

**The Role of DcR3 in
Systemic Lupus Erythematosus and
Islet β -Cell Viability and Function**

Bing Han

Département de Médecine

Faculté de Médecine

Université de Montréal

**Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiae Doctor (Ph.D.)
en Sciences Biomédicales**

July, 2009

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

« The Role of DcR3 in Systemic Lupus Erythematosus and
Islet β -Cell Viability and Function »

Présentée par:

Bing Han

A été évaluée par un jury composé des personnes suivantes:

Dr. Manuela Santos

.....

Président du jury

Dr. Jiangping Wu

.....

Directeur de recherché

Dr. Hongyu Luo

.....

Directeur de recherche

Dr. Janos G. Filep

.....

Membre du jury

Dr. Alain Lamarre

.....

Examineur externe

Dr. Daniel Lajeunesse

.....

Représentant du doyen de la FES

Summary

Decoy receptor 3 (DcR3) is a member of the tumor necrosis factor (TNF) receptor family, and is widely expressed in human normal tissues and malignant tumors. It is a decoy receptor of three TNF family members, i.e., FasL, LIGHT and TL1A. The interaction of DcR3 and its ligands will not transmit signal into cells via DcR3 because DcR3 is a soluble protein without a transmembrane and intracellular segment. Thereby, DcR3 competitively inhibits signaling through three functional receptors, i.e., Fas, HVEM/LT β R and DR3.

In previous studies, we found that DcR3 could modulate immune cell function, and protect islet viability. Herein, we generated DcR3 transgenic (Tg) mice driven by the human β -actin promoter to further investigate the function of DcR3.

Interestingly, the DcR3 Tg mice developed a lupus-like syndrome at 6 months of age. They presented a variety of autoantibodies including anti-nucleus and anti-dsDNA antibodies. They also manifested renal, dermal, hepatic and hematopoietic lesions. Compared to lpr and gld mouse lupus models, DcR3 Tg mice more closely resembled human SLE in terms of Th2-biased immune response and anti-Sm antibody production.

Furthermore, we found that DcR3-producing hematopoietic cell were sufficient to cause these pathological changes. Mechanistically, DcR3 may break T-cell homeostasis to interfere with peripheral tolerance, and then induce autoimmunity.

In humans, we detected high DcR3 levels in SLE patient sera. The high DcR3 levels were related to elevated IgE titer in some SLE patients, as was the case in the mouse model. Therefore, DcR3 may represent an important pathogenetic factor of human SLE.

Utilizing the DcR3 Tg mouse, we further elucidated the mechanism by which DcR3 protected islets from primary nonfunction (PNF). Blocking of LIGHT and TL1A signaling by DcR3 are involved in such protection. Moreover, by mRNA microarray we identified possible downstream molecules, which may mediate such protection. We confirmed that *Adcyap1* and *Bank1* played critical roles in mediating DcR3's effect in islet protection.

Our studies resolved a puzzle about the relationship between the Fas/FasL apoptosis signaling pathway and the pathogenesis of human SLE. DcR3 can block Fas/FasL pathway even if there is no genetic mutation in Fas and FasL. DcR3 can simultaneously interfere with LIGHT and TL1A signaling to cause a more complex phenotype than the simple Fas or FasL mutation

in patients. DcR3 can also be employed as a potential diagnostic parameter for SLE. The discovery of the mechanism of DcR3 in protecting islets allows us to explore novel therapeutic targets to protect islet graft.

Key words: DcR3; transgenic; systemic lupus erythematosus; islet transplantation; primary nonfunction (PNF).

Résumé

Le récepteur DcR3 (Decoy receptor 3) est un membre de la famille des récepteurs aux facteurs de nécrose tumorale (TNF). Il est fortement exprimé dans les tissus humains normaux ainsi que les tumeurs malignes. DcR3 est un récepteur pour trois ligands de la famille du TNF tels que FasL, LIGHT et TL1A. Étant une protéine soluble donc dépourvue de la portion transmembranaire et intracytoplasmique, le récepteur DcR3 est incapable d'effectuer une transduction de signal intracellulaire à la suite de son interaction avec ses ligands. De ce fait, DcR3 joue un rôle de compétiteur pour ces derniers, afin d'inhiber la signalisation via leurs récepteurs fonctionnels tels que Fas, HVEM/LT β R et DR3.

Lors de nos précédentes études, nous avons pu démontrer, que DcR3 pouvaist moduler la fonction des cellules immunitaires, et aussi protéger la viabilité des îlots de Langerhans. À la suite de ces résultats, nous avons généré des souris DcR3 transgéniques (Tg) en utilisant le promoteur du gène β -actine humaine afin d'étudier plus amplement la fonction de ce récepteur.

Les souris Tg DcR3 ont finalement développé le syndrome lupus-like (SLE) seulement après l'âge de 6 mois. Ces souris présentent une variété

d'auto-anticorps comprenant des anticorps anti-noyaux et anti-ADN. Elles ont également manifesté des lésions rénales, cutanées, hépatiques et hématopoïétiques. Contrairement aux modèles de lupus murin lpr et gld, les souris DcR3 sont plus proche du SLE humain en terme de réponse immunitaire de type Th2 et de production d'anticorps d'anti-Sm. En plus, nous avons constaté que les cellules hématopoïétiques produisant DcR3 sont suffisantes pour causer ces pathologies. DcR3 peut agir en perturbant l'homéostasie des cellules T pour interférer avec la tolérance périphérique, et ainsi induire l'autoimmunité.

Chez l'humain, nous avons détecté dans le sérum de patients SLE des niveaux élevés de la protéine DcR3. Chez certains patients, comme chez la souris, ces niveaux sont liés directement aux titres élevés d'IgE. Par conséquent, DcR3 peut représenter un facteur pathogénique important du SLE humain.

L'étude des souris Tg DcR3, nous a permis aussi d'élucider le mécanisme de protection des îlots de Langerhans. Le blocage de la signalisation des ligands LIGHT et TL1A par DcR3 est impliqué dans une telle protection. D'ailleurs, nous avons identifié par ARN microarray quelques molécules en aval de cette interaction, qui peuvent jouer un rôle dans le mécanisme

d'action. Nous avons par la suite confirmé que Adcyap1 et Bank1 joue un rôle critique dans la protection des îlots de Langerhans médiée par DcR3.

Notre étude a ainsi élucidé le lien qui existe entre la signalisation apoptotique médiée par Fas/FasL et la pathogénèse du SLE humain. Donc, malgré l'absence de mutations génétiques sur Fas et FasL dans le cas de cette pathologie, DcR3 est capable de bloquer cette signalisation et provoquer le SLE chez l'humain. Ainsi, DcR3 peut simultanément interférer avec la signalisation des ligands LIGHT et TL1A et causer un phénotype plus complexe que les phénotypes résultant de la mutation de Fas ou de FasL chez certains patients. DcR3 peut également être utilisé comme paramètre diagnostique potentiel pour le SLE. Les découvertes du mécanisme de protection des îlots de Langerhans par DcR3 ouvrent la porte vers de nouveaux horizons afin d'explorer de nouvelles cibles thérapeutiques pour protéger la greffe d'îlots.

Mots clés : DcR3; transgénique; systemic lupus erythematosus; transplantation d'îlots; PNF.

INDEX

<i>Summary</i>	<i>III</i>
<i>Résumé</i>	<i>VI</i>
<i>List of Figures</i>	<i>XIII</i>
<i>List of Abbreviations</i>	<i>XVI</i>
<i>Acknowledgements</i>	<i>XX</i>

I. INTRODUCTION	1
1. Decoy receptor 3	2
1-1 General information about DcR3.....	2
1-2 Biological function of DcR3 and its molecular mechanism.....	4
1-2-1 Fas and FasL.....	4
1-2-2 LIGHT and HVEM/LT β R	6
1-2-3 TL1A and DR3.....	7
1-3 Regulation of DcR3 expression.....	8
1-4 Immunity and DcR3.....	10
1-5 Cancer and DcR3.....	11
2. Systemic lupus erythematosus (SLE)	14
2-1 Epidemiology of SLE	14
2-2 Pathoetiology of SLE.....	15
2-2-1 Hormones.....	16
2-2-2 Genetic factors.....	17

2-2-3	Environment factors.....	18
2-2-4	Apoptosis and SLE.....	19
2-3	Mouse models of SLE.....	22
2-4	Clinical features of SLE.....	23
2-4-1	Symptoms.....	23
2-4-2	Diagnosis.....	23
2-4-3	Therapy.....	25
2-4-4	Prognosis.....	27
3.	Diabetes mellitus.....	28
3-1	The Epidemiology of Diabetes Mellitus.....	28
3-2	The pathoaetiology of Diabetes Mellitus.....	31
3-2-1	Genetic Factors.....	33
3-2-2	Environmental Factors.....	34
3-2-3	Accelerator Hypothesis.....	36
3-3	The clinical features of DM.....	37
3-3-1	Symptoms and complications.....	37
3-3-2	Diagnosis.....	39
3-3-3	Therapy.....	40
3-3-3-1	Diabetes education and lifestyle intervention.....	40
3-3-3-2	Insulin and other medications.....	40
3-3-3-3	Organ and cell transplantation.....	42
3-3-3-4	Gene therapy.....	45

Hypothesis and Objective	46
References for introduction	47
II. ARTICLES	66
Article 1: Overexpression of human decoy receptor 3 in mice results in a systemic lupus erythematosus-like syndrome.....	67
Article 2: DcR3 as a diagnostic parameter and risk factor for systemic lupus erythematosus.....	109
Article 3: DcR3 protects islet β -cells from apoptosis through modulating Adcyap1 and Bank1 expression.....	145
III. DISCUSSION	190
1. DcR3 and the pathogenesis of SLE	191
1-1 The DcR3 overexpression in self-reactive lymphocyte interferes with their anergy.....	192
1-2 DcR3 impairs the clearance of apoptotic cells and increases autoantigen presentation.....	194
1-3 DcR3 increases the memory T cell pool and enhances the secondary response to autoantigens.....	195
1-4 DcR3 disturbs B-cell homeostasis and tolerance.....	196
1-5 DcR3 favors Th2 development.....	197
2. The mechanisms by which DcR3 protects pancreatic islets	198
2-1 DcR3 Tg islet graft resisted PNF and presented long term normoglycemia in syngeneic diabetes mouse receiving a suboptimal dose of islets.....	198

2-2	DcR3 acts through the TL1A and LIGHT pathways.....	199
2-3	Using DNA microarray to identify molecules downstream of DcR3 action.....	200
	Conclusion and Future Perspectives.....	202
	References for discussion.....	203

LIST OF FIGURES AND TABLES

Introduction

Fig 1. The homology of the amino-acid sequences between DcR3 and OPG (osteoprotegerin)....	3
Fig 2. The apoptotic signal pathway of Fas / FasL (CD95/CD95L).....	5
Fig 3. Genomic structure of DcR3 and its adjacent genes.....	10
Fig 4. The susceptibility loci of SLE.....	18
Fig 5. The geographic distribution of DM prevalence.....	30
Fig 6. The pathogenesis of T1DM.....	31
Fig 7. Pathophysiology of T2DM.....	32
Table 1. Characteristics of lupus-prone mouse strains.....	22
Table 2. Diagnostic criteria of diabetes mellitus and other categories of hyperglycemia.....	39
Table 3. Medications for Diabetes.....	41

Article 1:

Fig 1. Generation and characterization of decoy receptor 3 (DcR3)-transgenic mice.....	99
Fig 2. Lymphadenopathy and survival rates in DcR3-Tg mice	100
Fig 3. Autoantibodies and B-1 cells in DcR3-Tg and WT mice.....	102
Fig 4. Tissue histopathology and urine and hematologic findings in aged DcR-Tg and WT mice.....	104
Fig 5. DcR3-induced protection of T cells against apoptosis.....	106

Fig 6. Expanded CD44 ^{high} ,CD62L ^{low} ,CD25- CD4 population in peripheral blood mononuclear cells (PBMCs) of DcR3-Tg mice.....	108
--	-----

Article 2:

Fig 1. Serum DcR3 levels and their relationship with SLEDAI in SLE patients.....	137
Fig 2. Relationship among medication, serum DcR3 levels and SLEDAI of SLE patients.....	139
Fig 3. Elevated serum IgE levels in SLE patients.....	140
Fig 4. T _H 2-prone immune responses in young DcR3 Tg mice.....	141
Fig 5. Development of an SLE-like syndrome in WBI-BMTx mice.....	142
Fig 6. Reduced survival rate in Tg BMTx recipients.....	144

Article 3:

Fig 1. DcR3 secretion by Tg islets.....	181
Fig 2. Tg islets present reduced apoptosis and maintain their function under cytokines assault...	182
Fig 3. Expression of TL1A and LIGHT pathway components in islets according to real-time PCR.....	183
Fig 4. Recombinant mouse LIGHT and TL1A induced lpr/lpr islet cell apoptosis.....	184
Fig 5. Reduced islet PNF in transplantation using DcR3 Tg islets.....	185
Fig 6. Adcyap1 overexpression protected β -cells from cytokine-induced apoptosis.....	186
Fig 7. Inhibition of Bank1 expression decreased cytokine-induced apoptosis in NIT-1 cells.....	188
Table 1. Real-time PCR primers used to quantify mRNA levels.....	178

Table 2. Genes identified by DNA microarray and verified by real-time PCR from IFN- γ plus IL-1 β -treated Tg versus WT islets.....179

Table 3. Genes identified by DNA microarray and verified by real-time PCR from TNF- α plus IL-1 β -treated Tg versus WT islets.....180

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
Adcyap1	adenylate cyclase activating polypeptide 1
AICD	activation-induced cell death
ALPS	lymphoproliferative syndrome
ANA	anti-nuclear antibody
AZA	azathioprine
BMTx	bone marrow transplantation
CAPN10	calpain 10
CARD	caspase-recruitment domain
CCR-7	CC-chemokine receptor-7
cDNA	complementary DNA
CFM	cyclophosphamide
CRD	cysteine-rich domains
CTLA4	cytotoxic lymphocyte antigen 4
DAPK	death-associated protein kinase
DcR3	Decoy receptor 3
DD	death domain
DED	death-effector domain
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DISC	death-inducing signalling complex
DR	death receptor
DR3	death domain-containing receptor 3

ds-DNA	double stranded DNA
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1 and 2
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FFA	free fatty acid
FLICE	FADD-like ICE
GAD	glutamic acid decarboxylase
GnRH	gonadotrophin releasing hormone
H&E	hematoxylin and eosin
HCQ	hydroxychloroquine
HLA	human leukocyte antigen
HPA	hypothalamo–pituitary–adrenal
HVEM	herpesvirus entry mediator protein
i.v.	intravenously
IEQ	islet equivalents
IFN-γ	Interferon- γ
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IL-2	Interleukin-2
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
JNK	c-Jun NH ₂ -terminal kinase
KIR	killer immunoglobulin-like receptors

LADA	latent autoimmune diabetes of the adult
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM
LTβR	lymphotoxin β receptor
MAPK	mitogen-activated protein kinases
MICA-5	MHC class I chain-related genes allele 5
mTOR	mammalian target of rapamycin
NF-κB	Transcription factor nuclear factor- κ B
NHL	novel helicase-like gene
NSAIDs	non-steroidal anti-inflammatory drugs
NZB	New Zealand Black
NZW	New Zealand White
OGTT	oral glucose tolerance test
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDN	prednisone
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol myristate acetate
PNF	primary non-function
RRE	Rta-responsive element
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
STAT	signal transducer and activator of transcription
STZ	streptozocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus

TCRβ	T cell receptor β
Tg	transgenic
TL1A	TNF-like molecule 1A
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
UV	Ultraviolet
VEGI	vascular endothelial growth inhibitor
WBI	whole-body irradiation
WT	wild type

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Jiangping Wu and Dr. Hongyu Luo for their scientific guidance and encouragement throughout this study and during the preparation of this thesis.

I would also like to thank all the colleagues in the lab for their splendid co-operation.

Thanks also extended to all my friends and those who always support me in different ways.

Finally, I would like to thank all of my family members for their understanding, patience, and their great support, which were critically important for me to complete this study.

I. Introduction

1. Decoy receptor 3

1-1. General information about DcR3

Decoy receptor 3 (DcR3) is a member of the tumor necrosis factor receptor (TNFR) family. Its full-length complementary DNA was first isolated from human fetal lung in 1998 by Pitti et al (1). The DcR3 gene is mapped to chromosome 20q13.3, a region associated with gene amplification and rearrangement in human cancer (2,3). The DcR3 cDNA encodes a 300-aa secreted protein that lacks transmembrane and intracellular domains in its sequence (1,4). It contains 4 conserved cysteine-rich domains (CRD) as other members of TNFR family, and one N-linked glycosylation site (Fig.1). There are two transcription variants of DcR3 at the 5' untranslated region but they encode the same protein (NM_032945.2 and NM_003823.2). DcR3 is expressed in humans, chicken (5), and Conger myriaster (6). The rodents do not have any homologue of DcR3 according to genome-wide computer search.

