitis and diabetes in animal models [149, 150]. To prevent these autoimmune responses, mammalian cells have evolved several mechanisms of control. These include increasing the discriminatory power of endogenous PRRs pathways [151] and the development of negative regulators of these pathways, for instance via 3' repair exonuclease 1 (Trex1) which metabolizes reverse transcribed DNA and thus prevent the IFN-stimulatory DNA response [152].

3.2.2. The role for PRRs in insulitis

Pancreatic islets of mice express the TLRs 2, 3, 4 and 9, with TLRs 2, 3 and 4 showing the highest level of expression in human and mouse islets [153, 154]. Confocal microscopic analysis confirmed a marked expression of TLR3 and TLR4 in both mouse and human beta cells [155]. TLR3 expression is up-regulated in beta cells exposed to double stranded RNA (dsRNA) [154, 156], a by-product of viral replication [157]. Human islets infected with Coxsackievirus B5 or exposed to the antiviral cytokine interferon-α, or a combination of interferon-γ + IL-1β, have increased expression of the transmembrane PRR TLR3 and of the cytosolic PRRs RIG-I and MDA5 [158, 159]. Intracellular dsRNA, from virus-infected cells, and extracellular dsRNA originated from damaged or dying cells following viral infection, can trigger beta cell apoptosis and cytokine/chemokine production after binding to TLR3. This is mediated at least in part through induction of the transcription factors NF-κB and IRF‑3 [156, 160-162]. Whereas extracellular dsRNA acts entirely via TLR3 in beta cells, intracellular dsRNA uses different pathways, which include the cytosolic PRRs RIG-I/MDA5 (Figure 4) [156] (and Article II).

Since each PRR only recognizes specific classes of microorganisms, different microbial components were used to evaluate their potential role as triggers of diabetes [154]. Among lipopolysaccharide, peptideoglycan and dsRNA, only dsRNA was able to cause beta

cell apoptosis and development of diabetes in the C57BL/6-rat insulin promoter-B7.1 mice, which do not normally develop the disease [154]. By adding immunization with the whole insulin protein, there was a significant increase in diabetes incidence, especially when immunization was initiated after dsRNA exposure. This suggests that dsRNA primes the mice to develop diabetes, probably by inducing beta cell apoptosis and exposure of autoantigens. These autoantigens can be then presented to the immune system. Of interest, the antigen-presenting capacity of the immune cells is also significantly induced by dsRNA [154]. Another study indicated that dsRNA, combined with insulin peptide B9-23 immunization in mice with RIP-B7.1 expressed on an $H2^d$ genetic background, induces the production of insulin autoantibodies and diabetes [163], reinforcing the importance of dsRNA and PRRs as inducers of autoimmunity.

Intracellular dsRNA promotes production of the antiviral type I interferons which, when prolonged or excessive, lead to beta cell apoptosis by mechanisms that may involve endoplasmic reticulum (ER) stress [162]. Of note, significant amounts of type 1 interferons were detected in the pancreas and in the islets of patients with T1D [164, 165], and IFN-α was involved in triggering hyperglycemia in experimental models of viral-induced diabetes [166, 167]. These findings indicate that activation of the PRRs TLR3 and/or MDA5/RIG-I in pancreatic beta cells promote several cellular responses that initiate by the activation of the key transcription factors NF-κB and IRF-3. This initial response is followed by type I interferon production which, in a paracrine way, promotes activation of the transcription factor STAT-1, overexpression of MHC class I molecules, further production of IFN-α/β and of several chemokines [156, 158, 160, 162, 168] (see below). The net result is the local attraction of immune cells that secrete pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ (Figure 6). This local inflammation, associated to cellular antiviral defenses, should in most cases eradicate the viral infection. However, in genetically susceptible individuals these

cellular responses aiming to eliminate the invading virus may produce unwanted results, leading to chronic inflammation and protracted beta cell loss (see below). How and why this takes place is a critical question that remains to be answered.

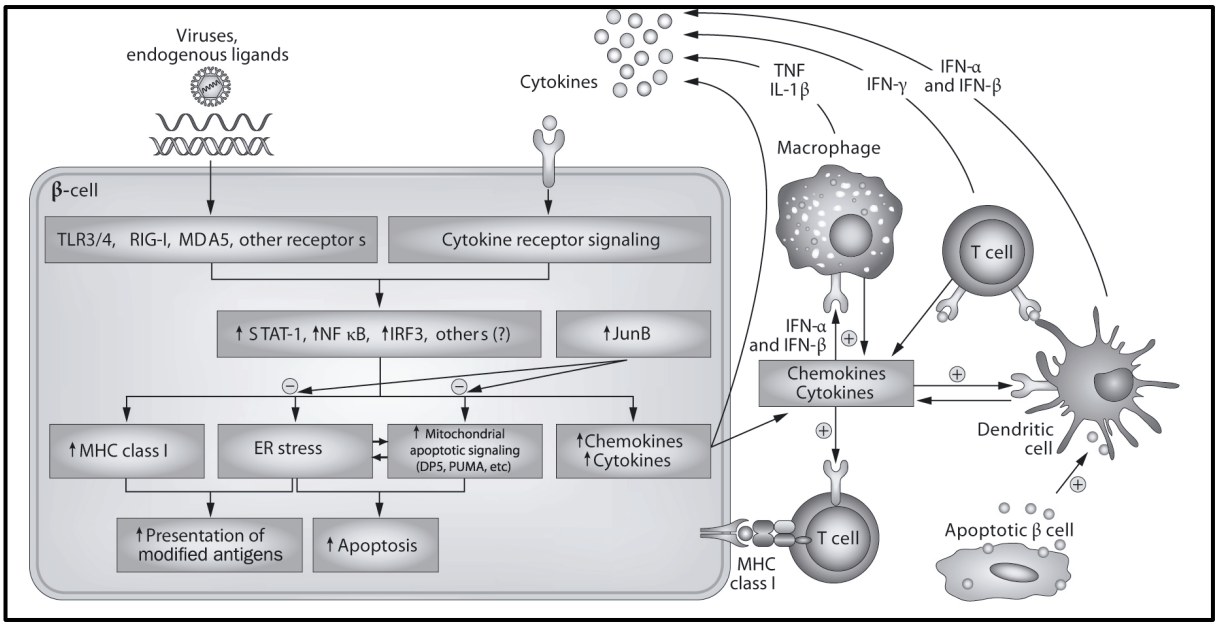

Figure 6. Cross-talk between pancreatic beta cells and the immune system during the development of T1D. Exogenous or endogenous ligands are recognized by the PRRs present in the beta cells, which lead to activation of key transcription factors. These transcription factors can trigger immune responses by promoting expression/secretion of several cytokines and chemokines, and attraction of cells from the immune system. The downstream signaling pathways also promote ER stress. ER stress, associated with an increased expression of MHC class I, might generate the presentation of modified autoantigens to cytotoxic T cells and consequently increase beta cell death. Of note, products of beta cell apoptosis caused by the original ligand or by cytotoxic T cells can be phagocysed by antigen-presenting cells. The infiltrating immune cells secrete additional proinflammatory cytokines (for instance, IL-1β, TNF and interferons) maintaining the vicious circle, which, if not controlled, will lead to persistent loss of beta cell and eventually diabetes. Actual beta cell death depends on both ER stress and activation of specific members of the Bcl2 family, such as DP5 [169] and PUMA [170]. To prevent this, pancreatic beta cells express negative regulators of these responses, such as the transcription factor JunB and the tyrosine phosphatase PTPN2, further discussed in Article I and II. (modified from [171]).

The present model considers exogenous ligands to the PRRs, in this case a virus, as

the initial trigger causing activation of the innate immune system in diabetes. This is supported by several studies demonstrating that viral infections, especially enteroviruses, play a role in the aetiology of T1D [172-174]. Some enterovirus strains, especially Coxsackievirus B4, have specific beta cell tropism and infection by these agents was detected in beta cells of patients with recent-onset T1D [175]. Another potential mechanism by which viruses might cause T1D is by molecular mimicry. In this case, viral antigens are supposed to share highly similar epitopes with autoantigens, leading the T cell receptor to fail in correctly differentiating between these molecules. These T cells are initially activated by both viral antigens and autoantigens and trigger an acute inflammatory response. Once the viral infection is solved, the persistence of the autoantigen might lead to a chronic autoimmune response. In fact, and as discussed below, molecular mimicry is probably more relevant during the amplification of an ongoing autoimmune process against beta cells [173].

Another possible mechanism of disease, which does not exclude viral infection as pathogenic agent in some individuals, is that endogenous ligands bind to PRRs. This may promote the inflammatory process [147], as already suggested for TLR4 and TLR9 in animal models of arthritis [176] and multiple sclerosis [177], respectively. Supporting this theory, apoptotic mouse beta cells undergoing secondary necrosis are able to trigger T cell immunity through a pathway that involves TLR2-MyD88-NF-κB. Moreover, autoimmune diabetes in two mouse models (multiple-low-dose streptozotocin and NOD) is significantly inhibited by TLR2 deletion, an effect secondary to impaired priming of diabetogenic T cells by antigen-presenting cells following beta cell injury [178]. This suggests that beta cell death, and its sensing by TLR2, is a putative initial event for the development of T1D. Polymorphisms in TLR2 were also significantly associated with asthma and T1D development in humans [179]. Moreover, islets isolated from TLR4-/- mice (C57BL/10ScNJ background), or islets infected with a virus encoding a dominant negative form of TLR4, are protected against allograft

rejection when transplanted in BALB/c recipients [180]. A study in T1D patients also evidenced an increase in TLR2 and 4 expression at the cell surface of peripheral monocytes compared with controls. The activity of several pathways downstream of these TLRs (NF-κB, IRAK1, MyD88, and TRIF) was also increased in T1D monocytes, suggesting that an overactivation of these pathways contributes to a proinflammatory state [181]. A recent experimental study, however, challenged the central role for TLRs 2 and 4 in diabetes. This study demonstrated that NOD mice deleted for TLRs 2 and 4 have the same prevalence of diabetes as wild type mice [180]. It was suggested instead that MyD88 modulates the normal intestinal microbiota and, by this indirect effect, regulates the progression of autoimmune diabetes in NOD mice [182].

Further studies are required to solve this question, and to determine the mechanisms by which changes in gut microbiota modulate the immune responses of NOD mice. There may be multiple pathways and diverse forms of human T1D from an immunological perspective [57, 171]. Inbred NOD mice and BB rats (another diabetes-prone animal strain) probably "freeze" one of these pathways, and it may be thus particularly difficult to extrapolate findings regarding the interaction between environment/innate immune response from these models to the heterogeneous human disease.

Polymorphisms of the cytosolic NLRs were previously associated with other inflammatory diseases, such as Crohn disease [183] and asthma [184], but their potential role in the pathogenesis of T1D remains to be determined. These receptors detect intracytoplasmic bacterial PAMPs and induce inflammation [146]. NLRs are highly expressed in the intestine, and NOD1 is important for the communication between intestinal bacteria and the immune system [185]. Therefore, NLRs are interesting candidates to study in the context of the interaction between the gut microenvironment and the immune response in NOD mice. Another possible explanation is that alterations in the gut microbiota, characterized by a

decrease in the "healthy" bacteria from the phylums *Bifidobacterium* and *Bacteriodes*, render animals more susceptible to immune diseases, such as colitis induced by dextran sulphate sodium (DSS) [186]. This could be explained by the fact that these bacteria metabolize dietary fibers and produce short-chain fatty acids (SCFA), as acetate and butyrate, which downregulate the immune system via binding to the receptor GPR43 [185]. In this case, changes in the gut microenvironment would lead to decreased expression of SCFA and prolonged activation of the immune system in susceptible individuals.

Recently, a genome-wide association study identified polymorphisms in *MDA5* in patients with T1D, and suggested that it is a candidate gene for T1D [187]. Deep sequencing of the *MDA5* region in the same database identified four rare polymorphisms that independently confer a significant protection against T1D [188]. This is probably due to an alteration in the structure of the MDA5 protein [188]. *In vitro* studies with fibroblasts double knockout for *RIG-I/MDA5* and transfected with plasmids expressing these polymorphisms confirmed that these cells express less type I interferon after dsRNA exposure [189]. Our own observations indicate that *MDA5*, but not *RIG-I*, knockdown in pancreatic beta cells significantly decreases the expression of several cytokines and chemokines (e.g. interferon beta, interleukin 15, CCL2, CCL5, CXCL10) after exposure to intracellular dsRNA (Article II). *MDA5* knockdown, however, does not prevent dsRNA-induced beta cell apoptosis (Article II).

3.2.3. Amplification of islet inflammation and the transition from innate to adaptive immunity

Important events, which probably take place in the early stages of insulitis, determine whether the above described inflammatory response will progress or not to a full adaptive immune response, with the potential to cause a prolonged autoimmune reaction [171]. The importance of adaptive immunity and T cells in T1D development is well recognized [56,

190, 191]. For instance, the removal or complete absence of mature T cells in rodent models prevents diabetes [192, 193], and a temporary preservation of beta cell function was reported in recent-onset T1D patients treated with anti-CD3 [194-196]. Adoptive transfer of T cells alone from RT6-depleted DR-BB rats to athymic WAG nude rats causes the development of diabetes [197]. Other two components of insulitis that may tip the balance in one or the other direction, i.e. amplification or resolution, are the "dialogue" between beta cells and the immune system via local production of chemokines/cytokines and the putative role for ER stress and other mediators of beta cell death on antigen presentation (see below, section 3.2.4).

3.2.4. The "dialogue" between beta cells and the immune system

Chemokines are molecules that promote leukocyte migration and activation during the early innate immune response, thus helping in the transition to an adaptive immunity [198]. Their specificity and complexity derives from the fact that they are released in diverse patterns in distinct inflammatory reactions ("chemokine signature"); there is also a regulation of their receptors in leukocytes, which vary in different immune responses [199-201]. The expression of chemokines and their receptors is altered in several autoimmune diseases, and this is also the case in T1D. Thus, increased levels of the Th-1-associated chemokines CCL3, CCL4 and CXCL10 were observed in the serum of recent-onset T1D patients [202-204] and a prospective study with 256 newly diagnosed T1D patients identified an inverse correlation between CCL3 and C-peptide [205]. There is, however, a major superposition between patients and controls. This reflects the difficulties in understanding proinflammatory mechanisms in early T1D by studies in the serum or peripheral cells using traditional approaches. Indeed, most of the "action" in early T1D takes place in the islet microenvironment and pancreatic draining-lymph nodes. These tissues are not easily accessible in humans and locally generated inflammatory signals are diluted in the general

circulation. In order to avoid these difficulties, a recent study used microarray analysis of peripheral blood mononuclear cells exposed to sera from T1D patients at different moments of the disease (recent onset and long-standing), healthy controls or at risk siblings of diabetic patients to identify circulating proinflammatory factors [206]. The serum from recent-onset T1D patients induced a specific signature, which included among others IL-1 family members and the chemokines CCL2 and CCL7 (both involved in monocyte/macrophage chemotaxis). This "proinflammatory signature" was demonstrated years before clinical disease in a small group of patients [206]. This interesting approach needs now to be confirmed in larger prospective studies.

Evaluation of pancreatic tissues obtained from recent-onset T1D patients confirmed CXCL10 expression by beta cells and its ligand, CXCR3, by the pancreatic-infiltrating lymphocytes, while they were not detected in pancreatic sections from non-diabetic organ donors [207]. Similar results were observed in another study in pancreas of patients with fulminant type 1 diabetes, a form of T1D probably caused by acute viral infection of the pancreas. Immunohistochemical analysis of these samples showed an extensive infiltration by CXCR3 receptor-bearing T cells and macrophages in the islets. CXCL10 was expressed in all the endocrine cells from the islets, but not in the exocrine pancreas, consistent with the less selective destruction of islet cells observed in this subtype of T1D [3].

Work in animal models show that islets from diabetes-prone NOD mice have increased levels of CXCL10, CCL2, CCL20 and IL-15 mRNAs and of CXCL10, CCL2 and several other chemokine proteins during the pre-diabetic period [199, 208-211]. Macrophages are the first cells to infiltrate the islets of NOD mice and BB rats, and depletion or inactivation of these cells prevent diabetes [212]. CXCL10 and CCL2 attract macrophages, and the early expression of CXCL10 and CCL2 in NOD islet cells probably contributes to the recruitment of macrophages during the early stages of insulitis. Transgenic expression of CCL2 in beta

cells leads to insulitis [213] and diabetes [211], and high basal CCL2 production by human islets correlates with a poor clinical outcome following islet transplantation in patients with T1D [214]. Regarding the adaptive immune response, diabetogenic CD4+ Th1 cells in NOD mice express CCR5 receptor and the CCR5 ligands CCL5 and CCL3, the CXCR3 ligand CXCL10, as well as XCL1, CCL2, CCL7 and CCL12 [215, 216]. CCL3 NOD knockout mice have reduced insulitis and are protected from diabetes [216], while deletion of the CCR5 receptor leads to a switch from a Th1 to a Th2 response, delaying islet allograft destruction in mice [217].

Pancreatic beta cells are an important source of chemokine production during insulitis. Thus, FACS-purified rat beta cells exposed to IL-1β + IFN-γ, or to dsRNA, have increased expression of mRNAs encoding for several cytokines and chemokines, such as CCL2, CXCL10, CCL20, CX3CL1 and IL-15 [161, 208, 218, 219]. Human islets treated with a combination of IL-1β plus IFN-γ significantly increase mRNA expression of IL-15, CXCL10, CCL2, CCL20 and CX3CL1 and secrete IL-15, CCL2, CXCL10, CXCL9, CXCL11 and CCL20 into the culture medium [208, 220]. The expression of chemokines by beta cells during inflammation is mainly regulated by the transcription factors NF-κB [221, 222] and STAT-1 [223, 224]. Of note, these transcription factors are also important mediators of cytokine-induced beta cell death [225] (see below).

The data discussed above suggest the presence of a "dialogue" between monocytes, dendritic cells and T cells and the target beta cells during the development of insulitis [171, 200] where activated immune cells produce cytokines such as IFN-γ, IL-1β and TNF-α, which stimulate beta cells to release chemokines and cytokines (Figure 6). This will attract more mononuclear cells that also release additional chemokines [200, 210]. If this vicious circle is not interrupted, there will be a progressive accumulation of activated macrophages and autoreactive T cells around and inside the islets. These observations indicate that a better

understanding of activation of chemokines and their receptors, or the transcription factors regulating them, may provide relevant information for the development of novel therapeutic approaches to prevent T1D.

3.2.5. The putative role for ER stress on beta cell death and antigen presentation

Apoptosis is probably the main form of beta cell death in autoimmune diabetes [212, 226]. As already discussed, transcription factors such as NF-κB and STAT-1 appear to regulate both chemokine production and the triggering of beta cell apoptosis [171, 225]. Thus, beta cell death will occur in the context of an intense inflammation in the islet microenvironment. After the death of beta cells, some of their components may be perceived as danger signals by the immune system, and antigens released from beta cells under attack - specially in the presence of inflammatory factors - may be taken by antigen-presenting cells (APC) in the pancreatic lymph node stimulating an autoimmune response [227] (Figure 6). In agreement with this hypothesis, caspase-3 knockout mice (caspase-3 is the major effector caspase downstream of the apoptotic pathway) are protected against diabetes induced by multiple-low-dose streptozotocin, suggesting that beta cell apoptosis is a necessary step for T cell priming [228]. Furthermore, apoptotic beta cells undergoing secondary necrosis can promote the production of TNF by macrophages and autoreactive T cells [178]. This "immune-enhancing" role of dying beta cells is most probably context-dependent. Thus, injection of low doses of streptozotocin in young NOD mice, at the pre-insulitis stage, decreases diabetes prevalence suggesting that the presence of low number of apoptotic beta cells in a non-inflammatory environment leads to tolerance instead of disease [227].

Inflammatory cytokines, metabolic stress or dsRNA induce ER stress in pancreatic beta cells, leading to accumulation of misfolded proteins in the ER and triggering of an unfolded protein response [162, 229, 230]. Prolonged or severe ER stress will eventually trigger beta cell apoptosis, both via Chop and caspase 12 activation [230], and via a cross-talk with the

mitochondrial pathways of cell death, including activation of the BH3-only proteins DP5 [169] and PUMA [170]. Dying cells can transfer information to APCs, signaling the cause of cell death and determining the immunological outcome of phagocytosis [231]. Peptides present inside of the ER from dying cells can make complexes with class I MHC molecules in dendritic cells without further cytosolic processing [232]. Thus, dying cells may signalize to APC about what happened just before death [231]. This may be beneficial in the context of a viral infection triggering apoptosis, but it may have negative consequences for the insulin-producing beta cells. As discussed above, insulin is a key antigen for autoimmune diabetes in both humans and NOD mice [71, 233], and it represents half of the total protein production by beta cells. Insulin accumulates in the ER during ER stress, partially in a misfolded configuration, which may augment it's antigenicity [230]. This may increase presentation of pro-insulin and insulin to APCs, especially in the context of inflammation [171]. Furthermore, misfolded proteins can be preferentially directed to proteossomal degradation, generating peptides that serve as ligands to class I MHC molecules [59] and increase the possibility of inadequate presentation of insulin to the immune system.

3.2.6. Maintenance or resolution of islet inflammation

The final stages of the natural history of beta cell loss are regulated by the adaptive immune response. This is characterized by stabilization and maintenance of insulitis and, in some cases, resolution. Inflammatory mediators continue to play a key role in this advanced stage of the disease [171].

Observations in NOD mice suggest that proinflammatory mediators contribute for both beta cell functional impairment and peripheral insulin resistance. Thus, NOD mice have a progressive glucose intolerance that follows the evolution of insulitis [234]. Islets isolated from these animals before hyperglycemia have impaired glucose metabolism and insulin release, but their function is restored after one week in culture [234] or when the mice are

pre-treated *in vivo* with monoclonal antibodies targeting the effector T cells [235]. Similar results were observed in islets isolated from one patient with recent-onset T1D [236]. This indicates that inflammation caused by infiltrating mononuclear cells induce a reversible impairment of beta cell function preceding beta cell destruction. This functional alteration is aggravated, at least in NOD mice, by the presence of inflammation-induced insulin resistance [237].

The inflammatory response may also affect beta cell proliferation. This was suggested by observations that beta cells from RIP-IFN-γ transgenic mice [238] and NOD mice in the pre-diabetic period [239, 240] have high rates of beta cell proliferation. Importantly, beta cell proliferation was also observed in humans with pancreatic inflammation secondary to prolonged life support [241]. Beta cell proliferation observed in mice is inhibited by agents that target the immune system, such as anti-CD3 antibodies [242], suggesting that inflammatory mediators induce beta cell proliferation during insulitis. On the other hand, *in vitro* evidence suggest that cytokines can "push back" newly developed beta cells into a de-differentiated state by both inducing re-expression of the Notch-delta pathway [243] and/or by inhibiting expression of relevant genes for the differentiated phenotype (*PDX1*, *ISL1*) and function of beta cells (*GLP-1* and *GH* receptors) [218, 219, 244]. Another beneficial role for inflammatory components was described in a model of pancreatitis: macrophages infiltrating the pancreas promoted islet angiogenesis and proliferation, helping to preserve beta cell mass [245]. Type 2 diabetes patients may also have a mild increase of islet-associated macrophages [246], and local expression of cytokines/chemokines [247], but this takes place in the absence of an autoimmune assault [226]. It is thus an intriguing possibility that in type 2 diabetes macrophages, rather than contributing to beta cell death as in T1D, may actually support beta cell survival. Further research is required to answer this question, and to determine which components of inflammation stimulate or prevent beta cell regeneration and survival.

It is probable that many individuals that develop mild insulitis are able to resolve the inflammation, and restore normal beta cell function. This possibility is supported by observations that: 1) some autoantibody positive individuals already presenting impaired beta cell function appear to recover beta cell function when followed prospectively [248]; 2) most islet autoantibody positive non-diabetic individuals do not show histological signals of insulitis [249]. Future additional information about mediators of insulitis resolution can help to identify subjects more susceptible to progress to T1D; in this case, treatment could be started earlier, and these individuals should be allocated preferentially to preventive studies.

4. Genetic susceptibility

As discussed above, T1D is chronic autoimmune disease in which both genetic and environmental factors contribute to the pathogenesis. Some rare forms of T1D have a monogenic heritage [250, 251], but most cases of T1D result from alterations in multiple genes and their interaction with environmental factors [252]. Studies in monozygotic twins reinforce the role of genetics factors by demonstrating concordance of T1D diagnosis between 30 to 68% [253-255]. The highest value was observed when twins were followed for nearly the whole life period [255]. To identify the genes involved in T1D development, two different approaches have been used: linkage studies (which evaluate pairs of relatives that are affect by T1D), and association studies (that can have case-control, cohort- or family-based design). Linkage studies usually evaluate large regions in the genome and compare how frequent are they shared between twins with T1D or individuals with high-risk markers of autoimmunity. Since these regions are broad, linkage studies can identify areas in the genome containing several genes, which makes it difficult to know the real causal gene. They are especially

useful in identifying genes with large effects, but with a relatively low frequency in the population (Figure 7).

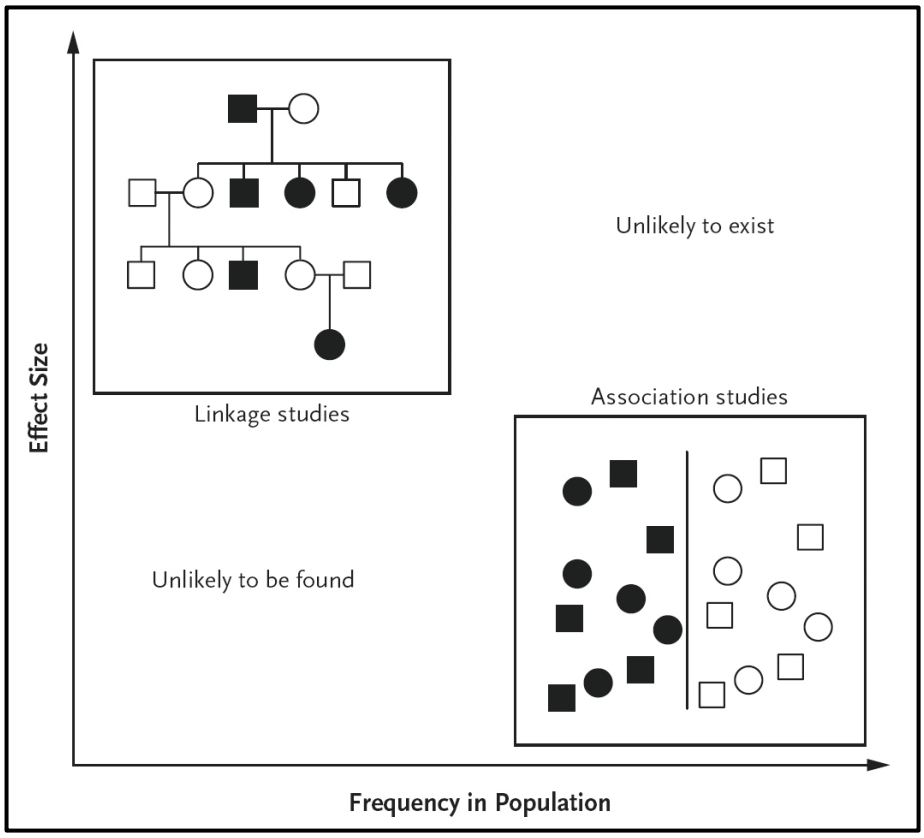

Figure 7. Different characteristics of the studies used to evaluate candidate genes for T1D. In complex disorders, such as T1D, genes with large effects on disease risk present a low frequency in populations. These genes are usually identified by linkage studies. On the other hand, genes with smaller effects occur more frequently in populations and are identified using association studies. (modified from [256])

The best available approach to identify genes with smaller effects are the association studies. These studies observe the nonrandomic occurrence of a genetic marker with a certain disease. They use high-throughput single-nucleotide polymorphisms (SNP) genotyping platforms and give relative dense information of the human genome. These new studies increased significantly the number of candidate genes related to T1D as illustrated in Figure 8. Despite the increase in the number of genes identified, the respective effect size of these genes is small when compared with the genes identified by the earlier linkage studies, ORs in

the range of respectively 1.2 - 2.0 vs. 2.5 - 6. The most important information provided by these new studies, however, is the demonstration that several signaling pathways present both in the immune system and in the target beta cells (for pathway information see: www.t1dbase.org) need to be compromised during the development of T1D, and further studies are needed to clarify these pathways.

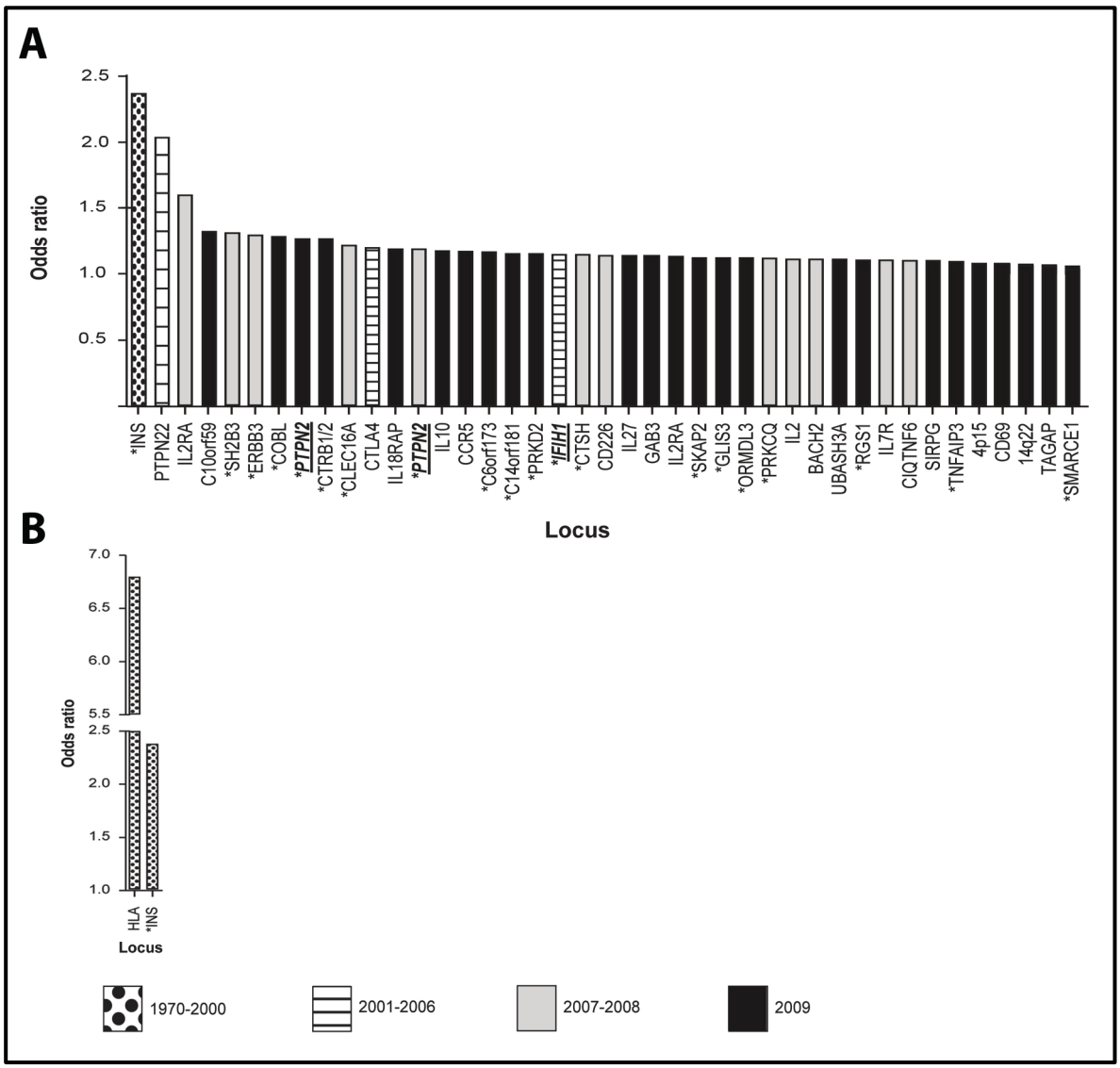

Figure 8. Estimated association (OR: odds ratio) and chronological description of candidate genes related to T1D identified by genome wide association (A, B) and linkage studies (B) (modified from [252]).