DcR3 is present in the normal human fetal lung, brain, and liver, also in the adult spleen, colon, lung, and activated T cells (1,7). More interestingly, it is highly expressed in many malignant tumors/cells. Overexpression of DcR3 has been reported in 34% (27/79) gastric carcinoma (8), in 44%

(30/68) gastrointestinal tract adenocarcinomas (9), in 63%-73% (185/294-163/223) colorectal tumors (10), in 60% (29/48) hepatocellular carcinoma (11), in 67% (10/15) pancreatic cancer (12), and in 83% (15/18) of high-grade gliomas (13). It is also found in lymphomas, renal cancers and ovarian cancers (14,15,16). According to our ELISA analysis, about 50–60% of various tumors overexpressed DcR3 (17).

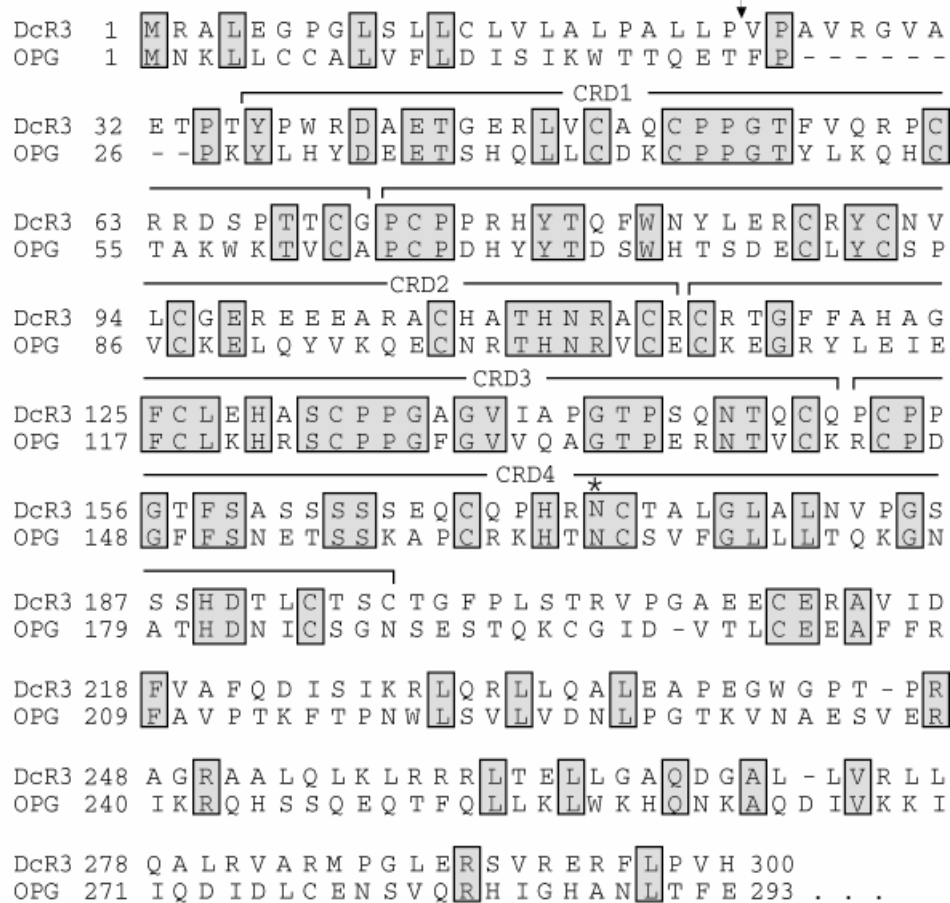


Figure 1. The homology of the amino-acid sequences between DcR3 and OPG (osteoprotegerin, a typical member of TNFR family) (source: ref.1)

Arrow: the putative signal cleavage site;

CRD: the cysteine-rich domains;

Asterisk: the N-linked glycosylation site.

1-2. Biological function of DcR3 and its molecular mechanism

Although the DcR3 does not transmit signal into cells, it is able to bind three TNF family members, i.e., FasL (Fas ligand), LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM) and TL1A (TNF-like molecule 1A) (1,4,9,18). Such competition will disturb the interaction of these molecules with their functional receptors, i.e., FasL with Fas, LIGHT with HVEM (herpesvirus entry mediator protein) (19,20) and LT β R (lymphotoxin β receptor) (21,22), and TL1A with DR3 (death domain-containing receptor 3) (18). As a consequence, DcR3 competitively suppresses signaling through these receptors, and interferes with their functions. A brief review of the functions of Fas, LIGHT and TL1A is given below.

1-2-1. Fas and FasL: Fas antigen, also known as CD95 or APO-1, is a principal death receptor to trigger apoptosis signaling pathway (Fig.2). Various types of cells express Fas, including most immune cells (i.e., T cells, activated B cells, mononuclear phagocytes) and some non-immune cells in the liver, lung, heart (23) and islets of Langerhans (24). Fas contains a conserved “death domain” in its cytoplasmic region. FasL is a homotrimeric membrane protein which is predominantly expressed on activated T cells. When FasL binds to Fas, it clusters 3 or more Fas

molecules to form the death-induced signaling complex (DISC). The death domain of the Fas polymer recruits Fas-associated death domain (FADD). The death effector domain of FADD consequently binds to FADD-like ICE (FLICE), or more commonly known as pro-caspase 8. FLICE can be cleaved into p10 and p18 subunits through self-proteolysis, and form an active heterotetramer enzyme. Active caspase-8 is then released into the cytoplasm to cleave and activate downstream effector caspases, such as caspases 3, 6, or 7. This eventually leads to DNA fragmentation, membrane blebbing, and cell death.

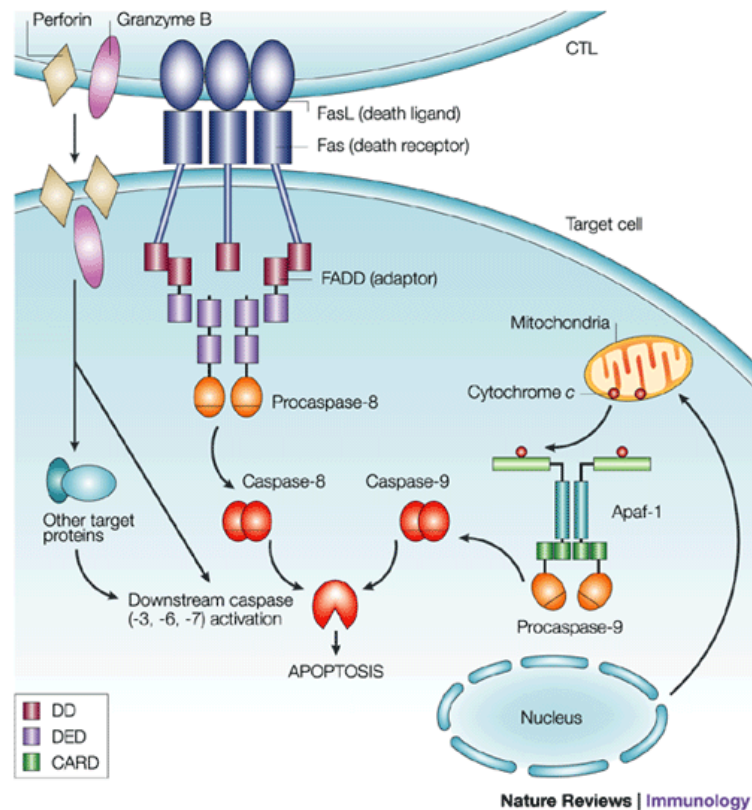


Figure 2: The apoptotic signal pathway of Fas / FasL (CD95/CD95L). (source: ref. 25)

The Fas/FasL apoptosis signaling pathway is involved in T-cell homeostasis, the establishment of immune privilege, cytotoxic T-cell activity (26) and tumor cell survival (27).

Besides triggering Fas positive cell to apoptosis, FasL may also act as a costimulator by retrograde signal transmission into FasL expressing cells by “reverse signaling” (28).

1-2-2. LIGHT and HVEM/LT β R: LIGHT is a type-II transmembrane protein (29), and is highly expressed on activated lymphocytes, CD8⁺ T cell lines, granulocytes, and monocytes (22). LIGHT, as a costimulator through the LIGHT/HVEM interaction, contributes to T-cell activation and modulates T-cell responses (30,31,32). The LIGHT/HVEM signaling has been involved in graft-versus-host diseases (33,34). On the other hand, the LIGHT/LT β R signaling plays various biological activities, including the induction of apoptosis (35,36,37), organogenesis of lymph nodes (38), restoration of secondary lymphoid structure and function (39,40), and production of cytokines (41). Moreover, LIGHT produced by activated lymphocytes can induce apoptosis of tumor cells expressing both LT β R and HVEM (22).

1-2-3. TL1A and DR3: TL1A (TNFSF15/VEGI) is the most recently identified member of the TNF superfamily. It has been shown to be highly expressed on the membrane of endothelial cells, dendritic cells and peripheral CD4⁺CCR9⁺ T cells (18,42). It is also a soluble protein (often called vascular endothelial growth inhibitor [VEGI]), which is present in body fluids or secreted by TNF- α -treated chondrocytes (43,44). DR3 is a high affinity receptor for TL1A (18) and contains a death domain similar to Fas. DR3 is not only a transmembrane receptor on lymphocytes, but also function as a "decoy" receptor when secreted without the transmembrane domain. The signaling through DR3 can either induce apoptosis or activate NF- κ B (45). In T cells, TL1A activates NF- κ B and cIAP-2 as a costimulator through ligation with DR3, and leads to elevated secretion of IFN- γ and GM-CSF, but not apoptosis (46). This mechanism is involved in certain inflammatory diseases (inflammatory bowel disease, mucosal inflammation, and atherogenesis) (47,48,49). Furthermore, the TL1A/DR3 signaling regulates osteoblast differentiation and apoptosis, and may contribute to arthritis and bone cancer (50).

In addition to FasL, LIGHT and TL1A, there is some indication that DcR3 might bind to other additional ligand(s) (51), but this has not been proven. Although mice do not express DcR3, studies by our team and Bossen et al. have showed that human that DcR3 can bind to mouse FasL, TL1A and LIGHT, and exert biological functions (4,52,53). DcR3 influences multiple physiological functions such as the induction of apoptosis (1,9,22,37,54), regulation of T-cell migration (55), regulation of angiogenesis (56), and modulation of macrophage and dendritic cell differentiation (57,58).

1-3. Regulation of DcR3 expression

The molecular mechanism regulating DcR3 expression in normal cells is not well elucidated. We know that DcR3 expression depends on the transcription factor NF- κ B and the activation of mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase 1 and 2 (ERK1/2) and c-Jun NH2-terminal protein kinase (JNK) (59). A very recent study showed that serum DcR3 levels have cyclic changes according to the menstrual cycle and DcR3 expression in endometrial cells is modulated by sex hormones (60). This study also suggested that the sex hormone-related signaling pathways may participate in the regulation.

In tumors, gene amplification and overexpression of DcR3 are frequently observed (1, 9). However, they are not always concurrent (9,61). Protein overexpression may not depend on genomic change, and the gene amplification may not cause the protein overexpression either. These suggest that the gene amplification is neither sufficient nor necessary for the overexpression of DcR3, and there are other mechanisms to modulate its expression level. Further studies found that insulin-like growth factor-1 induced activation of the PI3K/Akt/NF- κ B signaling pathway is important to regulate endogenous DcR3 expression in human pancreatic carcinoma (62). Interestingly, DcR3 overexpression positively relates to Epstein-Barr virus (EBV) infection in lymphomas (63). Here, DcR3 expression is induced by Rta, a transcriptional activator encoded by EBV (64). Rta not only directly binds to the Rta-responsive element (RRE) sequence located in the DcR3 promoter region, but also enhances PI3-K activity. Besides, the coordinate transcriptional regulation of overlapped genes may be involved in the mechanism. The DcR3 gene is located in a gene-rich cluster and partially overlaps with exons 32 through 35 of the novel helicase-like gene (NHL) (Fig. 3), which relates to multiple inherited human neoplastic disorders (9). Such overlapping structures could cause coordinate transcriptional regulation (65) that may contribute to the up-regulation of DcR3 in tumors or other diseases.

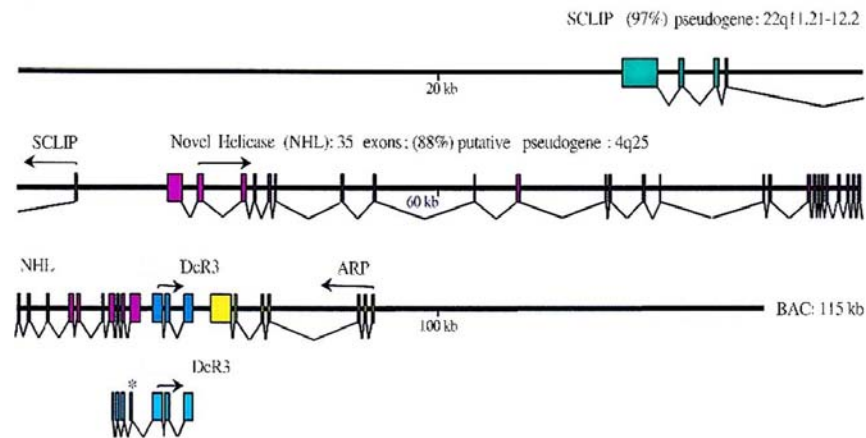


Figure 3. Genomic structure of DcR3 and adjacent genes. (source: ref. 9)

Shaded boxes: Exons, each color represents one gene;

Arrows: The translation initiation methionine and the direction of protein translation;

*: One of DcR3 transcript variants overlaps the NHL helicase sequence with exons 32 through 35.

1-4. Immunity and DcR3

DcR3 plays a complex role in immune modulation. DcR3 primarily downregulates immune function. It could suppress T- and B-cell activation and T-cell proliferation (66), inhibit T-cell and macrophage chemotaxis (13,55,66), impair macrophage function (67), as well as induce apoptosis of dendritic cells (68). On the other hand, activated T cells secrete DcR3, which can prevent T cells from activation-induced cell death (AICD) (1) and enhance T-cell activation and cytokine production through

costimulation (69,70,71). It also regulates the differentiation of dendritic cells and macrophages (57, 58), and the expression of adhesion molecules on endothelial cells (72). The complexity of the bioactivity of DcR3 hints that DcR3 may participate in multiple physiological procedures and precisely adjust different signaling pathways according to certain circumstances.

1-5. Cancer and DcR3

As described, DcR3 overexpression is found in diverse malignant tumors. Clinical studies also show relationship between DcR3 levels and malignancy, tumor stage and prognosis. Higher serum DcR3 levels are associated with poorer differentiation, later tumor stages and worse outcomes (8,15,73,74). These observations and in vitro experiments (1,12,75) support the notion that tumors may benefit from the elevation of DcR3 levels to overcome immune surveillance. Indeed, DcR3 may provide several advantages to tumors:

- 1) DcR3 may inhibit NK cell and cytotoxic T lymphocyte activity through blocking of the Fas/FasL, LIGHT/ HVEM-LT β R death signaling pathway;

- 2) DcR3 may suppress the tumor-reactive lymphocyte activation and cytokine production by repressing costimulation of LIGHT and TL1A, or by modulation of dendritic cells;
- 3) DcR3 may restrain T lymphocyte chemotaxis by the activation of LIGHT reverse signaling;
- 4) DcR3 may induce angiogenesis of tumors by neutralization of TL1A;
- 5) DcR3 may disturb mononuclear phagocyte differentiation, adhesion and antigen presenting functions (57,72).

Several questions remain to be answered. Which cells do contribute to increased serum DcR3 levels in tumor patients? Tumor cells, tumor-reactive immune cells, or both? Is DcR3 elevation a cause or a consequence of oncogenesis? In other words, could occasional elevated DcR3 levels induce malignant changes, or existing malignant cells and/or tumor-reactive immune cells over secrete DcR3? We do not have definitive answers to these questions.

In our study, DcR3 transgenic mice do not produce any conspicuous tumors when expressing folds higher endogenous DcR3 than those observed from tumor patients (data not shown). This suggests that sole DcR3 overexpression may not be sufficient to induce but functions as an accessory factor to carcinogenesis. Up-regulated DcR3 levels, which are

caused by virus infection, endocrine disturbances, improper gene regulation, may assist the malignantly transformed cells to escape immune surveillance. In any case, DcR3 is becoming a useful early diagnostic and prognostic marker of various malignant tumors.

In summary, DcR3 is a multifunctional regulator. It is not absolutely needed for normal physiological functions since rodents do not have it at all. Its biological function in humans might be fine tuning of certain signaling pathways. It may involve multiple organ development, immune system maturation, peripheral tolerance maintenance and tissue repair. Dysfunction of DcR3 may result in a variety of disorders in humans, and its expression could be used to monitor conditions of these diseases.

2. Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of many different autoantibodies against cell components, especially nuclear components, with involvement of multiple systems, and variable symptoms. Excessive autoantibodies lead to formation of immune complexes, with deposition in different tissues causing inflammation and consequent tissue damage. The clinical manifestations of SLE vary greatly. The course of the disease is characterized by alternative relapse and remission.