4.1. MHC genes

T cells, different from B cells which recognize soluble and cell-associated antigens, can only recognize antigens presented by other cells. The specificity of cell-associated antigen recognition is provided by the way they are presented by proteins encoded in the major histocompatibility complex (MHC). There are two main types of molecules encoded by the MHC region: 1) class I MHC molecules, which are expressed in all the nucleated cells and present antigens to CD8+ T cells, and 2) class II MHC molecules, which are expressed mainly in cells involved in antigen presentation, such as macrophages, dendritic cells, B cells, endothelial cells, and thymic epithelial cells, and present antigens to CD4+ T cells. These classes of MHC are highly polymorphic and allow the presentation of a significant number of antigens to T cells.

The MHC comprises a large region in the genome that, in addition to class I and II MHC molecules, encodes several genes involved in antigen processing. In humans the MHC is called human leukocyte antigens (HLA). [257]. MHC class I and II have similar structures with an extracellular peptide-biding cleft, a non-polymorphic Ig-like region, a transmembrane region, and a cytoplasmic region. The extracellular peptide-binding region includes polymorphic areas involved in antigen-specific binding (called pockets) and other polymorphic regions allowing recognition by T cells (Figure 9). The Ig-like region is where CD4 or CD8 co-receptors, respectively, bind to class II and I MHC molecules [258].

The importance of class II HLA in the pathogenesis of T1D has been known for more than 20 years [259]. Autoreactive T cells can recognize complexes formed of autoantigens plus HLA molecules and be positively or negatively regulated conferring, respectively, risk or protection against T1D development [260]. Each HLA molecule binds to some specific epitopes, and T cell responses against several autoantigens were observed in animal models and patients with T1D (see section 3.1.1). The class II HLA has the highest odds ratio (OR)

for T1D risk among all the genetic factors described until now (Figure 8). There is a strong correlation with class II HLA *DRB1*04* and *DQB1*0302* (also referred to as DR4-DQ8) in patients developing T1D during childhood, and presenting autoantibodies against insulin or proinsulin [62]. These HLA molecules can make complexes with an immunodominant peptide of insulin [261]. On the other hand, HLA allele *DQB1*0602* confers protection against T1D: the presence of this allele in a child with family history of T1D decreases to 0.1% the risk of disease, while another child in the same condition, except for the absence of this HLA allele, has a risk of 5% [54, 262].

Genotyping of HLA class II, associated with information of T1D family history, can be used as a predictive tool to determine the risk of developing T1D. This helps to select individuals for preventive studies or early interventions [54].

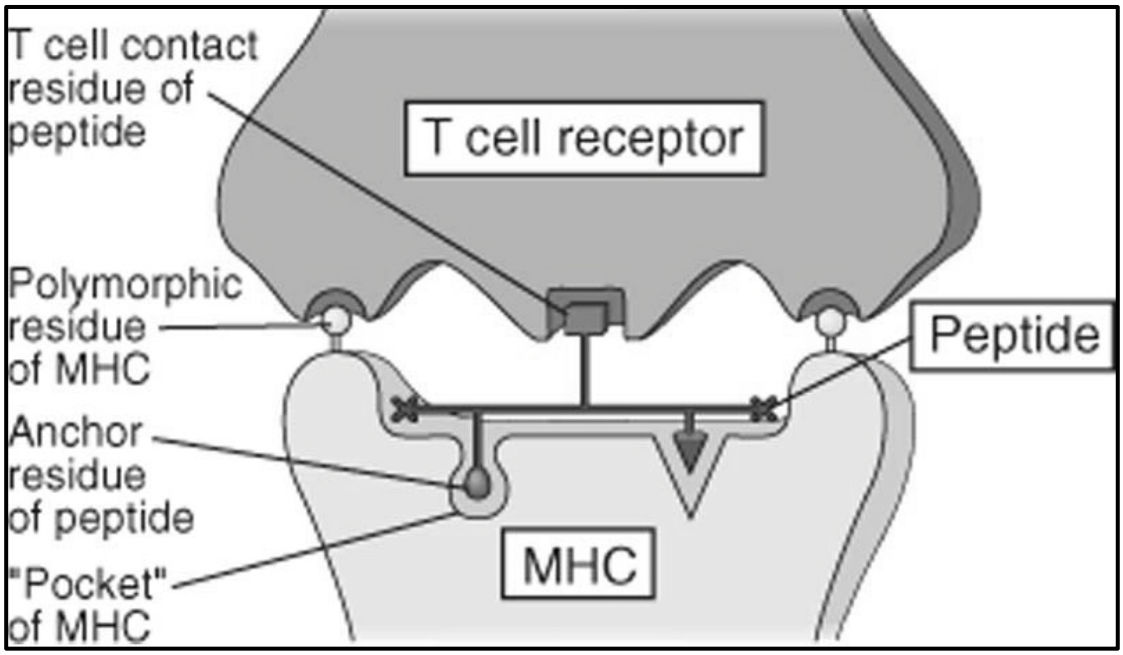

Figure 9. The recognition of MHC-peptide complex by T cells. In this model T cell receptor recognizes two polymorphic residues in the MHC and one in the peptide being presented. (modified from [257])

Class I HLA molecules are responsible for the presentation of autoantigens to CD8+ T cells. A recent study demonstrated that HLA-A2 efficiently present preproinsulin (PPI) to

cytotoxic CD8+ T cells [263]. These PPI-specific CD8+ T cells are able to kill human pancreatic beta cells, which may contribute for the release of additional autoantigens to the immune system. Further, hyperglycemia increased the presentation of PPI and the attack against beta cells [263]. This is in line with previous studies showing that exposure of human and rat beta cells to high glucose increases expression of the autoantigen GAD [264, 265]. Recently, two independent studies confirmed that class I HLA (HLA-A and HLA-B) is associated to T1D independently of class II HLA; as the former it can both confer risk or protection against T1D [266, 267]. The risk associated with class I HLAs, however, is lower as compared to class II limiting their use in predictive models.

4.2. Non-MHC genes

HLA genes are the candidate genes with the stronger association with T1D [256], but a significant decrease in the frequency of high-risk HLA in new-onset T1D patient has been observed in the last decade [268, 269]. This suggests that other genes and/or their interaction with environment factors might have a role in these patients. In agreement with this hypothesis, a recent study demonstrated that polymorphisms in the candidate gene *TCF7* are significantly related to T1D only in patients that do not have the high risk HLA-DR3/DR4 [270]. Computational analysis of the available genetic data suggest interactions between multiple genes and environmental factors in the pathogenesis of T1D, but these effects seems to be small and difficult to study [271]. In the search for these multiples genes, association studies have given important information. As shown in Figure 8, these studies increased the number of candidate genes from two, until 2000, to more than 50 in 2010.

Insulin gene *(INS)*: together with HLA, *INS* was one of the first T1D candidate genes to be identified [272]. The polymorphisms in *INS* are located in the variable number of tandem repeats (*VNTR*) locus. The *INS VNTR* class III is the protective genotype, and leads to increased expression of insulin mRNA and protein in the thymus in comparison with *INS*

VNTR class I, the genotype of risk for T1D [273, 274]. This decreased central expression of insulin probably enables some autoreactive T cells to escape from negative selection and enter the periphery. A recent study confirmed this theory by showing that 79% of subjects with *INS VNTR* class I have peripheral T cells reactive against a proinsulin tetramer, as compared with only 29% of the *INS VNTR* class III subjects [275].

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4): *CTLA4* encodes a co-stimulatory receptor that functions as a negative regulator of T cell activation [276, 277]. The variant leads to a decreased expression of a soluble form of *CTLA4* involved in the modulation of T regulatory cells [278]. Since T regulatory cells have a key role in peripheral tolerance, this explains why *CTLA4* polymorphisms are also related to several other immune diseases [277, 279].

Interleukin-2 receptor subunit-α (IL2RA): this gene encodes part of the IL2 receptor complex (also known as CD25) and is usually expressed on regulatory naïve T cells, memory T cells, and activated macrophages. CD25 controls T cell proliferation by an immunogenic stimulus. It was demonstrated that polymorphisms in *IL2RA* associated with increased risk of T1D induce less CD25 soluble in the serum [280], and decrease mRNA and surface expression of CD25 [281]. On the other hand, individuals with protective polymorphisms have more CD25 in memory T cells and secrete higher amount of IL2 after stimulus, which can modulate T regulatory cell function and T1D development [281]. *IL2RA* is also correlated with other autoimmune diseases in humans [282] and its ligand, interleukin 2 (IL2), with autoimmunity in mice [283].

Protein tyrosine phosphatase N22 (*PTPN22*): similar to *CTLA4* and *IL2RA*, variations in *PTPN22* have been associated to T1D and several other autoimmune diseases in humans [284-287]. *PTPN22* decreases the signaling of B and T cell receptors [288, 289] and also reduces T cell activation by interacting with the suppressor kinase C-terminal Src tyrosine

kinase [290]. Polymorphisms in *PTPN22* were initially thought to result in decreased function [291], but further studies demonstrated that it is in fact a gain-of-function [292]. This increase in *PTPN22* function leads to a reduced B and T cell activation, modified cytokine profile and probably reduced deletion of autoreactive B and T cells which favors the development of autoimmunity [288, 293].

Protein tyrosine phosphatase N2 (*PTPN2*): variations in a second protein tyrosine phosphatase, *PTPN2*, were recently associated with T1D [294, 295]. Initial studies suggested that *PTPN2* is mainly expressed in hematopoietic cells, but recent data from our group points to its expression and functional role in pancreatic beta cells (Articles I and II). *PTPN2* has two isoforms: the 45 kDa isoform, with mainly nuclear localization, and the 48 kDa isoform, located in the endoplasmic reticulum [296]. *PTPN2* was described as a regulator of several intracellular signaling pathways, including Janus kinases (JAKs), signal transducer and activator of transcription (STATs), extracellular signal–related kinase (ERK), epidermal growth factor receptor (EGFR), and insulin receptor β (IRβ) [297-300]. Mice knockout for *PTPN2* present a progressive systemic inflammatory disease [301] and deletions of *PTPN2* in humans are related to T cell acute lymphoblastic leukemia [302]. The known *PTPN2* polymorphisms associated with T1D are located upstream of the coding region or in introns, suggesting that either the real causal polymorphism remains to be identified or that noncoding variations can modify the expression of the spliced isoform 45 kDa [294].

Melanoma differentiation-associated gene 5 (*MDA5*): this gene encodes an helicase, also know as interferon induced with helicase C domain 1 (*IFIH1*), involved in the recognition of intracellular viral nucleic acids [141]. As discussed above (see section 3.2.2), fine-mapping of the *MDA5* region identified rare polymorphisms in this gene associated with protection against T1D [188], similar to observations made for *IL2RA* [281]. Since viruses are one of the putative environmental factors related to T1D [303], additional studies on the role of this gene

can provide important information on the mechanisms involved in T1D pathogenesis (Article II). Of note, this helicase recognizes Coxsakieviruses, the viruses most strongly associated with T1D [175].

Other candidate genes: as illustrated in Figure 8, several other candidate genes were recently identified and require additional studies to characterize their role in T1D. Interestingly, all of them, with the exception of *GLIS3* [304], are not associated with type 2 diabetes (T2D) confirming that these two forms of diabetes have different pathogenic mechanisms [226].

5. Environmental factors

As discussed above, T1D has an important genetic component. However, the concordance is far from 100% in monozygotic twins, indicating that environmental factors play a role in the development of T1D. Another evidence for environmental factors is the progressive increase in T1D incidence observed in the last decades, which is not compatible with the long time required for appearance of new genetic variants [7, 29]. Studies that evaluate the effects of migration [13] and the seasonality in the onset [40] also suggest environmental causes of T1D (see sections 1.2 and 1.4).

5.1. Perinatal factors

Intrautero maternal infections are potential causal agents for T1D development during childhood. This is suggested by the observation that around 20% of children born with congenital rubella later develop T1D [305]. The role for maternal enterovirus infection as a cause of T1D in children, however, remains unclear, with some studies demonstrating increased risk [306-308] but others not [309, 310]. Another perinatal factor associated with increased T1D risk is cesarean section delivery [35]. A possible explanation is that children born by cesarean section acquire the intestinal flora from the hospital instead from the mother

[311], and the gut microenvironment seems to be a potential regulator of immunity. High birth weight and increased weight gain during the first year of life are also associated to T1D risk [312]. Some authors suggested that the maternal transfer of autoantibodies could increase the risk for T1D [313]. Contrary to this idea, an elegant study demonstrated that children with autoantibodies at birth and without high-risk HLA genotype have in fact a lower risk of persistent autoantibodies compared to children born without autoantibodies [314]. Increasing maternal age [315], complicated delivery [316] and birth order [317] are also related to risk of islet autoimmunity and/or T1D.

5.2. Immunizations

The increase in T1D incidence in parallel with the widespread use of vaccination suggest a possible connection between these two events [9]. The recognition that vaccines can both induce or prevent autoimmunity in animal models [318, 319], and the possible role of infections in T1D [173] makes this an interesting hypothesis. Initial studies suggested that timing of vaccination could modify the risk of T1D development in children [320, 321]. In this case, vaccines given after two months of live potentially increased the risk of T1D [321]. On the other hand, several additional studies failed to identify a correlation between type of vaccine [322], timing of vaccination [323, 324], or specific genetic predisposition to vaccines [325] and development of T1D, indicating that either vaccination has no effect on diabetes or that it has a small effect, and not all studies have enough power to identify it. This last possibility seems unlikely taking into account the large number of cases evaluated in the negative studies [323, 325].

5.3. Components of the diet

Different diet components have been associated with increased risk of T1D for more than 20 years [326]. The most probable candidates for this correlation are: cow's milk, deficiency of vitamin D, cereals, omega-3 fatty acids, and nitrites.

Cow's milk: several components of the cow's milk, such as bovine serum albumin [327], beta-casein [328, 329], and beta-lactoglobulin [327, 330], have been implicated as potential triggers of autoimmune responses. An epidemiologic study from Finland indicated an increased risk of T1D related to the early introduction of cow's milk formula [327]. Other published studies confirmed this finding [331, 332], but others failed to support this correlation [333, 334]. A recent study evaluated the interaction between genetic predisposition (polymorphisms in the candidate gene *PTPN22*) and cow's milk introduction in the diet, potentially explaining these discrepancies. It was thus observed that the early introduction of cow's milk in the diet (< 6 months) only increases the risk of developing islet autoantibodies in children with specific polymorphisms in the gene *PTPN22* [335].

Vitamin D: besides their effects in calcium/phosphorus regulation, the active form of vitamin D, 1,25$(OH)_2$ vitamin D, has important immunomodulatory properties [336]. For instance, in NOD mice it promotes a more effective deletion of T cell in thymus and decreases the presence of apoptosis-resistant autoreactive T cells [337]. In addition, 1,25$(OH)_2$ vitamin D increases the number of regulatory T cells in pancreatic lymph nodes blocking progression of diabetes in NOD mice [338]. Beta cells express vitamin D receptors (VDRs), and *in vitro* treatment with 1,25$(OH)_2$ vitamin D significantly decreases the expression of chemokines, suggesting a local beneficial effect of vitamin D to decrease the risk of autoimmune diabetes [339].

Since the physiological levels of vitamin D are source of disagreement, some studies have focused on the possible role of supplementary doses in the incidence of T1D. Two large European studies demonstrated that early supplementation of vitamin D has a protective effect against T1D development [340, 341]. Despite some negative studies [308, 342], a recent meta-analysis including these later studies and others, reaching a total of 6,455 participants, demonstrated a 29% reduction in T1D development by vitamin D supplementation [343].

The genetic regulation of vitamin D may also have a role in this association: different polymorphisms in *VDR* are related to risk of T1D depending on the ultraviolet (UV) radiation in the geographical region evaluated [344].

Cereals: two prospective studies evaluated the timing of cereal introduction in the diet of children at risk of T1D (due to family history or high-risk HLA) and posterior appearance of islets autoantibodies. First exposure to cereals before age of three months [345, 346] or after seven months [345] increased the risk of autoantibodies. A recent study demonstrated that half of the T1D patients evaluated present increase of T cell reactivity to wheat, and that these cells secrete several proinflammatory cytokines. Almost all the subjects with this augmented T cell response were HLA *DR4-DQ8* [347], suggesting that presentation of wheat peptides in the context of class II HLA by susceptible individuals might collaborate to T1D development.

Other diet factors: omega-3 fatty acids [348] and nitrates [349] are two other components of the diet associated with risk of T1D in observational studies.

5.4. Viruses

Viruses are one of the most studied environmental factors related to development of T1D. Epidemiological, experimental and clinical data have provided strong indication that viruses might contribute to the pathogenesis of autoimmune diabetes.

5.4.1. Epidemiological studies

Enterovisuses (EV) such as Coxsackievirus B (CVB) [175], are the main candidates, but rotavirus [350, 351], mumps virus [352], and cytomegalovirus [353] have also been associated to T1D. The rubella virus is only related with diabetes when transmitted congenitally [305, 354] (see above).

Initial studies in the 60s suggested that the seasonal variability observed in T1D could be due to increased enteroviral infection, identified by the peripheral appearance of antibodies during the colder months of the year . Since serological studies have limitations in sensibility,

other molecular methods of diagnosis have been utilized, as RT-PCR assays. Using this technique, nine cross-sectional studies have evaluated the presence of EV genome in the blood of recent-onset T1D patients [355-363]. These studies demonstrated that 30% of the patients with recent-onset T1D are positive for EV RNA in comparison with 5% of the control group. However, it was not possible to determine by this approach if these infections were acute or chronic.

To answer this question, prospective studies were made. Studies done in Finland demonstrated that EV infections are more frequent in siblings that develop T1D than in siblings without the disease [307, 364]. Of interest, it was confirmed the positive correlation between the appearance of autoantibodies in children genetically susceptible and the seasonal variation of EV infections [365]. Furthermore, another study demonstrated a direct temporal correlation between the first autoantibodies development and EV RNA/serology appearance [366]. These positive associations were confirmed in other [366-368] but not in all studies [309, 369]. In respect to these negative studies, the longer time between collection of samples, and the less comprehensive diagnostic techniques used, may explain the observed lack of correlation between viral infection and T1D.

EV infections might be at least in part responsible for the increasing T1D incidence (see section 2.3). To correlate these two events, the EPIVIR project analyzed EV antibodies from pregnant women during the period of 1983-2001 in Finland and Sweden. Unexpectedly, there was a significant decrease in EV antibodies in this period [370] indicating that these infections are becoming less frequent while there is an increased incidence of T1D. Two hypotheses were proposed to explain these results. First, the “polio hypothesis” is based in observations done one century ago, when a decrease in poliovirus infection was paradoxically associated with an increase in paralytic complications. This was caused by a shift in the period of first infection, from the neonatal period to older ages, when maternal antibodies

have already disappeared [371]. The data from the EPIVIR project suggest a similar phenomenon in T1D, but further studies are necessary to confirm this concept. The second one is the "hygiene hypothesis", which postulates that repeated infections in early childhood train the immune system to better respond to future infection and also down-regulate autoimmune responses [371, 372]. In this scenario, a decrease in "in uterus" or early life infections with EV would predispose individuals to a late EV-induced autoimmunity against beta cells. Experimental data support this hypothesis: studies in NOD mice demonstrate that young animals infected with EV before insulitis are, in fact, protected against spontaneous autoimmune diabetes [373, 374].

Ongoing large prospective studies, such as The Environmental Determinants of Diabetes in the Young (TEDDY) study [375], will hopefully clarify the role of viruses in the development of T1D.

5.4.2. Experimental studies

The first observations suggesting a role for virus in the etiology of diabetes in animal models were done in the 60s and 70s. A correlation between foot-and-mouth disease in cattle and diabetes was described in Italy [376]. This was reinforced by studies showing that infection of certain mouse strains with encephalomyocarditis virus (EMCV) or CVB4 promotes destruction of pancreatic beta cells [377], hypoinsulinemia [378, 379] and diabetes [378, 379]. More interestingly, CVB4 was isolated from a patient with recent-onset T1D that died from ketoacidosis, and this virus was able to infect mouse pancreatic beta cells causing islet infiltration by inflammatory cells and diabetes [380]. Other viruses were also demonstrated to cause diabetes in animals, such as Killham's virus [381] and lymphocytic choriomeningitis virus (LCMV) [382]. This relation between viral infections and diabetes, however, seems to be time- and virus-dependent [173]. Thus, early infection by CVB4

protects NOD mice [383] and the B3 strain of CVB3 prevents autoimmune diabetes independently of timing [384] (see below).

There are two main mechanisms by which viruses can cause T1D: molecular mimicry and bystander activation of the immune system. These mechanisms are not mutually exclusive, since both can be involved in different models and phases of the disease.

Molecular mimicry: similarities between autoantigens and viral peptides can lead to the recognition of endogenous proteins by autoreactive T cells. This was first suggested for the autoantigen GAD and the CVB4 protein P2-C, which shares some resemblance [385]. In line with this, T cells from individuals at risk of T1D responded to a peptide derived from GAD, which has significant sequence similarity with P2-C protein, and the same T cells also cross-reacted with the viral protein [385]. GAD autoantibodies from T1D patients, however, did not cross-react with P2-C [386]. Studies in NOD mice demonstrated that this cross-reactivity depends on the MHC class II allele, suggesting that this only happens in susceptible individuals [131]. On the other hand, infection of BDC2.5 mice that do not recognize P2-C, but another autoantigen in beta cells (Chromogranin [58]), induce autoimmune diabetes, suggesting that diabetes induction does not depend on molecular mimicry in this model [387]. Furthermore, in the rat insulin promoter (RIP)-LMCV system, a model where the glycoprotein of LMCV is expressed in beta cells under the control of RIP, a modification of only one amino acid in the viral epitope significantly changed the rates at which these animals develop autoimmune diabetes [388]. Finally, a LCMV mimic ligand accelerated diabetes in mice already presenting autoimmunity, but did not initiate the disease [389]. These data indicate that molecular mimicry might have a more important role in amplifying the autoimmune assault in genetic predisposed individual, rather than in initiating the process [173]. A new model of molecular mimicry for studying autoimmune diseases described that similarity, instead of complete identity, produces a more severe form of autoimmune hepatitis

caused by virus [390]. These differences may be related to the presence of peripheral tolerance mechanisms that protect the liver from excessive autoimmune damage, which in this case could promote to deletion of autoreactive T cells against identical but not similar molecules [391].

Bystander mechanisms: the main role of viruses as initiators of autoimmunity might involve local damage to beta cells and the induction of proinflammatory mediators [171, 173, 387]. Thus, CVB-infected pancreatic beta cells can be phagocyted by both macrophages [392] and dendritic cells [393], leading to activation of the innate immune system, presentation of islet autoantigens and release of cytokines/chemokines. Local injury of beta cells induced by streptozotocin [394], or the viral by-product dsRNA [166], can also promote autoimmune diabetes in animal models. Of note, in the RIP-LCMV mouse model dendritic cells presenting autoantigen could both prime autoreactive T cells and maintain immune responses [395]. These immune cells also produce proinflammatory cytokines in the vicinity of the islets, which may induce beta cells apoptosis and activation of other immune cells (see above) [171]. Thus, the release of islet antigens in the context of an inflammatory milieu seems to be crucial for the development of autoimmune diabetes caused by viruses (Figure 6).

Additional effects of viral infection potentially promoting autoimmune diabetes in experimental models are:

Direct beta cell infection: infection of animals with high titers of EMCV causes massive destruction of beta cells and diabetes by a non-autoimmune mechanism [4]. In most cases, however, viruses cause a limited damage to beta cell directly or indirectly by the activation of antiviral responses [173]. CVB4 has a tropism for beta cells, and cause autoimmune diabetes in animal models The CVB3 strain, however, infects the acinar cells in the exocrine pancreas and prevents diabetes [373]. These different strains of CVB do not induce the same degree and type of damage in beta cells. This is an important factor in the development of

autoimmunity, since it was demonstrated in NOD mice that secondary beta cell necrosis following apoptosis, but not primary necrosis causes autoimmune diabetes [178].

Expression of MHC peptides: beta cells exposed to proinflammatory cytokines increase the expression of class I MHC, potentially "unmasking" these cells to autoreactive cytotoxic T cells which then directly kill them [167].

Timing: as discussed above, the ability of CVB4 to cause diabetes in animals depends on the timing of infection. This may be explained by the need for a critical mass of autoreactivity for the virus to initiate disease [396]. In other words, a virus may have a more diabetogenic effect if superimposed on the presence of a quiescent T cell population in the vicinity of the islets as is the case in young female NOD mice or adults male NOD mice.

Tolerance: in some cases, such as CVB3 infection, viruses can prevent instead of causing diabetes in NOD mice. In this context, early viral infections induce activation of T reg cells, which secrete transforming growth factor-β (TGF-β) which keeps under control the immune responses against viruses and autoimmunity [374]. Based on these findings, it was suggested that viral-induced autoimmune diabetes depends on the balance between beta cell injury and immunoregulation. Of note, in BB rats the Kilham virus induces diabetes without infecting beta cells; this is done by modifying the ratio T regulatory / autoreactive T effector cell [397], reinforcing the importance of the balance between these two populations of T cells for the immune homeostasis. Modifications in central tolerance may also have a role since CVB4 infects and proliferates in murine fetal thymus disturbing T cell maturation [398]. This intriguing observation needs additional *in vivo* studies.

Enhancing antibodies: an interesting mechanism for diabetes induction involves the antibody-dependent enhancement (ADE) of enterovirus infection in APCs. This may disseminate the viruses and aggravate lesion of the target organs, as already demonstrated in a model of myocarditis [399]. In agreement with this possibility peripheral blood mononuclear

cells pre-incubated with plasma or IgG from T1D patients and subsequently infected with CVB4 produce more type 1 interferons [400]. The target of these antibodies, present at high levels in the serum of T1D patients, is the capsid protein VP4 [401]. Further studies are necessary to clarify the role for ADEs in the pathogenesis of T1D.

Figure 10 presents a summary of the available information on the potential mechanisms by which viruses trigger T1D.

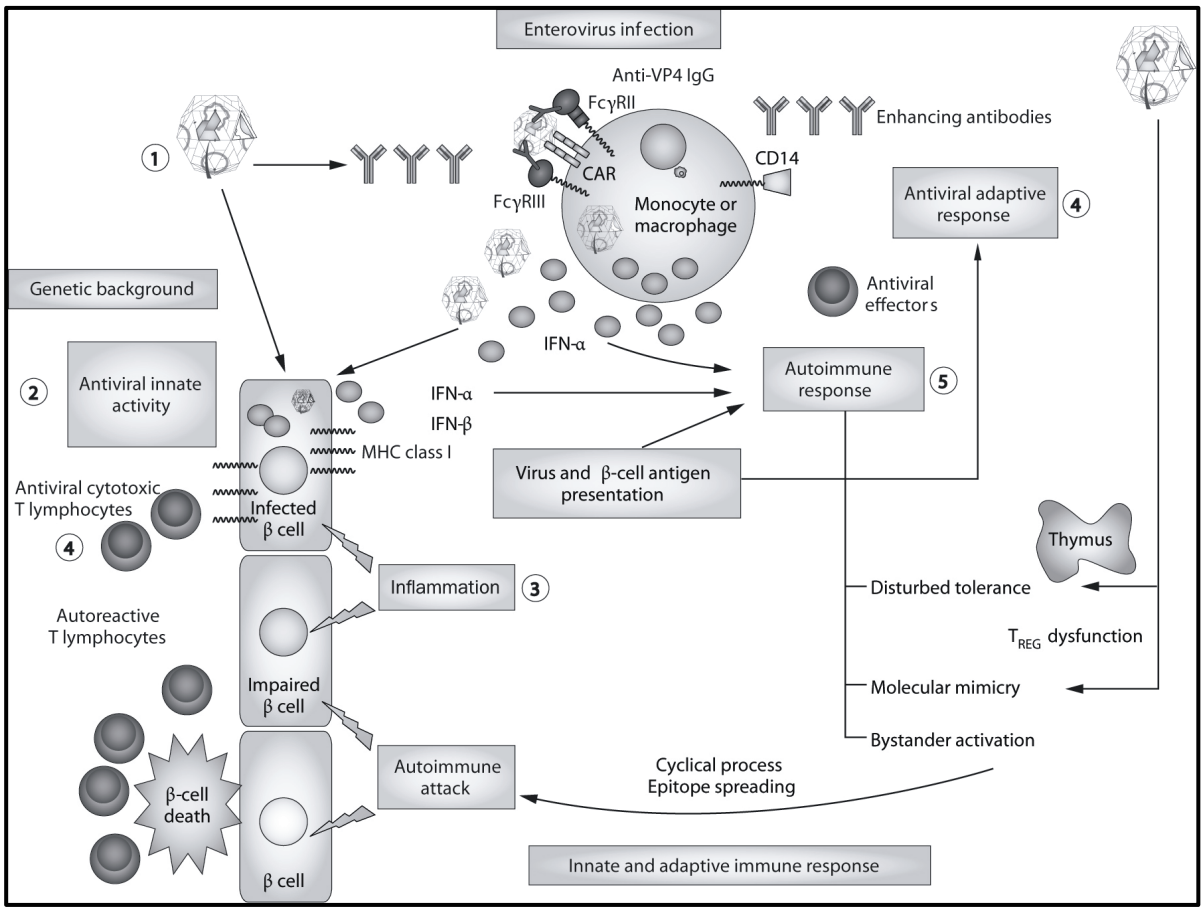

Figure 10. Model of virus-induced autoimmune diabetes. 1) Antibody-dependent enhancement of APCs increases the virus spreading to pancreatic beta cells which can be persistent or repeated. 2) Innate immune responses are activated, which in the context of some polymorphisms, such as in MDA5, can be modulated by decreasing expression of proinflammatory mediators (Article II). 3) Both beta cells and the infiltrating immune cells locally produce cytokines that compromise function and may kill beta cells. They also increase the expression of class I MHC and activate CD8+ T cells, and induce chemokine secretion which attracts additional immune cells. 4, 5) This inflammatory milieu and the release of islet antigens promotes the transition for an adaptive immune response, including

the activation and recruitment of autoreactive T cells and the triggering of a prolonged autoimmune assault. Disturbed tolerance, molecular mimicry, and bystander activation can cooperate in this process of auto-reactive T cell formation. This can be a chronic or recurrent process with wax and wane, leading to progressive increase in the number of autoantigens by epitope spreading. (CAR: Coxsackievirus receptor; FcγRII and FcγRIII: receptors for the Fc portion of the IgG molecules; IFN: interferon; MHC: major histocompatibility complex). (modified from [372]).

5.4.3. Human pancreas studies

Studies of human pancreatic biopsies confirmed data from peripheral markers (serology and genome RNA) by demonstrating direct and indirect signals of EV infection in patients with T1D [380, 402] or islet autoantibody positive [403]. A recent study evaluated the presence of the EV protein VP1 by immunohistochemistry in pancreas of 72 T1D patients and 50 controls; VP1 was identified it in 61% of the T1D patients, but in only 6% of the controls [404]. Since this technique can also induce some artifacts, these results were validated by the detection of a second marker of viral infection, namely the dsRNA-dependent protein kinase R (PKR) [404]. By using techniques that directly identify the virus, such as in situ hybridization, electron microscopy and viral isolation, another group, demonstrated viral infection in 3 out of 6 patients with recent-onset T1D, but in none of the 26 controls evaluated [175]. These studies used double staining for insulin and EV protein to show that the cells infected were indeed the beta cells [175, 404]. This reinforces the hypothesis of persistent or recurrent beta cell viral infections in individuals progressing to T1D.

6. Aims of the thesis

1- Compare information from genome-wide association studies and data from our previous arrays studies and online database (www.t1dbase.org) to identify T1D candidate genes expressed in beta cells and regulated by cytokines and dsRNA.

2- Evaluate the expression of T1D candidate genes, and downstream genes, in beta cells exposed or not to a combination of proinflammatory cytokines or a synthetic intracellular dsRNA (polyinosinic-polycitidilic acid (PIC)).

3- Design specific small-interference (si)RNAs against the selected T1D candidate genes to further study their role in beta cell responses to viral-mediated damage and inflammatory responses.

4- Integrate the data obtained to propose a model of the possible interaction between genetic predisposition (candidate genes selected) and environmental factors (viral infection) in the pathogenesis of T1D.