2-1. Epidemiology of SLE

Although about 20 percent of SLE patients are diagnosed before age 16 (76), SLE mainly affects child-bearing age females, and the disease lasts for life (77). It is more frequent among Asians, Afro-Americans and Afro-Caribbeans than Caucasians and Blacks in Africa (78,79,80). The prevalence of SLE is highly varied according to regions. In the USA, it is from 40 to 150 cases per 100,000 (81,82); in Asia, 50 to 100 cases per 100,000 (83); about 20 to 70 per 100,000 in Europe (84,85,86). The

incidence has nearly tripled in the past 40 years, but this maybe due to improved detection of mild forms of the disease (87).

2-2. Pathoetiology of SLE

Immune complex deposition, inflammation and vascular abnormalities are the basic pathological changes of SLE. Their central pathogenesis is the production of autoantibodies. Both T and B lymphocytes are necessary for the generation of autoantibodies. These antibodies aim several self molecules in the nucleus, cytoplasm, and cell surface, as well as some soluble molecules such as IgG and coagulation factors. Antinuclear antibodies are most common. The anti-double stranded DNA (ds-DNA) and anti-Sm antibodies are unique in SLE, and are thought to be the main reasons of tissue damage. When soluble self-antigens are exposed to autoantibodies, they form immune complexes, which are deposited in tissues, especially the joints, glomeruli and vascular wall. The deposited immune complexes will activate the complement system, which will then recruit and activate inflammatory cells, such as neutrophils and macrophages. These inflammatory cells will secrete reactive intermediates to mediate local inflammation. Subsequently, this leads to tissue damage and clinical symptoms.

In addition to T and B lymphocyte function which is important for autoantibody generation, other risk factors such as hormones, environmental elements and genetic susceptibility are also critical in SLE pathogenesis.

2-2-1. Hormones

Sex hormones are crucial in the pathogenesis of SLE. SLE is marked with female predominance. The female to male ratio of SLE patient rises from puberty (3: 1) and peak during child-bearing years (10-15: 1), it then decreases after menopause (8: 1) (88). High-estrogen levels, in the cases of early menarche, estrogen treatment, or Klinefelter's syndrome that are characterized by hypergonadotrophic hypogonadism, could significantly increase the risk of SLE (89,90). Moreover, abnormalities of sex hormone metabolism including redundancy of 16 hydroxyestrone (91) and reduction of androgens (testosterone, dihydrotestosterone, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S) (92,93)) are observed in both male and female SLE patients.

Besides sex hormones, gonadotrophin releasing hormone (GnRH) (94) and hormones in the hypothalamo-pituitary-adrenal (HPA) axis (such as

cortisol, prolactin and thyrotropin releasing hormone) (95,96) also influence the invasion and/or activity of SLE.

2-2-2. Genetic factors

SLE shows a strong familial aggregation (10–12%) among first degree relatives (97). Moreover, the concordance of SLE in identical twins is significantly higher than that in dizygotic twins (25–50% vs. 5%) (98). These observations suggest that the genetic factors play an important role in the pathogenesis of SLE. Although few SLE cases (<5%) are caused by single gene mutations (99), SLE is primarily a polygenic inheritance disease, which needs at least four susceptible genes to cause disease manifestation (100). More than 100 potential risk genes have been identified in the last few decades. Some of them were confirmed to have strong association with SLE. Those include: the genes of human leukocyte antigen (HLA) class II [HLA-DRB1*0301/*0302 (DR3), DRB1*1501/*1503 (DR2), DRB1*08 (DR8) (101,102,103)], genes of some classical complement activation pathway components [C1q, C1r/s (104), C2 (105,106), C4 (107,108,109)], the FCGR genes [FcγR IIa (110), IIb (111) and IIIa (112)], and some genes related to immune regulation, e.g., TNF- α (113,114), PDCD1 (115,116) and CTLA-4 (117,118).

In addition to these candidate genes, 13 susceptibility loci show significant linkage to SLE, according to a genome-wide linkage analysis (Fig. 4) (119). Eight of them (1q23, 1q25-31, 1q41-42, 2q35-37, 4p16-15.2, 6p11-21, 12q24, and 16q12) were confirmed by a number of studies (120,121,122,123, 124,125,126,127,128,129,130).

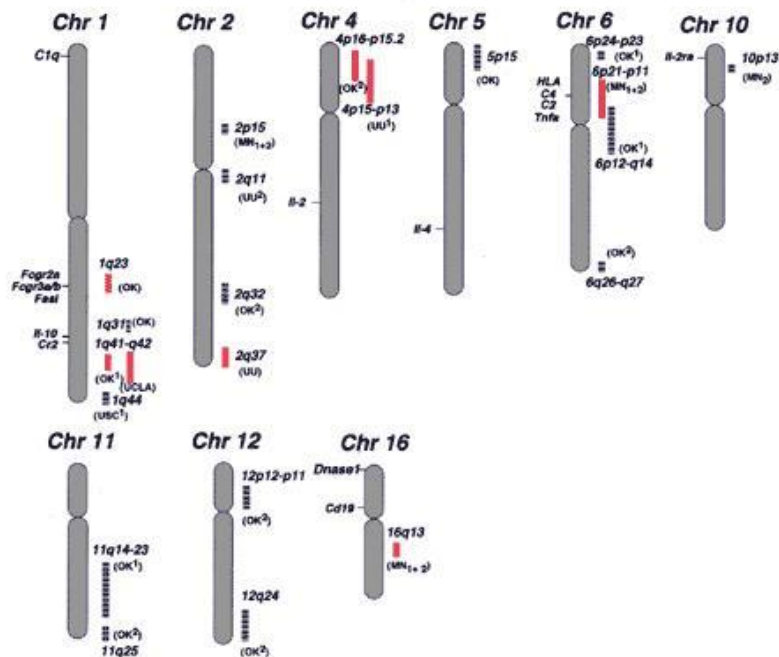


Fig. 4 The susceptibility loci of SLE (source: ref.131)

2-2-3. Environmental factors

The genetic idiosyncrasy and hormonal surroundings create a predisposition to SLE, while environmental factors can trigger the

initiation of SLE. Ultraviolet (UV) light, especially UVB, can bring about keratinocytes apoptosis, and then expose some self-antigens to the immune system triggering autoimmunity (132). Some chemicals, such as aromatic amines, hydrazines and their derivatives, are present in many drugs, agricultural and industrial products (even tobacco). They can potentially ignite a lupus-like syndrome (133). Infections in early childhood seem to affect the SLE risk in later life (134,135). Furthermore, the deficiency of vitamin D also has been found to be associated with SLE (136).

2-2-4. Apoptosis and SLE

Apoptosis, also called programmed cell death, is a process when cells undergo an ordered destruction and clearance, without releasing inflammatory intracellular contents into the extracellular environment. Apoptosis can be initiated by ligation of cell surface death receptors with their ligand(s), or by deficiency of survival stimuli. The former is called activation-induced cell death, and the latter is called passive cell death. In activation-induced cell death, ligated death receptor will activate caspase 8 (and caspase 10 in humans). While lack of survival stimuli will increase permeability of mitochondria and lead to release of cytochrome C.

Cytochrome C then couple with apoptosis activating factor-1 to induce activation of caspase 9. The activation of caspase 8 or caspase 9 is followed by the activation of an enzymatic cascade, nuclear condensation and fragmentation, and plasma membrane blebbing (137). Among all the cell death receptor/ligand signaling pathways, Fas/FasL-mediated apoptosis pathway is the best characterized (Fig 5). This pathway is crucial for the development of immune tolerance (138,139).

During the immune system maturation, self-tolerance is developed through apoptosis of auto-reactive lymphocytes in central lymphoid organs. Immature T/B cells with high affinity receptor of self-antigen will be induced to apoptosis. This is called central tolerance. But not all of the self-antigen can be presented in central lymphoid organs. Some auto-reactive lymphocytes can escape from negative selection. In peripheral, apoptosis of activated lymphocytes following an immune response maintains homeostasis of peripheral lymphocytes numbers, and contributes to peripheral tolerance to self-antigens (140,141). Disturbance in these apoptotic processes might break the balance present in the immune system and may predispose to autoimmunity. Some lupus-prone murine models support this assumption. The *lpr/lpr* and *gld/gld* mice, which have mutation in the Fas and FasL respectively, spontaneously develop

lymphadenopathy and lupus-like syndrome characterized by the presence of autoantibodies to nuclear antigens (142). Another model is the exogenous soluble Fas (sFas)-induced autoimmune symptoms in CD1 mouse (143). These models suggest that the disorder or blocking of Fas/FasL pathway will result in defect of peripheral autoreactive lymphocyte elimination that occurs through Fas-mediated apoptosis (144). However, in humans, Fas or FasL gene defects only cause the lymphoproliferative syndrome (ALPS) (140,145) with rare autoimmune manifestations (146). Most SLE patients do not have abnormality in their Fas/FasL gene structure and expression (147,148,149). Only sFas levels relate to SLE activity (150,151). Otherwise, apoptosis of peripheral lymphocytes in SLE patients is not compromised or even enhanced (152,153). This suggests that the relationship between apoptosis and self-tolerance in humans is much more complex than in mouse.

Apoptotic cells may also expose intracellular and cryptic epitopes to the immune system (154). The degradation and modification of cellular constituents during apoptosis could induce immunogenicity (155,156). If apoptotic residues can not be cleared in due time, e.g., due to deficiencies in complement factors or CD14 (157,158), autoimmunity may be stimulated.

2-3. Mouse models of SLE

Although no single animal model perfectly recapitulates human SLE, some animal models can help us understand the etiology and pathogenesis of this disease. There are many inbred strains of spontaneous SLE-prone mice, including MRL/lpr, MRL/gld, NZB (New Zealand Black), F1 hybrids of NZB×NZW (New Zealand White) (B/W F1), and BXSB mice. Each of these have their own genetic background and autoimmune characteristics (table 1). More gene manipulated models are being generated, such as transgenic or gene knockout mice, which may help clarify the significance of target molecules in SLE.

Strain	Photo-dermatitis	Histopathologic features				
		Sensitivity	Igs at DEJ	IC-GN	Arteritis	Arthritis
NZB	-	+	+ (IgM)	+	-	-
NZB/KN	+(alopecia)	unknown	+ (IgM)	+	-	+
B/W F1	-	+	++ (IgG)	+++	-	-
MRL/lpr	++	+++	+ (IgG)	+++	+	+
MRL/n	++ (aged)	-	+ (ANA)	+	+	+
BXSB	-	++	+ (IgG)	+++	-	-

Table 1. Characteristics of lupus-prone mouse strains (source: ref.159)

Igs at DEJ: immunoglobulins deposits at the dermoepidermal junction;

IC-GN: immune complex glomerulonephritis;

ANA: epidermal nuclear staining.

2-4. Clinical features of SLE

2-4-1. Symptoms

The clinical course and outcome of SLE are extremely variable. Glomerulonephritis, arthritis, systemic small arteries vasculitis, rashes, haemolytic anemia and thrombocytopenia, which are caused by immune complexes deposition and/or autoantibody-induced ADCC, are the most common clinical symptoms of SLE.

2-4-2. Diagnosis

The American College of Rheumatology (ACR) classification criteria, a generally-accepted diagnostic standard, were devised in 1982 and revised in 1997 (160). Any combination of 4 or more of the following 11 criteria, well-documented at any time during a patient's history, makes it likely that the patient has SLE (specificity and sensitivity are 95% and 75%, respectively).

1. Malar rash: fixed erythema, flat or raised, over the malar eminences;
2. Discoid rash: erythematous circular raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur;
3. Photosensitivity: exposure to ultraviolet light causes rash;

4. Oral ulcers: includes oral and nasopharyngeal ulcers, observed by a physician;
5. Arthritis: nonerosive arthritis of two or more peripheral joints, with tenderness, swelling, or effusion;
6. Serositis: pleuritis or pericarditis documented by ECG or rub or evidence of effusion;
7. Renal disorder: proteinuria >0.5 g/d or 3+, or cellular casts;
8. Neurologic disorder: seizures or psychosis without other causes;
9. Hematologic disorder: hemolytic anemia or leukopenia ($<4000/L$) or lymphopenia ($<1500/L$) or thrombocytopenia ($<100,000/L$) in the absence of offending drugs;
10. Immunologic disorder: anti-dsDNA, anti-Sm, and/or anti-phospholipid;
11. Antinuclear antibodies: an abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs.

2-4-3. Therapy

In the absence of specific therapy aiming at the pathogenesis, successful therapy of SLE depends on treating both underlying inflammation and symptoms. Currently immunosuppression is the major SLE therapy available. Four main classes of drugs, i.e., corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), antimalarials and cytotoxic agents, are used.

High doses of corticosteroids remain the first line of treatment for many manifestations of SLE, especially in severe disease with renal, CNS and hematological involvement. But it causes many hazardous side effects, such as infection, hyperlipidaemia, hypertension, osteoporosis, diabetes, and insomnia (161). The combined use of NSAIDs, antimalarials and cytotoxic agents can reduce the steroids' dosage and side effects to a relative low level.

Antimalarials are commonly employed to treat patients with fatigue, arthralgia/arthritis and rash, but without major organ damage. Antimalarials can interfere with immune cellular functions, affect immune responses, and modulate cytokine levels in SLE patients. Antimalarials also improve skin lesions against the damaging effects of ultraviolet light. In these ways, antimalarials have the potential to keep SLE in remission.

Active lupus with major organ involvement often requires prompt, aggressive therapy with cytotoxic agents. Cytotoxic agents were primarily known to interrupt nucleic acid and protein synthesis in malignant cell. Then its immune inhibition was discovered. The use of cytotoxic agents in SLE benefits controlling active disease, reducing the rate of disease flares, and reducing steroid requirements.

Currently, there are many new treatments under investigation (162). Some biological agents (e.g., antibodies) have been tested in clinical trials. They either modulate or inhibit T-cell activation, T- and B-cell interactions, anti-dsDNA antibody production, immune complexes deposition, complement activation/deposition, and cytokine activity. A hormonal modulator, DHEA, has also shown promising therapeutic effect in SLE patients. In addition, a small number of very severe refractory lupus patients (7 cases) received high-dose chemotherapy and autologous stem cell transplantation to gain remission (163).

Other than pharmacological treatment, management of daily life can help SLE patients to control the disease. These therapies include avoiding sunlight over-exposure, low saturated fat and a high fish oil diet, stress avoidance, and smoking cessation.

2-4-4. Prognosis

In many countries, the survival rate of SLE exceeded 80-90% in 5 years post-diagnosis, but decreases to 60-80% in next 10 years (164,165, 166,167). The major causes of mortality are organ failure (especially renal failure), thrombocytopenia, cardiovascular diseases, infections, high SLE disease activity index (SLEDAI) (165,168). Proper and timely treatment in experienced hospitals is very important to improve the long-term survival rate (169).

SLE is a common chronic disease that seriously imperils the survival and life quality of patients. There is no cure due to the ambiguous etiology. Investigation of SLE pathogenesis will help us to develop specific treatments.

3. Diabetes Mellitus

Diabetes mellitus is a syndrome (includes excessive urine production, increased fluid and food intake, body weight loss, changes in energy metabolism, even blurred vision and renal failure) as results of abnormal high blood glucose levels. This metabolism disorder has two most common forms: type 1 diabetes mellitus (T1DM) is caused by insufficient insulin production that mainly follows after autoimmune damage to pancreatic islets; type 2 diabetes mellitus (T2DM) is caused by failed response to insulin in effective organ (such as liver and muscle), but the level of insulin are normal or even elevated before the late stage of the disease. Besides, some pregnant women may suffer gestational diabetes mellitus due to carbohydrate intolerance.

3-1. The Epidemiology of Diabetes Mellitus

Nowadays, more than 170 million people live with diabetes worldwide (170), and that number will double in the next 20 years (171) (Fig. 5).

T1DM is one of the most common chronic childhood disease whose incidence is about 20 to 30 per 100 000 children per year in the United

Kingdom, Finland, Norway and Sweden, but is much lower in Asian, Indian, Middle Eastern and African populations (172,173). The incidence of T1DM is increasing rapidly worldwide, and is estimated to exceed 30-50 per 100,000 a year by 2010 (173). Studies showed 2.5-3% annual global increase rate of incidence of T1DM with a larger increase in some central and eastern European countries, some Asian countries and Australia (174,175). The largest rate of increase was seen in 0-4 year old children (173).

T2DM usually occurs after the age of 30, but it can exist without any symptoms for many years. The first diagnosis is sometimes made through incidental abnormal blood or urine glucose tests, or through associated complications (176). It constitutes more than 90% of overall diabetes cases in the world (177) with large geographical prevalence variation, even within the same or similar ethnic groups (178,179). The greatest increase of T2DM is in the developing countries of Asia, Africa, and South America, which are evolving with rapid cultural and social changes, ageing populations, urbanization, and unhealthy lifestyle and behavioral patterns (180). Another worrisome T2DM increase is in children and adolescents. The proportion of T2DM in new-onset diabetes during children and adolescents has increased more than 15 folds (<3% vs. 45%) in last 15

years (181). The increase of T2DM incidence closely parallels the increase of obesity. This unprecedented twin epidemic is termed as "diabesity" (171).

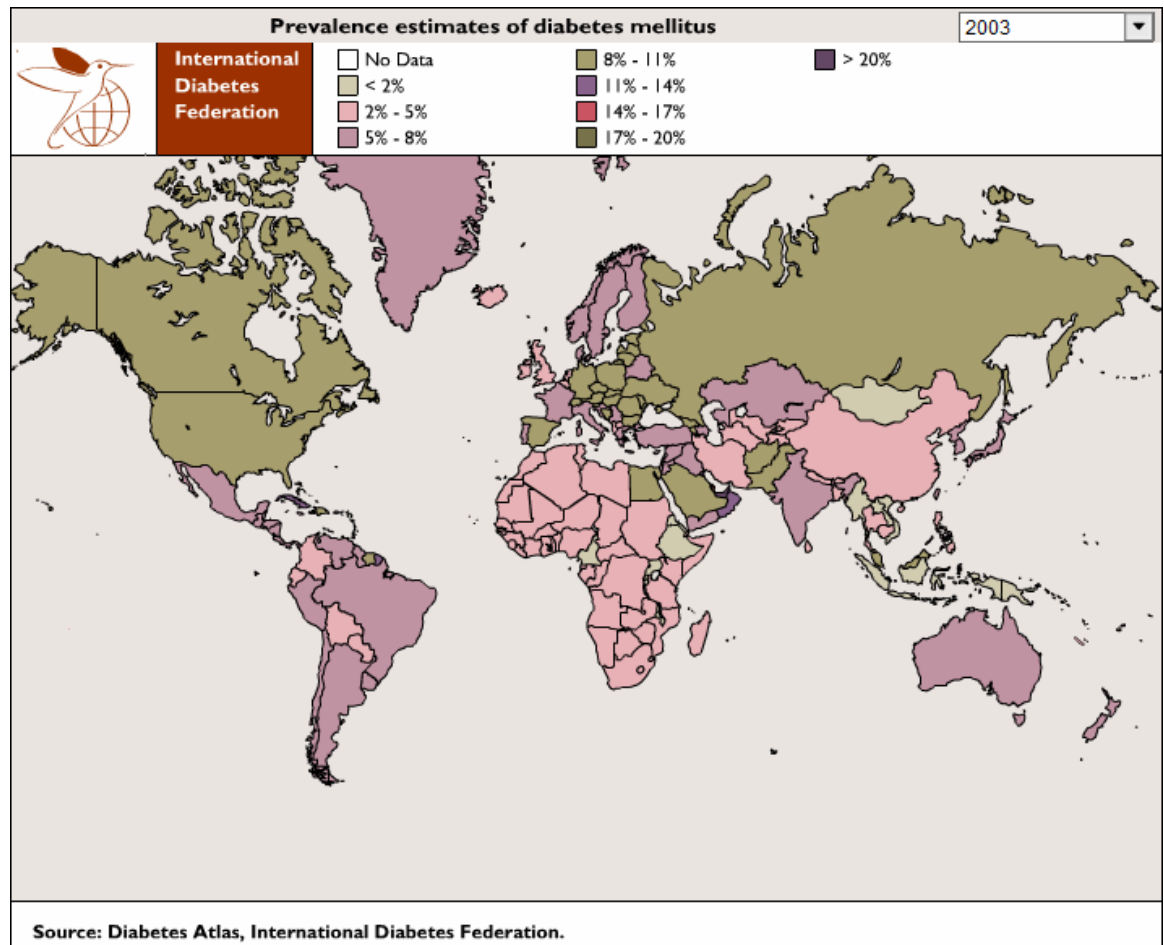


Figure 5. The geographic distribution of DM prevalence (source: <http://www.eatlas.idf.org/atlas.html?id=0>)

3-2. The pathoetiology of Diabetes Mellitus

T1DM had been defined as “insulin-dependent diabetes mellitus” due to the insufficient insulin in the body and the necessary treatment by artificial insulin even in its earliest stage. It is also called "juvenile diabetes" sometimes, because its onset is mainly in childhood although a few cases occur in adulthood, which is also called latent autoimmune diabetes of adults (182). T-cell-mediated autoimmune attack to the beta-cells of Langerhans islets is believed to be the major cause of the disease (Fig 6).

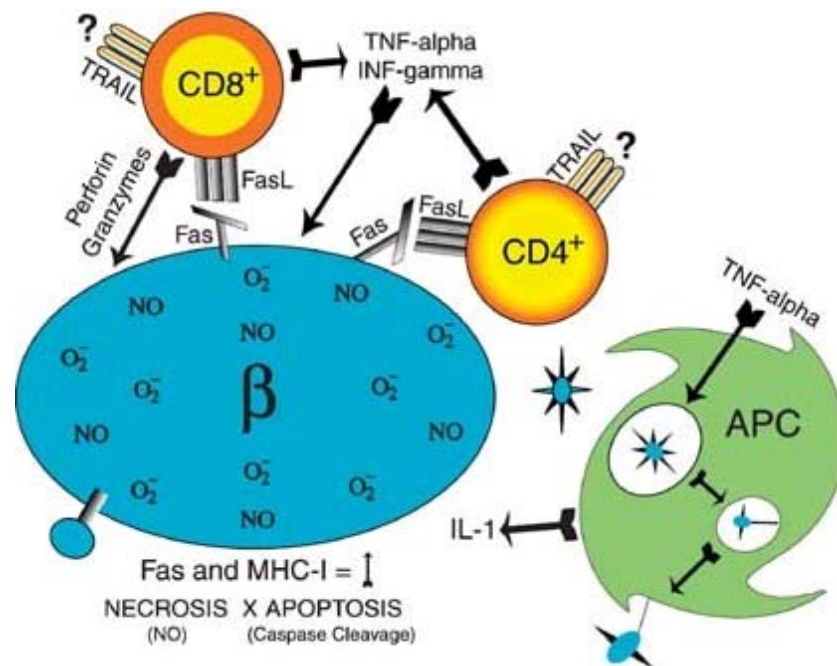


Fig. 6 The pathogenesis of T1DM. (source: ref. 183)

β -cell antigen is presented on the cell surface by MHC class I molecule. CD8⁺ T cells recognize this antigen and damage β -cell through the secretion of INF- γ or TNF/TRAIL or the perforin/granzyme system. The dendritic cells in islets take up cell components from dead β -cells, and present them to CD4⁺ T cells in lymph nodes. Active CD4⁺ T cells move into the islets to mediate killing through the Fas/FasL pathway.

T2DM was called “non-insulin-dependent diabetes” in contrast to T1DM. A deficient reaction to insulin in liver and muscle initiates this disease. In these tissues, glucose processing is compromised. Thereafter, unprocessed glucose accumulates in circulation and reduces islet beta cells’ response to glucose (Fig7). T2DM patients are not ketosis-prone and do not depend on exogenous insulin. However, they may need insulin treatment to control hyperglycemia if the diet and /or oral hypoglycemic agents do not work well.

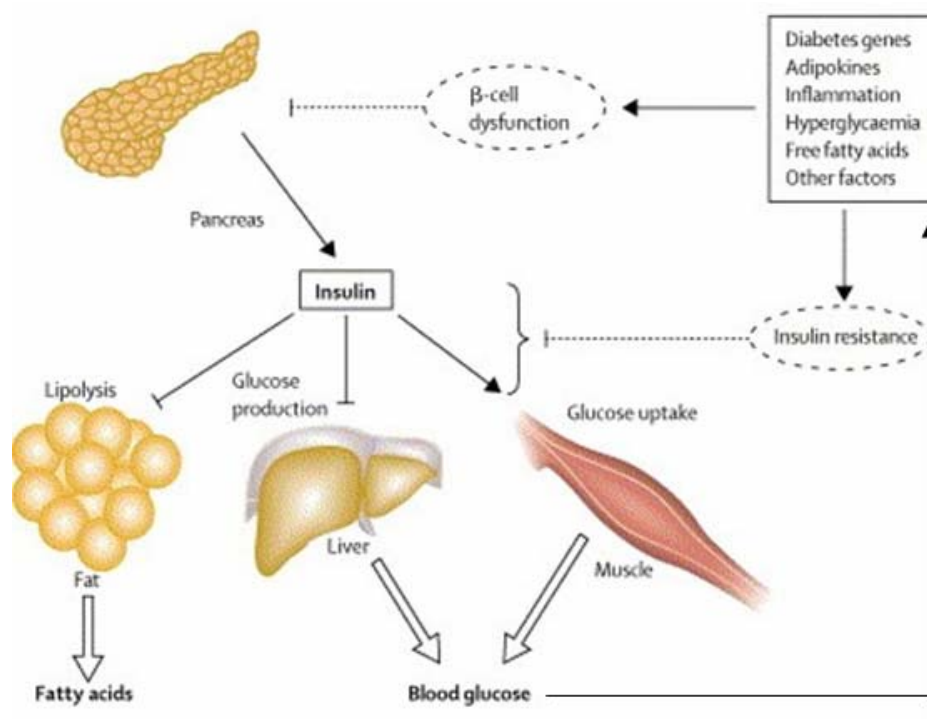


Figure 7. Pathophysiology of T2DM (source: ref. 170)

A variety of factors contribute to insulin resistance. Compromise of insulin action in major target tissues leads to increased circulating free fatty acids and hyperglycaemia, which will impair β -cell viability and worsen insulin resistance.

Like systemic lupus erythematosus, a combination of hereditary and environmental factors causes these metabolism dysfunctions, either in T1DM or T2DM. Clinical trials show that the concordance rates of monozygotic twins are around 50% in both T1DM and T2DM (184,185,186). These results suggest that the genetic factor is not the only protagonist in the etiology of DM. In other words, the trigger by environmental factors is also necessary to diabetes onset. T2DM seems to be familial (187,188). In addition to genetic factors, a similar lifestyle of members in the same family may also play a role.

3-2-1. Genetic Factors

Most diabetes, both T1DM and T2DM, are polygenic. There are only 1-5% cases (mainly neonatal diabetes mellitus and maturity-onset diabetes of the young) that are due to monogenic mutations (189). Although there are many diabetes risk genes, which have been identified by different studies, only a few of these findings are confirmed, i.e., HLA-DQ8 and DQ2, MICA-5 (MHC class I chain-related genes allele 5), KIR (killer immunoglobulin-like receptors) (190), CTLA4 (cytotoxic lymphocyte antigen 4), LYP (Lymphoid tyrosine phosphatase)/PTPN22 (191) to T1DM; TCF7L2 (Transcription Factor 7-Like 2) (192), CAPN10

(calpain 10) (193), PPAR γ (peroxisome proliferator-activated receptor gamma), KCNJ11 (194,195) to T2DM. As obesity contributes to T2DM, genes involved in obesity should also be considered risk factors for T2DM. More than 22 genes, which encode members of the leptin–melanocortin pathway, proinflammatory cytokines and uncoupling proteins (UCPs), are related to obesity risks (196).

3-2-2. Environmental Factors

Various environmental factors can influence diabetes morbidity.

a. Viral infection:

T1DM pathogenesis somewhat relates virus infection. Many viruses can trigger cell damaging processes in their host, such as Coxsackie B virus, mumps, echovirus, cytomegalovirus, Epstein-Barr virus (EBV), some retrovirus, rotavirus, parvovirus B19 and rubella virus (197). These viruses can induce autoimmunity through 1) direct infection of islets (198), 2) molecular mimicry, i.e., viral protein sharing similar peptide sequences with cellular autoantigens (199), or 3) activation of innate immunity (200).

b. Environmental contaminants and chemicals:

The increase of contaminants in the environment or occupational contact of certain chemicals could be potential risk factors of DM. For T1DM, higher intake of nitrates, nitrites, and *N*-nitroso compounds, as well as higher serum levels of polychlorinated biphenyls seem to link to higher incidence (201,202,203,204,205). Arsenic and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure is directly associated with T2DM risks (206,207,208,209).

c. Lifestyle and social factors:

Human behavior largely influences the incidence of DM (210). Decreased breastfeeding and early exposure to dietary cow-milk/cereals will raise the future risk of T1DM in the infants (211,212), while early supplement of vitamin D can decrease T1DM risks (213). The “Westernized food”, or energy-dense food, contributes to obesity, thereby increases the risk of T2DM (214). Physical activity is another effective behavior factor. Regular physical activity can reduce the risk of T2DM by 15-60% (195). Socio-economic and psychosocial factors could also indirectly affect the prevalence of DM through the change of lifestyle, nutrition, and natural environment (197,215,216).

d. Perinatal factors and postnatal growth:

Perinatal factors associated with increased T1DM risk to infant may include older maternal age at birth (217), excessive maternal weight gain, amniocentesis, preeclampsia (218), cesarean section delivery, complicated delivery, and maternal-fetal blood group incompatibility (219,220). Size at birth and early postnatal growth rates, which may reflect the fetal nutritional conditions, are significantly associated with the risk of diabetes, both T1DM and T2DM (221,222,223).

3-2-3. Accelerator hypothesis

Recent evidences indicate that incidence of both T1DM and T2DM increase in parallel with obesity (224,225). Meanwhile, the important role of insulin resistance in the early stages of T1DM development has been widely accepted (226,227). These findings led to a new theory called “accelerator hypothesis”. This hypothesis proposes that “T1DM and T2DM are the same disorder of insulin resistance set against different genetic backgrounds” (228). Excess bodyweight is the central point to the development of diabetes, both T1DM and T2DM. Weight gain increases insulin resistance and consequently results in hyperglycemia. Long-term high blood glucose induces apoptosis and immunogenicity of beta cells.

Based on these pathological changes, individual gene polymorphism and constitution, determine the tempo of beta cell loss and the age of onset, i.e., the manifestation of T1DM or T2DM. This hypothesis deems that T1DM and T2DM are subsets of the same disease. Thus, control of weight gain could prevent DM by slowing its progress.

3-3. The clinical features of DM

3-3-1. Symptoms and complications

Hyperglycemia is the principal cause of diabetes symptoms and complications. The classical diabetes triad symptoms are polyuria, polydipsia and polyphagia, i.e., frequent urination, increased thirst and fluid intake, increased appetite. Symptoms may develop quite rapidly (weeks or months) in T1DM, particularly in children. T1DM may also cause rapid weight loss and implacable fatigue. However, symptoms usually develop much slowly and mildly, sometimes may be even absent, in T2DM.

Ketoacidosis (DKA), an extreme state of metabolic dysregulation, may be the most common acute complication of DM. It is characterized by the acetone smell in the patient's breath, Kussmaul breathing, polyuria, nausea, vomiting and abdominal pain, and psychological disturbance. Severe DKA

can cause coma and even death (229). It is mainly present in patients with T1DM, but also observed in patients with T2DM under certain conditions. Another serious acute complication is hyperosmolar nonketotic state, which is more common in T2DM (230). It is mainly the result of dehydration due to loss of body water.

Prolonged high blood glucose levels along with abnormal lipid levels lead to blood vessels lesion (angiopathy), which subsequently induces chronic multiple organ complications, including blindness, renal failure, lower limb gangrene and cardiovascular diseases.

Nosogenetic effects of hyperglycemia mainly attribute to the formation of advanced glycation end-products (AGEs). Hyperglycemia in diabetes increases the formation and accumulation of AGEs. AGEs can interact with cell-surface receptor of AGE (RAGE), leading to cell activation and increasing expression of extracellular matrix proteins, vascular adhesion molecules, cytokines, growth factors, and the generation of reactive oxygen intermediates. Moreover, certain AGEs precursor can covalently crosslink proteins and change their structure and function. These pathologic changes result in almost all the diabetes complications, both micro- and macroangiopathies.

3-3-2. Diagnosis

The diagnostic criteria are based on the WHO recommendations of 1999. They incorporate both fasting criterion and 2-h-after-glucose-load (oral glucose tolerance test (OGTT)) criterion into a practicable diagnostic classification (table 2) (231). About 7% of people with impaired fasting glucose and impaired glucose tolerance will eventually progress to overt diabetes every year without proper treatment (232,233). If a pregnant woman has any two of the followings, she will be diagnosed as gestational diabetes: 1) fasting plasma glucose more than 5.3 mmol/dl, 2) 1-hour glucose level of OGTT more than 10 mmol/dl, 3) 2-hour glucose level of OGTT more than 8.6 mmol/dl (234).

There are some other accessorial clinical tests to value the status of DM. For examples, urine ketones test can indicate the severity of T1DM; the glycosylated hemoglobin test will give information about how well the blood glucose is controlled.