Article I

PTPN2, a candidate gene for type 1 diabetes, modulates interferon-gamma-induced pancreatic beta cell apoptosis.

Moore F, Colli ML, Cnop M, Esteve MI, Cardozo AK, Cunha DA, Bugliani M, Marchetti P, Eizirik DL.

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PTPN2, a Candidate Gene for Type 1 Diabetes, Modulates Interferon-γ–Induced Pancreatic β-Cell Apoptosis

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OBJECTIVE—The pathogenesis of type 1 diabetes has a strong genetic component. Genome-wide association scans recently identified novel susceptibility genes including the phosphatases PTPN22 and PTPN2. We hypothesized that PTPN2 plays a direct role in β-cell demise and assessed PTPN2 expression in human islets and rat primary and clonal β-cells, besides evaluating its role in cytokine-induced signaling and β-cell apoptosis.

RESEARCH DESIGN AND METHODS—PTPN2 mRNA and protein expression was evaluated by real-time PCR and Western blot. Small interfering (si)RNAs were used to inhibit the expression of PTPN2 and downstream STAT1 in β-cells, allowing the assessment of cell death after cytokine treatment.

RESULTS—PTPN2 mRNA and protein are expressed in human islets and rat β-cells and upregulated by cytokines. Transfection with PTPN2 siRNAs inhibited basal- and cytokine-induced PTPN2 expression in rat β-cells and dispersed human islets cells. Decreased PTPN2 expression exacerbated interleukin (IL)-1β + interferon (IFN)-γ–induced β-cell apoptosis and turned IFN-γ alone into a proapoptotic signal. Inhibition of PTPN2 amplified IFN-γ–induced STAT1 phosphorylation, whereas double knockdown of both PTPN2 and STAT1 protected β-cells against cytokine-induced apoptosis, suggesting that STAT1 hyperactivation is responsible for the aggravation of cytokine-induced β-cell death in PTPN2-deficient cells.

CONCLUSIONS—We identified a functional role for the type 1 diabetes candidate gene PTPN2 in modulating IFN-γ signal transduction at the β-cell level. PTPN2 regulates cytokine-induced apoptosis and may thereby contribute to the pathogenesis of type 1 diabetes. ***Diabetes*** **58:1283–1291, 2009**

Type 1 diabetes is a chronic autoimmune disease with a strong genetic etiology. Genetic predisposition to type 1 diabetes depends on a small number of genes having large effects and a larger number of genes having small effects (1). These genes interact with putative environmental factors, which may include viral infections, triggering insulitis and eventually diabetes (2). Recent genome-wide association studies have shown association between type 1 diabetes and four chromosome regions, pointing to several new candidate genes for the disease (3). Most of the newly and previously identified genes are assumed to regulate immune function. This contrasts with type 2 diabetes, where similar studies indicate a major role for genes regulating β-cell function (4).

We have presently evaluated whether the recently identified candidate genes for type 1 diabetes (3,5) are expressed in pancreatic β-cells and whether their expression is modulated by proinflammatory cytokines. This was done by examining our previous array analyses of cytokine-treated or virus-infected rodent and human β-cells/pancreatic islets (6–10; complete information on these arrays is available at the Beta Cell Gene Expression Bank [11]) and new array analyses performed in our laboratory using the new Affymetrix rat array Genechip 230.2.0 (unpublished data). This analysis identified β-cell expression of the candidate gene protein tyrosine phosphatase (PTP)N2.

PTPN2 (also known as TC-PTP or PTP-S2) is a member of the first nontransmembrane (NT1) subfamily of PTPs. PTPs are a superfamiliy of enzymes with opposite roles compared with protein tyrosine kinases (12). PTPN2 is expressed in immune cells, and its expression is modulated by cell cycle, mitogenic agents, and cytokines (13). PTPN2 exists as two isoforms generated from alternative splicing: a major TC45 isoform (45 kDa) containing a nuclear localization sequence and that shuttles between the nucleus and the cytoplasm and a less abundant TC48 isoform (48 kDa) that is anchored to the endoplasmic reticulum (13). Many targets have already been identified for TC45, including Janus kinases (JAKs) and signal transducer and activator of transcription (STATs), p42/44 mitogen-activated protein kinase (MAPK) (extracellular signal–related kinase [ERK]), epidermal growth factor receptor (EGFR), and insulin receptor β (IRβ) (14–17). Several of these pathways have been implicated in the control of β-cell physiology, survival, and expansion (18–20).

We have previously suggested that islet inflammation and subsequent β-cell death develops in the context of a "dialogue" between the immune system and β-cells (21). Thus, β-cells exposed to viral agents (6,22), or to endogenous Toll-like receptor ligands (23), release cytokines and chemokines that attract and activate macrophages, T-cells, and B-cells. These immune cells will then trigger β-cell apoptosis via contact mediators such as Fas-FasL and/or via secretion of proinflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α (21,24). IFN-γ has a key role in this process. Thus, neither IL-1β nor TNF-α alone induce

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human or rodent β-cell death, but combinations of these cytokines with IFN-γ lead to 50% β-cell death after 6–9 days (21,24). IFN-γ signal transduction involves activation of the tyrosine kinases JAK1 and JAK2 that phosphorylate STAT1, which then dimerizes and translocates to the nucleus where it binds γ-activated sites of diverse genes (21). Excessive activation of JAK/STAT signaling may lead to cell death, and STAT1 transcriptional activity is regulated by multiple negative feedback mechanisms, including inhibition of JAKs by specific phosphatases and by the suppressors of cytokine signaling and inhibition of STAT1 activity by protein inhibitor of activated STAT (PIAS) proteins (25). β-Cells from STAT1-deficient mice ($STAT1^{-/-}$) are protected against IL-1β + IFN-γ–induced apoptosis, and $STAT1^{-/-}$ mice are more resistant to diabetes induced by multiple low doses of streptozotocin or after backcross into NOD mice (18,26). This protective effect takes place at the β-cell level, since islets from $STAT1^{-/-}$, but not from wild-type mice, prevent diabetes when transplanted into wild-type mice subsequently treated with multiple low doses of streptozotocin (27). However, it still remains unclear whether modulation of phosphatases modifies IFN-γ–induced β-cell apoptosis.

We presently report that PTPN2 is regulated by the cytokines IL-1β, IFN-γ, and TNF-α in rodent and human pancreatic islet cells. Importantly, small interfering (si)RNA-mediated PTPN2 knockdown increases STAT1 activation and aggravates cytokine-induced β-cell apoptosis in a STAT1-dependent manner. These observations indicate that one of the new candidate genes for type 1 diabetes may act at the immune and β-cell level, exacerbating cytokine-induced β-cell apoptosis under inflammatory conditions.

RESEARCH DESIGN AND METHODS

Culture of primary fluorescence-activated cell–sorted rat β-cells, human islets, and INS-1E cells. Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Islets were isolated by collagenase digestion and handpicked under a stereomicroscope. β-Cells were purified by autofluorescence-activated cell sorting (FACSAria; BD Bioscience, San Jose, CA) (28,29). The preparations contained 90.4 ± 3.2% β-cells (n = 4). β-Cells were cultured for 2 days in Ham's F-10 medium containing 10 mmol/l glucose, 2 mmol/l glutaMAX, 50 μmol/l 3-isobutyl-1-methylxanthine, 5% fetal bovine serum (FBS), 0.5% charcoal-absorbed BSA (Boehringer, Indianapolis, IN), 50 units/ml penicillin, and 50 μg/ml streptomycin (29,30). During cytokine exposure, cells were cultured in the same medium but without serum.

Human islets were isolated from 11 nondiabetic organ donors (age 63 ± 5 years; BMI 25.1 ± 0.8 kg/m^2) in Pisa, Italy, with the approval of the local ethics committee. Islets were isolated by enzymatic digestion and density-gradient purification (31) and cultured in M199 medium containing 5.5 mmol/l glucose. Their functional status was determined using glucose-stimulated insulin release and was 2.8 ± 0.4 (expressed as stimulation index). The human islets were shipped to Brussels within 1–5 days of isolation. After overnight recovery in Ham's F-10 containing 6.1 mmol/l glucose, 10% FBS, 2 mmol/l GlutaMAX, 50 μmol/l 3-isobutyl-1-methylxanthine, 1% BSA, 50 units/ml penicillin, and 50 μg/ml streptomycin, islets were exposed to cytokines in the same medium without FBS for 2 days. The percentage of β-cells, examined in the 11 dispersed islet preparations by staining with anti-insulin antibody (1:1,000; Sigma, Bornem, Belgium) and donkey anti-mouse IgG rhodamine (1:200; Lucron Bioproducts, De Pinte, Belgium), was 49 ± 5%.

The rat insulin-producing INS-1E cell line (a gift from Dr. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured as previously described (32,33).

RNA interference. The siRNAs used in this study are listed in Supplementary Table A1 (available in an online-only appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1510/DC1). The best settings for the transfection of siRNAs in INS-1E cells, primary fluorescence-activated cell–sorted rat β-cells, and dispersed human islets were established by using a fluorescein isothiocyanate (FITC)-conjugated siRNA (siGLO Green Transfection Indicator, Thermo Scientific). Different transfection methods were tested, including electroporation, and several lipid reagents. Among those, DharmaFECT 1 (Thermo Scientific) was selected for its high transfection efficiency, namely 95 ± 2.7% in INS-1E cells and 80.7 ± 5.6% in primary rat β-cells. No cell toxicity was observed with this transfection method (% cell death: 9.4 ± 1.7% after transfection vs. 8.4 ± 2.2% in control for INS-1E cells and 9.8 ± 1.3% after transfection vs. 7.4 ± 1.7% in control for primary rat β-cells). Cells were cultured in antibiotic-free medium for at least 24 h before transfection. For transfection, the siRNA and the DharmaFECT were diluted separately in OptiMEM medium and incubated at room temperature for 5 min. Lipid-siRNA complexes were then formed at room temperature for 20 min in a proportion of 0.75, 1.25, and 1.75 μl DharmaFECT to 150 nmol/l of siRNA for INS-1E cells, primary β-cells, and dispersed human islets, respectively. The complexes were diluted five times in antibiotic-free medium and added to the cells at a final concentration of 30 nmol/l siRNA (except when indicated otherwise) for overnight transfection. The concentration of 30 nmol/l was selected after dose-response studies (data not shown). Afterward, cells were cultured for a 48-h recovery period and subsequently exposed to cytokines.

Cell treatment and nitric oxide measurement. The following cytokine concentrations were used, based on previous dose-response experiments (10,24,33): recombinant human IL-1β (specific activity 1.8 × 10^7 units/mg; a gift from C.W. Reinolds, National Cancer Institute, Bethesda, MD) at 10, 50, or 100 units/ml as indicated; recombinant murine TNF-α (specific activity: 2 × 10^8 units/mg; Innogenetics, Gent, Belgium) at 1,000 units/ml; and recombinant rat and human IFN-γ (specific activity: 2 × 10^7 units/mg; R&D Systems, Abingdon, U.K.) at 100 and 1,000 units/ml for rat cells and human islets, respectively. When cells were treated with cytokines, culture supernatants were collected for nitrite determination (nitrite is a stable product of nitric oxide [NO] oxidation) at $OD^{540\ nm}$ using the Griess method (34). Palmitate (sodium salt, Sigma) was dissolved in 90% ethanol, heated to 60°C, and used at a final concentration of 0.5 mmol/l in medium containing 1% BSA (35). The SERCA blocker CPA (Sigma) was dissolved in DMSO and used at a concentration of 25 μmol/l.

Assessment of cell viability. The percentage of viable, apoptotic, and necrotic cells was determined after a 15-min incubation with the DNA-binding dyes propidium iodide (PI, 5 μg/ml; Sigma) and Hoechst 33342 (HO, 5 μg/ml; Sigma) (10,29,36). A minimum of 500 cells was counted in each experimental condition. Viability was evaluated by two independent observers, one of them being unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percent apoptosis, calculated as number of apoptotic cells/total number of cells × 100. In some experiments, apoptosis was confirmed using the Cell Death Detection ELISAplus kit (Roche Diagnostics, Vilvoorde, Belgium), which detects cytoplasmic fragmented DNA.

mRNA extraction and real-time PCR. Poly(A)$^+$ mRNA was isolated from INS-1E cells, rat primary β-cells, and human islets using the Dynabeads mRNA DIRECT kit (Invitrogen) and reverse transcribed as previously described (10,29,37). The real-time PCR amplification reaction was done as described (29,37), using SYBR Green and compared with a standard curve (38). Expression values were corrected for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). IL-1β alone does not modify GAPDH expression, whereas exposure to IL-1β + IFN-γ reduces its expression according to cell death in INS-1E cells (Supplementary Fig. A1 and 7,10). The primers used in this study are detailed in Supplementary Table A2 (online appendix).

Western blot analysis. Cells were washed with cold PBS and lysed with either Laemmli buffer or phospho lysis buffer (the compositions of the buffers are provided in Supplementary Table A3). Lysates were then resolved by 8–10% SDS-PAGE and transferred to a nitrocellulose membrane. The antibodies used in this study are listed in Supplementary Table A4. Immunoreactive bands were revealed using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific), detected using a LAS-3000 charge-coupled device camera and quantified with the Aida Analysis software (Fujifilm).

Immunofluorescence. INS-1E cells were seeded onto glass coverslips and treated as indicated. Cells were washed with cold PBS, fixed for 10 min in 100% methanol at −20°C, washed three times with PBS, quenched 5 min in PBS/0.1% sodium borohydride, washed twice with PBS, and incubated for 5 min in PBS containing 0.2% Triton X-100 (PBST). After a 1-h blocking with 5% normal goat serum in PBST, cells were incubated overnight with PTPN2 antibodies (0.7 μg/ml) in PBST/0.5% normal goat serum (Supplementary Table A4). Cells were washed three times with PBST, and FITC-conjugated goat anti-mouse antibodies (Lucron Bioproducts) were applied for 2 h at 1:200 in PBST. After two washes with PBST, nuclei were counterstained with Hoechst 33342 (HO) for 5 min and washed three times with PBS. Coverslips were mounted in mounting medium (Dakocytomation), and immunofluorescence was visualized on a Zeiss microscope.

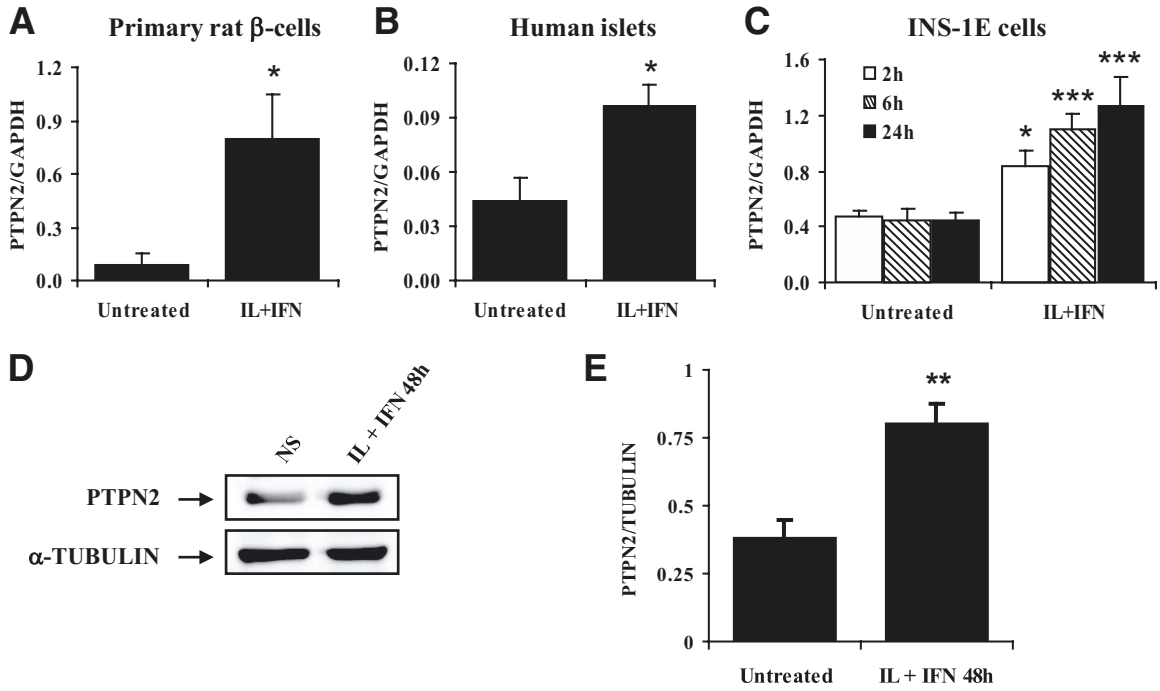

FIG. 1. Cytokines upregulate PTPN2 expression in primary fluorescence-activated cell–sorted rat β-cells, human islets, and INS-1E cells. *A*: Rat β-cells were cultured for 2 days and subsequently left untreated or treated with the combination of IL-1β (10 units/ml) + IFN-γ (100 units/ml) for 24 h. *B*, *D*, and *E*: Hand-picked human islets were cultured overnight and then left untreated or exposed to IL-1β (50 units/ml) + IFN-γ (1,000 units/ml) for 48 h. *C*: INS-1E cells were left untreated or treated with IL-1β (10 units/ml) + IFN-γ (100 units/ml) for 2, 6, and 24 h as indicated. *A–C*: PTPN2 mRNA expression was assayed by RT-PCR and normalized for the housekeeping gene GAPDH. *D*: PTPN2 and α-tubulin expression in human islets were evaluated by Western blot. *E*: Mean optical density measurements of PTPN2 Western blots corrected for protein loading by α-tubulin (representative figure in *D*). Results are means ± SE of three to five independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. untreated cells by Student's *t* test. NS, nonstimulated (untreated).

Statistical analysis. Data are presented as means ± SE. Comparisons were performed by two-tailed paired Student's *t* test or by ANOVA followed by Student's *t* test with Bonferroni correction. A P value <0.05 was considered statistically significant.

RESULTS

Cytokines upregulate PTPN2 mRNA and protein expression in primary rat β-cells, human islets, and INS-1E cells. We first evaluated the expression of PTPN2 mRNA (both TC45 and TC48 isoforms) in primary fluorescence-activated cell–sorted rat β-cells, human islets, and rat insulin–producing INS-1E cells and tested whether cytokine treatment would affect its expression. PTPN2 mRNA was expressed in untreated primary rat β-cells and human islets cells, and IL-1β + IFN-γ exposure upregulated its expression by 9.6-fold (rat β-cells) and 2.2-fold (human islets) after 24 and 48 h, respectively (Fig. 1*A* and *B*). A time course of IL-1β + IFN-γ treatment in INS-1E cells indicated that PTPN2 mRNA was already induced after 2 h, increasing progressively up to 24 h (Fig. 1*C*). We then confirmed by Western blot that PTPN2 was upregulated by two- and threefold, respectively, in human islets and INS-1E cells after IL-1β + IFN-γ treatment (Fig. 1*D* and *E* and Fig. 3*B* and *C*). In accordance with previous reports (13), we observed TC45 (*arrow*, Figs. 1*D* and 3*B*) to be the major PTPN2 isoform expressed in β-cells, with TC48 being poorly expressed (Fig. 1*D* and 3*B*). In contrast, the expression of PTPN22 (another phosphatase associated with type 1 diabetes risk; 39) was only present in samples from rat spleen and lymph nodes (used as positive controls), whereas no or marginal PTPN22 expression was observed in primary β-cells, human islets, and INS-1E cells. The expression of PTPN22 was not modified by cytokines (Supplementary Fig. A2 in the online appendix).

PTPN2 subcellular distribution. PTPN2 was mainly located in the nucleus under unstimulated conditions (Fig. 2, *left panels*). However, a 15-min treatment with IFN-γ alone or in combination with IL-1β or TNF-α resulted in redistribution of the protein between the cytoplasm and the nucleus (Fig. 2, *middle* and *right panels*).

siRNA targeting PTPN2 inhibits basal and cytokine-induced PTPN2 expression and exacerbates cytokine-induced apoptosis in INS-1E cells, primary rat β-cells, and dispersed human islets. We next examined the role of PTPN2 in β-cells. To this end, INS-1E cells were left untransfected, transfected with an irrelevant control siRNA (siCtrl), or with an siRNA targeting both PTPN2 isoforms (siPTPN2). Cells were then left untreated, or treated for 24 h with IL-1β, IFN-γ, TNF-α, or with the combination of IL-1β + IFN-γ or TNF-α + IFN-γ. PTPN2 mRNA expression was significantly upregulated after IL-1β and IL-1β + IFN-γ treatment in untransfected and siCtrl-transfected INS-1E cells (Fig. 3*A*). The expression of PTPN2 proteins was increased by all cytokines tested, alone or in combination, in both untransfected and siCtrl-transfected cells (Fig. 3*B* and *C*), suggesting that PTPN2 expression is regulated at both transcriptional and post-transcriptional levels. Transfection with PTPN2 siRNA potently inhibited basal and cytokine-induced PTPN2 expression at the mRNA and protein level (Fig. 3*A–C*). We then evaluated whether siRNA-mediated PTPN2 inhibition affects cytokine-induced apoptosis in INS-1E cells. As expected, a 24-h treatment with IL-1β + IFN-γ or TNF-α + IFN-γ induced apoptosis in untransfected and siCtrl-trans-

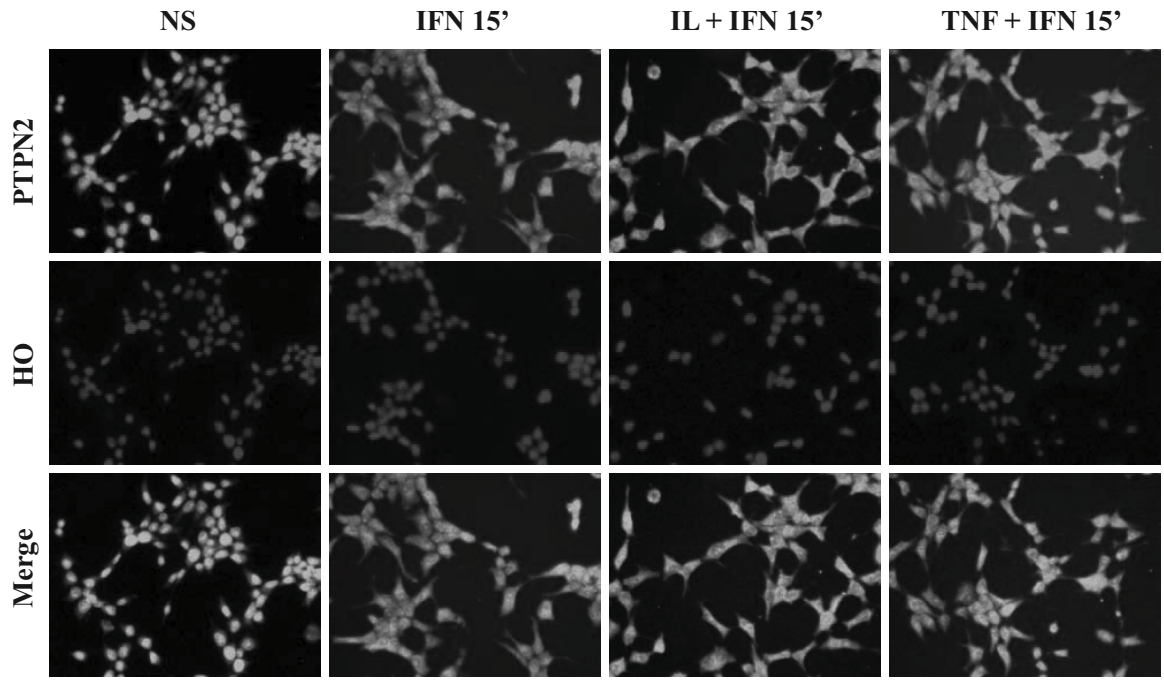

FIG. 2. Effects of cytokine exposure on PTPN2 subcellular distribution. INS-1E cells were plated onto glass coverslips and left untreated or treated for 15 min with IFN-γ (100 units/ml), IL-1β (10 units/ml) + IFN-γ (100 units/ml), or TNF-α (1,000 units/ml) + IFN-γ (100 units/ml). Cells were then fixed and processed for immunofluorescence as described in RESEARCH DESIGN AND METHODS. The figure is representative of five independent experiments. (A high-quality digital representation of this figure is available in the online issue.)

fected cells, whereas each individual cytokine did not (Fig. 4*A*; of note, the necrotic component was <2% under all experimental conditions tested; data not shown). The inhibition of PTPN2 exacerbated β-cell apoptosis after exposure to both IL-1β + IFN-γ and TNF-α + IFN-γ treatments. Importantly, PTPN2 inhibition also rendered IFN-γ treatment toxic to the cells, whereas this cytokine alone failed to induce apoptosis in both control counterparts (Fig. 4*A*). These results were confirmed using a second method to evaluate apoptosis, namely quantification of cytoplasmic fragmented DNA (Fig. 4*B*), and also with a second siRNA targeting PTPN2 (see below). The increased apoptosis in cytokine-treated PTPN2-deficient INS-1E was not accompanied by a more severe decrease in Ins1 and Ins2 mRNA contents in these cells (Supplementary Fig. A3) and could not be explained by a higher release of NO, since nitrite was similarly produced by untransfected, siCtrl-, and siPTPN2-transfected cells after IL-1β, IL-1β + IFN-γ, and TNF-α + IFN-γ treatments (Fig. 4*C*). Blocking PTPN2 using siRNA also augmented IFN-γ–, IL-1β + IFN-γ–, and TNF-α + IFN-γ–induced apoptosis in primary rat β-cells (Fig. 4*D*). Similar observations were made in dispersed human islet cells, in which siRNA-mediated PTPN2 inhibition exacerbated IL-1β + IFN-γ–induced cell death by 30–38% (Fig. 4*E* and *F*). This was again independent of NO production (data not shown). This effect seems to be specific for cytokines, since PTPN2 inhibition did not increase β-cell apoptosis in response to palmitate, high glucose, or the SERCA blocker CPA (Supplementary Fig. A4).

PTPN2 inhibition increases IFN-γ–induced STAT1 and STAT3 phosphorylation. Taking into account that STAT1 is a substrate of PTPN2 in other cell types (13) and that it is an important mediator of cytokine-induced β-cell apoptosis (18), we next examined the effect of PTPN2 inhibition on the kinetics and magnitude of IFN-γ–induced STAT1 phosphorylation. STAT1 phosphorylation was highly induced after 15 min of IFN-γ treatment in both untransfected and siCtrl-transfected controls, slowly decreasing between 2 and 24 h (Fig. 5*A* and *B*). However, IFN-γ–induced STAT1 phosphorylation was markedly enhanced in cells lacking PTPN2, reaching a peak at 1–2 h and slowly decreasing afterward (Fig. 5*A* and *B*). We confirmed that the increased STAT1 phosphorylation in PTPN2-deficient cells was not due to an augmentation of total STAT1 content in these cells (Fig. 5*A*). Comparable results were observed for STAT3, another target of PTPN2 phosphatase activity, with clearly increased STAT3 phosphorylation in PTPN2-deficient cells (Fig. 5*A* and *C*). These data demonstrate that the phosphatase PTPN2 is a major modulator of IFN-γ–induced STAT1 and STAT3 activity in β-cells. We also evaluated the p42/44 MAPK (ERK), EGFR, and IRβ activation pathways, previously described as PTPN2 targets in other cell types (14,16,40). Neither ERK nor EGFR signaling pathways were affected after PTPN2 inhibition in INS-1E cells (Supplementary Fig. A5). On the other hand, there was an increase in IRβ phosphorylation after 30 min of cytokine treatment in PTPN2-inhibited INS-1E cells that lasted until 14 h (Supplementary Fig. A5).

Double knockdown of PTPN2 and STAT1 protects INS-1E cells against cytokine-induced apoptosis. To evaluate the role of STAT1 in the exacerbation of cytokine-induced β-cell apoptosis observed after PTPN2 inhibition, we additionally interfered with STAT1 in a double knockdown approach. We first confirmed by Western blot that both PTPN2 and STAT1 siRNAs adequately inhibited their respective targets without affecting the expression of the other protein and also that the double transfection of PTPN2 and STAT1 siRNAs potently inhibited both target proteins (Fig. 6*A*). As previously shown (Fig. 4), IL-1β + IFN-γ or TNF-α + IFN-γ induced apoptosis to a similar degree in both control conditions, and PTPN2 inhibition exacerbated cytokine-induced apoptosis, also rendering treatment with IFN-γ alone toxic to the cells (Fig. 6*B*).

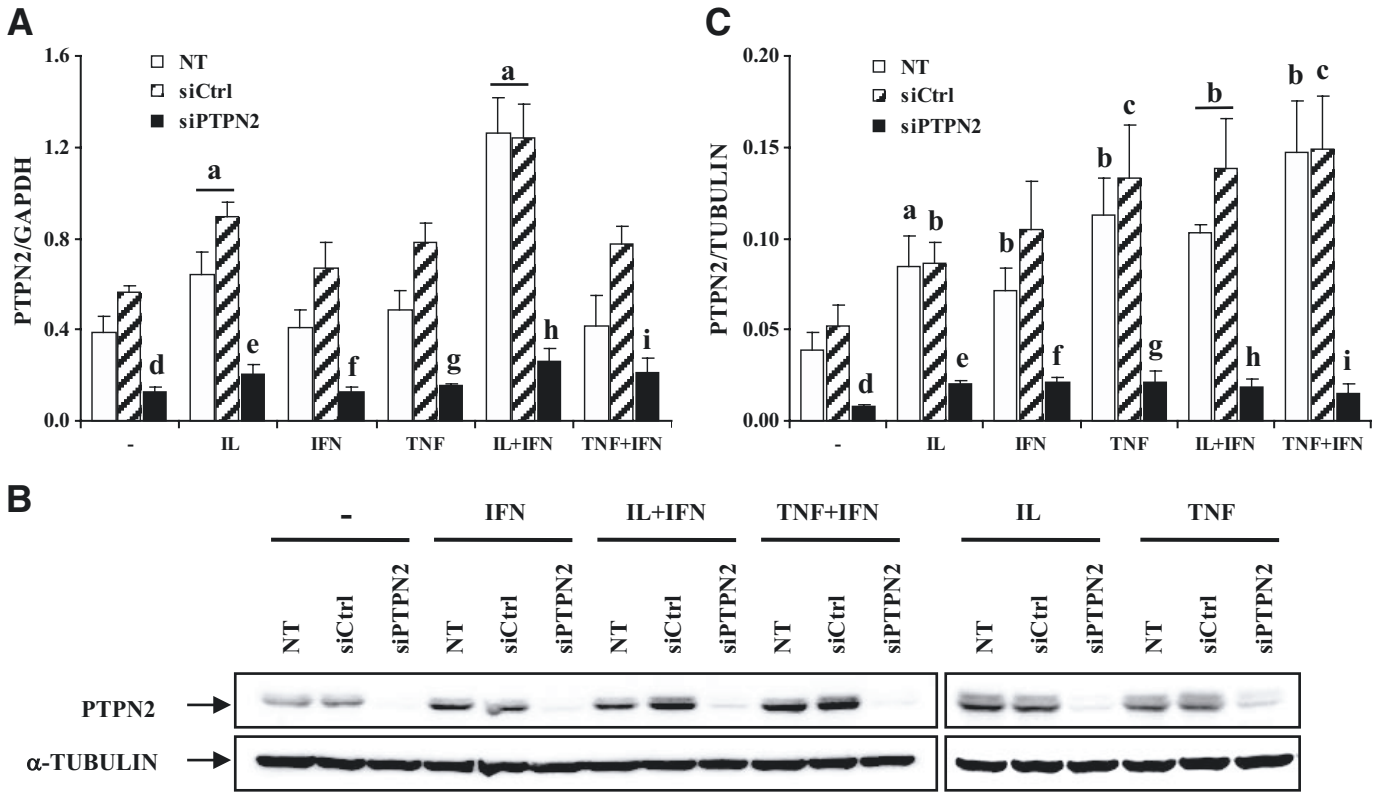

FIG. 3. A siRNA targeting PTPN2 inhibits basal and cytokine-induced PTPN2 expression in INS-1E cells. INS-1E cells were left untransfected (NT □) or transfected with 30 nmol/l of either a control siRNA (siCtrl, ▨) or a pool of siRNAs targeting PTPN2 (siPTPN2, ■). After 2 days of recovery, cells were left untreated, or treated for 24 h with IL-1β (100 units/ml), IFN-γ (100 units/ml), TNF-α (1,000 units/ml), IL-1β (10 units/ml) + IFN-γ (100 units/ml), or TNF-α (1,000 units/ml) + IFN-γ (100 units/ml). *A*: PTPN2 mRNA expression was assayed by RT-PCR and normalized for the housekeeping gene GAPDH. Results are means ± SE of four independent experiments. *B*: PTPN2 and α-tubulin protein expression were evaluated by Western blot. The results are representative of five independent experiments. *C*: Mean optical density measurements of PTPN2 Western blots, corrected for protein loading by α-tubulin. Results are means ± SE of five independent experiments; *a*: $P < 0.001$, *b*: $P < 0.01$, and *c*: $P < 0.05$ vs. untreated NT or untreated transfected with the same siRNA; *d*: $P < 0.01$ vs. untreated NT and siCtrl; *e*: $P < 0.01$ vs. IL-1β–treated NT and siCtrl; *f*: $P < 0.01$ vs. IFN-γ–treated NT and siCtrl; *g*: $P < 0.01$ vs. TNF-α–treated NT and siCtrl; *h*: $P < 0.001$ vs. IL-1β + INF-γ–treated NT and siCtrl, *i*: $P < 0.01$ vs. TNF-α + INF-γ–treated NT and siCtrl; ANOVA followed by Student's *t* test with Bonferroni correction.