	Glucose concentration in venous plasma (mmol/L)
Diabetes mellitus	Fasting ≥ 7.0 or 2-h post-glucose load ≥ 11.1
Impaired glucose tolerance	Fasting (if measured) < 7.0 and 2-h post-glucose load ≥ 7.8 and < 11.1
Impaired fasting glucose	Fasting ≥ 6.1 and < 7.0 and 2 h post-glucose load (if measured) < 7.8

Table 2. Diagnostic criteria of diabetes mellitus and other categories of hyperglycemia (source: ref. 231)

Glucose load=75 g glucose orally.

3-3-3. Therapy

Diabetes mellitus is a chronic disease currently without a cure. The goal of therapy is to maintain blood glucose level as close to normal as possible to prevent either acute or chronic complications.

3-3-3-1. Diabetes education and lifestyle intervention

Self-monitoring the blood glucose of diabetes patient is very important to keep both short-term and long-term blood glucose levels within acceptable bounds. Proper diet, regular physical exercise, moderate body weight and refrain from smoke and alcohol will help the patient to control the blood sugar and greatly reduce the risk of progression (232, 233). It is very necessary to impart related knowledge to the diabetes patients and help them modify their lifestyle.

3-3-3-2. Insulin and other medications

T1DM patients, as well as T2DM patients who do not respond to oral medications, need insulin therapy to survive. There are several different insulin types and administration methods available currently.

Besides insulin, many choices of drugs can be used to modulate glucose levels, especially for T2DM. The mechanism and active site of these medications are shown in Table 3

Additionally, since cardiovascular disorders, such as hypertension, coronary artery disease and cerebrovascular disease, occur very often in T2DM (235), the management of cardiovascular risk factors is necessary and beneficial (236).

Classification	Medication	Route	The way it works
Sulfonylureas	Glimepiride, Glipizide, Glipizide ER, Glyburide	Oral	Increases insulin production
Biguanides	Glucophage, Glucophage XR	Oral	Lowers glucose from digestion
Alpha-Glucosidase Inhibitors	Glyset and Precose	Oral	Slows digestion, slows glucose production
Thiazolidinediones	Actos and Avandia	Oral	Lowers glucose production
Meglitinides	Prandin and Starlix	Oral	Increases insulin production
DPP-4 Inhibitors	Januvia	Oral	Lowers glucose by blocking an enzyme
Incretin Mimetics	Byetta	Injectable	Helps the pancreas make insulin, slows digestion
Anti-hyperglycemic	Symlin	Injectable	Controls postprandial blood glucose

Table 3. Medications for Diabetes (source: ref.237)

3-3-3-3. Organ and cell transplantation

The DM, in particular T1DM, has no cure. A potential cure for it is transplantation, either at the organ level or cell level.

a. Pancreas transplantation

Pancreas transplantation is an effective treatment for diabetes mellitus, especially for the end-stage renal complication. It was first successfully performed in combination with kidney transplant in 1966 (238). The improvements in surgery and immunosuppressors have significantly increased the graft survival rate; the 5-year graft survival rate is around 50%-70% (239). Pancreas transplant can achieve long-term normoglycemia, reduce mortality, and ameliorate diabetic complications (240). However, pancreas transplantation is still a high-risk operation with some severe postoperative complications. Moreover, the availability of organ donors and side effects of immunosuppressants are also limiting factors for popularization of this treatment.

b. Islet transplantation

Islet transplantation was applied to treat insulin-dependent diabetes since the 1970's, but it had hardly achieved desirable long-term effectiveness

until the advent of a steroid-free immunosuppressive protocol in 2000 (241). The adoption of the Edmonton protocol, which uses steroid-free immunosuppression, has elevated the success of islet transplantation greatly. Most recipients could attain an insulin-independence immediately after transplantation (242,243). However, a large islet mass ($> 10,000$ islet equivalents (IEQ)/kg recipient body weight) derived from multiple donors (2-4) is necessary to achieve insulin independence (244) because of the loss of a large number of transplanted islets in the first 10–14 days after transplantation despite efficient immunosuppressive regimens (245,246). Experimental models also show that early damage results in 60% loss of the transplanted syngeneic islet mass in the peri-transplant period (247,248). This early stage non-rejective graft failure or the primary non-function (PNF) is becoming a bottleneck for the development of islet transplantation since the improvement of immunosuppressive agents. PNF reduces effective grafted islet mass, and then increases the metabolic load to surviving islets. This overloading reduces islet survival time and diminishes the long-term insulin independence, which is frequently (~90%) lost in 5 years after islet transplantation (249,250).

The reasons for PNF include oxidative stress in the islets during pretransplant manipulations (251,252), loss of trophic factors for the

isolated islets (253), host innate immunity and inflammatory responses (254), and failure of revascularization of islet graft (255, 256). Proinflammatory cytokines (e.g., TNF- α , IL-1, and IFN- γ)-triggered multiple signaling pathways (e.g., c-Jun NH2-terminal kinase (JNK1) (257), Fas/FasL (258), nuclear factor- κ B, and transcription factor signal transducer and activator of transcription (STAT) (259)) contribute to the occurrence of PNF. Prevention of islet graft PNF through improvement of islet isolation and culture technology, application of inflammation inhibitors, and genetic modification to repress islet apoptosis would likely make a significant impact on the efficacy of islet transplantation.

c. Stem cell transplantation

Recent research showed that embryonic, fetal and adult stem/progenitor cells including putative multipotent pancreatic stem/progenitor cells have a potential for self-renewal of islet β -cells (260,261). The transplantation of insulin-producing β -cell derived from either wild-type or genetically modified stem/progenitor cells, or the expansion and differentiation of putative multipotent pancreatic stem/progenitor cells in vivo, may be promising alternative therapies for T1DM or T2DM in humans.

3-3-3-4. Gene therapy

Gene therapy of DM has been under investigation intensely in recent years. Studies include genetic modification of graft islets, genetically engineered ectopic insulin production, preventing or curing autoimmunity (262). All these therapies require an effective gene delivery system, which is the bottleneck of the gene therapy. The disadvantages (such as oncogenicity, efficacy and immunogenicity) of viral gene delivery systems limit its clinical usefulness. With future advances in gene delivery technologies, gene therapy may be a potential cure to DM.

Hypothesis and Objective

The etiology of SLE remains unclear. Disorder of lymphocyte apoptosis can lead to lupus-like syndromes in mouse models. But in humans, the relationship between SLE and lymphocyte apoptosis is a puzzle. DcR3, a native block of apoptosis, may play a role in pathogenesis of human SLE via interruption of lymphocyte apoptosis.

Our group pioneered the research on the biological functions of DcR3. We discovered its roles in the modulation of T-lymphocyte functions (7,55) and reported its expression in varied malignant tumors (17). Moreover, we showed protective effect of DcR3 on islet transplantation (53).

As part of my Ph.D. Program, the important- role of DcR3 in the pathogenesis of SLE both in a mouse model and in patients has been investigated. The mechanisms of DcR3's effect on islet protection have been further studied. The details are provided in the following 3 chapters.

References

- 1 Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 1998 Dec 17;396(6712):699-703.
- 2 Shinomiya T, Mori T, Ariyama Y, Sakabe T, Fukuda Y, Murakami Y, et al. Comparative genomic hybridization of squamous cell carcinoma of the esophagus: the possible involvement of the DPI gene in the 13q34 amplicon. *Genes Chromosomes Cancer* 1999 Apr;24(4):337-44.
- 3 Muleris M, Almeida A, Gerbault-Seureau M, Malfoy B, Dutrillaux B. Identification of amplified DNA sequences in breast cancer and their organization within homogeneously staining regions. *Genes Chromosomes Cancer* 1995 Nov;14(3):155-63.
- 4 Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, et al. Modulation of T-cell responses to alloantigens by TR6/DcR3. *J Clin Invest* 2001 Jun;107(11):1459-68.
- 5 Bridgham JT, Johnson AL. Characterization of chicken TNFR superfamily decoy receptors, DcR3 and osteoprotegerin. *Biochem Biophys Res Commun* 2003 Aug 8;307(4):956-61.
- 6 Tsutsui S, Yoshino Y, Matsui S, Nakamura O, Muramoto K, Watanabe T. Isolation of epidermal cells and cDNA cloning of TNF decoy receptor 3 of conger eel, *Conger myriaster*. *Fish Shellfish Immunol* 2008 Mar;24(3):366-71.
- 7 Wan X, Shi G, Semenuk M, Zhang J, Wu J. DcR3/TR6 modulates immune cell interactions. *J Cell Biochem* 2003 Jun 1;89(3):603-12.
- 8 Chen G, Luo D. Over-expression of decoy receptor 3 in gastric precancerous lesions and carcinoma. *Ups J Med Sci* 2008;113(3):297-304.
- 9 Bai C, Connolly B, Metzker ML, Hilliard CA, Liu X, Sandig V, et al. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc Natl Acad Sci U S A* 2000 Feb 1;97(3):1230-5.
- 10 Mild G, Bachmann F, Boulay JL, Glatz K, Laffer U, Lowy A, et al. DCR3 locus is a predictive marker for 5-fluorouracil-based adjuvant chemotherapy in colorectal cancer. *Int J Cancer* 2002 Nov 20;102(3):254-7.
- 11 Shen HW, Gao SL, Wu YL, Peng SY. Overexpression of decoy receptor 3 in hepatocellular carcinoma and its association with resistance to Fas ligand-mediated apoptosis. *World J Gastroenterol* 2005 Oct 14;11(38):5926-30.

12 Tsuji S, Hosotani R, Yonehara S, Masui T, Tulachan SS, Nakajima S, et al. Endogenous decoy receptor 3 blocks the growth inhibition signals mediated by Fas ligand in human pancreatic adenocarcinoma. *Int J Cancer* 2003 Aug 10;106(1):17-25.

13 Roth W, Isenmann S, Nakamura M, Platten M, Wick W, Kleihues P, et al. Soluble decoy receptor 3 is expressed by malignant gliomas and suppresses CD95 ligand-induced apoptosis and chemotaxis. *Cancer Res* 2001 Mar 15;61(6):2759-65.

14 Chang PM, Chen PM, Hsieh SL, Tzeng CH, Liu JH, Chiou TJ, et al. Expression of a soluble decoy receptor 3 in patients with diffuse large B-cell lymphoma predicts clinical outcome. *Int J Oncol* 2008 Sep;33(3):549-54.

15 Macher-Goeppinger S, Aulmann S, Wagener N, Funke B, Tagscherer KE, Haferkamp A, et al. Decoy receptor 3 is a prognostic factor in renal cell cancer. *Neoplasia* 2008 Oct;10(10):1049-56.

16 Simon I, Liu Y, Krall KL, Urban N, Wolfert RL, Kim NW, et al. Evaluation of the novel serum markers B7-H4, Spondin 2, and DcR3 for diagnosis and early detection of ovarian cancer. *Gynecol Oncol* 2007 Jul;106(1):112-8.

17 Wu Y, Han B, Sheng H, Lin M, Moore PA, Zhang J, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 2003 Jul 10;105(5):724-32.

18 Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, et al. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 2002 Mar;16(3):479-92.

19 Montgomery RI, Warner MS, Lum BJ, Spear PG. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 1996 Nov 1;87(3):427-36.

20 Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol* 1991;9:617-48.

21 Crowe PD, VanArsdale TL, Walter BN, Ware CF, Hession C, Ehrenfels B, et al. A lymphotoxin-beta-specific receptor. *Science* 1994 Apr 29;264(5159):707-10.

22 Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, et al. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest* 1998 Sep 15;102(6):1142-51.

23 Krammer PH. CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol* 1999;71:163-210.

24 Augstein P, Elefanty AG, Allison J, Harrison LC. Apoptosis and beta-cell destruction in pancreatic islets of NOD mice with spontaneous and cyclophosphamide-accelerated diabetes. *Diabetologia* 1998 Nov;41(11):1381-8.

25 Thome M, Tschopp J. Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev*

Immunol 2001 Oct;1(1):50-8.

26 Andersen MH, Schrama D, Thor SP, Becker JC. Cytotoxic T cells. *J Invest Dermatol* 2006 Jan;126(1):32-41.

27 Igney FH, Krammer PH. Tumor counterattack: fact or fiction? *Cancer Immunol Immunother* 2005 Nov;54(11):1127-36.

28 Ulisse S, Cinque B, Silvano G, Rucci N, Biordi L, Cifone MG, et al. Erk-dependent cytosolic phospholipase A2 activity is induced by CD95 ligand cross-linking in the mouse derived Sertoli cell line TM4 and is required to trigger apoptosis in CD95 bearing cells. *Cell Death Differ* 2000 Oct;7(10):916-24.

29 Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* 1998 Jan;8(1):21-30.

30 Harrop JA, McDonnell PC, Brigham-Burke M, Lyn SD, Minton J, Tan KB, et al. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. *J Biol Chem* 1998 Oct 16;273(42):27548-56.

31 Tamada K, Shimosaki K, Chapoval AI, Zhai Y, Su J, Chen SF, et al. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J Immunol* 2000 Apr 15;164(8):4105-10.

32 Granger SW, Rickert S. LIGHT-HVEM signaling and the regulation of T cell-mediated immunity. *Cytokine Growth Factor Rev* 2003 Jun;14(3-4):289-96.

33 Ye Q, Fraser CC, Gao W, Wang L, Busfield SJ, Wang C, et al. Modulation of LIGHT-HVEM costimulation prolongs cardiac allograft survival. *J Exp Med* 2002 Mar 18;195(6):795-800.

34 Kosuge H, Suzuki J, Kakuta T, Haraguchi G, Koga N, Futamatsu H, et al. Attenuation of graft arterial disease by manipulation of the LIGHT pathway. *Arterioscler Thromb Vasc Biol* 2004 Aug;24(8):1409-15.

35 Chen MC, Hwang MJ, Chou YC, Chen WH, Cheng G, Nakano H, et al. The role of apoptosis signal-regulating kinase 1 in lymphotoxin-beta receptor-mediated cell death. *J Biol Chem* 2003 May 2;278(18):16073-81.

36 Browning JL, Miatkowski K, Sizing I, Griffiths D, Zafari M, Benjamin CD, et al. Signaling through the lymphotoxin beta receptor induces the death of some adenocarcinoma tumor lines. *J Exp Med* 1996 Mar 1;183(3):867-78.

37 Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, et al. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem* 2000 May 12;275(19):14307-15.

38 Scheu S, Alferink J, Potzel T, Barchet W, Kalinke U, Pfeffer K. Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. *J Exp Med* 2002 Jun 17;195(12):1613-24.

-
- 39 Wang J, Foster A, Chin R, Yu P, Sun Y, Wang Y, et al. The complementation of lymphotoxin deficiency with LIGHT, a newly discovered TNF family member, for the restoration of secondary lymphoid structure and function. *Eur J Immunol* 2002 Jul;32(7):1969-79.
- 40 Gommerman JL, Browning JL. Lymphotoxin/light, lymphoid microenvironments and autoimmune disease. *Nat Rev Immunol* 2003 Aug;3(8):642-55.
- 41 Degli-Esposti MA, vis-Smith T, Din WS, Smolak PJ, Goodwin RG, Smith CA. Activation of the lymphotoxin beta receptor by cross-linking induces chemokine production and growth arrest in A375 melanoma cells. *J Immunol* 1997 Feb 15;158(4):1756-62.
- 42 Papadakis KA, Zhu D, Prehn JL, Landers C, Avanesyan A, Lafkas G, et al. Dominant role for TL1A/DR3 pathway in IL-12 plus IL-18-induced IFN-gamma production by peripheral blood and mucosal CCR9+ T lymphocytes. *J Immunol* 2005 Apr 15;174(8):4985-90.
- 43 Lehmann W, Edgar CM, Wang K, Cho TJ, Barnes GL, Kakar S, et al. Tumor necrosis factor alpha (TNF-alpha) coordinately regulates the expression of specific matrix metalloproteinases (MMPS) and angiogenic factors during fracture healing. *Bone* 2005 Feb;36(2):300-10.
- 44 Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. *Trends Biochem Sci* 2002 Jan;27(1):19-26.
- 45 Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, et al. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. *Science* 1996 Nov 8;274(5289):990-2.
- 46 Wen L, Zhuang L, Luo X, Wei P. TL1A-induced NF-kappaB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells. *J Biol Chem* 2003 Oct 3;278(40):39251-8.
- 47 Bamias G, Martin C, III, Marini M, Hoang S, Mishina M, Ross WG, et al. Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* 2003 Nov 1;171(9):4868-74.
- 48 Prehn JL, Mehdizadeh S, Landers CJ, Luo X, Cha SC, Wei P, et al. Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation. *Clin Immunol* 2004 Jul;112(1):66-77.
- 49 Kang YJ, Kim WJ, Bae HU, Kim DI, Park YB, Park JE, et al. Involvement of TL1A and DR3 in induction of pro-inflammatory cytokines and matrix metalloproteinase-9 in atherogenesis. *Cytokine* 2005 Mar 7;29(5):229-35.
- 50 Robinson LJ, Borysenko CW, Blair HC. Tumor necrosis factor family receptors regulating bone turnover: new observations in osteoblastic and osteoclastic cell lines. *Ann N Y Acad Sci* 2007 Nov;1116:432-43.

51 Hsu TL, Chang YC, Chen SJ, Liu YJ, Chiu AW, Chio CC, et al. Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J Immunol* 2002 May 15;168(10):4846-53.

52 Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, et al. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem* 2006 May 19;281(20):13964-71.

53 Wu Y, Han B, Luo H, Roduit R, Salcedo TW, Moore PA, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* 2003 Sep;52(9):2279-86.

54 Yu KY, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J Biol Chem* 1999 May 14;274(20):13733-6.

55 Shi G, Wu Y, Zhang J, Wu J. Death decoy receptor TR6/DcR3 inhibits T cell chemotaxis in vitro and in vivo. *J Immunol* 2003 Oct 1;171(7):3407-14.

56 Yang CR, Hsieh SL, Teng CM, Ho FM, Su WL, Lin WW. Soluble decoy receptor 3 induces angiogenesis by neutralization of TL1A, a cytokine belonging to tumor necrosis factor superfamily and exhibiting angiostatic action. *Cancer Res* 2004 Feb 1;64(3):1122-9.

57 Chang YC, Chen TC, Lee CT, Yang CY, Wang HW, Wang CC, et al. Epigenetic control of MHC class II expression in tumor-associated macrophages by decoy receptor 3. *Blood* 2008 May 15;111(10):5054-63.

58 Wu SF, Liu TM, Lin YC, Sytwu HK, Juan HF, Chen ST, et al. Immunomodulatory effect of decoy receptor 3 on the differentiation and function of bone marrow-derived dendritic cells in nonobese diabetic mice: from regulatory mechanism to clinical implication. *J Leukoc Biol* 2004 Feb;75(2):293-306.

59 Kim S, Fotiadu A, Kotoula V. Increased expression of soluble decoy receptor 3 in acutely inflamed intestinal epithelia. *Clin Immunol* 2005 Jun;115(3):286-94.

60 Chen HF, Chen JS, Shun CT, Tsai YF, Ho HN. Decoy receptor 3 expression during the menstrual cycle and pregnancy, and regulation by sex steroids in endometrial cells in vitro. *Hum Reprod* 2009 Feb 26.

61 Arakawa Y, Tachibana O, Hasegawa M, Miyamori T, Yamashita J, Hayashi Y. Frequent gene amplification and overexpression of decoy receptor 3 in glioblastoma. *Acta Neuropathol* 2005 Mar;109(3):294-8.

62 Chen PH, Yang CR. Decoy receptor 3 expression in AsPC-1 human pancreatic adenocarcinoma cells via the phosphatidylinositol 3-kinase-, Akt-, and NF-kappa B-dependent pathway. *J Immunol*

2008 Dec 15;181(12):8441-9.

63 Ohshima K, Haraoka S, Sugihara M, Suzumiya J, Kawasaki C, Kanda M, et al. Amplification and expression of a decoy receptor for fas ligand (DcR3) in virus (EBV or HTLV-I) associated lymphomas. *Cancer Lett* 2000 Nov 10;160(1):89-97.

64 Ho CH, Hsu CF, Fong PF, Tai SK, Hsieh SL, Chen CJ. Epstein-Barr virus transcription activator Rta upregulates decoy receptor 3 expression by binding to its promoter. *J Virol* 2007 May;81(9):4837-47.

65 Herzog H, Darby K, Ball H, Hort Y, Beck-Sickinger A, Shine J. Overlapping gene structure of the human neuropeptide Y receptor subtypes Y1 and Y5 suggests coordinate transcriptional regulation. *Genomics* 1997 May 1;41(3):315-9.

66 Ka SM, Sytwu HK, Chang DM, Hsieh SL, Tsai PY, Chen A. Decoy receptor 3 ameliorates an autoimmune crescentic glomerulonephritis model in mice. *J Am Soc Nephrol* 2007 Sep;18(9):2473-85.

67 Chang YC, Hsu TL, Lin HH, Chio CC, Chiu AW, Chen NJ, et al. Modulation of macrophage differentiation and activation by decoy receptor 3. *J Leukoc Biol* 2004 Mar;75(3):486-94.

68 You RI, Chang YC, Chen PM, Wang WS, Hsu TL, Yang CY, et al. Apoptosis of dendritic cells induced by decoy receptor 3 (DcR3). *Blood* 2008 Feb 1;111(3):1480-8.

69 Wan X, Zhang J, Luo H, Shi G, Kapnik E, Kim S, et al. A TNF family member LIGHT transduces costimulatory signals into human T cells. *J Immunol* 2002 Dec 15;169(12):6813-21.

70 Shi G, Luo H, Wan X, Salcedo TW, Zhang J, Wu J. Mouse T cells receive costimulatory signals from LIGHT, a TNF family member. *Blood* 2002 Nov 1;100(9):3279-86.

71 Lee CS, Hu CY, Tsai HF, Wu CS, Hsieh SL, Liu LC, et al. Elevated serum decoy receptor 3 with enhanced T cell activation in systemic lupus erythematosus. *Clin Exp Immunol* 2008 Mar;151(3):383-90.

72 Yang CR, Hsieh SL, Ho FM, Lin WW. Decoy receptor 3 increases monocyte adhesion to endothelial cells via NF-kappa B-dependent up-regulation of intercellular adhesion molecule-1, VCAM-1, and IL-8 expression. *J Immunol* 2005 Feb 1;174(3):1647-56.

73 Takahama Y, Yamada Y, Emoto K, Fujimoto H, Takayama T, Ueno M, et al. The prognostic significance of overexpression of the decoy receptor for Fas ligand (DcR3) in patients with gastric carcinomas. *Gastric Cancer* 2002;5(2):61-8.

74 Wu Y, Guo E, Yu J, Xie Q. High DcR3 expression predicts stage pN2-3 in gastric cancer. *Am J Clin Oncol* 2008 Feb;31(1):79-83.

75 Li W, Zhang C, Chen C, Zhuang G. Correlation between expression of DcR3 on tumor cells and sensitivity to FasL. *Cell Mol Immunol* 2007 Dec;4(6):455-60.

76 Schaller J. Lupus in childhood. *Clin Rheum Dis* 1982 Apr;8(1):219-28.

-
- 77 Rothfield N. Clinical features of systemic lupus erythematosus. In: Kelley W, Harris E, Ruddy S, Sledge C, editors. *Textbook of Rheumatology*. Philadelphia: WB Saunders; 1981.
- 78 Rus V, Maury E, Hochberg M. The epidemiology of systemic lupus erythematosus. In: Wallace D, Hahn B, editors. *Dubois' Lupus Erythematosus*. Philadelphia: Lippincott Williams and Wilkins; 2002.
- 79 Hochberg MC. The incidence of systemic lupus erythematosus in Baltimore, Maryland, 1970-1977. *Arthritis Rheum* 1985 Jan;28(1):80-6.
- 80 Symmons DP. Frequency of lupus in people of African origin. *Lupus* 1995 Jun;4(3):176-8.
- 81 Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, et al. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum* 1998 May;41(5):778-99.
- 82 Chakravarty EF, Bush TM, Manzi S, Clarke AE, Ward MM. Prevalence of adult systemic lupus erythematosus in California and Pennsylvania in 2000: estimates obtained using hospitalization data. *Arthritis Rheum* 2007 Jun;56(6):2092-4.
- 83 Feng PH. Systemic lupus erythematosus: the face of Asia. *Ann N Y Acad Sci* 2007 Jun;1108:114-20.
- 84 Hopkinson ND, Doherty M, Powell RJ. Clinical features and race-specific incidence/prevalence rates of systemic lupus erythematosus in a geographically complete cohort of patients. *Ann Rheum Dis* 1994 Oct;53(10):675-80.
- 85 Voss A, Green A, Junker P. Systemic lupus erythematosus in Denmark: clinical and epidemiological characterization of a county-based cohort. *Scand J Rheumatol* 1998;27(2):98-105.
- 86 Stahl-Hallengren C, Jonsen A, Nived O, Sturfelt G. Incidence studies of systemic lupus erythematosus in Southern Sweden: increasing age, decreasing frequency of renal manifestations and good prognosis. *J Rheumatol* 2000 Mar;27(3):685-91.
- 87 Uramoto KM, Michet CJ, Jr., Thumboo J, Sunku J, O'Fallon WM, Gabriel SE. Trends in the incidence and mortality of systemic lupus erythematosus, 1950-1992. *Arthritis Rheum* 1999 Jan;42(1):46-50.
- 88 Lahita RG. The role of sex hormones in systemic lupus erythematosus. *Curr Opin Rheumatol* 1999 Sep;11(5):352-6.
- 89 Costenbader KH, Feskanich D, Stampfer MJ, Karlson EW. Reproductive and menopausal factors and risk of systemic lupus erythematosus in women. *Arthritis Rheum* 2007 Apr;56(4):1251-62.
- 90 French MA, Hughes P. Systemic lupus erythematosus and Klinefelter's syndrome. *Ann Rheum Dis* 1983 Aug;42(4):471-3.
- 91 Lahita RG, Bradlow HL, Kunkel HG, Fishman J. Alterations of estrogen metabolism in systemic lupus erythematosus. *Arthritis Rheum* 1979 Nov;22(11):1195-8.

-
- 92 Lahita RG, Bradlow HL, Ginzler E, Pang S, New M. Low plasma androgens in women with systemic lupus erythematosus. *Arthritis Rheum* 1987 Mar;30(3):241-8.
- 93 Jungers P, Nahoul K, Pelissier C, Dougados M, Tron F, Bach JF. Low plasma androgens in women with active or quiescent systemic lupus erythematosus. *Arthritis Rheum* 1982 Apr;25(4):454-7.
- 94 Jacobson JD, Ansari MA, Kinealy M, Muthukrishnan V. Gender-specific exacerbation of murine lupus by gonadotropin-releasing hormone: potential role of G alpha(q/11). *Endocrinology* 1999 Aug;140(8):3429-37.
- 95 Lechner O, Dietrich H, Oliveira dos SA, Wieggers GJ, Schwarz S, Harbutz M, et al. Altered circadian rhythms of the stress hormone and melatonin response in lupus-prone MRL/MP-fas(Ipr) mice. *J Autoimmun* 2000 Jun;14(4):325-33.
- 96 Gutierrez MA, Garcia ME, Rodriguez JA, Rivero S, Jacobelli S. Hypothalamic-pituitary-adrenal axis function and prolactin secretion in systemic lupus erythematosus. *Lupus* 1998;7(6):404-8.
- 97 Alarcon-Segovia D, Arcon-Riquelme ME, Cardiel MH, Caeiro F, Massardo L, Villa AR, et al. Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum* 2005 Apr;52(4):1138-47.
- 98 Pisetsky DS. Systemic lupus erythematosus. In: Klippel JH, editor. *Primer on the rheumatic diseases*. 11th ed. Georgia, USA: Arthritis Foundation; 1997. p. 246-51.
- 99 Walport MJ, Davies KA, Botto M. C1q and systemic lupus erythematosus. *Immunobiology* 1998 Aug;199(2):265-85.
- 100 Schur PH. Genetics of systemic lupus erythematosus. *Lupus* 1995 Dec;4(6):425-37.
- 101 Howard PF, Hochberg MC, Bias WB, Arnett FC, Jr., McLean RH. Relationship between C4 null genes, HLA-D region antigens, and genetic susceptibility to systemic lupus erythematosus in Caucasian and black Americans. *Am J Med* 1986 Aug;81(2):187-93.
- 102 Woodrow JC. Immunogenetics of systemic lupus erythematosus. *J Rheumatol* 1988 Feb;15(2):197-9.
- 103 Reveille JD, Moulds JM, Ahn C, Friedman AW, Baethge B, Roseman J, et al. Systemic lupus erythematosus in three ethnic groups: I. The effects of HLA class II, C4, and CR1 alleles, socioeconomic factors, and ethnicity at disease onset. LUMINA Study Group. *Lupus in minority populations, nature versus nurture*. *Arthritis Rheum* 1998 Jul;41(7):1161-72.
- 104 Sullivan KE, Winkelstein JA. Genetically determined deficiencies of the complement system. In: Ochs HD, Smith C, Puck J., editors. *Primary Immunodeficiency Diseases: A Molecular and Genetic Approach*. New York: Oxford University Press; 1999. p. 397-416.
- 105 Atkinson JP. Complement activation and complement receptors in systemic lupus

erythematosus. Springer Semin Immunopathol 1986;9(2-3):179-94.

106 Agnello V. Association of systemic lupus erythematosus and SLE-like syndromes with hereditary and acquired complement deficiency states. Arthritis Rheum 1978 Jun;21(5 Suppl):S146-S152.

107 Hauptmann G, Tappeiner G, Schifferli JA. Inherited deficiency of the fourth component of human complement. Immunodeficiency Rev 1988;1(1):3-22.

108 Hartung K, Baur MP, Coldewey R, Fricke M, Kalden JR, Lakomek HJ, et al. Major histocompatibility complex haplotypes and complement C4 alleles in systemic lupus erythematosus. Results of a multicenter study. J Clin Invest 1992 Oct;90(4):1346-51.

109 Manderson AP, Botto M, Walport MJ. The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol 2004;22:431-56.

110 Smyth LJ, Snowden N, Carthy D, Papasteriades C, Hajeer A, Ollier WE. Fc gamma RIIa polymorphism in systemic lupus erythematosus. Ann Rheum Dis 1997 Dec;56(12):744-6.

111 Chu ZT, Tsuchiya N, Kyogoku C, Ohashi J, Qian YP, Xu SB, et al. Association of Fc gamma receptor IIb polymorphism with susceptibility to systemic lupus erythematosus in Chinese: a common susceptibility gene in the Asian populations. Tissue Antigens 2004 Jan;63(1):21-7.

112 Karassa FB, Trikalinos TA, Ioannidis JP. The role of Fc gamma RIIA and IIIA polymorphisms in autoimmune diseases. Biomed Pharmacother 2004 Jun;58(5):286-91.

113 Sullivan KE, Wooten C, Schmeckpeper BJ, Goldman D, Petri MA. A promoter polymorphism of tumor necrosis factor alpha associated with systemic lupus erythematosus in African-Americans. Arthritis Rheum 1997 Dec;40(12):2207-11.

114 Danis VA, Millington M, Hyland V, Lawford R, Huang Q, Grennan D. Increased frequency of the uncommon allele of a tumour necrosis factor alpha gene polymorphism in rheumatoid arthritis and systemic lupus erythematosus. Dis Markers 1995 Mar;12(2):127-33.

115 Prokunina L, Gunnarsson I, Sturfelt G, Truedsson L, Seligman VA, Olson JL, et al. The systemic lupus erythematosus-associated PDCD1 polymorphism PD1.3A in lupus nephritis. Arthritis Rheum 2004 Jan;50(1):327-8.

116 Prokunina L, Castillejo-Lopez C, Oberg F, Gunnarsson I, Berg L, Magnusson V, et al. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. Nat Genet 2002 Dec;32(4):666-9.

117 Liu MF, Wang CR, Lin LC, Wu CR. CTLA-4 gene polymorphism in promoter and exon-1 regions in Chinese patients with systemic lupus erythematosus. Lupus 2001;10(9):647-9.

118 Hudson LL, Rocca K, Song YW, Pandey JP. CTLA-4 gene polymorphisms in systemic lupus erythematosus: a highly significant association with a determinant in the promoter region. Hum Genet 2002 Oct;111(4-5):452-5.

119 Castro J, Balada E, Ordi-Ros J, Vilardell-Tarres M. The complex immunogenetic basis of systemic lupus erythematosus. *Autoimmun Rev* 2008 May;7(5):345-51.

120 Gaffney PM, Kearns GM, Shark KB, Ortmann WA, Selby SA, Malmgren ML, et al. A genome-wide search for susceptibility genes in human systemic lupus erythematosus sib-pair families. *Proc Natl Acad Sci U S A* 1998 Dec 8;95(25):14875-9.

121 Moser KL, Neas BR, Salmon JE, Yu H, Gray-McGuire C, Asundi N, et al. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. *Proc Natl Acad Sci U S A* 1998 Dec 8;95(25):14869-74.

122 Gaffney PM, Ortmann WA, Selby SA, Shark KB, Ockenden TC, Rohlf KE, et al. Genome screening in human systemic lupus erythematosus: results from a second Minnesota cohort and combined analyses of 187 sib-pair families. *Am J Hum Genet* 2000 Feb;66(2):547-56.

123 Gray-McGuire C, Moser KL, Gaffney PM, Kelly J, Yu H, Olson JM, et al. Genome scan of human systemic lupus erythematosus by regression modeling: evidence of linkage and epistasis at 4p16-15.2. *Am J Hum Genet* 2000 Dec;67(6):1460-9.

124 Lindqvist AK, Steinsson K, Johanneson B, Kristjansdottir H, Arnasson A, Grondal G, et al. A susceptibility locus for human systemic lupus erythematosus (hSLE1) on chromosome 2q. *J Autoimmun* 2000 Mar;14(2):169-78

125 Shai R, Quismorio FP, Jr., Li L, Kwon OJ, Morrison J, Wallace DJ, et al. Genome-wide screen for systemic lupus erythematosus susceptibility genes in multiplex families. *Hum Mol Genet* 1999 Apr;8(4):639-44.