Inhibition of STAT1 protected the cells against IL-1β + IFN-γ- and TNF-α + IFN-γ–induced apoptosis (by 56 and 67%, respectively). STAT1 knockdown abrogated the proapoptotic effect of PTPN2 inhibition in cells exposed to IFN-γ or to combinations of cytokines (Fig. 6*B*). These results were confirmed using a second siRNA targeting PTPN2 expression (Supplementary Fig. A6) and suggest that increased STAT1 activity contributes to the aggravation of cytokine-induced apoptosis in PTPN2-deficient β-cells.

DISCUSSION

We presently show that the phosphatase PTPN2 is expressed in human islets and rat primary and immortal β-cells and that its expression is regulated by the proinflammatory cytokines IL-1β, IFN-γ, and TNF-α in these cells. PTPN2 was first identified in humans as a T-cell PTP (41) with two spliced variants, namely TC45 and TC48 (13,42). In rat β-cells and human islets, the TC45 isoform is by far the most prevalent (Fig. 1*D* and 3*B*), and it is able to shuttle between the nucleus and the cytoplasm after cytokine exposure (Fig. 2). This change in subcellular location of PTPN2 has been shown previously in other cell types (14,16) and allows the phosphatase to target numerous cellular substrates, including cytoplasmic JAK tyrosine kinases, EGFR, the IRβ and nuclear STAT1, STAT3, STAT5, and STAT6 (43,44). Because of its modulatory role in a wide variety of signaling pathways, perturbations in PTPN2 expression are associated with diverse pathogenic processes, including ABC-like diffuse large B-cell lymphomas (45), inflammation-associated tumorigenesis (44), and several autoimmune disorders (see below).

PTPN2 inhibition in β-cells results in an early and more intense STAT1 and STAT3 activation after IFN-γ treatment, suggesting that this phosphatase plays an important role in the dephosphorylation and consequent inactivation of these transcription factors in β-cells. This early regulation of IFN-γ–induced STAT1 activation seems to be critical for the subsequent triggering of β-cell apoptosis, since PTPN2-deficient β-cells show an aggravation of cytokine-induced apoptosis, which is reverted by the combined inhibition of PTPN2 and STAT1. Importantly, PTPN2 inhibition unmasked the proapoptotic effect of IFN-γ alone in both INS-1E and primary β-cells, while it failed to augment palmitate- or CPA-induced β-cell death. Taken together, these observations indicate two interesting aspects that require additional investigation: *1*) The crucial negative feedback of PTPN2 on the STAT1 signaling pathway suggests that PTPN2 is part of the "defense mechanisms" triggered by β-cells in response to cytokines. It remains to be clarified why this and other defense mechanisms (24) are not sufficient to protect β-cells against apoptosis. *2*) IFN-γ alone induces STAT1 activation, but this only leads to apoptosis when PTPN2 activa-

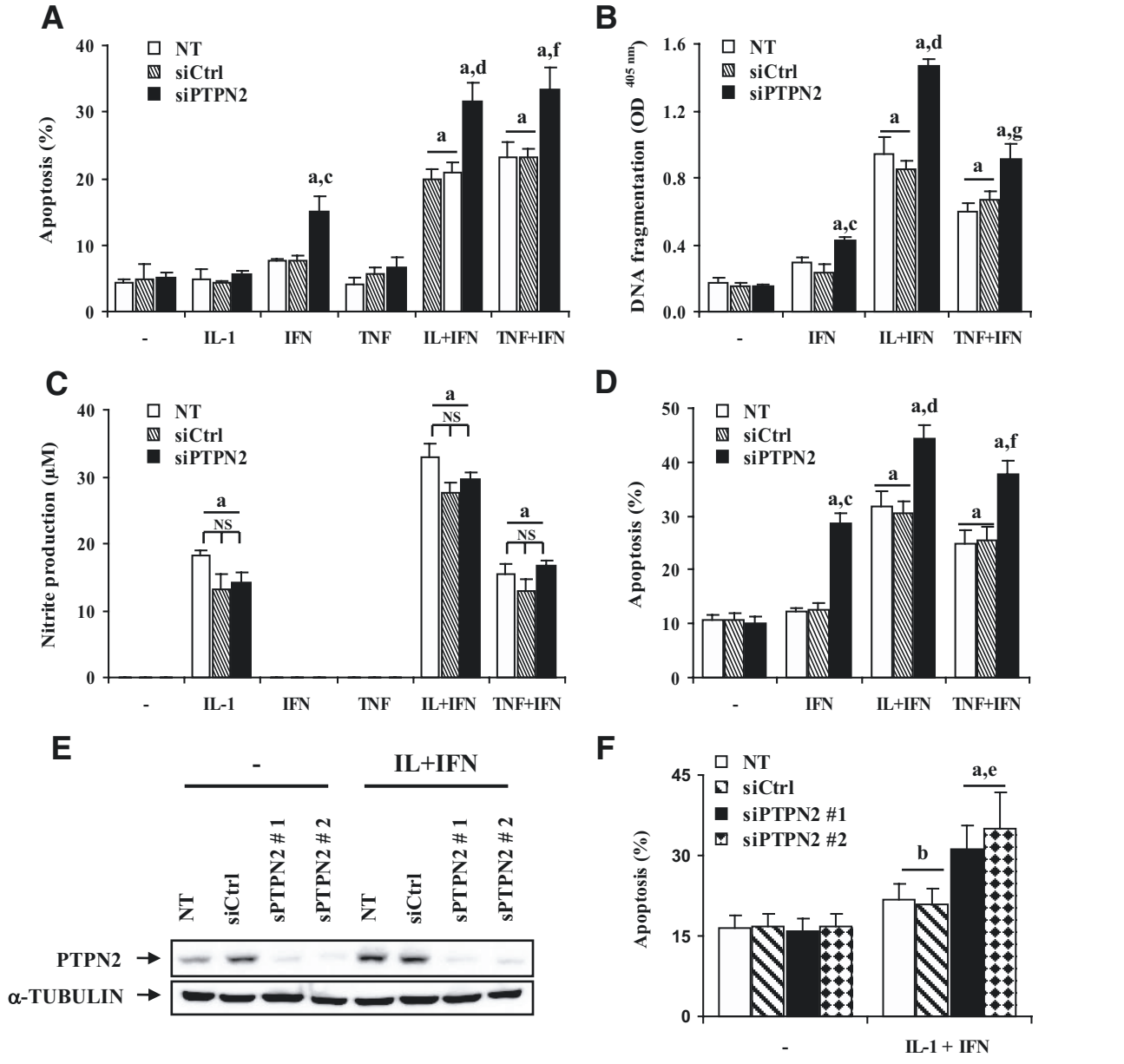

FIG. 4. siRNA-mediated PTPN2 inhibition exacerbates cytokine-induced apoptosis in INS-1E cells, primary rat β-cells, and dispersed human islets, independently of NO production. INS-1E cells were transfected and treated as described in Fig. 3. Apoptosis was evaluated after 24 h using HO/PI staining (*A*) and a Cell Death Detection ELISAplus kit (*B*). *C*: INS-1E cells were transfected and treated as described in Fig. 3, and nitrite concentrations in supernatants were measured as described in RESEARCH DESIGN AND METHODS (*D*). Primary fluorescence-activated cell–sorted rat β-cells were cultured for 2 days and then transfected as described in Fig. 3. After 2 days of recovery, cells were left untreated (NT) or treated for 72 h with IFN-γ (100 units/ml), IL-1β (10 units/ml) + IFN-γ (100 units/ml), or TNF-α (1,000 units/ml) + IFN-γ (100 units/ml). *E* and *F*: Dispersed human islets were left untransfected, or transfected with 30 nmol/l of siCtrl or human siPTPN2 #1 or #2 and cultured for a 48-h recovery period. Cells were then treated with IL-1β (50 units/ml) + IFN-γ (1,000 units/ml) for 48 h when PTPN2 and α-tubulin were evaluated by Western blot (*E*) and 96 h when apoptosis was evaluated by HO/PI staining (*F*). Results are means ± SE of four experiments; *a*: $P < 0.001$ and *b*: $P < 0.05$ vs. untreated NT or untreated transfected with the same siRNA; *c*: $P < 0.001$ vs. IFN-γ–treated NT and siCtrl, *d*: $P < 0.001$ and *e*: $P < 0.05$ vs. IL-1β + INF-γ–treated NT and siCtrl, *f*: $P < 0.001$ and *g*: $P < 0.05$ vs. TNF-α + INF-γ–treated NT and siCtrl; ANOVA followed by Student's *t* test with Bonferroni correction.

tion is prevented. This suggests that STAT1 must cross a "nuclear activation threshold" before becoming proapoptotic to β-cells. It remains to be clarified whether this threshold depends on the intensity/length and/or periodicity of STAT1 activation, as previously suggested for nuclear factor κB (33).

The exacerbation of apoptosis in β-cells with decreased PTPN2 expression is independent of augmented NO production. This contrasts with observations reporting that PTPN2-deficient macrophages produce higher amounts of nitric oxide under inflammatory conditions (40,46), highlighting the differential regulation of inflammation-associated genes in cells from different backgrounds.

As discussed above, the transcription factor STAT1 has a major role in cytokine-induced β-cell apoptosis in vitro (18) and in vivo (27). It is thus conceivable that a genetically determined modification in the expression and/or the function of PTPN2 may sensitize β-cells to IFN-γ/STAT1–driven proapoptotic signaling, amplifying β-cell loss under inflammatory conditions, and in some cases, contributing to diabetes. The potential effect of the transient hyper-

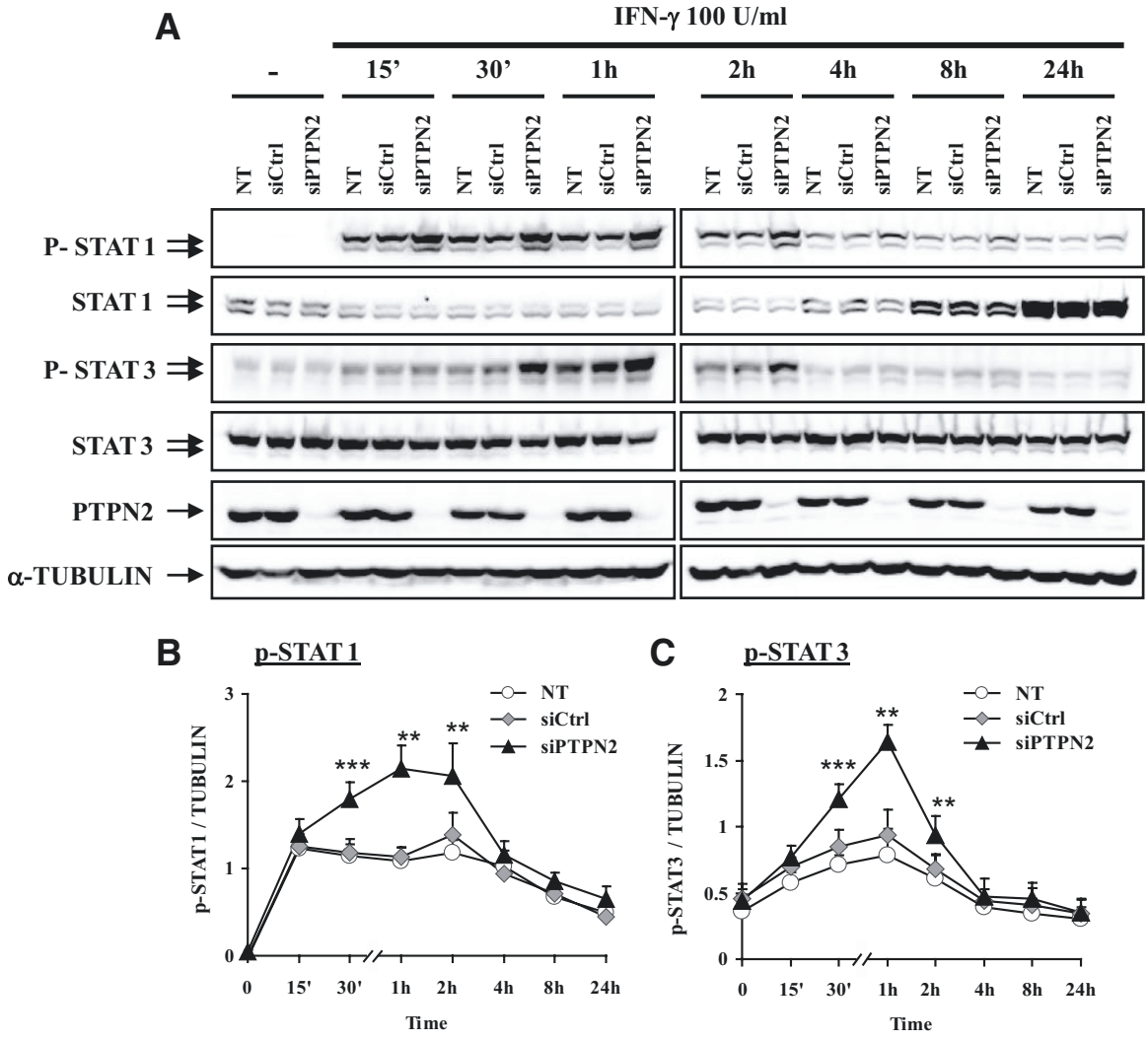

FIG. 5. PTPN2 inhibition increases IFN-γ–induced STAT1 and STAT3 phosphorylation. INS-1E cells were left untransfected (NT) or transfected with 30 nmol/l of either a control siRNA (siCtrl) or with a pool of siRNAs targeting PTPN2 (siPTPN2). After 2 days of recovery, cells were left untreated or treated with IFN-γ (100 units/ml) for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h. *A*: phospho-STAT1, total STAT1, phospho-STAT3, total STAT3, PTPN2, and α-tubulin proteins were evaluated by Western blot. These results are representative of five independent experiments. *B* and *C*: Mean optical density measurements of phospho-STAT1 (*B*) and phospho-STAT3 (*C*) Western blots corrected for protein loading by α-tubulin. Results are means ± SE of five independent experiments; ***P* < 0.01 and ****P* < 0.001 vs. NT and siCtrl at the same time point, ANOVA followed by Student's *t* test with Bonferroni correction.

phosphorylation of STAT3 or IRβ in PTPN2-inhibited cells remains to be clarified. STAT3 has been reported to be dispensable for β-cell development and function (47), but it is generally associated with increased survival of tumoral cells (48), whereas the insulin receptor may exert positive feedback on β-cell function and survival (20).

Over the past few years, several new candidate genes have been identified in human type 1 diabetes. Most of these genes are expressed in the immune system, including major histocompatibility complex (e.g., HLA-DRB1), CTLA4, IL-2Rα (CD25), IFIH1 (MDA5), and several protein tyrosine phosphatases, namely PTPN22, PTPN11, and the recently reported PTPN2 (3,49). Modified function or regulation of these genes could contribute to the development of an autoimmune response. Thus, nonsynonymous single-nucleotide polymorphisms (SNPs) in PTPN22 (human LYP) have been associated with type 1 diabetes and other autoimmune processes, probably secondary to defective deletion of autoreactive T-cells during thymic selection (39). The fact that PTPN22 is not expressed in β-cells (present data) reinforces the likelihood that this is solely due to effects on the immune system. PTPN2-null mice present severe abnormalities in the immune system, resulting in a systemic inflammatory disease and widespread tissue infiltration by mononuclear cells (13,46,50). Moreover, the *PTPN2* gene is associated with several other autoimmune disorders besides type 1 diabetes, including Crohn's disease, ulcerative colitis, and rheumatoid arthritis (49,51,52). It is currently unknown whether the SNPs identified in the PTPN2 gene on chromosome 18p11 will lead to a gain or a loss of function of the protein. PTPN2 overexpression in other cell types has been shown to induce a p53- and caspase 1–dependent apoptosis (53), whereas suppression of PTPN2 sensitize β-cells to cytokine-induced apoptosis (present data). In experiments on INS-1E cells, we observed that DNA vector–based overexpression of PTPN2 induced 30–50% of apoptosis 36 h after transfection, whereas there was <8% apoptotic cells in control-transfected cells (data not shown). These preliminary results require experimental confirmation in primary rat and human β-cells using adenoviral vectors to overex-

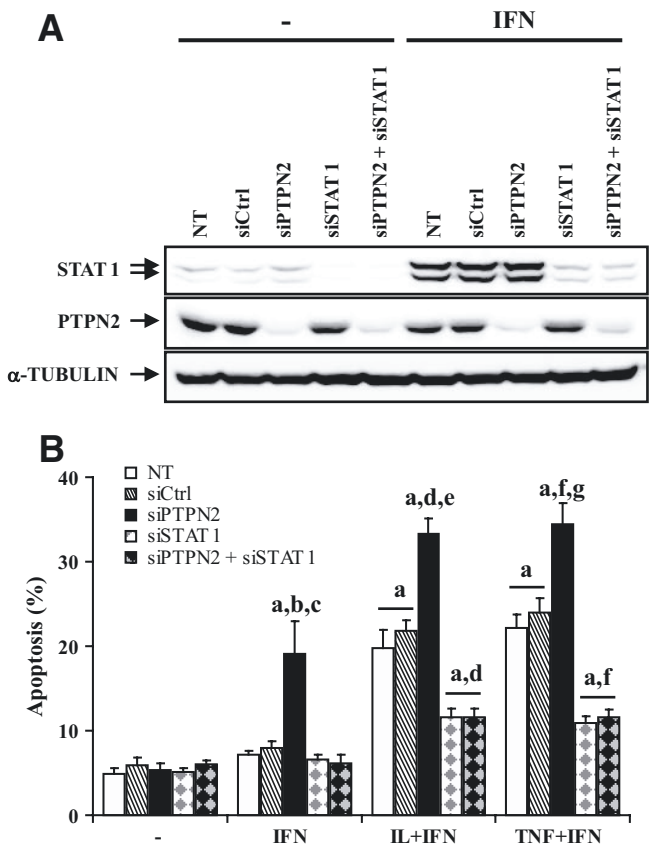

FIG. 6. Double knockdown of PTPN2 and STAT1 protects INS-1E cells from cytokine-induced apoptosis. INS-1E cells were left untransfected (NT) or were transfected with 60 nmol/l of a control siRNA (siCtrl), or with 30 nmol/l of either a pool of siRNAs targeting PTPN2 (siPTPN2), or a siRNA targeting STAT1 (siSTAT1), or double transfected with 30 nmol/l of both siPTPN2 and siSTAT1. After 2 days of recovery, cells were left untreated, or treated for 24 h with IFN-γ (100 units/ml), IL-1β (10 units/ml) + IFN-γ (100 units/ml), or TNF-α (1,000 units/ml) + IFN-γ (100 units/ml) as indicated. *A*: Expression of STAT1, PTPN2, and α-tubulin proteins were evaluated by Western blot. The results are representative of three independent experiments. *B*: Apoptosis was evaluated using HO/PI staining. Results are means ± SE of four independent experiments; *a*: $P < 0.001$ vs. untreated NT or untreated transfected with the same siRNA; *b*: $P < 0.001$ vs. IFN-γ–treated NT and siCtrl; *c*: $P < 0.001$ vs. IFN-γ–treated siSTAT1 and siPTPN2 + siSTAT1; *d*: $P < 0.001$ vs. IL-1β + INF-γ–treated NT and siCtrl; e: $P < 0.001$ vs. IL-1β + INF-γ–treated siSTAT1 and siPTPN2 + siSTAT1; *f*: $P < 0.001$ vs. TNF-α + INF-γ–treated NT and siCtrl; *g*: $P < 0.001$ vs. TNF-α + INF-γ–treated siSTAT1 and siPTPN2 + siSTAT1; ANOVA followed by Student's *t* test with Bonferroni correction.

press PTPN2, but they suggest that PTPN2 must be tightly regulated to avoid deleterious effects.

Pancreatic β-cells themselves may play an active role in the pathogenesis of type 1 diabetes (21), and at least three of the type 1 diabetes–associated genes are expressed in β-cells, namely insulin, IFIH1 (MDA5) (6,54), and PTPN2 (present study). β-Cells have been shown to participate actively in the recruitment and activation of the immune system by secreting various chemokines and cytokines under inflammatory conditions (18,21,55) and by providing "danger signals" to the immune system during apoptosis, especially in the context of local inflammation (56). We presently demonstrate that changes in PTPN2 function in β-cells sensitize these cells to surrounding proapoptotic inflammatory signals (e.g., cytokines), potentially amplifying β-cell loss and insulitis. This suggests that PTPN2, a type 1 diabetes–associated gene, may modulate β-cell apoptosis in early type 1 diabetes independently of its potential effects on the immune system.

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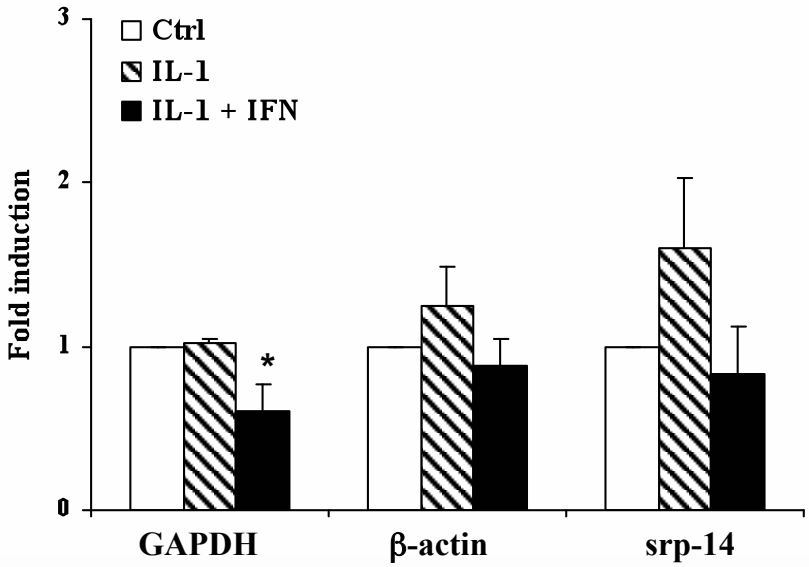
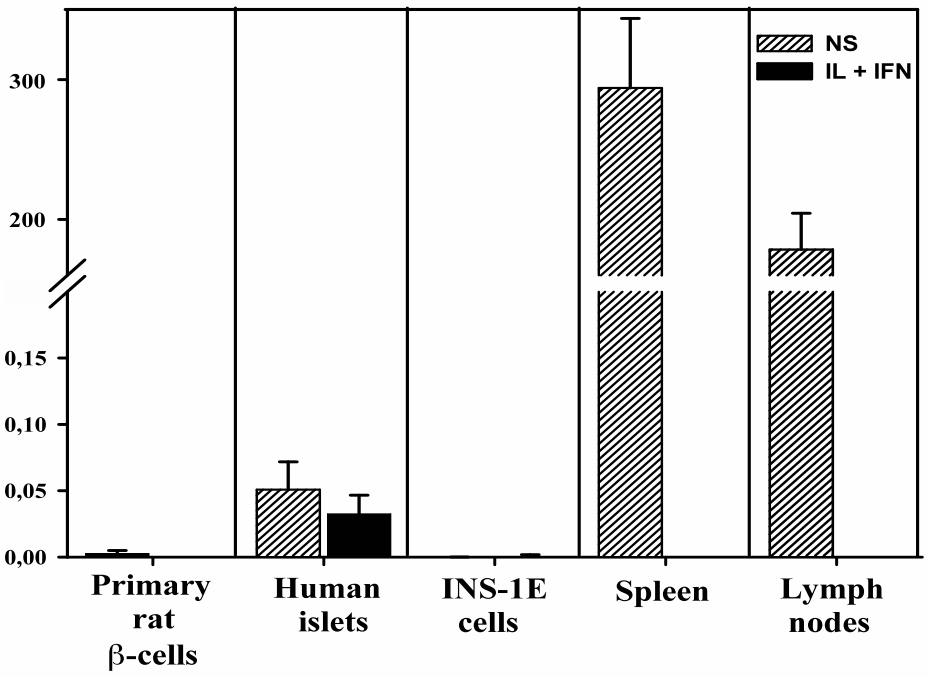
Supplementary material from article I:

PTPN2, a candidate gene for type 1 diabetes, modulates interferon-gamma-induced pancreatic beta cell apoptosis. Moore F, Colli ML, Cnop M, Esteve MI, Cardozo AK, Cunha DA, Bugliani M, Marchetti P, Eizirik DL. *Diabetes*. 2009: 58:1283-91.

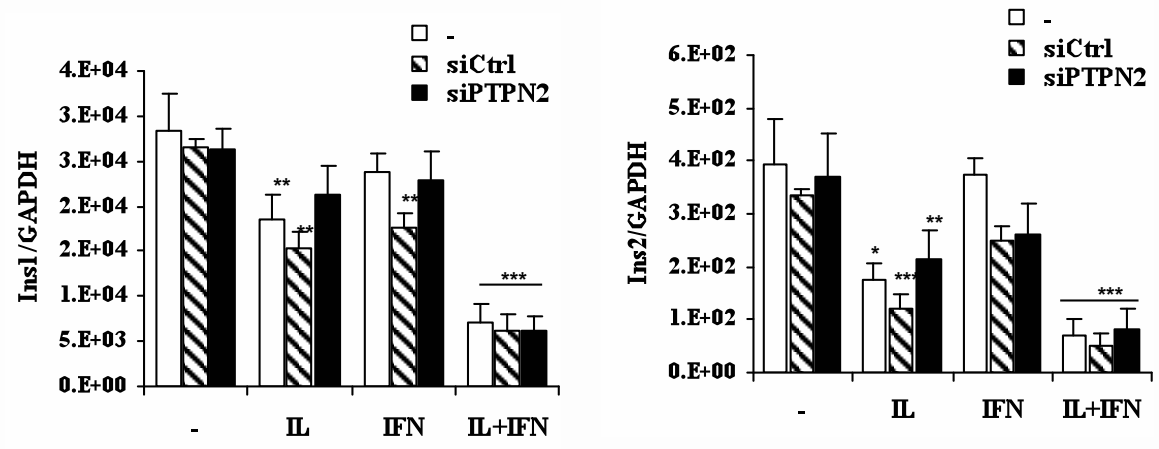
Supplementary Figure A1. Comparison between the expression of different housekeeping genes in INS-1E cells. INS-1E cells were left untreated or treated with either IL-1β (100 U/ml) or IL-1β (10 U/ml) + IFN-γ (100 U/ml) for 24h. GAPDH, β-actin and srp-14 mRNA expression were assayed by real time PCR. Results are mean ± SEM of 4 independent experiments; * $p<0.05$ vs untreated cells by Student's *t* test.



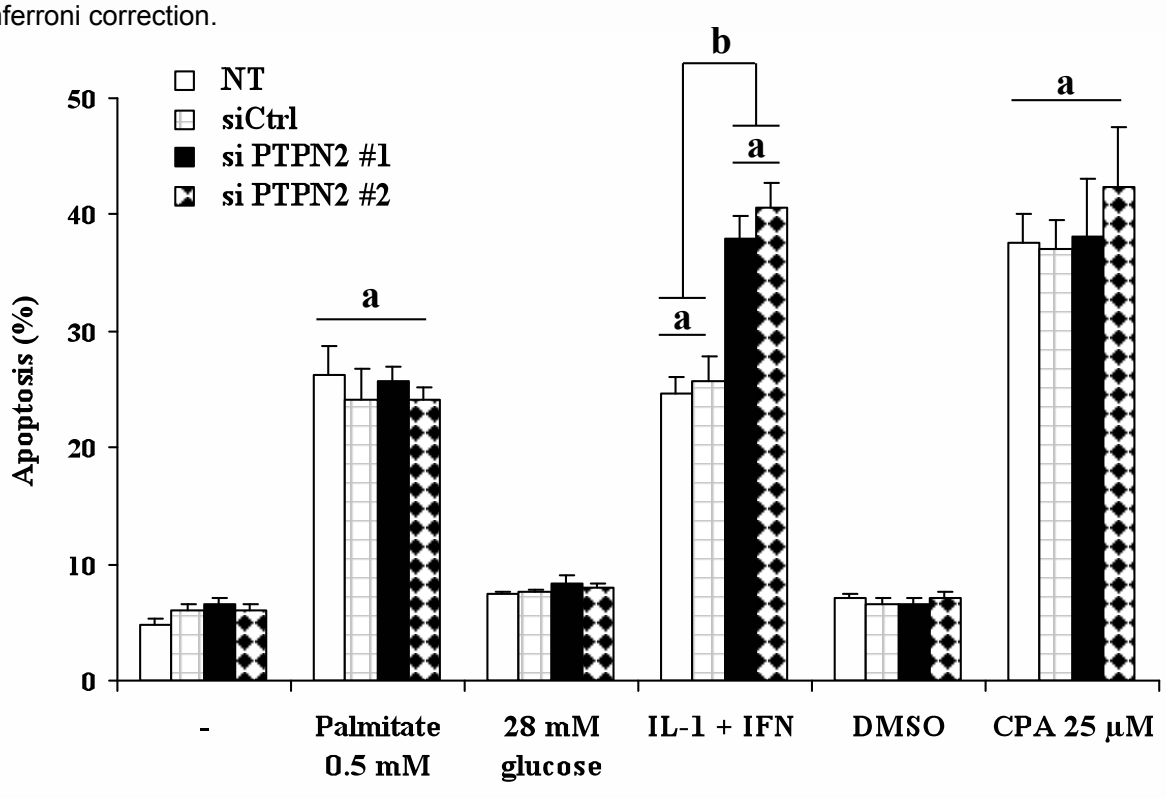
Supplementary Figure A2. PTPN22 is not or poorly expressed in primary FACS-purified rat β-cells, human islets or INS-1E cells. PTPN22 mRNA expression was assayed in the same samples as in Fig. 1 and in rat spleen and lymph nodes, used as positive controls. Results are mean ± SEM of 3-5 independent experiments. N.S., non stimulated controls.