126 Nath SK, Quintero-Del-Rio AI, Kilpatrick J, Feo L, Ballesteros M, Harley JB. Linkage at 12q24 with systemic lupus erythematosus (SLE) is established and confirmed in Hispanic and European American families. *Am J Hum Genet* 2004 Jan;74(1):73-82.

127 Cantor RM, Yuan J, Napier S, Kono N, Grossman JM, Hahn BH, et al. Systemic lupus erythematosus genome scan: support for linkage at 1q23, 2q33, 16q12-13, and 17q21-23 and novel evidence at 3p24, 10q23-24, 13q32, and 18q22-23. *Arthritis Rheum* 2004 Oct;50(10):3203-10.

128 Tsao BP, Cantor RM, Grossman JM, Shen N, Teophilov NT, Wallace DJ, et al. PARP alleles within the linked chromosomal region are associated with systemic lupus erythematosus. *J Clin Invest* 1999 Apr;103(8):1135-40.

129 Moser KL, Gray-McGuire C, Kelly J, Asundi N, Yu H, Bruner GR, et al. Confirmation of genetic linkage between human systemic lupus erythematosus and chromosome 1q41. *Arthritis Rheum* 1999 Sep;42(9):1902-7.

130 Johanneson B, Lima G, von SJ, Alarcon-Segovia D, Alarcon-Riquelme ME. A major susceptibility locus for systemic lupus erythematosus maps to chromosome 1q31. *Am J Hum Genet* 2002 Nov;71(5):1060-71.

131 Hirose S, Jiang Y, Hamano Y, Nishimura H. Genetic basis of systemic lupus erythematosus. *Drugs Today (Barc)* 2002 Mar;38(3):167-84.

132 Casciola-Rosen L, Rosen A. Ultraviolet light-induced keratinocyte apoptosis: a potential mechanism for the induction of skin lesions and autoantibody production in LE. *Lupus* 1997;6(2):175-80.

133 Reidenberg MM, Drayer DE, Lorenzo B, Strom BL, West SL, Snyder ES, et al. Acetylation phenotypes and environmental chemical exposure of people with idiopathic systemic lupus erythematosus. *Arthritis Rheum* 1993 Jul;36(7):971-3.

134 Molokhia M, Hoggart C, Patrick AL, Shriver M, Parra E, Ye J, et al. Relation of risk of systemic lupus erythematosus to west African admixture in a Caribbean population. *Hum Genet* 2003 Mar;112(3):310-8.

135 Greenwood BM. Autoimmune disease and parasitic infections in Nigerians. *Lancet* 1968 Aug 17;2(7564):380-2.

136 Huisman AM, White KP, Algra A, Harth M, Vieth R, Jacobs JW, et al. Vitamin D levels in women with systemic lupus erythematosus and fibromyalgia. *J Rheumatol* 2001 Nov;28(11):2535-9.

137 Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251-306.

138 Famularo G, Nucera E, Marcellini S, De SC. Fas/Fas ligand on the road: an apoptotic pathway common to AIDS, autoimmunity, lymphoproliferation and transplantation. *Med Hypotheses* 1999 Jul;53(1):50-62.

139 Nagata S. Fas ligand-induced apoptosis. *Annu Rev Genet* 1999;33:29-55.

140 Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998 Aug 28;281(5381):1305-8.

141 Van PL, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 1998 Apr 10;280(5361):243-8.

142 Cohen PL, Eisenberg RA. The *lpr* and *gld* genes in systemic autoimmunity: life and death in the Fas lane. *Immunol Today* 1992 Nov;13(11):427-8.

143 Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* 1994 Mar 25;263(5154):1759-62.

144 Russell JH, Rush B, Weaver C, Wang R. Mature T cells of autoimmune *lpr/lpr* mice have a defect in antigen-stimulated suicide. *Proc Natl Acad Sci U S A* 1993 May 15;90(10):4409-13.

145 Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, et al. Dominant

interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 1995 Jun 16;81(6):935-46.

146 Bettinardi A, Brugnoli D, Quiros-Roldan E, Malagoli A, La GS, Corra A, et al. Missense mutations in the Fas gene resulting in autoimmune lymphoproliferative syndrome: a molecular and immunological analysis. *Blood* 1997 Feb 1;89(3):902-9.

147 McNally J, Yoo DH, Drappa J, Chu JL, Yagita H, Friedman SM, et al. Fas ligand expression and function in systemic lupus erythematosus. *J Immunol* 1997 Nov 1;159(9):4628-36.

148 Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J Clin Invest* 1996 Sep 1;98(5):1107-13.

149 Kojima T, Horiuchi T, Nishizaka H, Sawabe T, Higuchi M, Harashima SI, et al. Analysis of fas ligand gene mutation in patients with systemic lupus erythematosus. *Arthritis Rheum* 2000 Jan;43(1):135-9.

150 Bijl M, van LT, Limburg PC, Spronk PE, Jaegers SM, Aarden LA, et al. Do elevated levels of serum-soluble fas contribute to the persistence of activated lymphocytes in systemic lupus erythematosus? *J Autoimmun* 1998 Oct;11(5):457-63.

151 Papo T, Parizot C, Ortova M, Piette JC, Frances C, Debre P, et al. Apoptosis and expression of soluble Fas mRNA in systemic lupus erythematosus. *Lupus* 1998;7(7):455-61.

152 Perniok A, Wedekind F, Herrmann M, Specker C, Schneider M. High levels of circulating early apoptotic peripheral blood mononuclear cells in systemic lupus erythematosus. *Lupus* 1998;7(2):113-8.

153 Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. *Ann Rheum Dis* 1999 May;58(5):309-14.

154 Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994 Apr 1;179(4):1317-30.

155 Utz PJ, Anderson P. Posttranslational protein modifications, apoptosis, and the bypass of tolerance to autoantigens. *Arthritis Rheum* 1998 Jul;41(7):1152-60.

156 Utz PJ, Hottelet M, Schur PH, Anderson P. Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. *J Exp Med* 1997 Mar 3;185(5):843-54.

157 Steinbach F, Henke F, Krause B, Thiele B, Burmester GR, Hiepe F. Monocytes from systemic lupus erythematosus patients are severely altered in phenotype and lineage flexibility. *Ann Rheum Dis* 2000 Apr;59(4):283-8.

158 Kovacs B, Szentendrei T, Bednarek JM, Pierson MC, Mountz JD, Vogelgesang SA, et al. Persistent expression of a soluble form of Fas/APO1 in continuously activated T cells from a patient

with SLE. *Clin Exp Rheumatol* 1997 Jan;15(1):19-23.

159 Furukawa F, Yoshimasu T. Animal models of spontaneous and drug-induced cutaneous lupus erythematosus. *Autoimmun Rev* 2005 Jul;4(6):345-50.

160 Petri M. Review of classification criteria for systemic lupus erythematosus. *Rheum Dis Clin North Am* 2005 May;31(2):245-54, vi.

161 Ioannou Y, Isenberg DA. Current concepts for the management of systemic lupus erythematosus in adults: a therapeutic challenge. *Postgrad Med J* 2002 Oct;78(924):599-606.

162 Giovanni Franchin, Elena Peeva, Betty Diamond. Pathogenesis of SLE: implications for rational therapy. *Drug Discovery Today: Disease Mechanisms* 2004 Dec;1(3):303-8.

163 Traynor AE, Schroeder J, Rosa RM, Cheng D, Stefka J, Mujais S, et al. Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. *Lancet* 2000 Aug 26;356(9231):701-7.

164 Ward MM, Pyun E, Studenski S. Long-term survival in systemic lupus erythematosus. Patient characteristics associated with poorer outcomes. *Arthritis Rheum* 1995 Feb;38(2):274-83.

165 Bono L, Cameron JS, Hicks JA. The very long-term prognosis and complications of lupus nephritis and its treatment. *QJM* 1999 Apr;92(4):211-8.

166 Jacobsen S, Petersen J, Ullman S, Junker P, Voss A, Rasmussen JM, et al. Mortality and causes of death of 513 Danish patients with systemic lupus erythematosus. *Scand J Rheumatol* 1999;28(2):75-80.

167 Peschken CA, Esdaile JM. Rheumatic diseases in North America's indigenous peoples. *Semin Arthritis Rheum* 1999 Jun;28(6):368-91.

168 Hellmann DB, Petri M, Whiting-O'Keefe Q. Fatal infections in systemic lupus erythematosus: the role of opportunistic organisms. *Medicine (Baltimore)* 1987 Sep;66(5):341-8.

169 Ward MM. Hospital experience and mortality in patients with systemic lupus erythematosus. *Arthritis Rheum* 1999 May;42(5):891-8.

170 Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 2005 Apr 9;365(9467):1333-46.

171 Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature* 2001 Dec 13;414(6865):782-7.

172 Onkamo P, Vaananen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 1999 Dec;42(12):1395-403.

173 Devendra D, Liu E, Eisenbarth GS. Type 1 diabetes: recent developments. *BMJ* 2004 Mar 27;328(7442):750-4.

-
- 174 Dahlquist G, Mustonen L. Analysis of 20 years of prospective registration of childhood onset diabetes time trends and birth cohort effects. Swedish Childhood Diabetes Study Group. *Acta Paediatr* 2000 Oct;89(10):1231-7.
- 175 Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet* 2000 Mar 11;355(9207):873-6.
- 176 Zimmet P, Dowse G, Finch C, Serjeantson S, King H. The epidemiology and natural history of NIDDM--lessons from the South Pacific. *Diabetes Metab Rev* 1990 Mar;6(2):91-124.
- 177 Shaw JE, Chisholm DJ. 1: Epidemiology and prevention of type 2 diabetes and the metabolic syndrome. *Med J Aust* 2003 Oct 6;179(7):379-83.
- 178 King H, Rewers M. Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO Ad Hoc Diabetes Reporting Group. *Diabetes Care* 1993 Jan;16(1):157-77.
- 179 Adegate E, Schattner P, Dunn E. An update on the etiology and epidemiology of diabetes mellitus. *Ann N Y Acad Sci* 2006 Nov;1084:1-29.
- 180 World Health Organization. Prevention of diabetes mellitus. Geneva: WHO; 1994. Report No.: Technical Report Series no. 844.
- 181 Type 2 diabetes in children and adolescents. American Diabetes Association. *Diabetes Care* 2000 Mar;23(3):381-9.
- 182 Ola TO, Gigante A, Leslie RD. Latent autoimmune diabetes of adults (LADA). *Nutr Metab Cardiovasc Dis* 2006 Apr;16(3):163-7.
- 183 Sanlioglu AD, Griffith TS, Omer A, Dirice E, Sari R, Altunbas HA, et al. Molecular mechanisms of death ligand-mediated immune modulation: a gene therapy model to prolong islet survival in type 1 diabetes. *J Cell Biochem* 2008 Jun 1;104(3):710-20.
- 184 Kaprio J, Tuomilehto J, Koskenvuo M, Romanov K, Reunanen A, Eriksson J, et al. Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland. *Diabetologia* 1992 Nov;35(11):1060-7.
- 185 Barnett AH, Eff C, Leslie RD, Pyke DA. Diabetes in identical twins. A study of 200 pairs. *Diabetologia* 1981 Feb;20(2):87-93.
- 186 Poulsen P, Kyvik KO, Vaag A, Beck-Nielsen H. Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study. *Diabetologia* 1999 Feb;42(2):139-45.
- 187 Tattersal RB, Fajans SS. Prevalence of diabetes and glucose intolerance in 199 offspring of thirty-seven conjugal diabetic parents. *Diabetes* 1975 May;24(5):452-62.

188 Pierce M, Keen H, Bradley C. Risk of diabetes in offspring of parents with non-insulin-dependent diabetes. *Diabet Med* 1995 Jan;12(1):6-13.

189 Hattersley A, Bruining J, Shield J, Njolstad P, Donaghue K. ISPAD Clinical Practice Consensus Guidelines 2006-2007. The diagnosis and management of monogenic diabetes in children. *Pediatr Diabetes* 2006 Dec;7(6):352-60.

190 Sanjeevi CB. Genes influencing innate and acquired immunity in type 1 diabetes and latent autoimmune diabetes in adults. *Ann N Y Acad Sci* 2006 Oct;1079:67-80.

191 Bartsocas CS, Gerasimidi-Vazeou A. Genetics of type 1 diabetes mellitus. *Pediatr Endocrinol Rev* 2006 Aug;3 Suppl 3:508-13.

192 Sale MM, Smith SG, Mychaleckyj JC, Keene KL, Langefeld CD, Leak TS, et al. Variants of the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in an African-American population enriched for nephropathy. *Diabetes* 2007 Oct;56(10):2638-42.

193 Saez ME, Gonzalez-Sanchez JL, Ramirez-Lorca R, Martinez-Larrad MT, Zabena C, Gonzalez A, et al. The CAPN10 gene is associated with insulin resistance phenotypes in the Spanish population. *PLoS ONE* 2008;3(8):e2953.

194 Flanagan SE, Clauin S, Bellanne-Chantelot C, de LP, Harries LW, Gloyn AL, et al. Update of mutations in the genes encoding the pancreatic beta-cell K(ATP) channel subunits Kir6.2 (KCNJ11) and sulfonylurea receptor 1 (ABCC8) in diabetes mellitus and hyperinsulinism. *Hum Mutat* 2009 Feb;30(2):170-80.

195 Qi L, Hu FB, Hu G. Genes, environment, and interactions in prevention of type 2 diabetes: a focus on physical activity and lifestyle changes. *Curr Mol Med* 2008 Sep;8(6):519-32.

196 Walley AJ, Blakemore AI, Froguel P. Genetics of obesity and the prediction of risk for health. *Hum Mol Genet* 2006 Oct 15;15 Spec No 2:R124-R130.

197 Peng H, Hagopian W. Environmental factors in the development of Type 1 diabetes. *Rev Endocr Metab Disord* 2006 Sep;7(3):149-62.

198 Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N Engl J Med* 1979 May 24;300(21):1173-9.

199 Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 1992 Jan;89(1):283-92.

200 Ellerman KE, Richards CA, Guberski DL, Shek WR, Like AA. Kilham rat triggers T-cell-dependent autoimmune diabetes in multiple strains of rat. *Diabetes* 1996 May;45(5):557-62.

201 Parslow RC, McKinney PA, Law GR, Staines A, Williams R, Bodansky HJ. Incidence of

childhood diabetes mellitus in Yorkshire, northern England, is associated with nitrate in drinking water: an ecological analysis. *Diabetologia* 1997 May;40(5):550-6.

202 Wilson GL, Mossman BT, Craighead JE. Use of pancreatic beta cells in culture to identify diabetogenic N-nitroso compounds. *In Vitro* 1983 Jan;19(1):25-30.

203 van Maanen JM, Albering HJ, de Kok TM, van Breda SG, Curfs DM, Vermeer IT, et al. Does the risk of childhood diabetes mellitus require revision of the guideline values for nitrate in drinking water? *Environ Health Perspect* 2000 May;108(5):457-61.

204 Helgason T, Ewen SW, Ross IS, Stowers JM. Diabetes produced in mice by smoked/cured mutton. *Lancet* 1982 Nov 6;2(8306):1017-22.

205 Longnecker MP, Klebanoff MA, Brock JW, Zhou H. Polychlorinated biphenyl serum levels in pregnant subjects with diabetes. *Diabetes Care* 2001 Jun;24(6):1099-101.

206 Rahman M, Axelson O. Diabetes mellitus and arsenic exposure: a second look at case-control data from a Swedish copper smelter. *Occup Environ Med* 1995 Nov;52(11):773-4.

207 Tseng CH, Tai TY, Chong CK, Tseng CP, Lai MS, Lin BJ, et al. Long-term arsenic exposure and incidence of non-insulin-dependent diabetes mellitus: a cohort study in arseniasis-hyperendemic villages in Taiwan. *Environ Health Perspect* 2000 Sep;108(9):847-51.

208 Henriksen GL, Ketchum NS, Michalek JE, Swaby JA. Serum dioxin and diabetes mellitus in veterans of Operation Ranch Hand. *Epidemiology* 1997 May;8(3):252-8.

209 Pesatori AC, Zocchetti C, Guercilena S, Consonni D, Turrini D, Bertazzi PA. Dioxin exposure and non-malignant health effects: a mortality study. *Occup Environ Med* 1998 Feb;55(2):126-31.

210 Wing RR, Goldstein MG, Acton KJ, Birch LL, Jakicic JM, Sallis JF, Jr., et al. Behavioral science research in diabetes: lifestyle changes related to obesity, eating behavior, and physical activity. *Diabetes Care* 2001 Jan;24(1):117-23.

211 Sipetic S, Vlajinac H, Kocev N, Bjekic M, Sajic S. Early infant diet and risk of type 1 diabetes mellitus in Belgrade children. *Nutrition* 2005 Apr;21(4):474-9.