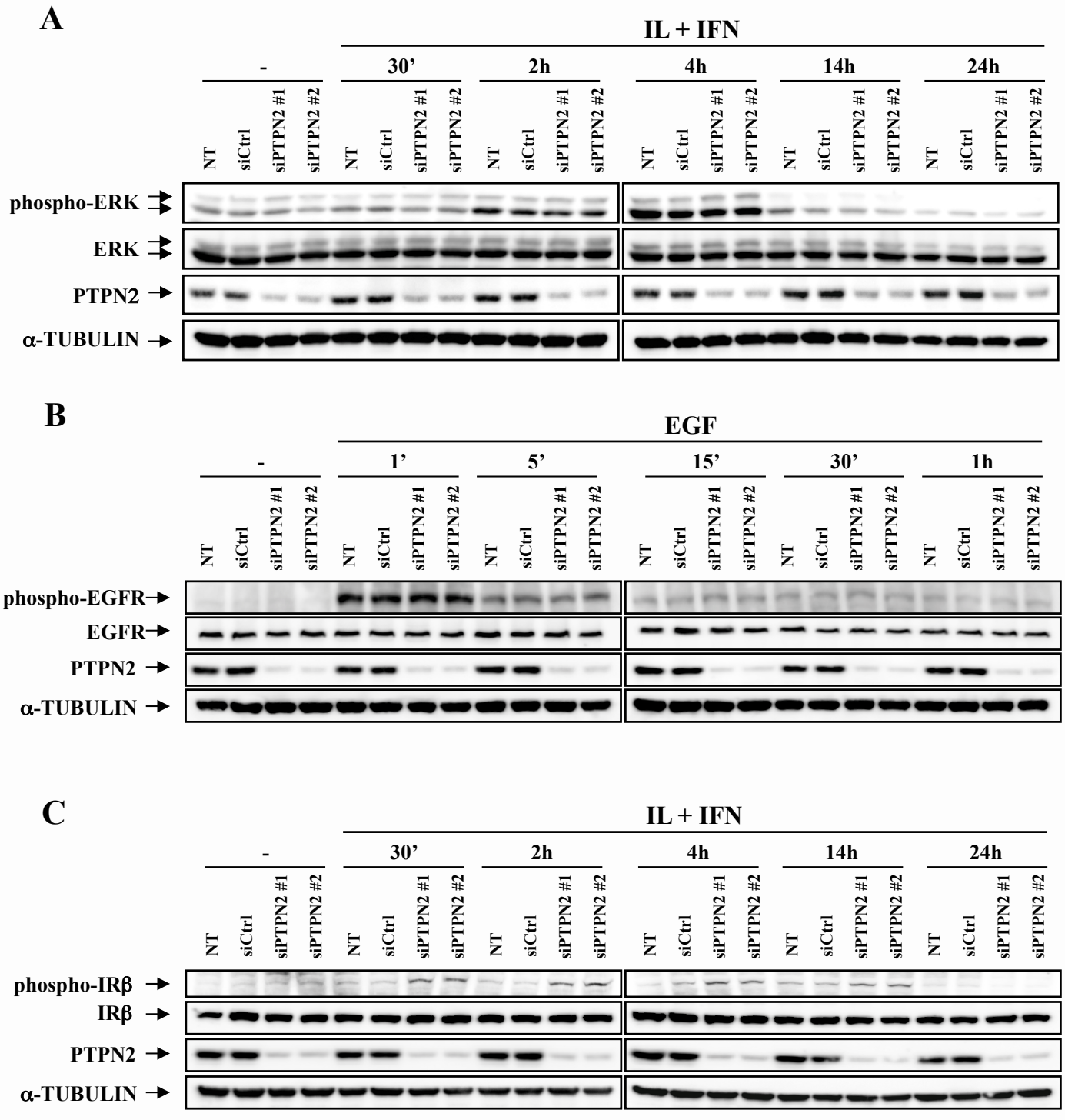
Supplementary Figure A3. PTPN2 inhibition does not influence insulin mRNA expression in INS-1E cells. INS-1E were transfected as described in Fig. 3 and left untreated or treated with either IL-1β (100 U/ml), IFN-γ (100 U/ml) or IL-1β (10 U/ml) + IFN-γ (100 U/ml) for 24h. Insulin 1 and insulin 2 mRNA expression were assayed by RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs untreated cells by Student's *t* test.



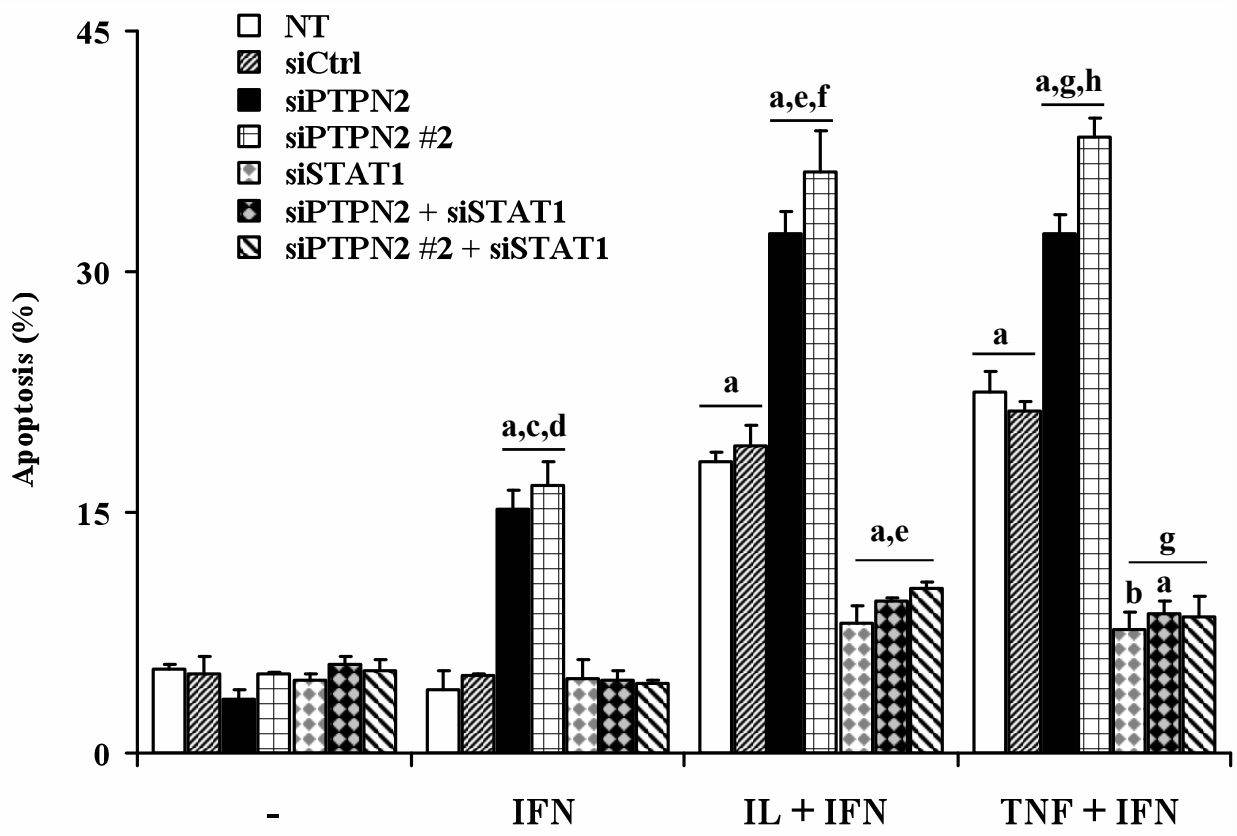
Supplementary Figure A4. PTPN2 inhibition does not exacerbate palmitate or CPA-induced cell death in INS-1E cells. INS-1E cells were left untransfected (NT), or transfected with 30 nM of either siCtrl, siPTPN2 #1 or siPTPN2 #2. After 2 days of recovery, cells were left untreated, or treated for 24h with 0.5 mM palmitate, 28 mM glucose, IL-1β (10 U/ml) + IFN-γ (100 U/ml) or 25 µM CPA as indicated. The control condition for CPA (DMSO) contained a similar dilution of DMSO. Apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of 4 experiments; a: $p<0.001$ vs untreated NT or untreated transfected with the same siRNA; b: $p<0.001$ vs IL-1β + INF-γ-treated NT & siCtrl; ANOVA followed by Student's *t* test with Bonferroni correction.



Supplementary Figure A5. siRNA-mediated PTPN2 knockdown does not influence ERK or EGFR phophorylation, but increases IRβ phosphorylation. INS-1E cells were left untransfected (NT), or were transfected with 30 nM of either siCtrl, siPTPN2 #1 or siPTPN2 #2. After 2 days of recovery, cells were left untreated, or treated for different time points with IL-1β (10 U/ml) + IFN-γ (100 U/ml) or rrEGF (100 ng/ml) as indicated. (A) phospho-ERK and total ERK; (B) phosphor-EGFR and total EGFR; (C) phospho-IRβ and total IRβ were evaluated by Western blot. PTPN2 and α-tubulin proteins were probed to ascertain accurate inhibition and equal loading respectively. Each result is representative of 4 independent experiments.



Supplementary Figure A6. Double knockdown of PTPN2 (by two different siRNAs) and STAT1 protects INS-1E cells from cytokine-induced apoptosis. INS-1E cells were left untransfected (NT), or were transfected with 60 nM of a control siRNA (siCtrl) or with 30 nM of either a pool of siRNAs targeting PTPN2 (siPTPN2), or another siRNA targeting PTPN2 (siPTPN2 #2), or a siRNA targeting STAT1 (siSTAT1), or double transfected with 30 nM of both siPTPN2 and siSTAT1, or siPTPN2 #2 and siSTAT1. After 2 days of recovery, cells were left untreated, or treated for 24h with IFN-γ (100 U/ml), IL-1β (10 U/ml) + IFN-γ (100 U/ml) or TNF-α (1000 U/ml) + IFN-γ (100 U/ml). Apoptosis was then evaluated using HO/PI staining. Results are mean ± SEM of 4 independent experiments; a: $p<0.001$ and b: $p<0.01$ vs untreated NT or untreated transfected with the same siRNA; c: $p<0.001$ vs IFN-γ-treated NT & siCtrl; d: $p<0.001$ vs IFN-γ-treated siSTAT1, siPTPN2 + siSTAT1 & siPTPN2 #2 + siSTAT1; e: $p<0.001$ vs IL-1β + INF-γ-treated NT & siCtrl; f: $p<0.001$ vs IL-1β + INF-γ-treated siSTAT1, siPTPN2 + siSTAT1 & siPTPN2 #2 + siSTAT1; g: $p<0.001$ vs TNF-α + INF-γ-treated NT & siCtrl; h: $p<0.001$ vs TNF-α + INF-γ-treated siSTAT1, siPTPN2 + siSTAT1 & siPTPN2 #2 + siSTAT1; ANOVA followed by Student's *t* test with Bonferroni correction.



Article II

MDA5 and *PTPN2*, two candidate genes for type 1 diabetes, modify pancreatic beta cell responses to the viral by-product double-stranded RNA.

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MDA5 and PTPN2, two candidate genes for type 1 diabetes, modify pancreatic β-cell responses to the viral by-product double-stranded RNA

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β-Cell destruction in type 1 diabetes (T1D) is at least in part consequence of a 'dialog' between β-cells and immune system. This dialog may be affected by the individual's genetic background. We presently evaluated whether modulation of MDA5 and PTPN2, two candidate genes for T1D, affects β-cell responses to double-stranded RNA (dsRNA), a by-product of viral replication. These genes were selected following comparison between known candidate genes for T1D and genes expressed in pancreatic β-cells, as identified in previous array analysis. INS-1E cells and primary fluorescence-activated cell sorting-purified rat β-cells were transfected with small interference RNAs (siRNAs) targeting MDA5 or PTPN2 and subsequently exposed to intracellular synthetic dsRNA (polyinosinic–polycitidilic acid—PIC). Real-time RT–PCR, western blot and viability assays were performed to characterize gene/protein expression and viability. PIC increased MDA5 and PTPN2 mRNA expression, which was inhibited by the specific siRNAs. PIC triggered apoptosis in INS-1E and primary β-cells and this was augmented by PTPN2 knockdown (KD), although inhibition of MDA5 did not modify PIC-induced apoptosis. In contrast, MDA5 silencing decreased PIC-induced cytokine and chemokine expression, although inhibition of PTPN2 induced minor or no changes in these inflammatory mediators. These findings indicate that changes in MDA5 and PTPN2 expression modify β-cell responses to dsRNA. MDA5 regulates inflammatory signals, whereas PTPN2 may function as a defence mechanism against pro-apoptotic signals generated by dsRNA. These two candidate genes for T1D may thus modulate β-cell apoptosis and/or local release of inflammatory mediators in the course of a viral infection by acting, at least in part, at the pancreatic β-cell level.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease with a strong inflammatory component. Islet inflammation (insulitis) probably takes place in the context of a 'dialog' between invading immune cells and the target β-cells. This dialog is partially mediated by cytokines and chemokines released by both β-cells and immune cells and by immunogenic signals delivered by dying β-cells. This leads to induction and amplification or, in some cases, resolution of insulitis (1). The evolution of islet inflammation, and its potential progression to clinical diabetes, probably depends on the interplay between the patient's genetic background and environmental triggers, such as viral infections and/or dietetic components (1–4).

Identification of genetic-based pathways for complex diseases, such as T1D, provides the initial framework for investigations of environmental influences on a given genetic background (5). The relevance of this approach has already been shown in rheumatoid arthritis (6), and was recently confirmed in the context of T1D by a study showing interaction between polymorphisms in the candidate gene PTPN22 and the early introduction of cow's milk in the emergence of islet autoantibodies and diabetes in a Finnish population (7). These population studies, however, cannot clarify the molecular mechanisms involved in the interactions between the

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genetic background and environmental factors. To address this issue in the context of candidate genes that may affect pancreatic β-cell survival and insulitis development in T1D, we used a three-pronged strategy.

(1) Compare the list of known candidate genes for T1D (8–12) with genes expressed in pancreatic β-cells and modified by inflammatory cytokines and/or double-stranded (ds) RNA/virus as determined by our previous microarray analysis (13–18). We observed that at least 30% of the candidate genes for T1D are expressed in β-cells (data not shown), confirming that these cells may have an active role in the emergence of insulitis (1). Two of the identified candidate genes were of particular interest, namely MDA5 (melanoma differentiation-associated gene 5; also known as *IFIH1*) and PTPN2 (protein tyrosine phosphatase N2; also known as TC-PTP or PTP-S2). MDA5 is a cytoplasmic receptor for viral nucleic acids involved in the innate immune response to viruses (19). Rare polymorphisms of MDA5, leading to inhibited function, decrease the risk of T1D by nearly 50% (20). Since MDA5 is a member of the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, we investigated in parallel the role of the other helicase, RIG-I, previously shown to be involved in the recognition of cytoplasmic 5′-triphosphate single-stranded RNA and double-stranded RNA (dsRNA) (21), and of some of the key signalling patterns involved in dsRNA-induced β-cell apoptosis. The phosphatase PTPN2 functions as a negative regulator of several signalling pathways, including Janus kinases (JAKs), signal transducer and activator of transcription (STATs), p42/44 MAPK (ERK), epidermal growth factor receptor and insulin receptor β, and modulates β-cell apoptosis induced by interferon (IFN)-γ (18);
(2) Design specific small interference RNAs (siRNAs) targeting these genes to evaluate their function in β-cells, with special focus on the induction of apoptosis and production of inflammatory cytokines and chemokines;
(3) Expose β-cells transfected with siRNAs targeting the candidate genes to intracellular double-stranded (ds) RNA. dsRNA is a by-product generated during replication and transcription of both RNA and DNA viruses and an efficient inducer of apoptosis, type I interferons and other cytokines/chemokines important for the host immune response to viral infection (22,23). We thus aimed to model *in vitro* and under well-controlled conditions the putative genetic/environmental interactions that may take place in early T1D.

The data obtained suggest that MDA5 and PTPN2 are induced by dsRNA in pancreatic β-cells. Of interest, blocking MDA5 expression prevents dsRNA-induced expression of cytokines and chemokines, important mediators of insulitis. PTPN2 seems to play a different role in this process, since PTPN2 silencing sensitized β-cells to dsRNA-induced apoptosis but had limited effects on the expression of inflammatory mediators. These observations indicate that two candidate genes for T1D may act, at least in part, at the β-cell level, modulating apoptosis and the generation of inflammatory signals in the course of a viral infection.

RESULTS

siRNAs against MDA5 and RIG-I prevent polyinosinic–polycitidilic acid-induced activation of interferon β/NF-κB and chemokines, but not apoptosis in insulin-producing cells

We used the synthetic dsRNA polyinosinic–polycitidilic acid (PIC) with different lengths (21) to selectively evaluate the role of MDA5 and RIG-I (Supplementary Material, Fig. S1A). The largest PIC (>2000 bp; PIC2) preferentially induced MDA5, whereas the PIC with <2000 bp (PIC1) induced mostly RIG-I (data not shown). Both forms of PIC induced a significant increase in apoptosis in INS-1E cells detected 12 h after exposure [Supplementary Material, Figs S1B and S2C; there was no increase in apoptosis at 6 h (data not shown)]. Transfection with respective PICs increased the expression of MDA5 (Fig. 1A and Supplementary Material, Fig. S2A) and RIG-I (Fig. 1B) by 23- and 40-fold after 24 h. To evaluate the functional role of MDA5 and RIG-I, INS-1E cells were pre-treated either with an inactive siRNA (siControl) or with siRNAs targeting MDA5 (siMDA5) or RIG-I (siRIG-I). The siControl has been validated by previous studies from our group (18,24), and a recent array analysis indicated that it does not modify the expression of MDA5, RIG-I or other genes related to β-cell function and survival (unpublished data). PIC-induced MDA5 or RIG-I expression was prevented by >80% by the specific siRNAs against MDA5 or RIG-I, but not by the siControl (Fig. 1A and B). To further confirm the efficiency of these KDs, we performed reporter assays using an IFN-β promoter reporter or a reporter containing multiple NF-κB-binding sites. Both reporters were activated by PIC, and MDA5 KD partially inhibited their activation (Fig. 1C and D). Similar results were observed with siRNA-mediated inhibition of RIG-I, with the difference that siRIG-I did not prevent NF-κB induction (Supplementary Material, Fig. S3A and B). We additionally studied the effect of knocking down these helicases in PIC-induced apoptosis. Both PICs induced apoptosis, but neither MDA5 nor RIG-I silencing prevented cell death 24 h after PIC transfection (Fig. 1E and F). To exclude that this absence of protection was due to redundancy between the two helicases (i.e. MDA5 would compensate for the absence of RIG-I in the recognition of dsRNA and vice versa), we performed double-KD of MDA5 and RIG-I but this also failed to prevent PIC-induced apoptosis (data not shown). This suggests that both helicases are not crucial regulators of intracellular PIC-induced β-cell death.

One of the mechanism by which viruses may contribute to T1D is by promoting the expression of pro-inflammatory mediators (1,25). Against this background, we next examined the effect of MDA5 and RIG-I KD on PIC-induced expression of cytokines and chemokines in INS-1E cells. There was a marked induction of the expression of mRNAs encoding for the cytokines IFN-β and interleukin (IL) 15 and the chemokines CCL2, CCL5 and CXCL10 after 24 h of PIC exposure compared with untreated cells (Fig. 2A and B). MDA5 KD significantly reduced PIC-induced expression of all cytokines and chemokines studied, whereas RIG-I KD decreased PIC-induced expression of IFN-β, CCL2 and CXCL10, but not IL15 or CCL5 (Fig. 2B). A similar pattern was observed

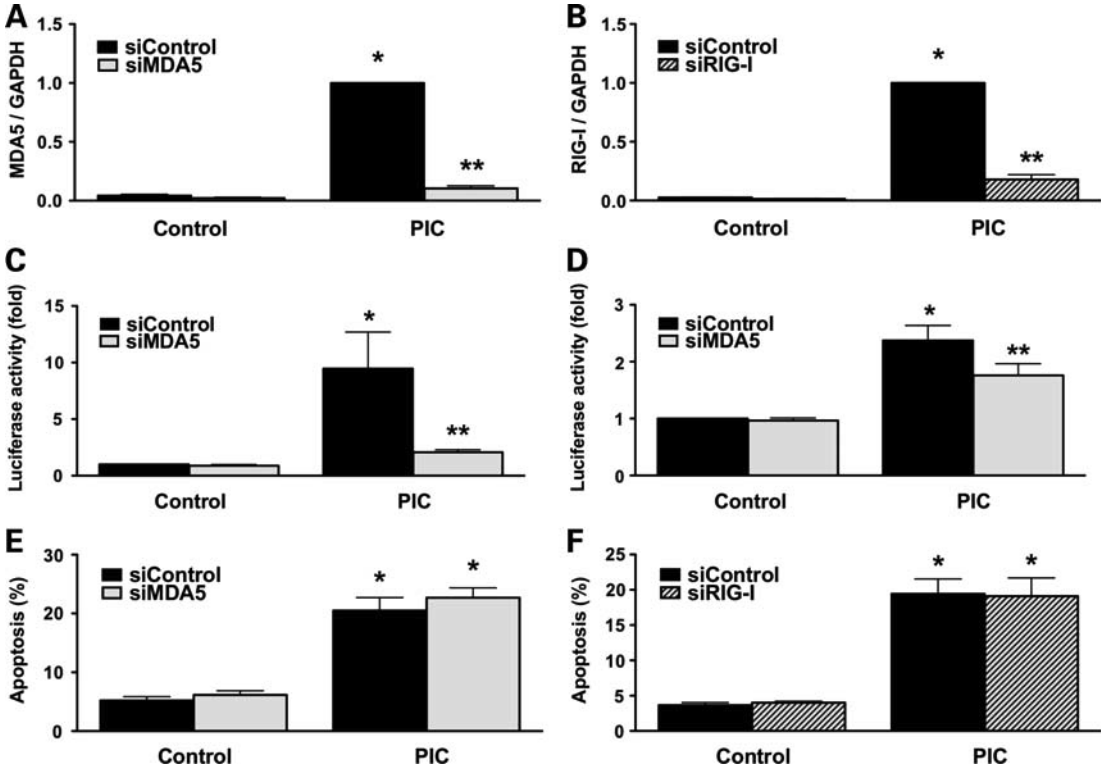

Figure 1. siMDA5 and siRIG-I prevent PIC-induced activation of interferon-β promoter and NF-κB reporter, but do not modify INS-1E cell death. INS-1E cells were transfected with either an siControl (black bars), or siMDA5 (grey bars) or siRIG-I (striped bars). After 24 h of recovery, cells were left untreated or transfected with PIC1 (<2000 bp, used for siRIG-I experiments) or PIC2 (>2000 bp, siMDA5 experiments) as described in Materials and Methods. (**A** and **B**) MDA5 and RIG-I mRNA expression was assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC; ANOVA. (**C** and **D**) Twenty-four hours after transfection with siMDA5, cells were transfected with IFN-β (C) or NF-κB (D) reporters plus a pRL-CMV plasmid (used as internal control); cells were then exposed to internal PIC for 24 h and luciferase activity was assayed. The values were corrected for the activity of the internal control, pRL-CMV, and are presented as fold induction in relation to siControl. Results are mean ± SEM of 5–11 independent experiments; $^{*}P < 0.05$ versus siControl, $^{**}P < 0.05$ versus siControl+PIC, paired *t*-test. (**E** and **F**) INS-1E cells were transfected with siMDA5 or siRIG-I and then PIC as described earlier. Apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of five independent experiments; $^{*}P < 0.01$ versus siControl; ANOVA.

at the protein level for CCL5 (Fig. 3). These observations were confirmed by the use of a second siRNA against MDA5 (Supplementary Material, Fig. S4).

Primary β-cells, different from INS-1E cells, express an additional receptor for dsRNA, namely the toll-like receptor 3 (TLR3) (26), potentially creating a more complex response to dsRNA. Similar to INS-1E cells, PIC induced a marked increase of MDA5 and RIG-I expression in primary β-cells. siMDA5 and siRIG-I prevented PIC-induced MDA5 and RIG-I mRNA expression by >80% (Fig. 4A and B). In line with the observations made in INS-1E cells, KD of both helicases did not modify β-cells apoptosis after PIC exposure (Fig. 4C and D). Expression of mRNAs encoding for IFN-β, IL15, CCL2, CCL5 and CXCL10 was also induced in β-cells, and this was partially prevented by KD of MDA5, except for IL15 (Fig. 4E). On the other hand, RIG-I KD failed to prevent PIC-induced expression of the cytokines and chemokines studied (Fig. 4F). These findings were confirmed at the protein level for the chemokine CCL5 (Supplementary Material, Fig. S5A and B), and as whole they suggest that MDA5 plays a more important role than RIG-I for the recognition of intracellular dsRNA in β-cells.

Mechanisms contributing for PIC-induced apoptosis in insulin-producing cells

Since KD of the helicases MDA5 and RIG-I failed to prevent β-cell apoptosis induced by intracellular dsRNA (Figs 1 and 4), we next examined the putative role of the dsRNA-dependent protein kinase (PKR) in this process. PKR was previously shown to contribute for external dsRNA+-IFN-γ-induced apoptosis in mouse islet cells (27). PIC induced a 20-fold increase in PKR expression, which was prevented by >80% using a specific siRNA against PKR (Supplementary Material, Fig. S6A). PKR KD, however, did not prevent PIC-induced apoptosis (Supplementary Material, Fig. S6B) or the induction of cytokines or chemokines (Supplementary Material, Fig. S6C) in primary β-cells. Unexpectedly, PKR KD augmented PIC-induced CCL2 expression (Supplementary Material, Fig. S5C).

These, and our previous observations, suggest that the pattern recognition receptors TLR3 (28), MDA5, RIG-I and PKR (present data) are not the main mediators of internal dsRNA-induced β-cell apoptosis. Of note, we have previously shown that TLR-3 plays a key role for β-cell apoptosis triggered by external PIC+IFN-γ (26). To further clarify

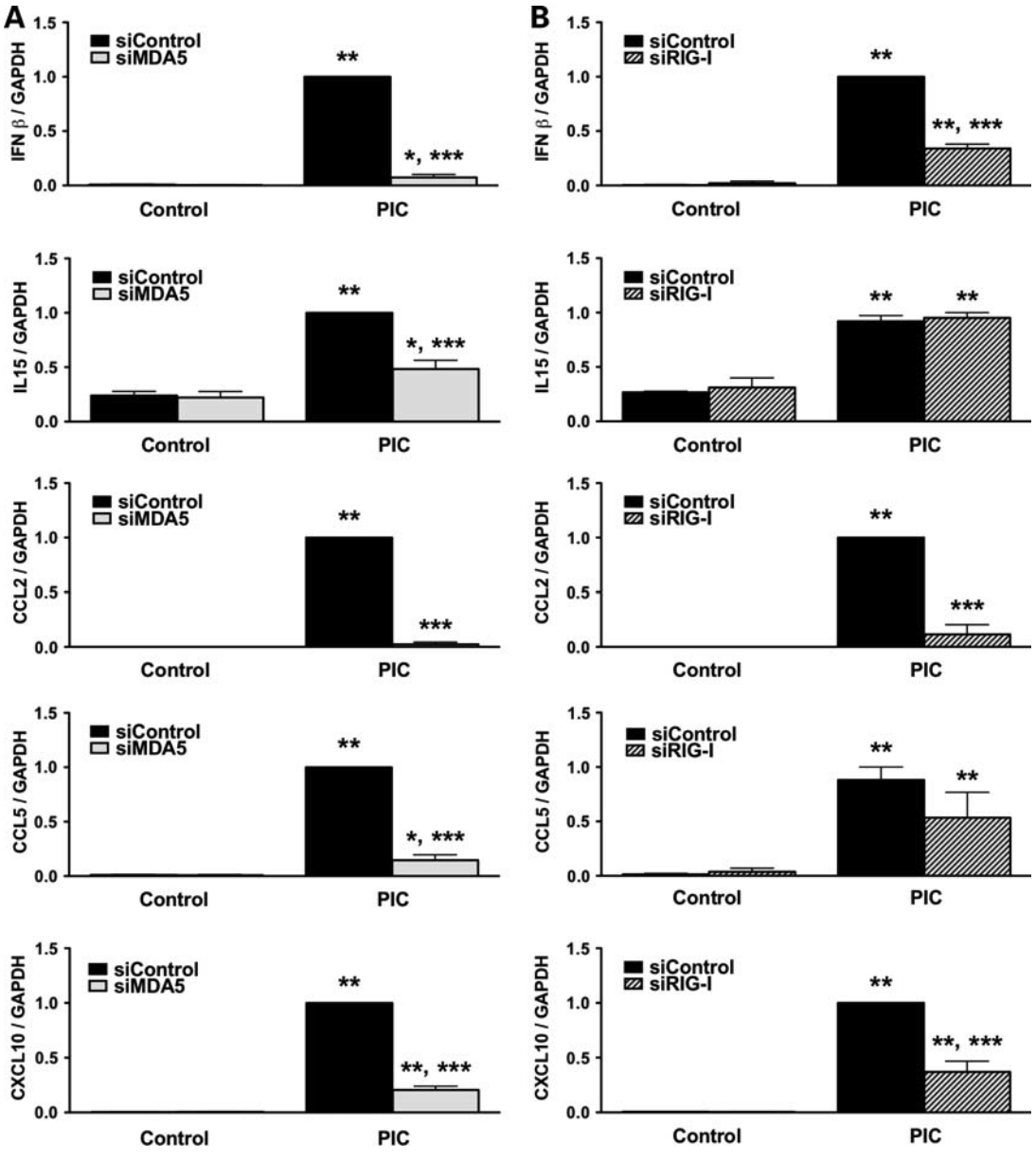

Figure 2. MDA5 or RIG-I KD partially prevents PIC-induced upregulation of mRNA expression of cytokines and chemokines in INS-1E cells. INS-1E cells were transfected with siControl (black bars), siMDA5 (grey bars) or siRIG-I (striped bars), and after recovery left untreated or exposed to different internal PICs (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments) for 24 h. Poly(A)+ mRNA was extracted, assayed by real-time RT–PCR for rat IFN-β, IL15, CCL2, CCL5 and CXCL10 mRNAs and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.05$ versus siControl, $^{**}P < 0.01$ versus siControl, $^{***}P < 0.01$ versus siControl+PIC, ANOVA.

the mechanisms involved in internal PIC-induced β-cell apoptosis, we next examined the downstream apoptosis regulators Jun N-terminal kinase (JNK)/JunB (29). PIC transfection induced concomitant JNK phosphorylation and decreased JunB protein expression (Fig. 5A and B). To investigate whether the decline in JunB expression was secondary to JNK activation, as previously shown in other experimental models (30), we used the JNK inhibitor SP600125. SP600125 decreased PIC-induced JNK phosphorylation and prevented PIC-induced decline in JunB protein expression (Fig. 5C). We have previously observed a similar phenomenon in β-cells treated with cytokines (29), suggesting that JunB degradation is indeed secondary to JNK activation in β-cells.

JNK activation is one of the mechanisms by which dsRNA induces apoptosis in other cell types (31). In line with these observations, two different JNK inhibitors, namely the chemical inhibitor SP600125 and the D-TAT JNKi peptide (32), induced a partial protection against PIC-induced apoptosis in INS-1E cells (Fig. 5D and E). JunB reduces cytokine-mediated endoplasmic reticulum (ER) stress and prevents apoptosis in pancreatic β-cells (29); since one of the effects of JNK activation is to reduce JunB protein levels (Fig. 5B and C), we next examined whether decreased JunB expression indeed contributes for PIC-induced apoptosis. For this purpose, JunB was knocked down by the use of a specific and previously validated siRNA (29). KD of JunB (Fig. 6A and B)

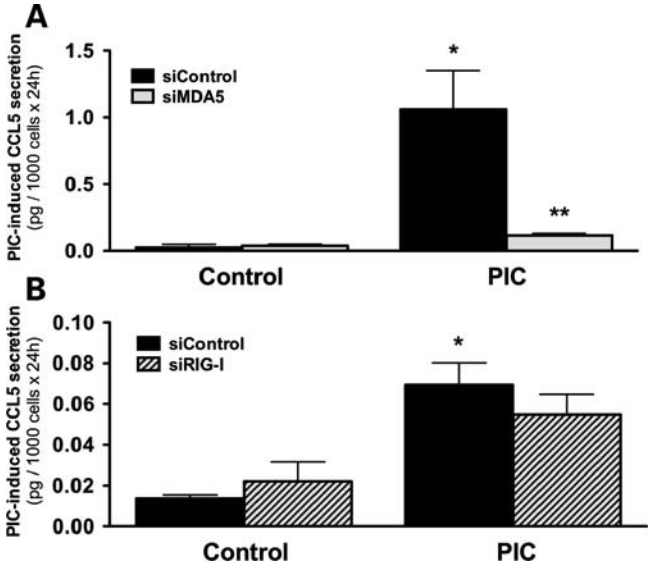

Figure 3. MDA5 but not RIG-I KD prevents PIC-induced CCL5 secretion in INS-1E cells. INS-1E cells were transfected with siControl (black bars), siMDA5 (**A**, grey bars) or siRIG-I (**B**, striped bars) and then treated as described in Figure 2. CCL5 secretion was evaluated in the supernatant by ELISA 24 h after internal PIC exposure (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments), as described in Materials and Methods. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

increased caspase-3 cleavage (Supplementary Material, Fig. S7) and aggravated PIC-induced apoptosis in both INS-1E cells and autofluorescence-activated cell sorting (FACS)-purified β-cells (Fig. 6C and D). These results were confirmed with a second siRNA against JunB (data not shown). To determine whether increase in cell death was NO- and ER stress dependent, we evaluated the mRNA expression of XBP1s, CHOP and iNOS, and nitrite production. There was no difference between expression of these markers in cells treated with siJunB or siControl plus PIC (data not shown), suggesting that JunB protects β-cells against PIC-induced apoptosis by an ER stress- and NO-independent mechanism that remains to be clarified.

JunB is one of the subunits of the transcription factor activator protein-1 (AP-1). Besides its role in the process of apoptosis, AP-1 also regulates the expression of cytokines and chemokines in other cell types (33). We did not, however, observe any effect of JunB KD on PIC-induced expression of cytokines and chemokines (Supplementary Material, Fig. S8).

PTPN2 modulates PIC-induced β-cell apoptosis

PIC induced expression of PTPN2 at both mRNA and protein levels in INS-1E cells and FACS-purified rat β-cells (Fig. 7 A, B and E). The upregulation of PTPN2 mRNA was significant after 6 h of PIC transfection and remained increased up to 24 h (Supplementary Material, Fig. S2B). A previously validated siRNA against PTPN2 (18) decreased its basal mRNA expression by >60 and >80% in INS-1E and primary β-cells, respectively, and blocked PTPN2 induction after PIC exposure in both cell types (Fig. 7A and B). Of relevance, PTPN2 KD sensitized INS-1E cells (Fig. 7C and F) and primary β-cells (Fig. 7D) to PIC-induced apoptosis, indicating a potential modulatory function for this candidate gene in β-cell death during viral infection. On the other hand, PTPN2 had no or mild effect on the expression of chemokines and cytokines following transfection with PIC (Supplementary Material, Fig. S9). These results were confirmed with a second siRNA against PTPN2 (data not shown).

DISCUSSION

We presently show that two candidate genes for T1D, namely MDA5 and PTPN2, modulate β-cell responses to dsRNA, a by-product of viral infection. The findings suggest that MDA5 controls the local expression of cytokines and chemokines during a viral infection, whereas PTPN2 might protect β-cells from virus-induced apoptosis.

Viruses are one of the putative environmental factors associated with T1D in genetically susceptible individuals (1,2). As the first line of defence against pathogens, non-immune cells (present data) and cells from the innate immune system detect viral infections by pattern-recognition receptors (PRRs) that identify pathogen-associated molecular patterns, such as dsRNAs. Both RNA and DNA viruses produce dsRNA as an intermediate product of their replication. Human and rodent pancreatic islets express PRRs for dsRNA, namely TLR3, RIG-I and MDA5 (17,26,34,35; present data). Studies in knockout animals demonstrated that the helicases RIG-I and MDA5 recognize different types of viruses, with MDA5 acting as the main receptor for cytoplasmic dsRNA in astrocytes (36) and β-cells (present data). Of particular relevance in the context of T1D, MDA5 recognizes members of the picornavirus family, including Coxsackievirus B4, Encephalomyocarditis virus and Theiler's virus (37). Coxsackievirus B4 was recently isolated from the pancreas of patients with new onset T1D (38,39), and polymorphisms leading to loss of function of MDA5 confer resistance against T1D (20).

To study the mechanisms by which changes in MDA5 function may contribute to insulitis and β-cell apoptosis, we combined the use of siRNA-mediated inhibition of MDA5 with transfection of β-cells with synthetic dsRNA (PIC). Decrease in the expression of MDA5 or the related helicase RIG-I did not protect β-cells against PIC-induced apoptosis, but KD of MDA5 inhibited expression of the inflammatory mediators CXCL10, CCL2, CCL5, IFN-β and IL15. The chemokine CXCL10 attracts monocytes, T lymphocytes and natural killer (NK) cells (40), and islet-specific expression of CXCL10 in a mouse model of autoimmune diabetes caused by viruses [rat insulin promotor (RIP)-LMCV] accelerates autoimmunity by enhancing the migration of antigen-specific lymphocytes (41). On the other hand, neutralization of CXCL10 (42) or its receptor (CXCR3) (43) prevents autoimmune disease in the same mouse model (RIP-LCMV). CCL2 attracts monocytes and T lymphocytes and may thus contribute to the migration of mononuclear cells to pancreatic islets in early T1D (44). CCL5 attracts activated T-cells, dendritic cells and monocytes during inflammation and early immune responses (45). Individuals with specific polymorphisms in this gene present lower

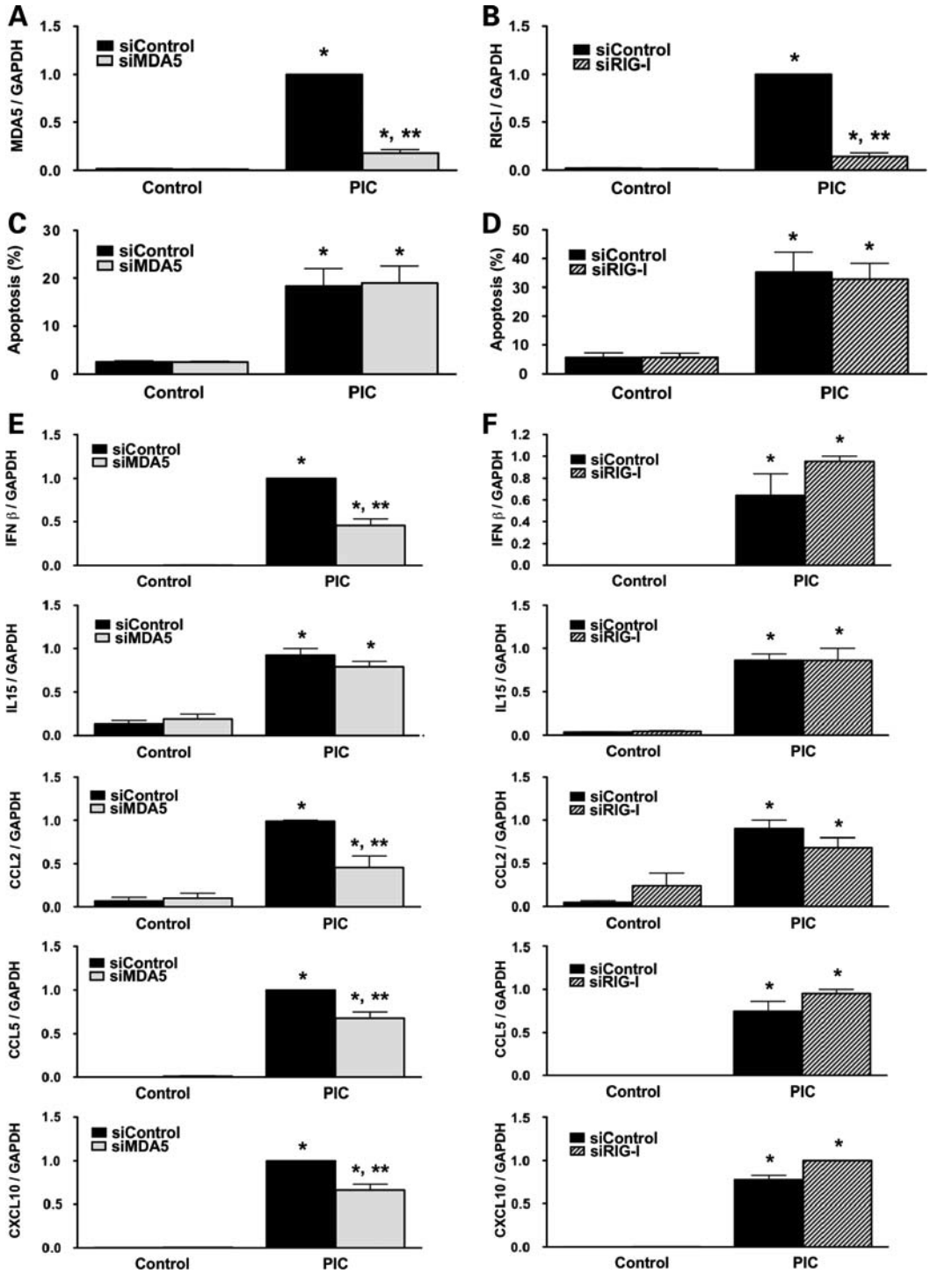

Figure 4. siMDA5 or siRIG-I prevent PIC-induced upregulation of cytokines and chemokines, but not apoptosis, in primary rat β-cells. FACS-purified rat β-cells were transfected with siControl (black bars), siMDA5 (grey bars) or siRIG-I (striped bars) as described in Materials and Methods. After 48 h of recovery, cells were left untreated or transfected with different PICs (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments) for 24 h. (**A** and **B**) MDA5 and RIG-I mRNA expression was assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (**C** and **D**) Apoptosis was evaluated 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of three to five independent experiments; $^{*}P < 0.01$ versus siControl, ANOVA. (**E** and **F**) INF-β, IL15, CCL2, CCL5 and CXCL10 mRNA expression were assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

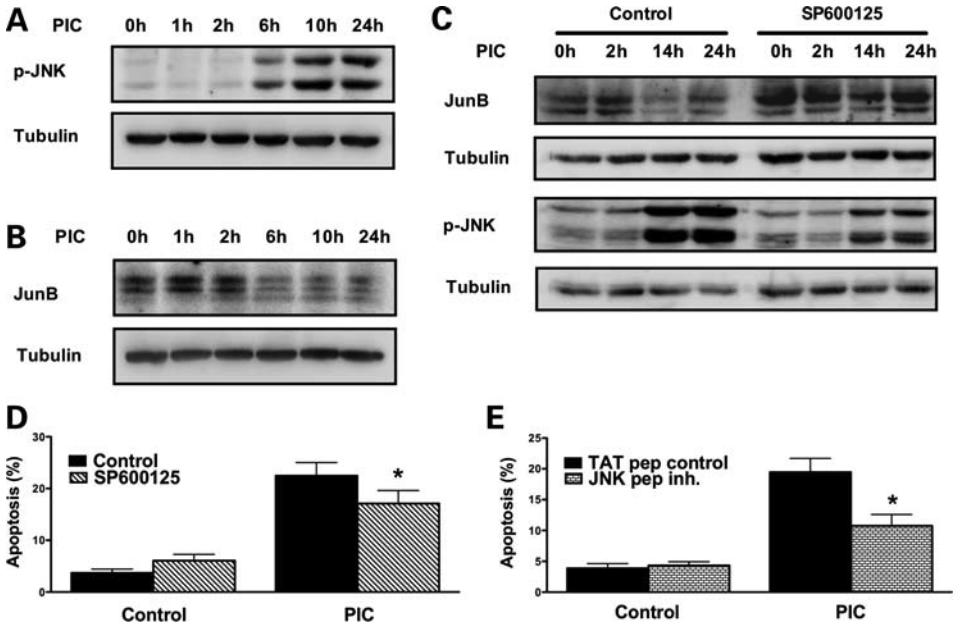

Figure 5. Inhibition of JNK activation partially prevents PIC-induced JunB degradation and apoptosis in INS-1E cells. INS-1E cells were transfected with PIC1 for the indicated time points. (**A** and **B**) Expression of phospho-JNK, JunB and α-tubulin were evaluated by western blot. Pictures are representative of three independent experiments. (**C**) INS-1E cells were pre-treated with 10 μM of the JNK inhibitor SP600125 for 4 h and then transfected with PIC before being retrieved for protein extraction at the indicated time points; SP600125 was maintained in the medium following PIC treatment. Phospho-JNK, JunB and α-tubulin protein were evaluated by western blot. Pictures are representative of three independent experiments. (**D** and **E**) INS-1E cells were treated with the JNK chemical inhibitor SP600125 (D) or the JNK peptide inhibitor (E) for 4 h, and then transfected with PIC plus SP600125 or JNK peptide inhibitor. Apoptosis was evaluated 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of four independent experiments; $^{*}P < 0.05$ versus siControl+PIC, paired *t*-test.

serum levels of CCL5 and may be protected against T1D (46). IL15 enhances the cytolytic function of T and NK cells and induces IFN-γ production by NK cells; it also functions as a potent growth factor of T-, B-, and NK cells (47). Studies in NOD mice have demonstrated elevated expression of CXCL10, CCL2 and IL15 mRNAs and/or protein in pancreatic islets during the prediabetic stage (1,48,49). The type I interferon IFN-β is a cytokine produced by most cells during viral infections, promoting the expression of diverse genes relevant for the antiviral response in target cells; and it also modulates the adaptive immune system by activating dendritic cells, T- and B-cells (23,50). Of relevance, type I IFNs have been detected in the islets of patients with recent onset T1D (51,52), and transgenic expression of IFN-β in mouse β-cells leads to diabetes (53). The present observation that MDA5 modulates dsRNA-induced expression of these cytokines and chemokines in INS-1E cells and primary β-cells reinforces the hypothesis that this candidate gene has a key role in the regulation of local islet inflammation during viral infection.

Since there was no protection against PIC-induced apoptosis after MDA5 and/or RIG-I KD in our model, we searched for alternative pathways that may explain the pro-apoptotic effects of intracellular PIC in β-cells. The main finding was that JNK, an activator of the transcription factor AP-1, contributes for apoptosis after PIC transfection, an effect mediated at least in part via JunB protein degradation. The role of JNK activation in PIC-induced apoptosis has been shown previously in HeLa cells (31), but to our knowledge there are no previous reports on the putative role for JunB degradation on this process. JunB degradation also plays a role in cytokine-induced β-cell apoptosis (29), emphasizing the relevant role of JunB in preserving β-cell viability. In the case of cytokine-triggered apoptosis, however, part of the protective effects of JunB are due to inhibition of NO formation and induction of ER stress (29). NO and ER stress do not play a major role for intracellular PIC-induced β-cell death (data not shown), suggesting that JunB acts by another mechanism in this context. The nature of this mechanism remains to be clarified.

As mentioned earlier, type I IFNs are important cytokines for the host immune response against viral infections (23). In addition to antiviral properties, these cytokines have the potential to induce apoptosis and/or promote systemic autoimmunity (50). To limit these effects of IFNs, organisms have developed several negative regulators of the IFN responses (54). One of these regulators is the phosphatase PTPN2, which prevents sustained IFN signalling by dephosphorylating JAKs and STATs (55). We have recently shown that KD of PTPN2 in pancreatic β-cells exposed to IFN-γ augments STAT1 signalling and increases apoptosis (18). In line with these findings, STAT1 knockout prevents both IL-1β+IFN-γ (56) and external dsRNA plus IFN-γ-induced apoptosis in β-cells (26) and abolishes the pro-apoptotic effect of PTPN2 KD in the presence of IFN-γ (18). We presently demonstrate that, similar to cytokines, PTPN2 KD exacerbates dsRNA-induced apoptosis, suggesting a potential interaction between an environmental agent (dsRNA, a viral by product) and a candidate gene for T1D (PTPN2). This type of interaction may explain why viral infections only cause diabetes in some individuals or mouse strains that overreact to the viral challenge. In line with this hypothesis, PIC only induces diabetes in C57BL/6 mice when

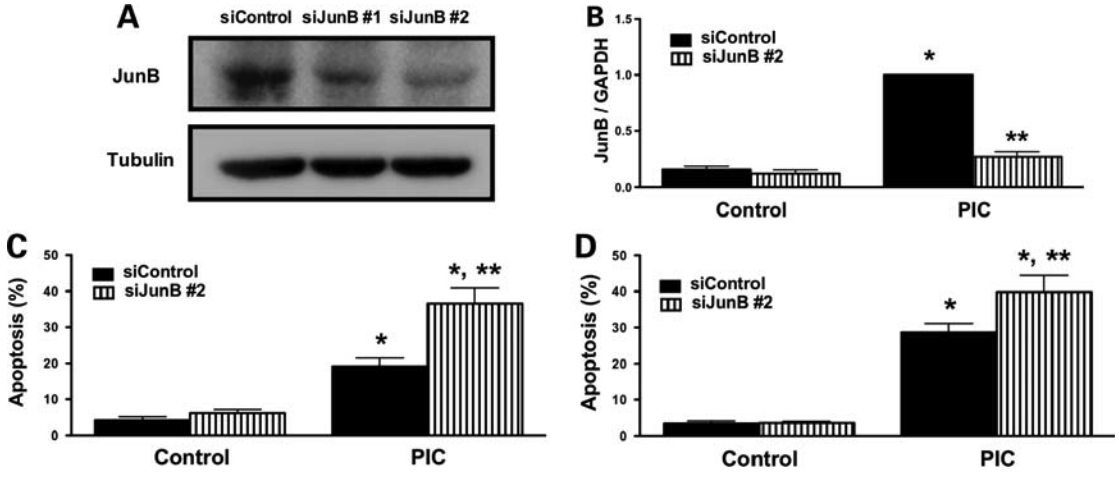

Figure 6. JunB KD augments PIC-induced β-cell death. INS-1E cells (**A**–**C**) and primary rat β-cells (**D**) were transfected with siControl, siJunB 1 or siJunB 2 and then treated as described in Figures 2 and 4. (A) JunB protein expression was evaluated after transfection with two specific siRNAs. Pictures are representative of three independent experiments. (B) PIC-induced JunB mRNA expression was blocked by siRNA against JunB. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (C and D) Apoptosis was evaluated in INS-1E cells (C) or FACS-purified rat β-cells (D) 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

these mice are backcrossed onto strains that express the co-stimulatory molecule B7.1 in the islets (57). The B7.1 mice produce higher levels of type I IFNs than wt C57BL/6, and administration of an antibody against type I IFN prevents diabetes (57). The protective effects of PTPN2 against PIC-induced apoptosis seems, however, to be transitory or insufficient, since 12 h after PIC transfection β-cells start to die independent of the continuous increase in PTPN2 expression. These results are in line with previous studies showing that cytokine-induced increase in protective molecules such as SOCS-3 (58) or JunB (29) is not sufficient to prevent β-cell death induced by protracted pro-apoptotic stimulus.

MDA5 and PTPN2 are expressed also in other tissues, including in cells from the immune system (18,19). T1D is a chronic autoimmune disease in which macrophages, NK and T-cells play a crucial role in disease development (1). Thus, different levels of MDA5 and PTPN2 expression in immune cells may also play a role in the triggering and progression of insulitis. The relative contribution of candidate gene expression in immune cells or β-cells needs to be clarified by future studies using tissue-specific knockout models.

In conclusion, we demonstrate that two candidate genes for T1D, namely MDA5 and PTPN2, may contribute to the pathogenesis of diabetes by modifying the β-cell responses to a viral infection. MDA5 potentially modulates the cross-talk between β-cells and the innate/adaptive immune system through the local production of cytokines and chemokines, whereas PTPN2 may protect β-cells against apoptosis due to negative feedback on IFN signalling. Single or, most probably, combined genetically determined changes in the expression of these and other relevant genes in β-cells and the immune system may lead to an 'unlucky genetic combination', which causes, for instance, an exaggerated inflammatory response to a β-cell viral infection. If this response is coupled to defective β-cell protective mechanisms, it may result in excessive and/or protracted β-cell death, leading to amplification of insulitis and eventually causing enough destruction of β-cells to trigger clinical diabetes.

MATERIALS AND METHODS

Culture of primary FACS-purified rat β-cells and INS-1E cells

Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Rat islets were isolated by collagenase digestion followed by hand-picking under a stereomicroscope. For β-cell isolation, islets were dispersed and β-cells purified by FACS (FACSAria, BD Bioscience, San Jose, CA, USA) (26,59). The preparations used in the present study contained 89.4 ± 1.2% β-cells ($n = 22$). Purified β-cells were cultured for 2 days in Ham's F-10 medium containing 10 mM glucose, 2 mM glutamine, 50 μM 3-isobutyl-L-methylxanthine, 5% heat-inactivated fetal bovine serum (FBS), 0.5% charcoal-absorbed bovine serum albumin (BSA Fraction V, Boehringer, Indianapolis, IN, USA), 50 U/ml penicillin and 50 μg/ml streptomycin before use in subsequent experiments (26,60). During dsRNA and siRNAs transfection, cells were cultured in the same medium but without antibiotics or BSA.

The rat insulin-producing INS-1E cell line (a kind gift from Dr C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in RPMI 1640 GlutaMAX-I, 5% FBS, 10 mM HEPES, 1 mM Na-pyruvate and 50 μM 2-mercaptoethanol (61). INS-1E cells (passages 57–75) were plated for 48 h prior to transfection.

RNA interference

The following siRNAs were used in this study: Allstars Negative Control siRNA (Qiagen, Venlo, Netherlands),

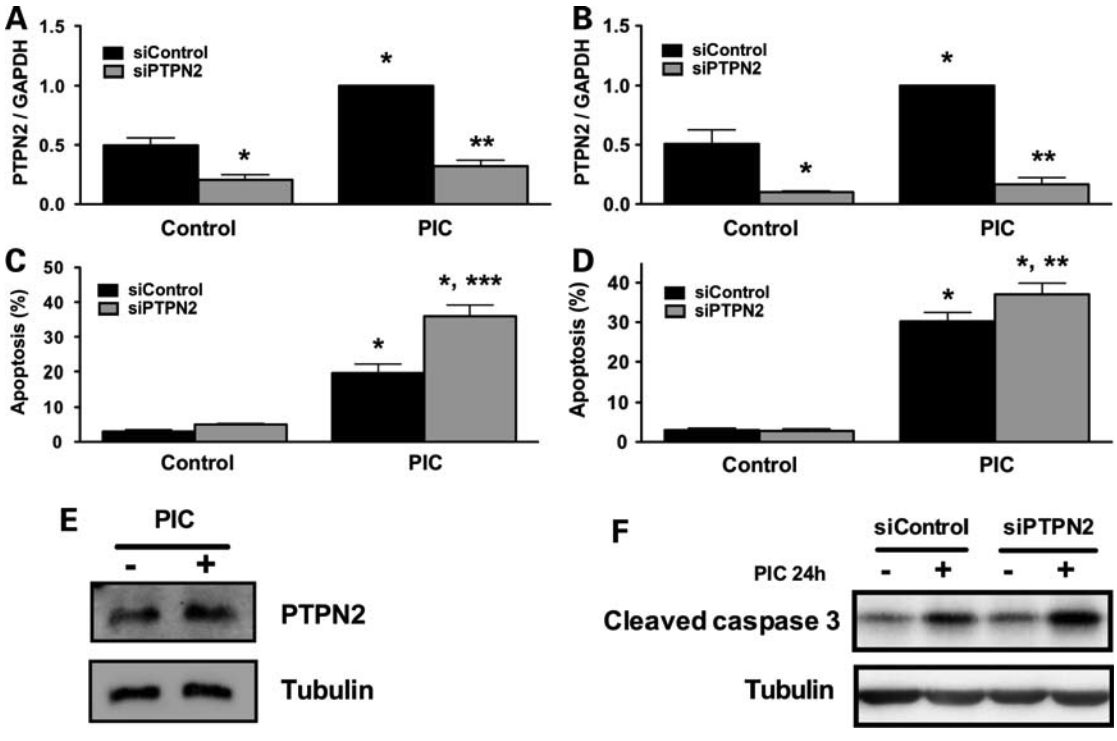

Figure 7. KD of PTPN2 sensitizes INS-1E cells and primary β-cells to PIC-induced apoptosis. INS-1E cells (**A**) or FACS-purified rat β-cells (**B**) were transfected with siControl (black bars) or siPTPN2 (grey bars) and 48 h after recovery exposed to PIC1 for 24 h. (A and B) PTPN2 mRNA expression was evaluated by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to five independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (**C** and **D**) INS-1E cells (C) or primary β-cells (D) were transfected with siControl or siPTPN2 and 48 h after recovery exposed to internal PIC1 for 24 h. Apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of six independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.05$ versus siControl+PIC, $^{***}P < 0.01$ versus siControl+PIC, ANOVA. (**E**) PTPN2 and α-tubulin protein expression was assessed by western blot in FACS-purified rat β-cells exposed to internal PIC1 for 24 h; the picture is representative of two independent experiments. (**F**) INS-1E cells were transfected with control siRNA, or with an siRNA targeting PTPN2 as described in Materials and Methods, and 48 h after recovery exposed to internal PIC1 for 24 h. Cleavage of caspase-3 was observed by western blot. The picture is representative of two independent experiments.

ON-TARGETplus rat MDA5 SMARTpool® (siMDA5 1; Thermo Scientific, Chicago, IL, USA), Silencer® Select Predesigned siRNA rat MDA5 (siMDA5 2; Applied Biosystems, Austin, TX, USA), Silencer® Select Custom Designed siRNA rat RIG-I (Applied Biosystems), ON-TARGETplus rat PTPN2 SMARTpool® (Thermo Scientific), Silencer® Select Predesigned siRNA rat PTPN2 (Applied Biosystems), rat JunB 1 and rat JunB 2 (Invitrogen, Carlsbad, CA, USA) and ON-TARGETplus rat PKR SMARTpool® (Thermo Scientific) (sequences are provided in Supplementary Material, Table S1). The optimal settings for the transfection of siRNAs in both INS-1E cells and primary FACS-purified rat β-cells were first established by using an FITC-coupled siRNA (siGLO Green Transfection Indicator, Thermo Scientific), and the transfection procedure performed using DharmaFECT 1 (Thermo Scientific) as described (18). The concentration of 30 nM was selected after dose–response studies (18; data not shown). Afterwards, cells were cultured for a 24–48 h recovery period and subsequently transfected with the synthetic dsRNA, PIC, as described in the following.

dsRNA transfection and JNK inhibition

The synthetic dsRNAs, PIC, were from Sigma-Aldrich (PIC1; St Louis, MO, USA) and Invivogen (PIC2; San Diego, CA, USA) and used at the final concentration of 1 μg/ml (28). All experiments were performed with intracellular PIC, obtained via cell transfection. For PIC transfection, the same conditions described for siRNA were utilized, except that the DharmaFECT:PIC ratio used was 0.8 and 1.25 μl of DharmaFECT to 5 μg of PIC for INS-1E cells and primary β-cells, respectively. Since it was demonstrated that helicases recognize dsRNA molecules on the basis of their length (21), a PIC with >2000 bp (PIC2) was used for experiments targeting MDA5, whereas all other experiments were done with PIC1, with <2000 bp, as in our previous studies (26,28).

The JNK inhibitor SP600125 (Sigma-Aldrich) was dissolved in DMSO and used at a concentration of 10 μM (24). The peptide JNK inhibitor D-TAT-JNKi (a kind gift of C. Bonny and M. Mathieu; XigenPharma, Lausanne, Switzerland) and the control peptide D-TAT were dissolved in culture medium and used at the concentration of 10 μM (32). Both inhibitors were added to the cell culture 4 h before PIC transfection and kept in the medium during PIC exposure.

Assessment of cell viability

The percentage of viable, apoptotic and necrotic cells was determined following 15 min of incubation with the DNA-binding dyes propidium iodide (PI, 5 μg/ml, Sigma) and Hoechst 33342 (HO, 5 μg/ml, Sigma). This method is

quantitative and has been validated for use in pancreatic β-cells and INS-1E cells by systematic comparison with electron microscopy, caspase-3 activation and DNA laddering (14,18,24,26,62). A minimum of 500 cells was counted in each experimental condition. Viability was evaluated by two independent observers, one of them being unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percentage of apoptosis and were calculated as number of apoptotic cells/total number of cells × 100. In some experiments, the presence of apoptosis was confirmed by western blot analysis of activated (cleaved) caspase-3 (see what follows).

mRNA extraction and real-time PCR

Poly(A)+ mRNA was isolated from INS-1E cells and rat primary β-cells using the Dynabeads mRNA DIRECT™ kit (Invitrogen, Paisley, UK), and reverse-transcribed as described previously (14,26,44). The real-time PCR amplification reaction was done as described (26,44), using SYBR Green and compared with a standard curve (63). Expression values were then corrected for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized by the highest value of each experiment considered 1; PIC treatment does not modify GAPDH expression in insulin-producing cells (64,65). The primer sequences for rat PTPN2, MDA5, RIG-I, PKR, INF-β, IL15, CCL2, CCL5, CXCL10, CHOP, XBP1-s, iNOS, JunB, GAPDH are described in Supplementary Material, Table S2. The cytokines and chemokines to be studied were selected on the basis of our previous array analysis (15) and studies focused (26,28,64,65) as representative of β-cell inflammatory responses to dsRNA.

Promoter studies

A total of 10^5 INS-1E cells were plated in 24-well plates and transfected with siRNAs against MDA5, RIG-I or control as described earlier. After 24 h of recovery, plasmid constructs containing the firefly luciferase gene under the control of either multiple copies of the NF-κB consensus sequence (BD Biosciences Clontech, Mountain View, CA USA) or the mouse IFN-β promoter (66) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. Twelve hours later, cells were exposed overnight to internal PIC (1 μg/ml). Luciferase activities were assayed 24 h after PIC treatment using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) (26,65). Test values were corrected for the luciferase activity of the internal control plasmid, pRL-CMV, and shown as fold induction compared with siControl.

Assessment of CCL5 protein and nitrite production

After 24 h of PIC transfection, cell supernatants were retrieved and rat CCL5 was measured by enzyme-linked immunosorbent assay (Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA).

In some experiments, culture supernatants were also collected for nitrite determination (nitrite is a stable product of nitric oxide oxidation) at OD_{540nm} using the Griess reagent (67).

Western blot analysis

Cells were washed once with cold PBS and directly lysed with either Laemmli buffer (25 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 350 mM 2-mercaptoethanol, 175 mM dithiothreitol, 0.005% bromophenol blue completed by a protease inhibitor cocktail—Roche Diagnostics, Vilvoorde, Belgium) or phospho lysis buffer (1% NP40, 25 mM Hepes, pH 7.8, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). Lysates were then resolved by 8–12% SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with antibodies targeting PTPN2 (R&D Systems), JunB, dsRNA-dependent protein kinase (PKR) (Santa Cruz Biotechnology, CA, USA), phospho-JNK, cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) or α-tubulin (Sigma, Bornem, Belgium), used as the housekeeping protein. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG was used as a secondary antibody (Lucron Bioproducts, De Pinte, Belgium). Immunoreactive bands were revealed using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific), detected using an LAS-3000 CCD camera and quantified with the Aida Analysis software (Fujifilm).

Statistical analysis

Data are presented as mean ± SEM. Comparisons were performed by two-tailed paired Student's *t*-test or by ANOVA, followed by Student's *t*-test with Bonferroni correction as indicated. A *P*-value <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Supplementary material from article II:

MDA5 and *PTPN2*, two candidate genes for type 1 diabetes, modify pancreatic beta cell responses to the viral by-product double-stranded RNA. Colli ML, Moore F, Gurzov EN, Ortis F, Eizirik DL. *Hum Mol Genet*. 2010: 19:135-46.

SUPPLEMENTARY MATERIAL

FIGURE LEGENDS

Figure S1. PICs with different lengths induce apoptosis in INS-1E cells. (A) 5 µg of PICs 1 and 2 were analyzed in an ethidium-bromide stained 1% agarose gel. (B) 10^4 INS-1E cells were left untreated (Control) or transfected with PIC1 (< 2,000 bp) or PIC2 (> 2,000 bp) and apoptosis was evaluated 24h after transfection using HO/PI staining as described in Methods. Results are mean ± SEM of 3 independent experiments. *: $P < 0.01$ vs Control, ANOVA.

Figure S2. PIC induces a time-dependent increase in MDA5 and PTPN2 mRNA expression and apoptosis in INS-1E cells. INS-1E cells were transfected with PIC1 and mRNA expression of MDA5 (A) and PTPN2 (B) was evaluated at different time points by real-time RT-PCR. (C) Apoptosis was evaluated using HO/PI staining as described in Methods. Results are mean ± SEM of 4 independent experiments. *: $P < 0.05$ vs Control, **: $P < 0.01$ vs Control, paired t test.

Figure S3. siRNA targeting RIG-I partially prevents activation of the interferon-β promoter. INS-1E cells were transfected with control siRNA (siControl - black bars), or with siRNA targeting RIG-I (siRIG-I - striped bars) as described in Methods. After 24h of recovery cells were transfected with IFN-β (A) or NF-κB (B) promoter reporters plus a pRL-CMV plasmid (used as internal control). Cells were then exposed to internal PIC1 for 24h and luciferase activity was assayed. The values were corrected for the activity of the internal control, pRL-CMV and are presented as fold induction in relation to

siControl. Results are mean ± SEM of 3-5 independent experiments; *: $P < 0.05$ vs siControl, **: $P < 0.05$ vs siControl + PIC, ANOVA.

Figure S4. Two different siRNAs against MDA5 induce similar effects in INS-1E cells viability and mRNA expression of cytokines and chemokines. INS-1E cells were transfected with either a control siRNA (siControl - black bars), or two different siRNAs targeting MDA5 (siMDA5 #1 - light gray bars; siMDA5 #2 - striped bars). After 24h of recovery, cells were left untreated or transfected with PIC2 (> 2,000 bp) as described in Methods. (A) MDA5 mRNA expression was assayed by real time RT-PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments; *: $P < 0.01$ vs siControl; **: $P < 0.01$ vs siControl + PIC; ANOVA. (B) INS-1E cells were transfected with siRNAs and PIC, and apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of 4 independent experiments; *: $P < 0.01$ vs siControl; ANOVA. (C) mRNA expression of rat IFN-β, IL15, CCL2, CCL5 and CXCL10 was analyzed by real time RT-PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments; *: $P < 0.01$ vs siControl, **: $P < 0.01$ vs siControl + PIC, ANOVA.

Figure S5. siMDA5, but not siRIG-I, decreases CCL5 secretion after exposure to internal PIC in FACS-purified rat β-cells.

FACS-purified rat β-cells were transfected with siControl (black bars), siMDA5 (A – grey bars) or siRIG-I (B – striped bars) and treated as described in Figure 4. CCL5 secretion was evaluated in the supernatant by ELISA 24h after internal PICs exposure (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments), as described in Methods. Results are mean ± SEM of 3-6 independent experiments; *: $P < 0.01$ vs siControl, **: $P < 0.01$ vs siControl + PIC, paired *t* test.

Figure S6. Knockdown of PKR neither prevents PIC-induced apoptosis nor expression of cytokines / chemokines. FACS-purified rat β-cells were transfected with control siRNA (siControl – black bars), or with siRNA targeting PKR (siPKR – doted bars) as described in Methods, and 48h after recovery exposed to PIC1 for 24h. (A and C) mRNA expression of PKR and selected cytokines (IFN-β and IL15) and chemokines (CCL2, CCL5 and CXCL10) were evaluated by real time RT-PCR. Results are mean ± SEM of 4 independent experiments; *: $P < 0.01$ vs siControl, **: $P < 0.01$ vs siControl + PIC, ANOVA. (B) Apoptosis was estimated 24h after PIC using HO/PI staining. Results are mean ± SEM of 3 independent experiments. *: $P < 0.01$ vs Control, ANOVA.

Figure S7. Knockdown of JunB increases caspase-3 activation after PIC transfection. (A) INS-1E cells were transfected with control siRNA (siControl – black bars), or with siRNAs targeting JunB (siJunB #1 - grey bars, or siJunB #2 – striped bars) as described in Methods, and 24h after recovery exposed to internal PIC1 for 24h. Cleavage of caspase-3 was evaluated by Western blot. Results are mean ± SEM of 3 independent experiments and are presented as fold induction compared to siControl; *: $P < 0.01$ vs siControl + PIC, ANOVA.

Figure S8. Knockdown of JunB does not modify expression of cytokines / chemokines in FACS-purified rat β-cells. Primary rat β-cells were transfected with control siRNA (siControl – black bars), or with siRNA targeting JunB (siJunB #2 – striped bars) and 48h after recovery exposed to PIC1 for 24h. mRNA expression of selected cytokines (IFN-β and IL15) and chemokines (CCL2, CCL5 and CXCL10) was evaluated by real time RT-PCR. Results are mean ± SEM of 4 independent experiments; *: $P < 0.01$ vs siControl, ANOVA.

Figure S9. Inhibition of PTPN2 expression induces no or minor changes in the expression of cytokines / chemokines after PIC exposure. (A) INS-1E cells or (B) FACS-purified rat β-cells were transfected with control siRNA (siControl – black bars), or with siRNA targeting PTPN2 (siPTPN2 – grey bars) as described in Methods, and 48h after recovery exposed to PIC1 for 24h. mRNA expression of selected cytokines (IFN-β and IL15) and chemokines (CCL2, CCL5 and CXCL10) was evaluated by real time RT-PCR. Results are mean ± SEM of 3-5 independent experiments; *: $P < 0.05$ vs siControl; **: $P < 0.001$ vs siControl; ***: $P < 0.001$ vs siControl + PIC, ANOVA.

Figure S1

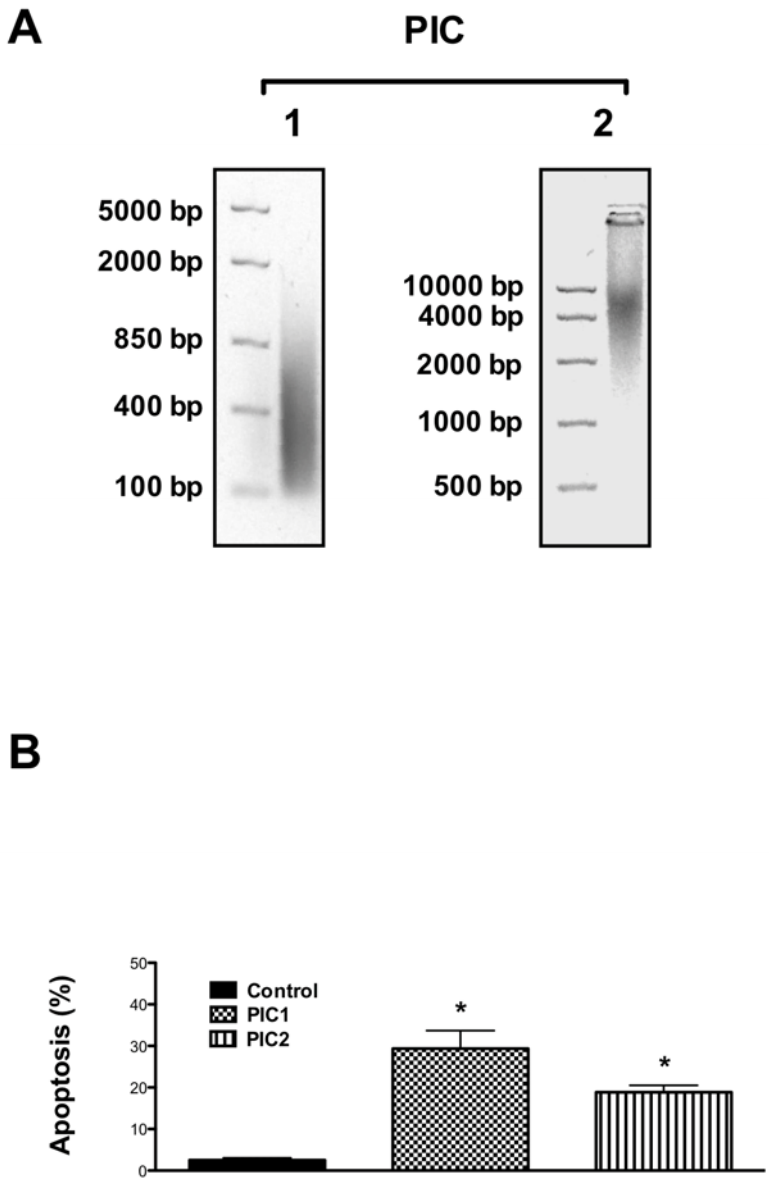
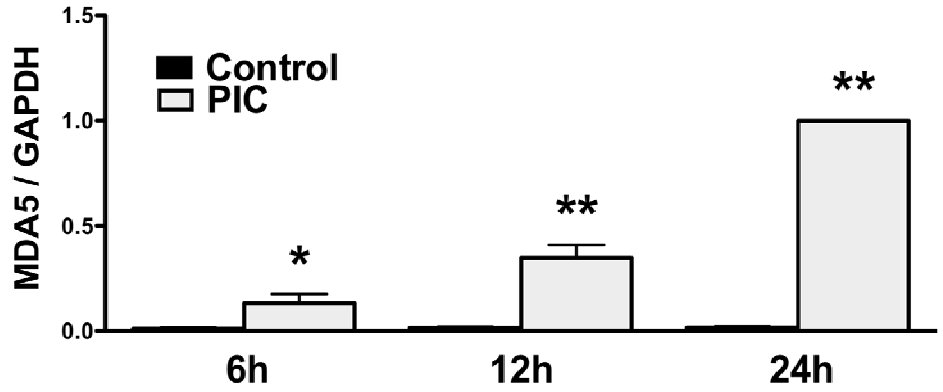
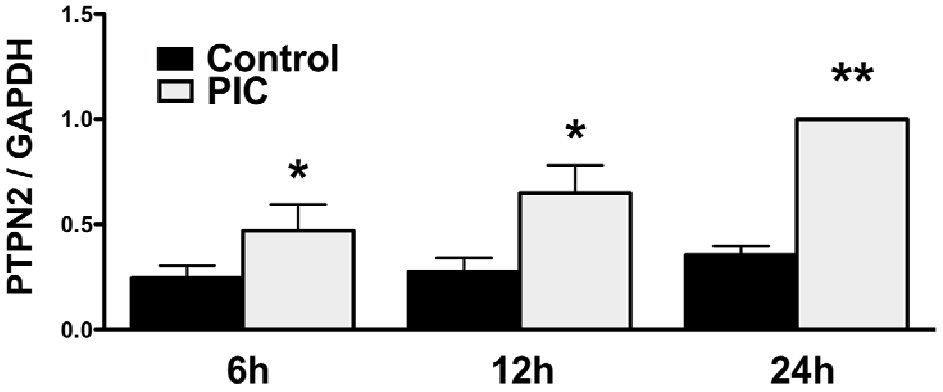

A
PIC
1
2
5000 bp
2000 bp
850 bp
400 bp
100 bp
10000 bp
4000 bp
2000 bp
1000 bp
500 bp
B
Apoptosis (%)
50
40
30
20
10
0
Control
PIC1
PIC2
*
*

Figure S2

A



B



C

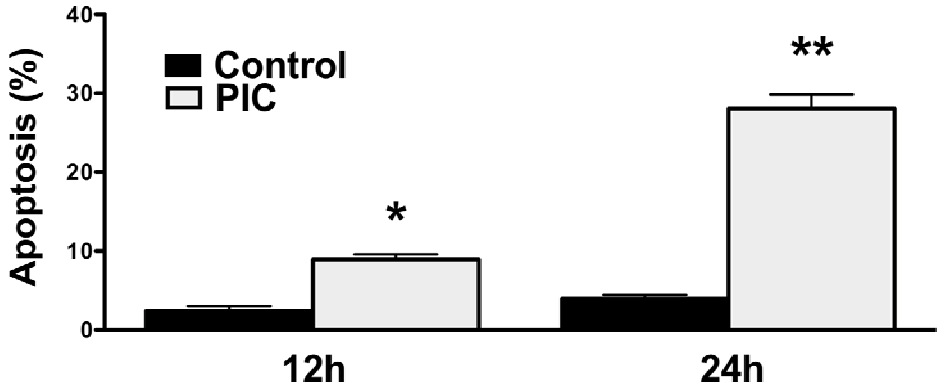


Figure S3

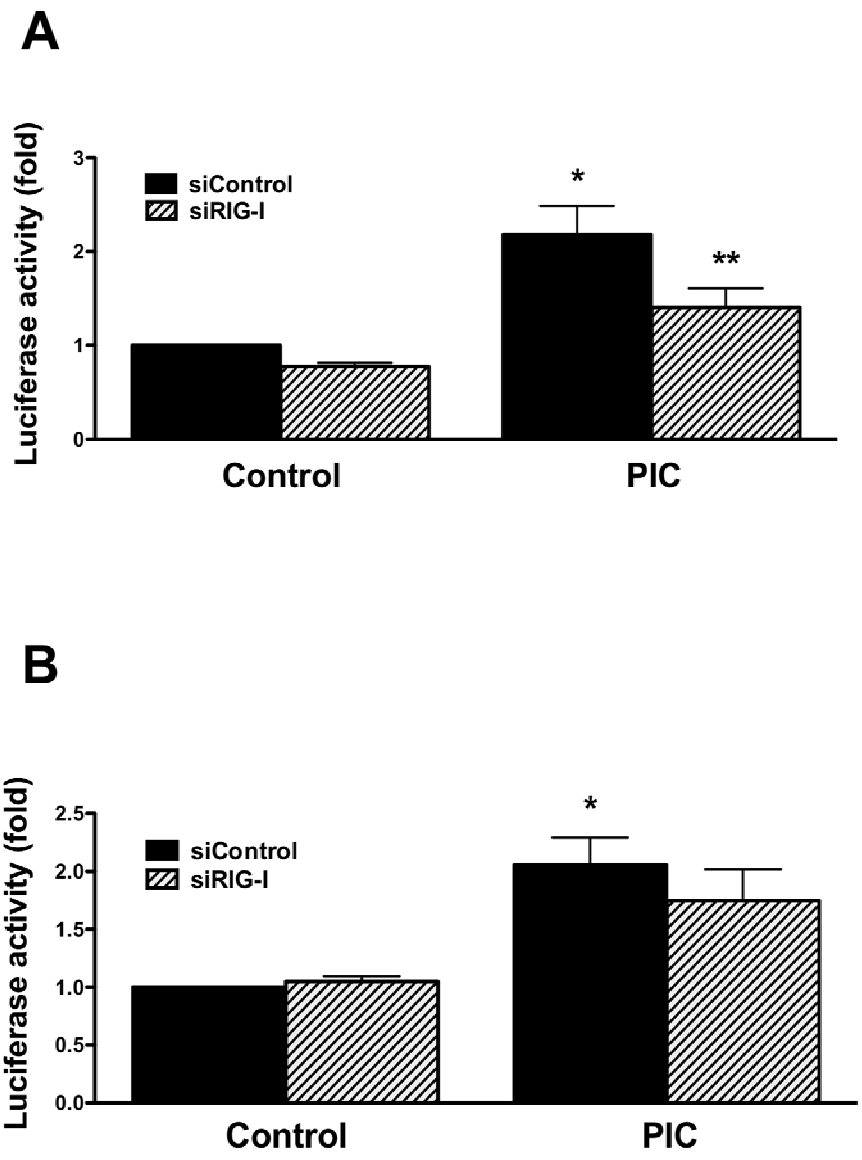

A
Luciferase activity (fold)
3
2
1
0
siControl
siRIG-I
*
**
Control
PIC
B
Luciferase activity (fold)
2.5
2.0
1.5
1.0
0.5
0.0
siControl
siRIG-I
*
Control
PIC

Figure S4

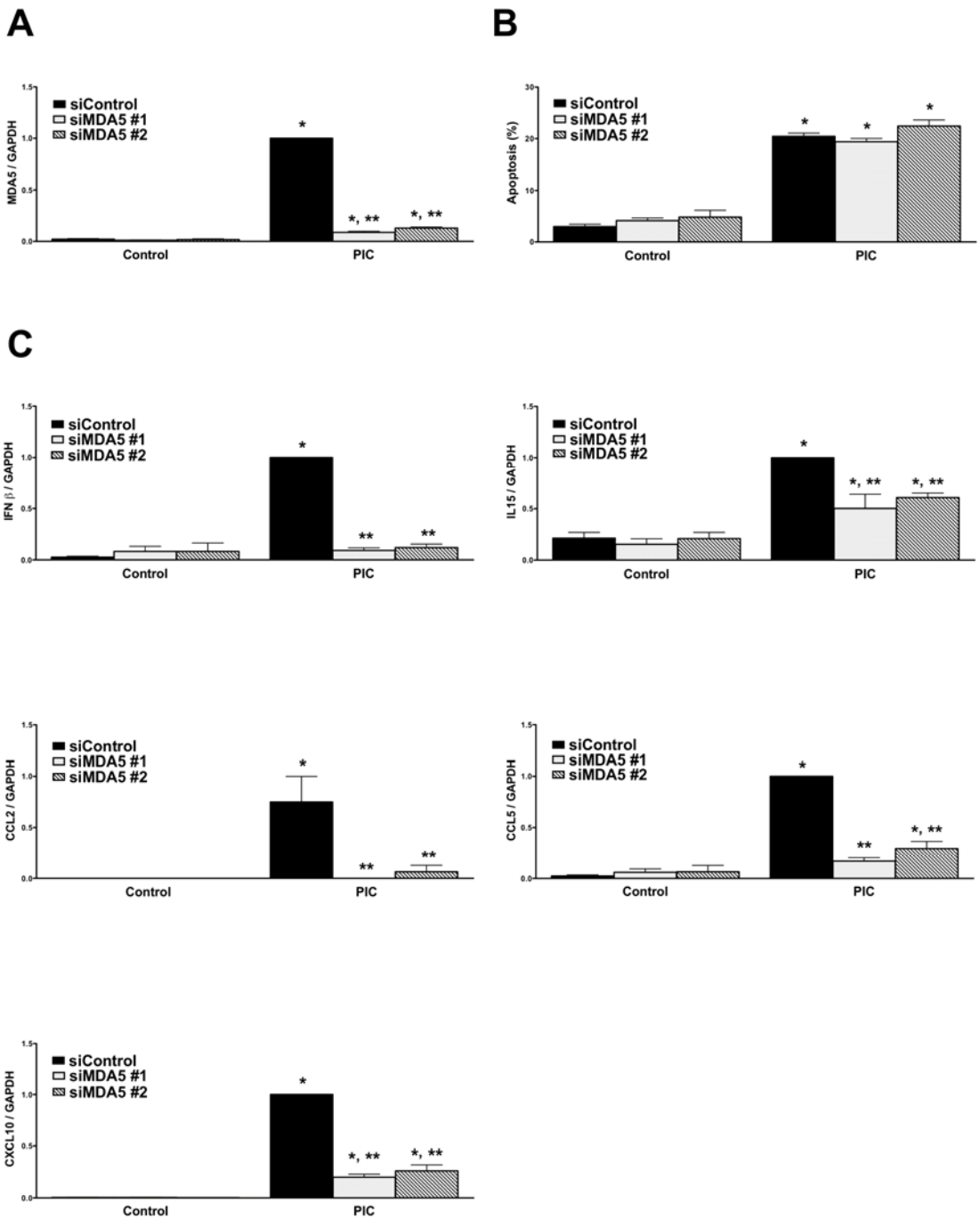

A
B
C
siControl
siMDA5 #1
siMDA5 #2
MDA5 / GAPDH
Apoptosis (%)
IFN β / GAPDH
IL15 / GAPDH
CCL2 / GAPDH
CCL5 / GAPDH
CXCL10 / GAPDH
Control
PIC
*
**
*, **

Figure S5

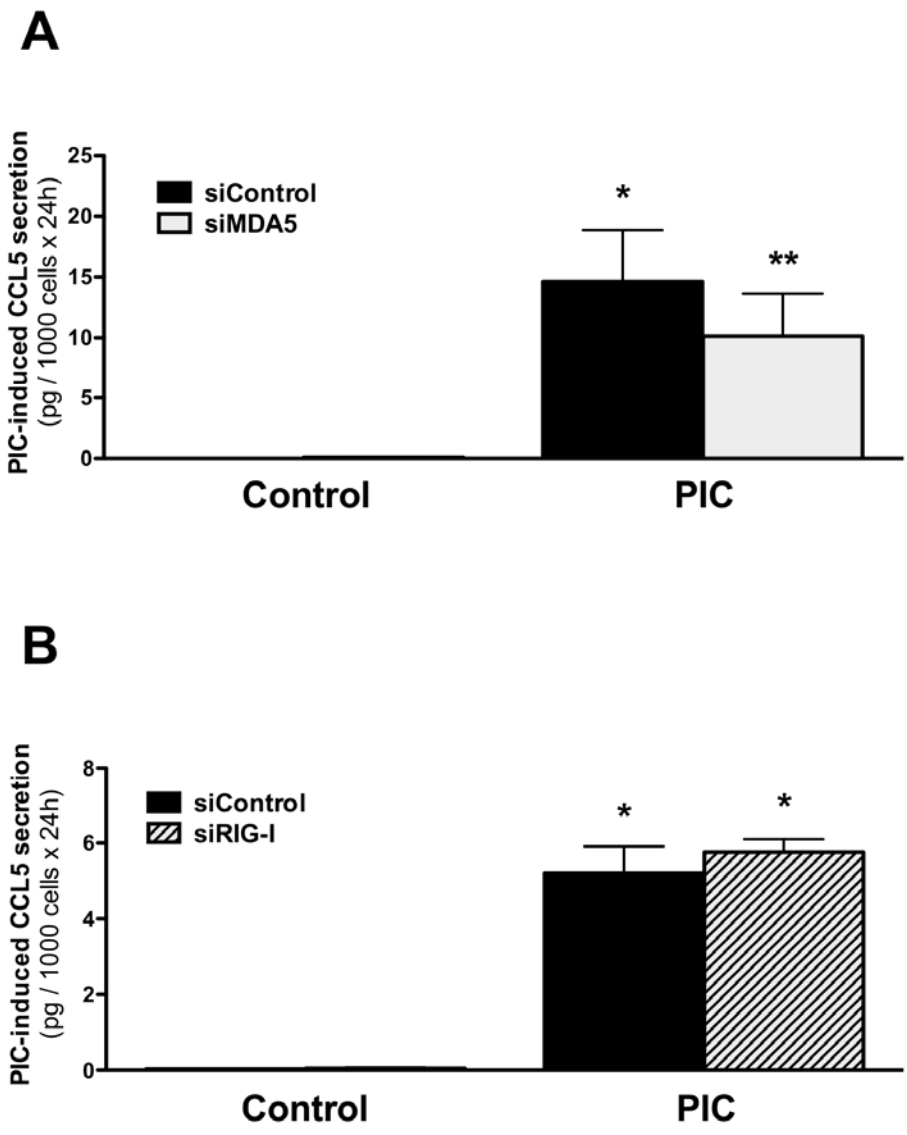

A
PIC-induced CCL5 secretion
(pg / 1000 cells x 24h)
25
20
15
10
5
0
siControl
siMDA5
*
**
Control
PIC
B
PIC-induced CCL5 secretion
(pg / 1000 cells x 24h)
8
6
4
2
0
siControl
siRIG-I
*
*
Control
PIC

Figure S6

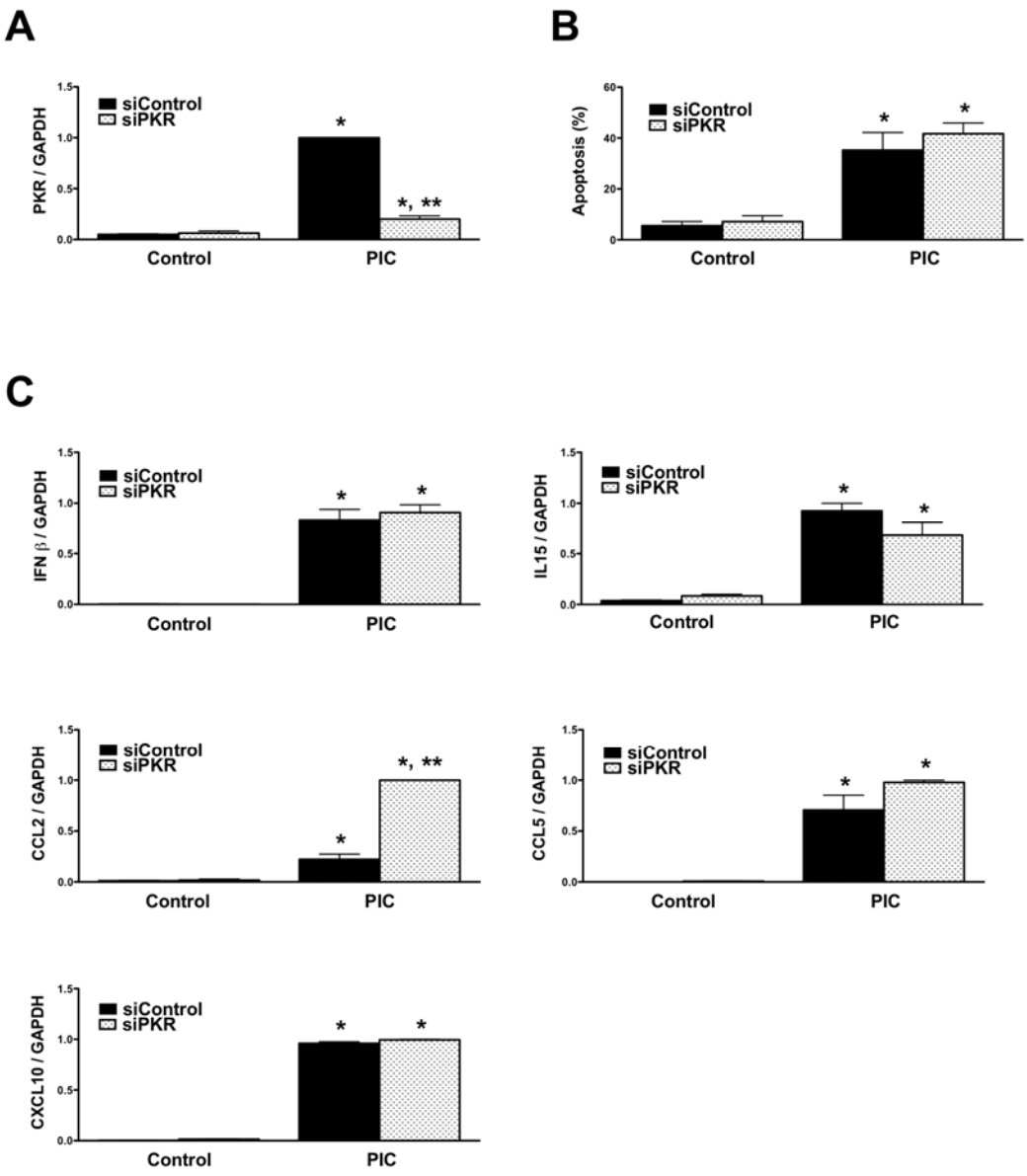

A
B
C
siControl
siPKR
PKR / GAPDH
Apoptosis (%)
IFN β / GAPDH
IL15 / GAPDH
CCL2 / GAPDH
CCL5 / GAPDH
CXCL10 / GAPDH
Control
PIC
*
*, **

Figure S7

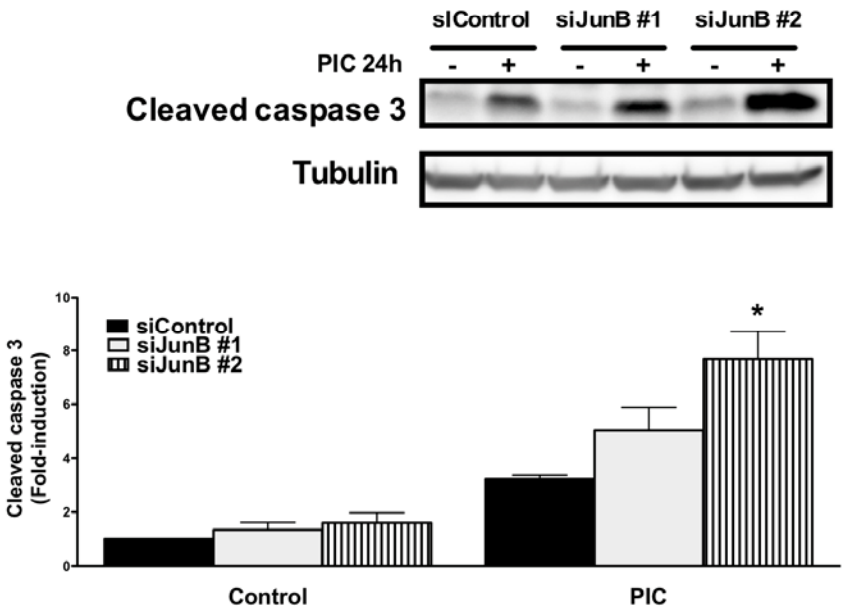

siControl
siJunB #1
siJunB #2
PIC 24h
- + - + - +
Cleaved caspase 3
Tubulin
Cleaved caspase 3
(Fold-induction)
siControl
siJunB #1
siJunB #2
0
2
4
6
8
10
*
Control
PIC

Figure S8

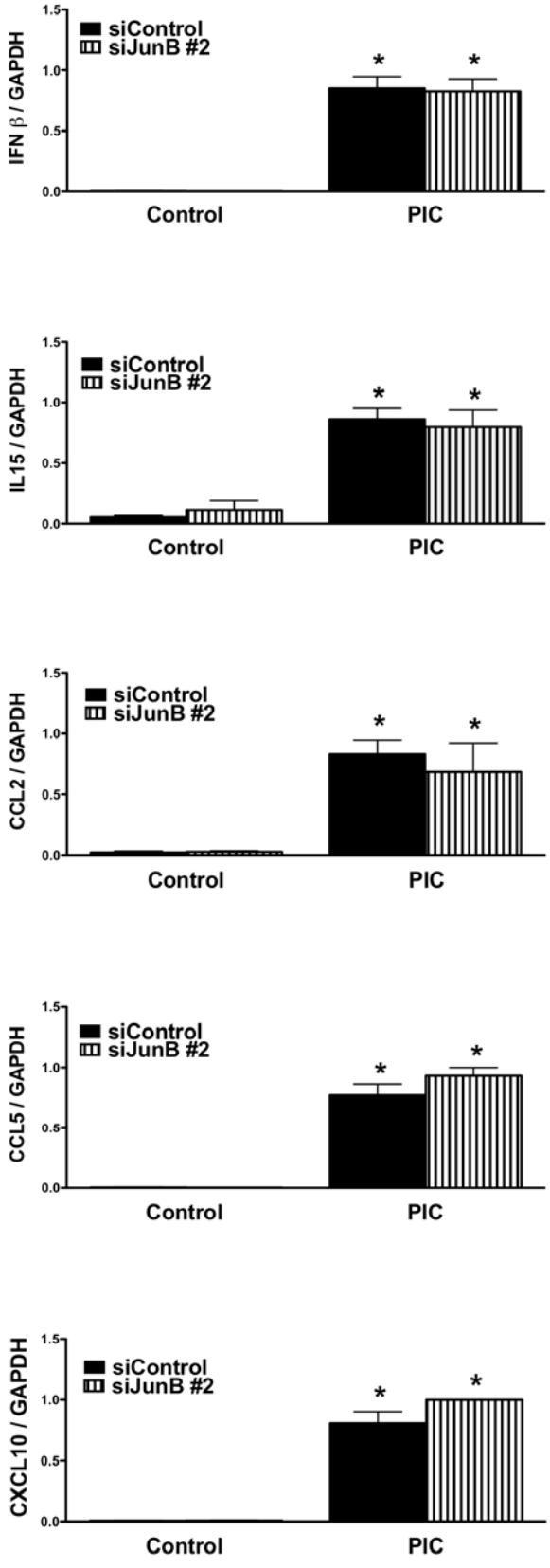
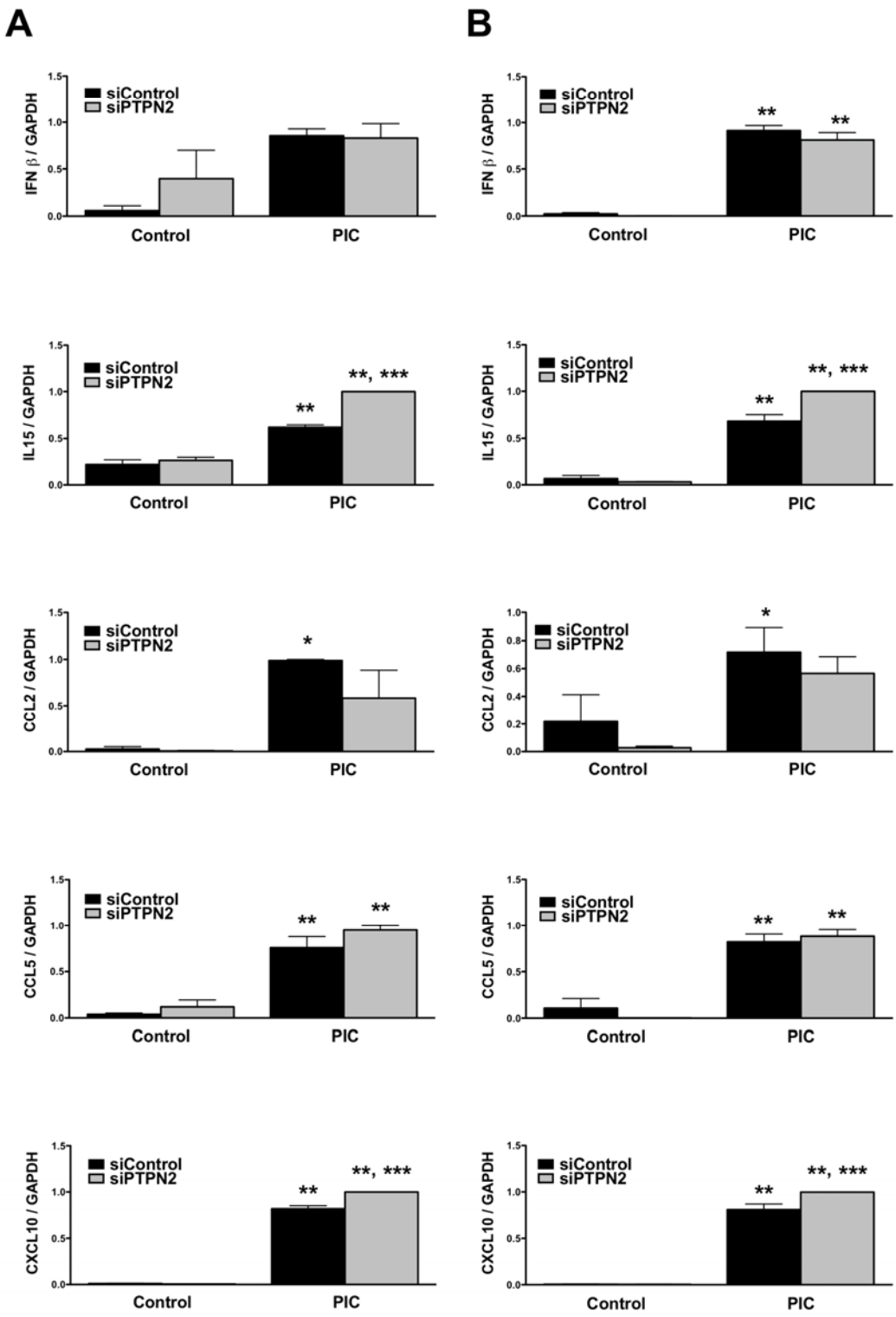

IFN β / GAPDH
siControl
siJunB #2
1.5
1.0
0.5
0.0
*
*
Control
PIC
IL15 / GAPDH
siControl
siJunB #2
1.5
1.0
0.5
0.0
*
*
Control
PIC
CCL2 / GAPDH
siControl
siJunB #2
1.5
1.0
0.5
0.0
*
*
Control
PIC
CCL5 / GAPDH
siControl
siJunB #2
1.5
1.0
0.5
0.0
*
*
Control
PIC
CXCL10 / GAPDH
siControl
siJunB #2
1.5
1.0
0.5
0.0
*
*
Control
PIC

Figure S9


A
B
siControl
siPTPN2
IFN β / GAPDH
IL15 / GAPDH
CCL2 / GAPDH
CCL5 / GAPDH
CXCL10 / GAPDH
Control
PIC
*
**
, *

8. Discussion and Conclusions

In complex autoimmune diseases the interaction between genes with different effect sizes and putative environmental factors can lead to tissue damage and development of the disease. In the case of T1D, this combination of factors produces a chronic autoimmune assault against the insulin-producing pancreatic beta cells. This, associated to the fact that these cells do not proliferate [405] or do so only under special conditions [241], leads to a progressive and permanent decrease in beta cell mass. The individuals affected by the disease are mainly children and young adults and require insulin replacement for life.

The existence of genetic components in T1D has been known for years [53]. The concordance for T1D among monozygotic twins (30-70%) is one of the best clues for this genetic association, but the segregation in families does not fit in well-defined patterns of inheritance [253, 406]. Despite the knowledge that genetics factors are involved in T1D pathogenesis, until recently only two genes have been associated with the disease, the class II *HLA* and *INS* genes [256]. The development of new technologies for genotyping, coupled to the increase in the number of samples available from international consortiums in the late 90s and 2000s, such as The International Type 1 Diabetes Genetics Consortium (T1DGC) and The Wellcome Trust Case Control Consortium (WTCCC), made it possible the identification of several new candidate genes for T1D [187, 294, 295, 407, 408]. Thus, in addition to class II HLA, the main locus related to T1D, more than 50 new regions have been identified as significantly associated to autoimmune diabetes [252]. In these regions many genes with putative pathophysiological role were localized and are presently under study [252]. Some regions, however, contain either genes with unknown function or no known annotated genes. In these "deserts", genes actually related to diabetes can be located at a distant region. In this case, the real genes can be part of some regulatory circuit under the control of the long-range *cis*-regulatory element from the region where the polymorphism is localized. This mechanism

was recently demonstrated for the 8q24 gene desert variant, which controls the nearby proto-oncogene *MYC* and increases the risk of prostate cancer [409].

Since genetic studies only identify potential candidate genes, but do not demonstrate how they modify/promote disease, functional studies play an important role in the characterization of these new variants. Most of the risk genes for T1D described up to now are supposed to exert their effects at the immune system. However, by correlating data available from T1D genome-wide association studies [187, 188, 294, 295] with our previous array studies of beta cells [158, 168, 218], we noticed that >30% of the T1D candidate genes are also expressed by pancreatic beta cells.

The autoimmune attack in T1D is selectively directed to pancreatic beta cells. Several lines of evidence suggest the presence of a cross-talk between beta cells and the immune cells, promoting the initiation and/or maintenance of this selective assault [171]. This was already suggested by the presence of autoantibodies and T cell reactivity against specific beta cell antigens, and by the important role of local production of chemokines/cytokines by the beta cells during the insulitis [171]. Based on these information, we decided to further study the biological role of the newly discovered T1D candidate genes at the beta cell level. Initially, we analyzed the expression of these genes in pancreatic beta cells exposed to proinflammatory cytokines and/or dsRNA/virus. Using this approach, we identified several candidate genes that are well expressed in beta cells and regulated by cytokines/dsRNA (data not shown). We next selected two of these candidates considered of particular interest, namely *PTPN2*, also know as *TC-PTP* or *PTP-S2*, and *MDA5,* also known as *IFIH1,* for additional studies.

The region where *PTPN2* is located was identified by two independent genome-wide association studies for T1D candidate genes [294, 295], which support the possibility that it confers risk for T1D. Genome-wide association studies usually identify smaller regions than

linkage studies, but these regions often contain more than one potential candidate gene. It is thus sometimes difficult to know for certain which of the genes is the one conferring diabetes risk. Reinforcing *PTPN2* as a T1D candidate gene, the region identified on 18p11 has *PTPN2* as the only gene present [294, 295]. Using the online beta cell database (www.t1dbase.org) we noticed that mRNA expression of *PTPN2* in FACS-purified rat beta cells is half of *glucokinase* expression (*glucokinase* is a well expressed gene in beta cells, and we usually utilize it as a "reference gene" to evaluate expression) [410]. *PTPN2* is also well expressed in the beta cell linage INS-1E and in human islets; of note, in human islets *PTPN2* expression is even higher than *glucokinase*. We confirmed these array data by evaluating the presence of *PTPN2* at mRNA and protein level, by respectively real time RT-PCR and Western blot, in INS-1E cells, rat beta cells and human islet cells (Article I). Moreover, the array data suggested that proinflammatory cytokines, the viral mimetic dsRNA and Coxsackievirus B5 induced 1.6 to 2.3-fold increase in *PTPN2* mRNA expression in beta cells [410], an observation again confirmed in independent experiments at both mRNA and protein level (Articles I and II). This confirms that array data provide useful tools to identify candidate genes expressed in beta cells for further functional studies. This is in line with previous findings from our group, showing that >90% of cytokine-modified genes identified by array analysis are confirmed by RT-PCR analysis in independent samples [219, 411]. PTPN2 is a protein tyrosine phosphatase involved in the regulation of several signaling pathways, such as janus kinases (JAKs), signal transducer and activator of transcription (STATs), p42/44 mitogen-activated protein kinase (MAPK) (extracellular signal-related kinase (ERK)), epidermal growth factor receptor (EGFR), and insulin receptor β (IRβ) [412]. From the known signaling pathways targeted by *PTPN2*, STAT1 seems of particular relevance. STAT1 is a key transcription factor, previously shown by our group to be involved in beta cell apoptosis both *in vitro* [223] and *in vivo* [224] .

In beta cells exposed to cytokines, the knockdown of *PTPN2* significantly exacerbated cytokine-induced apoptosis and STAT1 phosphorylation (Article I). These events are probably correlated, since the double KD of STAT1 and *PTPN2* prevented this increase in apoptosis (Article I). Since *PTPN2* has other targets, we evaluated the activation of ERK and EGFR, but there was no change (Supplementary material Article I). STAT3 and IRβ, on the other hand, presented an increase in their activation in the context of *PTPN2* silencing (Supplementary Material Article I). It is unlikely, however, that these effects contribute for the observed increase in beta cell apoptosis, since silencing of SOCS3, and consequent overactivation of STAT3, leads to protection against diabetes in streptozotocin- treated mice [413], and selective knockdown of STAT3 in beta cells does not affect their physiology or survival [414]. The increase in IRβ activity observed in previous studies actually amplified insulin signaling and increased insulin content without affecting cell viability [300, 415, 416]. The role of STAT1 in autoimmune diabetes may go beyond the direct control on beta cells apoptosis. Data from a recent array study from our group indicate that STAT1 is an important positive regulator of chemokine production in beta cells treated with proinflammatory cytokines (Moore F, Naamane N, Colli ML, Bouckenooghe T, Ortis F, Gurzov EN, Igoillo-Esteve M, Mathieu C, Bontempi G, Thykjaer T, Ørntoft TF, Eizirik DL, submitted for publication), a finding supported by previous *in vivo* studies [223, 224]. In our array data, however, *PTPN2* knockdown did not modify to any major degree the expression of proinflammatory cytokines and chemokines in beta cells exposed to IL-1β + IFN-γ (Article I) or to dsRNA (Article II). One explanation might also come from the recent array study with STAT1 silencing (Moore F, Naamane N, Colli ML, Bouckenooghe T, Ortis F, Gurzov EN, Igoillo-Esteve M, Mathieu C, Bontempi G, Thykjaer T, Ørntoft TF, Eizirik DL, submitted). In this study, we observed that knockdown of the transcription factor IRF1, a target gene of STAT1, significantly prolongs STAT1 activation and chemokine expression. Thus, increased

activity of STAT1 secondary to *PTPN2* KD could also promote increased IRF1 expression, leading to a negative feedback on chemokine expression. Another important factor is timing, since the increased activation of STAT1 was mainly observed at earlier time points (30-120 min) while the peak of chemokine induction is at 12h and 24h after cytokines and intracellular dsRNA exposure, respectively. Other mechanisms may be involved in this unexpected disparity between increased STAT1 activation and apoptosis on one side, and lack of modifications in chemokine expression on the other; this interesting finding deserves further studies.

Cytokines downregulate the expression of several genes involved in the maintenance of beta cell function and phenotype, including *INS1* and *INS2* [219], and STAT1 is probably the master regulator of this "de-differentiating" " effect (Moore F, Naamane N, Colli ML, Bouckenooghe T, Ortis F, Gurzov EN, Igoillo-Esteve M, Mathieu C, Bontempi G, Thykjaer T, Ørntoft TF, Eizirik DL, submitted). In our experiments we observed that at least *INS1* and *INS2* were not modified by knocking-down *PTPN2* in the presence of cytokines (Supplementary material Article I), again pointing to a dissociation between pro-apoptotic and functional effects downstream of STAT-1.

The augmentation of apoptosis caused by knockdown of *PTPN2* in pancreatic beta cells exposed to intracellular dsRNA (Article II) has a different mechanism as compared to cytokines, since this viral mimetic does not directly activate STAT1. Transfection of beta cells with dsRNA promotes the expression and secretion of type I interferons [162]. These cytokines bind to their own receptors in an autocrine/paracrine way, promoting the activation of a complex that includes STAT1, STAT2, and IRF9 (also known as ISGF3), and the expression of several downstream genes, many of them proapoptotic [417]. The knockout of STAT1 prevents beta cell apoptosis induced by extracellular dsRNA + IFN-γ [156]. On the other hand, silencing of *PTPN2* amplifies STAT1 phosphorylation after interferon β treatment

in other cell types [418], and mutations on the STAT2 SH2 domain makes it resistant to *PTPN2* dephosphorylation, increasing expression of interferon-stimulated genes (ISGs) and apoptosis induced by type I interferons [419].

The exposure of BL/6 mice expressing the co-stimulatory molecule B7.1 to synthetic dsRNA induces diabetes in 78% of the animals by the age of 20 week [166]. There is an inverse correlation between the peak of IFN-α and the time to onset of diabetes, and IFN-α neutralizing antibodies significantly decrease diabetes prevalence after dsRNA exposure [166]. Taken this into account, we propose a model (Figure 11) to explain the effects of *PTPN2* knockdown in intracellular dsRNA- and cytokine-induced apoptosis. Based on this model, we assume that beta cells evolved negative regulators to decrease the proapoptotic effects of excessive activation of type I interferons (induced by viral dsRNA) or cytokines; one of these regulators is *PTPN2*. It is thus conceivable that *PTPN2* polymorphisms that reduce its function may lead to an overactivation of STATs and triggering/augmentation of beta cell apoptosis during viral infections or in a proinflammatory environment (insulitis). These apoptotic beta cells can be then phagocyzed by APCs and presented to autoreactive T cells, amplifying the process of autoimmunity.

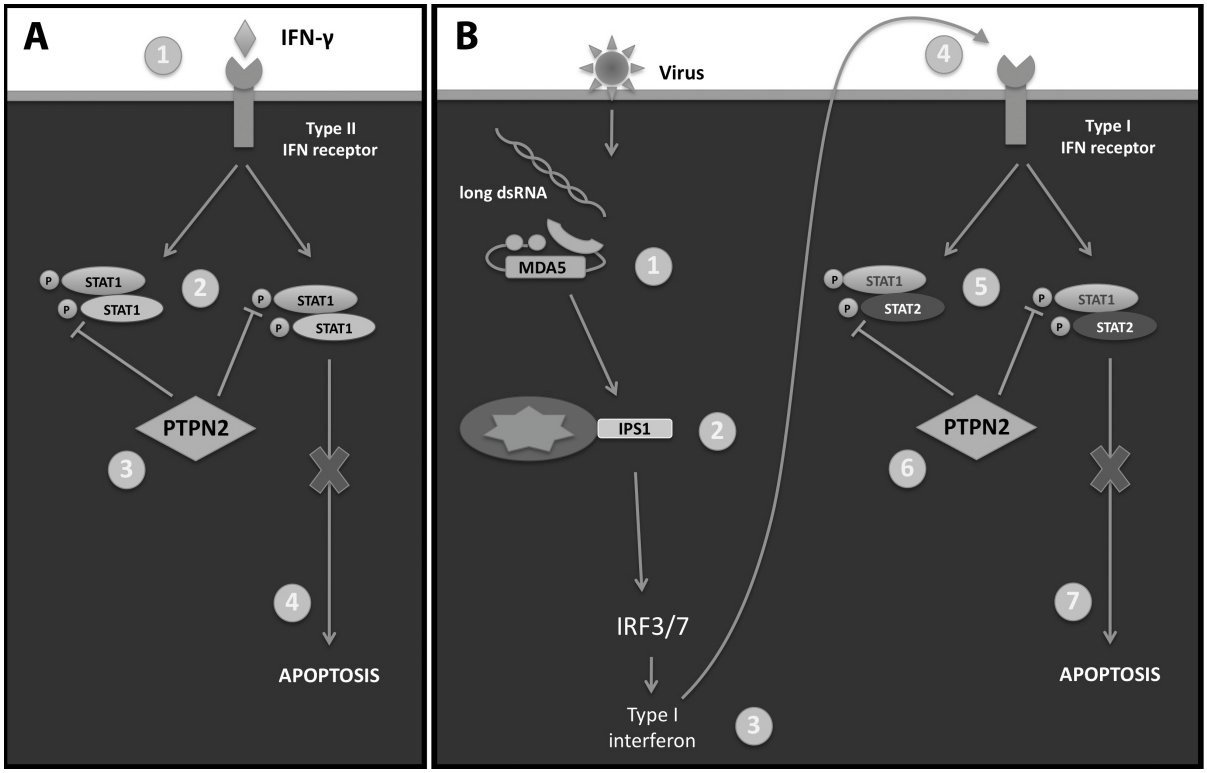


Figure 11. Pathways controlled by PTPN2 in beta cells exposed to cytokines (A) or intracellular dsRNA (B).

A- 1) Interferon-γ binds to its receptor on the beta cell surface, 2) STAT1 phosphorylation is activated, 3) PTPN2 dephosphorylates STAT1 as part of feedback mechanisms to prevent apoptosis, 4) if PTPN2 fails to modulate STAT1 activation, beta cell apoptosis is induced.

B- 1) dsRNA produced during replication and transcription of both RNA and DNA viruses is recognized mainly by MDA5 in beta cells. After this, a complex of kinases is activated and binds to the mitochondrial adaptor IPS1. 2) This adaptor promotes the activation of interferon regulatory factors (IRFs), 3) IRF3/7 induce the expression of the anti-viral type I interferons, 4) type I interferons bind to its receptor in an autocrine/paracrine way and 5) activates the ISGF3 complex (STAT1/STAT2/IRF9), 6) to control the activity of ISGF3 the beta cells increase the expression of PTPN2 that dephosphorylates STAT1 and 2, 7) preventing the induction of apoptosis potentially caused by an excessive or prolonged ISGF3 activation.

The model proposed for PTNP2 actions in beta cells (Figure 11) is based on the idea that polymorphisms in this gene lead to loss of function. The SNPs already described for *PTPN2* are localized upstream of the coding region of the gene or in introns, and further

studies are necessary to confirm the effect of these variants in *PTPN2* function. Study in *PTPN2* knockout mice demonstrated that these animals have altered hematopoiesis and develop a severe systemic inflammatory disease within two weeks of birth, characterized by chronic myocarditis, gastritis, and nephritis [301]. Furthermore, the heterozygous *PTPN2* mutant mice are more susceptible to dextran sulphate sodium-induced colitis, an experimental model of inflammatory colitis [420]. These information indicate that loss of function might be the possible effect of the *PTPN2* polymorphisms observed in T1D patients, but also in Celiac disease [421] and Crohn's disease [295].

The second candidate gene selected for functional evaluation was *MDA5*; it was also identified by comparison between genome-wide association studies and beta cell array studies (www.t1dbase.org), as described above. The disease-associated region on chromosome 2q24.3, where *MDA5* is localized, has three additional T1D candidate genes: fibroblast activation protein (*FAP*), grancalcin (*GCA*), and the potassium voltage-gated channel and subfamily H member 7 (*KCNH7*) [187]. Since *MDA5* is an helicase involved in the recognition of viral infection, and viruses are among the most probable environmental factors related to T1D [173, 174, 371], there was great interest in confirming its role as a T1D candidate gene. To answer this question, a recent study re-sequenced the *MDA5* gene in 480 T1D patients and 480 healthy controls; this confirmed the previous SNPs and identified four new rare variants as associated to T1D risk [188]. These rare *MDA5* variants present stronger protective effects (OR: 0.51-0.74) as compared to the previously identified polymorphisms (OR: 0.86) [188]. Computational analysis suggested that some of the variants modify the MDA5 protein, compromising its function [188]. These findings were confirmed by *in vitro* studies using double KO cells (RIG-I and *MDA5*) transfected with plasmids expressing the previously described variants [189].

MDA5 expression at basal level in human islets is similar to *glucokinase*, and it presents a 4.5-11-fold or 8.3-12.9-fold up-regulation after exposure to cytokines or viral infection, respectively [410]. We observed similar effects in INS-1E cells and FACS-purified rat beta cells (Article II). Differently from *PTPN2* knockdown, however, *MDA5* silencing did not modify beta cells apoptosis induced by intracellular dsRNA (Article II), demonstrating that this gene alone is not a key regulator of beta cell death. A possible explanation is that recognition of dsRNA is a redundant system, in which RIG-I, PKR, NALP3 and TLR3 can recognize the viral by-product besides *MDA5* [135, 422]. Primary beta cells express all these receptors [410], and the same is true for INS-1E cells, with the exception of TLR3 [156]. TLR3 is involved mainly in apoptosis caused by extracellular dsRNA [156, 162]. We used a combination of *MDA5* + *RIG-I* siRNAs without observing any additional protection against beta cell death (Article II). This suggests that another combination of receptors may be involved and that multiple knockdowns (more then 3 genes) are necessary to protect beta cells against intracellular dsRNA. Of note, our experiments were done using knockdown of these genes by specific siRNAs that led to an 80% inhibition (Article II). This residual function of the KD genes could still convey part of the pro-apoptotic stimuli, but it seems unlikely that an 80% blocking, which suffices to nearly completely prevent the pro-inflammatory response (see below), would not induce at least a partial protection against apoptosis if this was indeed a downstream effect of *MDA5* in beta cells. (Article II). The existence of unknown receptors cannot be excluded since the recognition of endogenous and exogenous stimulus by PRRs is a field in constant progress [133].

Despite the fact that polymorphisms in *MDA5* probably does not augment T1D risk via increasing beta cell death, we identified a major role for this gene in controlling the expression of several chemokines and cytokines (Article II). Among the chemokines downregulated by knockdown of *MDA5*, CXCL10 is of special interest. Two independent

studies identified CXCL10 expression in islets of recent-onset T1D, with [3, 207] or without accompanying viral infection of beta cells [207]. Importantly CXCR3, the CXCL10 receptor, was present in the pancreas-infiltrating lymphocytes [207], suggesting a "dialog" between CXCL10-producing beta cells and T cells expressing the chemokine receptor. CXCL10 is increased in the serum of T1D patients at the moment of the diagnosis; it decreases after 16 months of follow-up, but still remains higher than in healthy controls [423]. Long-lasting T1D patients (> 1 year) have similar increase in CXCL10 in the serum, and hyperglycemia further augments the secretion of CXCL10 by monocytes collected from these individuals [424]. Blockage of CXCL10 [425] or CXCR3 knockout [426] prevent viral-induced autoimmune diabetes in RIP-LCMV mice.

Another chemokine, CCL2, was observed to be under control of *MDA5* after beta cell exposure to dsRNA (Article II). An increase in CCL2 in the serum of T1D patients was detected in some [427] but not other studies [423]. As commented on section 3.2.4, these discrepant results may be caused by differences in timing of disease, dilution of the chemokine in the circulation or other methodological problems. NOD mice knockout for CCR2, the receptor for CCL2, have a significant delay in the development of diabetes, but reach a similar incidence of diabetes at 30 weeks [428]. This suggests that CCL2 has a more important role at early time points of insulitis, while the intense pro-inflammatory milieu of chemokines/cytokines and T cell infiltration present in islets at later stages of insulitis is sufficient for triggering disease.

Type 1 interferons are key cytokines in mounting cellular responses against viruses [417, 429]. They "prepare" the immune system to fight against viral infections by promoting maturation of dendritic cells, activating Natural Killer cells and cytotoxic T cells [429]. Furthermore, these cytokines have direct effects in the viral-infected cells by increasing the expression of class I MHC and consequently presentation of viral peptides and endogenous

antigens to CD8+ T cells [429]. The persistent infection of beta cells by viruses may lead to a chronic local production of IFN-α/β and protracted presentation of different autoantigens to autoreactive T cells [174, 175]. The decrease in dsRNA-induced IFN-β expression induced by silencing *MDA5* (Article II) might be one of the mechanisms responsible for the protection against T1D observed in individuals with loss-of-function *MDA5* polymorphisms. Since these variants do not completely abrogate *MDA5* function, they probably decrease local inflammation to an extent that is sufficient to prevent local damage but still allow a sufficient anti-viral response to avoid disseminated/chronic infection. Our data, using siRNAs to knockdown *MDA5*, demonstrate a significant but not complete reduction in the expression of interferons and several chemokines/cytokines (Article II), which may be reminiscent of the putative *in vivo* situation. These other cytokines/chemokines regulated by *MDA5* are further discussed in section 3.2.4.

PTPN2 and *MDA5* are also expressed in cells from the immune system [141, 430], and future experiments involving conditional knockout in both pancreatic beta cells and/or immune cells are required to provide a complete idea on the role of these genes in beta cells and the immune system during diabetes development.

Taken into consideration our results (Articles I and II) and previously published studies [188, 299, 301, 420], we hereby propose a unified model to explain the roles of *PTPN2* and *MDA5* in pancreatic beta cells infected by viruses (Figure 12).

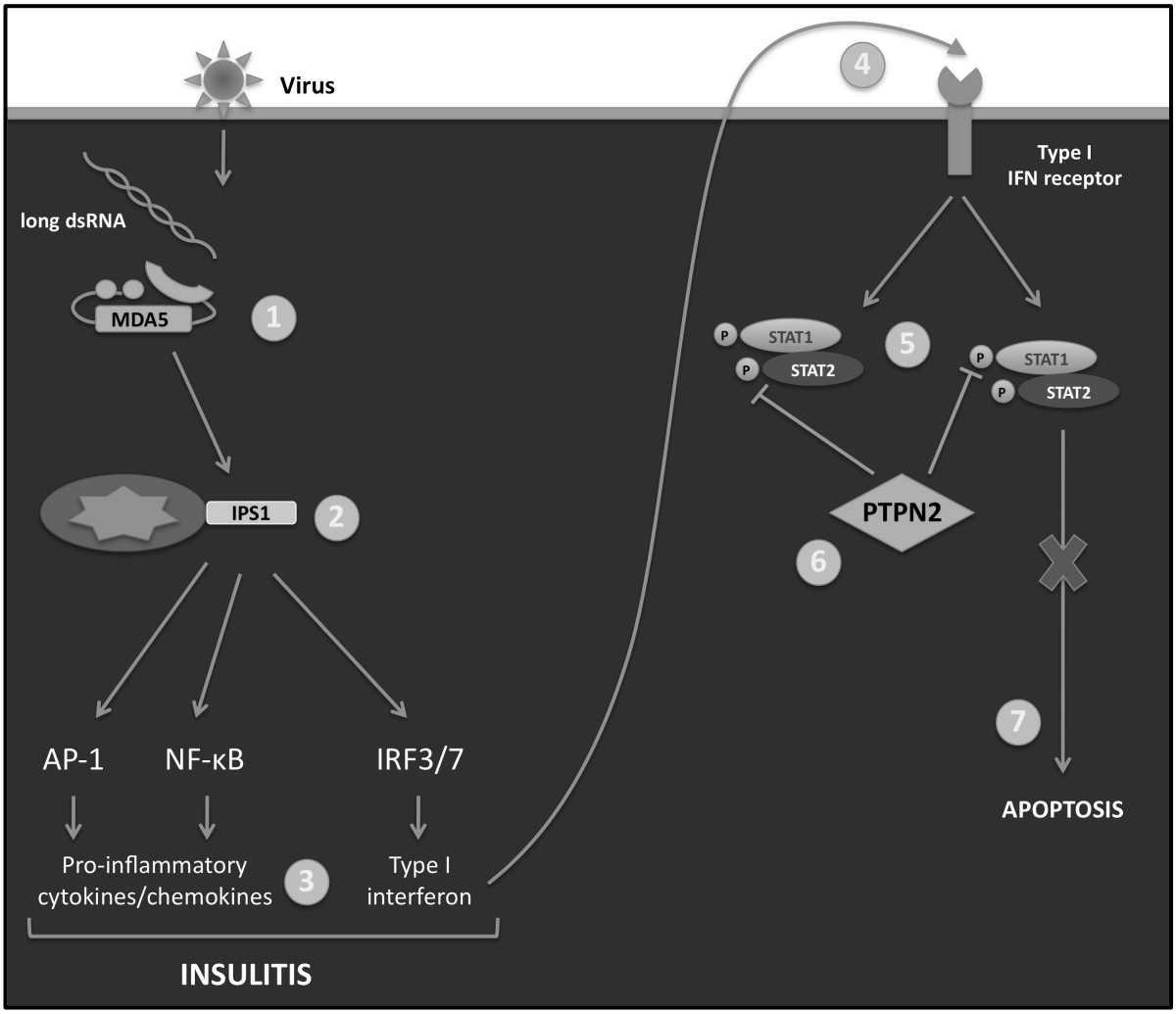


Figure 12. Effects of PTPN2 and MDA5, two candidate genes for T1D, in pancreatic beta cells exposed to intracellular dsRNA. 1) During viral infection of beta cells, the viral by-product dsRNA is released into the cytoplasm. Coxsackieviruses, the main viruses associated with T1D, usually generate long dsRNAs (>2,000 bp) [431] which are recognized by the helicase MDA5. 2) MDA5 then activates several kinases that produce a complex which binds to the mitochondrial adaptor IPS1. 3) IPS1 activates several transcription factors, such as AP-1, NF-κB and IRF3/7, leading to expression of proinflammatory cytokines/chemokines. 4) Type I interferons act in a autocrine/paracrine way in its receptor and, 5) promote phosphorylation of STAT1/2 and the assembly of ISGF3. 6) To control the excessive activation of STAT1/2, which may trigger apoptosis, beta cells increase the expression of PTPN2 that dephosphorylates these molecules. 7) If PTPN2 is not present, or is present in insufficient amounts, overactivation of STAT1/2 leads to beta cell death.

In summary, our results were the first to demonstrate that the T1D candidate genes *PTPN2* and *MDA5* have a direct role in beta cells exposed to proinflammatory cytokines and/or intracellular dsRNA, besides their effects in the immune system. These findings are potentially important because they reinforce the active role of pancreatic beta cells during the process of insulitis, a long-neglected aspect of T1D research, and provide a possible biological explanation for genetic findings from genome-wide association studies; this is an essential part in the confirmation and characterization of new candidate genes for T1D. We demonstrated with this approach that *PTPN2* is a regulator of beta cells apoptosis caused by proinflammatory cytokines and intracellular dsRNA, while *MDA5* seems to control local infiltration by immune cells via regulation of chemokine/cytokine expression, and possibly indirectly via class I MHC antigen presentation.

9. Future perspectives

Our results raised some important questions that require further studies. We emphasize here four of them:

1) Which are the mechanisms involved in pancreatic beta cells apoptosis induced by intracellular dsRNA? We have presently excluded *MDA5* and *RIG-I* as mediators of apoptosis, and additional pathways now need to be examined.

2) Do proinflammatory mediators and dsRNA modify the way beta cells present autoantigens to the immune system? Does *MDA5* knockdown, by decreasing cytokine/chemokine expression, decrease this antigenic presentation?

3) Besides *PTPN2* and *MDA5*, which are the other candidate genes expressed by beta cells, and what are their biological functions on beta cell survival and the triggering of insulitis?

4) Are there interactions between these and other candidate genes which lead to modification/potentiation of their effects on beta cell responses to proinflammatory cytokines and intracellular dsRNA, and on the "amplifying dialog" with the immune system?

To address these questions some experiments are already ongoing in our group. Thus, to identify the mechanisms of dsRNA-induced cell death we are presently evaluating the intrinsic and extrinsic pathways of apoptosis, which culminate in activation of caspase 3, the main effector caspase. The extrinsic pathway involves caspase 8 activation, and it is mitochondrial independent [432]. The intrinsic pathway produces major mitochondrial modifications that lead to caspase 9 activation and apoptosis [432].

The role of antigen presentation will be pursued in collaboration with the laboratory of Prof. Chantal Mathieu (KUL-Belgium). We intend to evaluate whether "danger signals" (proinflammatory cytokines, dsRNA, metabolic stress, etc) increase or modify the antigens that are presented to the immune system. It will be also interesting to examine whether *MDA5*

additionally modulates the cross-talk with the immune system via controlling how much beta cells "present themselves" to autoreactive T cells.

We are already looking for further candidate genes expressed in pancreatic beta cells and potentially involved in responses to proinflammatory cytokines and dsRNA, starting with *GLIS3*. For this purpose, we are using the same approach followed in this thesis.

Finally, using data from this thesis and from still to be studied candidate genes, we hope to unveil cellular networks connecting candidate genes and leading to putative modification/potentiation in their effects by potential co-inheritance. These studies will require both multiple knockdown of these genes *in vitro* and the development of several novel transgenic mouse models.

10.References

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