212 Malcova H, Sumnik Z, Drevinek P, Venhacova J, Lebl J, Cinek O. Absence of breast-feeding is associated with the risk of type 1 diabetes: a case-control study in a population with rapidly increasing incidence. *Eur J Pediatr* 2006 Feb;165(2):114-9.

213 Stene LC, Joner G. Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. *Am J Clin Nutr* 2003 Dec;78(6):1128-34.

214 Zimmet PZ, McCarty DJ, de Court. The global epidemiology of non-insulin-dependent diabetes mellitus and the metabolic syndrome. *J Diabetes Complications* 1997 Mar;11(2):60-8.

215 Thernlund GM, Dahlquist G, Hansson K, Ivarsson SA, Ludvigsson J, Sjoblad S, et al. Psychological stress and the onset of IDDM in children. *Diabetes Care* 1995 Oct;18(10):1323-9.

216 Kisch ES. Stressful events and the onset of diabetes mellitus. *Isr J Med Sci* 1985

Apr;21(4):356-8.

217 Dahlquist G, Kallen B. Maternal-child blood group incompatibility and other perinatal events increase the risk for early-onset type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1992 Jul;35(7):671-5.

218 Svensson J, Carstensen B, Mortensen HB, Borch-Johnsen K. Early childhood risk factors associated with type 1 diabetes--is gender important? *Eur J Epidemiol* 2005;20(5):429-34.

219 Stene LC, Barriga K, Norris JM, Hoffman M, Klingensmith G, Erlich HA, et al. Symptoms of common maternal infections in pregnancy and risk of islet autoimmunity in early childhood. *Diabetes Care* 2003 Nov;26(11):3136-41.

220 Stene LC, Barriga K, Norris JM, Hoffman M, Erlich HA, Eisenbarth GS, et al. Perinatal factors and development of islet autoimmunity in early childhood: the diabetes autoimmunity study in the young. *Am J Epidemiol* 2004 Jul 1;160(1):3-10.

221 Dahlquist GG, Patterson C, Soltesz G. Perinatal risk factors for childhood type 1 diabetes in Europe. The EURODIAB Substudy 2 Study Group. *Diabetes Care* 1999 Oct;22(10):1698-702.

222 Stene LC, Magnus P, Lie RT, Sovik O, Joner G. Birth weight and childhood onset type 1 diabetes: population based cohort study. *BMJ* 2001 Apr 14;322(7291):889-92.

223 Ong KK, Dunger DB. Birth weight, infant growth and insulin resistance. *Eur J Endocrinol* 2004 Nov;151 Suppl 3:U131-U139.

224 Hypponen E, Kenward MG, Virtanen SM, Piitulainen A, Virta-Autio P, Tuomilehto J, et al. Infant feeding, early weight gain, and risk of type 1 diabetes. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes Care* 1999 Dec;22(12):1961-5.

225 Hypponen E, Virtanen SM, Kenward MG, Knip M, Akerblom HK. Obesity, increased linear growth, and risk of type 1 diabetes in children. *Diabetes Care* 2000 Dec;23(12):1755-60.

226 Greenbaum CJ. Insulin resistance in type 1 diabetes. *Diabetes Metab Res Rev* 2002 May;18(3):192-200.

227 Leslie RD, Taylor R, Pozzilli P. The role of insulin resistance in the natural history of type 1 diabetes. *Diabet Med* 1997 Apr;14(4):327-31.

228 Wilkin TJ. The accelerator hypothesis: weight gain as the missing link between Type I and Type II diabetes. *Diabetologia* 2001 Jul;44(7):914-22.

229 Fasanmade OA, Odeniyi IA, Ogbera AO. Diabetic ketoacidosis: diagnosis and management. *Afr J Med Med Sci* 2008 Jun;37(2):99-105.

230 Nugent BW. Hyperosmolar hyperglycemic state. *Emerg Med Clin North Am* 2005 Aug;23(3):629-48, vii.

231 Definition, diagnosis and classification of diabetes mellitus and its complications. Geneva : World Health Organization; 1999.

-
- 232 Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 2001 May 3;344(18):1343-50.
- 233 Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 2002 Feb 7;346(6):393-403.
- 234 Gestational diabetes mellitus. *Diabetes Care* 2003 Jan;26 Suppl 1:S103-S105.
- 235 Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002 May 15;287(19):2570-81.
- 236 Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 1998 Sep 12;352(9131):837-53.
- 237 Manzella D. Medication reference chart for type 2 diabetes- The new, and the tried and true. http://diabetes.about.com/od/equipmentandbreakthroughs/a/med_ref_chart.htm
- 238 Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. Allograft transplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* 1967 Jun;61(6):827-37.
- 239 Health Resources and Services Administration HSBDoT. 2006 Annual report of the US Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: transplant data 1996 2005. US Department of Health and Human Services; 2006.
- 240 Dean PG, Kudva YC, Stegall MD. Long-term benefits of pancreas transplantation. *Curr Opin Organ Transplant* 2008 Feb;13(1):85-90.
- 241 Vaithilingam V, Sundaram G, Tuch BE. Islet cell transplantation. *Curr Opin Organ Transplant* 2008 Dec;13(6):633-8.
- 242 Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000 Jul 27;343(4):230-8.
- 243 Robertson RP. Islet transplantation as a treatment for diabetes - a work in progress. *N Engl J Med* 2004 Feb 12;350(7):694-705.
- 244 Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 2006 Sep 28;355(13):1318-30.
- 245 Makhlof L, Kishimoto K, Smith RN, Abdi R, Koulmanda M, Winn HJ, et al. The role of autoimmunity in islet allograft destruction: major histocompatibility complex class II matching is necessary for autoimmune destruction of allogeneic islet transplants after T-cell costimulatory blockade. *Diabetes* 2002 Nov;51(11):3202-10.
- 246 Evgenov NV, Medarova Z, Pratt J, Pantazopoulos P, Leyting S, Bonner-Weir S, et al. In vivo imaging of immune rejection in transplanted pancreatic islets. *Diabetes* 2006 Sep;55(9):2419-28.

-
- 247 Linn T, Schmitz J, Hauck-Schmalenberger I, Lai Y, Bretzel RG, Brandhorst H, et al. Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. *Clin Exp Immunol* 2006 May;144(2):179-87.
- 248 Gimi B, Leoni L, Oberholzer J, Braun M, Avila J, Wang Y, et al. Functional MR microimaging of pancreatic beta-cell activation. *Cell Transplant* 2006;15(2):195-203.
- 249 Nanji SA, Shapiro AM. Advances in pancreatic islet transplantation in humans. *Diabetes Obes Metab* 2006 Jan;8(1):15-25.
- 250 Balamurugan AN, Bottino R, Giannoukakis N, Smetanka C. Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes. *Pancreas* 2006 Apr;32(3):231-43.
- 251 Murdoch TB, McGhee-Wilson D, Shapiro AM, Lakey JR. Methods of human islet culture for transplantation. *Cell Transplant* 2004;13(6):605-17.
- 252 Lee TC, Barshes NR, Brunnicardi FC, Alejandro R, Ricordi C, Nguyen L, et al. Procurement of the human pancreas for pancreatic islet transplantation. *Transplantation* 2004 Aug 15;78(3):481-3.
- 253 Rosenberg L. Clinical islet cell transplantation. Are we there yet? *Int J Pancreatol* 1998 Dec;24(3):145-68.
- 254 Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, et al. Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes* 1999 Oct;48(10):1907-14.
- 255 Carlsson PO, Palm F, Mattsson G. Low revascularization of experimentally transplanted human pancreatic islets. *J Clin Endocrinol Metab* 2002 Dec;87(12):5418-23.
- 256 Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* 2002 May;51(5):1362-6.
- 257 Noguchi H, Nakai Y, Ueda M, Masui Y, Futaki S, Kobayashi N, et al. Activation of c-Jun NH2-terminal kinase (JNK) pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor. *Diabetologia* 2007 Mar;50(3):612-9.
- 258 McKenzie MD, Dudek NL, Mariana L, Chong MM, Trapani JA, Kay TW, et al. Perforin and Fas induced by IFN γ and TNF α mediate beta cell death by OT-I CTL. *Int Immunol* 2006 Jun;18(6):837-46.
- 259 Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002 Sep;3(9):651-62.
- 260 Mimeault M, Batra SK. Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. *Stem Cell Rev* 2008;4(1):27-49.
- 261 Mimeault M, Batra SK. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. *Stem Cells* 2006 Nov;24(11):2319-45.
- 262 Xu R, Li H, Tse LY, Kung HF, Lu H, Lam KS. Diabetes gene therapy: potential and challenges. *Curr Gene Ther* 2003 Feb;3(1):65-82.

II. ARTICLES

Article 1.

Overexpression of human decoy receptor 3 in mice results in a systemic lupus erythematosus-like syndrome

Bing Han , Paul A. Moore, Jiangping Wu , Hongyu Luo

Arthritis Rheum. 56(11):3748-58, 2007.

Summary: In this paper, we showed that overexpression of DcR3 induced lupus-like syndromes in transgenic mouse model. We also found enlarged memory T cell pool in DcR3 transgenic mouse. Furthermore, we demonstrated that DcR3 could disturb AICD of T lymphocytes. Our results imply that DcR3 may play an important role in SLE pathogenesis through interfering lymphocyte homostasis.

Bing Han performed all assays and experiments, and processed and analyzed data; Paul Moore constructed DcR3 plasmid and synthesized recombinant DcR3; Jiangping Wu and Hongyu Luo directed the experiment design and data analysis.

Overexpression of human decoy receptor 3 in mice results in a systemic lupus erythematosus-like syndrome

Bing Han¹, Paul A. Moore², Jiangping Wu^{1*}, Hongyu Luo^{1*}

¹ Centre hospitalier de l'Université de Montréal, and Notre-Dame Hospital, Montréal, Quebec, Canada

² Human Genome Sciences, Rockville, Maryland

* Correspondence to Jiangping Wu, Laboratory of Immunology, Research Centre, CHUM, Notre-Dame Hospital, Pavillion DeSève, Room Y-5612, 1560 Sherbrooke Street East, Montréal, Quebec H2L 4M1, Canada

* Correspondence to Hongyu Luo, Laboratory of Immunology, Research Centre, CHUM, Notre-Dame Hospital, Pavillion DeSève, Room Y-5612, 1560 Sherbrooke Street East, Montréal, Quebec H2L 4M1, Canada

† Drs. Han, Wu, and Luo are co-inventors of and have a patent pending for the use of serum decoy receptor 3 as a diagnostic marker for systemic lupus erythematosus.

ABSTRACT

Objective

Decoy receptor 3 (DcR3), a tumor necrosis factor receptor family member, is a secreted protein that can enhance cell survival by interfering with multiple apoptosis pathways. This study was undertaken to investigate the role of DcR3 in the pathogenesis of autoimmune disease.

Methods

We generated transgenic mice with actin promoter-driven expression of human DcR3 and investigated the development of autoimmune disease in these mice.

Results

T cell immune responses were compromised in young DcR3-transgenic mice. Beyond 5-6 months of age, transgenic mice developed a systemic lupus erythematosus (SLE)-like syndrome, with numerous features of the disease. They produced autoantibodies against double-stranded DNA. Their kidneys showed pathologic changes indicative of glomerular nephritis and IgG and C3 deposition, and proteinuria, leukocyturia, and hematuria, were evident. Aged transgenic mice also developed skin lesions

and lymphocyte infiltration in the liver, and exhibited leukopenia, anemia, and thrombocytopenia. The SLE-like syndrome penetrance in DcR3-transgenic mice was sex associated, occurring in ~60% of females versus 20% of males. Exogenous recombinant DcR3 or endogenous DcR3 produced by transgenic T cells effectively protected T cells against activation-induced apoptosis in vitro. Probably as a consequence of this, CD4 cells with a phenotype of previous activation were increased in the peripheral blood of transgenic mice beyond 6 months of age.

Conclusion

These results show that DcR3 overexpression could lead to an SLE-like syndrome in mice.

Systemic lupus erythematosus (SLE) is a potentially severe autoimmune disease of undetermined etiology, with genetic, environmental, and sex factors as contributing elements. It is a polygenic disease, and as many as 30 susceptibility loci with possible links to its pathogenesis have been identified in mice (1). Among suspected pathogenic genes, a sizable number are in the apoptosis pathway (2). Indeed, in prototypical murine SLE models (i.e., *lpr/lpr* and *gld/gld* mice), the animals are defective in Fas and FasL, respectively, which are critical elements in T cell apoptosis. In contrast, SLE patients rarely have Fas or FasL mutations (3,4). This prompted us to examine other genes in the apoptosis pathway, with regard to their roles in SLE pathogenesis.

Human decoy receptor 3 (DcR3) belongs to the tumor necrosis factor receptor (TNFR) family (5), but lacks transmembrane and cytoplasmic domains in its sequence. It is thus a secreted protein (6). DcR3 can bind to the TNF family members FasL (5), LIGHT (6,7), and TNF-like molecule 1A (TL1A) (8) and block their interaction with their respective receptors, i.e., Fas, herpesvirus entry mediator (HVEM) protein, and the death domain-containing receptor DR3 (6-8). DcR3 does not bind to other known TNF family members (9), but findings of one study have suggested the existence of additional DcR3-binding ligand(s) (10). The mouse does not have an orthologous counterpart of the human DcR3 gene according to

a genome-wide computer search, but studies by our group and by Bossen et al have established that human DcR3 can bind to mouse FasL and LIGHT (6,9,11). This allows human DcR3 to function in mouse models, both in vitro and in vivo.

DcR3 has a clear role in apoptosis. FasL is a well-known molecule involved in apoptosis. LIGHT is a ligand for HVEM protein and lymphotoxin β receptor (LT β R), in addition to being a ligand for DcR3 (12). LIGHT can induce apoptosis in cells expressing both HVEM protein and LT β R (13) or LT β R alone (14). TL1A, a newly recognized member of the TNF family, can evoke apoptosis via its receptor, DR3 (8). Consequently, the interaction of DcR3 with FasL, LIGHT, and TL1A blocks apoptosis mediated by Fas, HVEM protein, LT β R, and DR3 (8).

Normal T cells express low levels of DcR3 (15), and healthy individuals have near-background serum DcR3 levels (16). DcR3 expression is augmented in activated T cells (15); this probably represents a fine-tuning mechanism to balance the need for clonal expansion and subsequent massive activation-induced cell death (AICD) of T cells. Malfunction of this balance due to failed AICD might lead to pathologic consequences, such as autoimmune diseases, including SLE. Of interest, overexpression

of DcR3 messenger RNA in peripheral blood mononuclear cells (PBMCs) of SLE patients compared with healthy individuals has been reported (17).

In this study, to explore the possible role of DcR3 in SLE pathogenesis, we generated transgenic mice with actin promoter-driven expression of human DcR3. These DcR3-transgenic mice manifested an SLE-like syndrome after 5-6 months of age.

MATERIALS AND METHODS

Generation of transgenic mice with actin promoter-driven DcR3 expression.

Human full-length DcR3 complementary DNA (cDNA) was cloned into *Bam* HI and *Xba* I sites in pAC vector between the human β -actin promoter and β -actin poly(A) signals. The resulting construct was named pAC-DcR3. The 5.7-kb *Cla* I/*Cla* I fragment containing the β -actin promoter, DcR3 cDNA, and β -actin poly(A) signal was excised and injected into fertilized eggs from C3H \times C57BL/6 mice or C57BL/6 mice. The transgenic mice were genotyped by polymerase chain reaction (PCR), for which the 5' primer (5'-GAGCGCTTCCTCCCTGTGCAC) was derived from the DcR3 sequence and the 3' primer (5'-GAATGCAATTGTTGTTGGTAACTTG) from the actin sequence downstream of DcR3. Amplification conditions were as follows: 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and 1 cycle of 72°C for 5 minutes. The PCR results were confirmed by enzyme-linked immunosorbent assay (ELISA) for serum DcR3.

DcR3 ELISA.

The DcR3 ELISA has been described elsewhere (16). A monoclonal antibody specific for DcR3 was used for coating, and a biotinylated affinity-purified rabbit antibody against DcR3 was used as the detecting antibody. The sensitivity of the assay was 6 pg/ml.

Flow cytometry.

Two- or 3-color flow cytometry was performed to determine the expression of Thy1.2, CD4, CD8, CD3, T cell receptor β (TCR β), CD62L, CD44, CD19, CD5, CD25, and annexin V, as described previously (18).

Western blotting to detect serum autoantibodies against various organs.

The organs were homogenized in phosphate buffered saline (PBS) and centrifuged for 15 minutes at 3,000g. The cleared lysates were passed through a 0.22- μ m microfilter and resolved by sodium dodecyl sulfate-8% polyacrylamide gel electrophoreses (60 μ g/lane) overnight at 20V. The proteins were transferred to polyvinylidene difluoride membranes, which were then blocked with Tris buffered saline (TBS) blocking buffer (100 mM Tris·HCl, 0.9% NaCl [pH 7.5]) containing 5% milk powder. The membranes were reacted with transgenic or wild-type mouse serum (1:500 dilution in blocking buffer) overnight at 4°C and washed 3 times at room

temperature with TBS containing 0.05% Tween 20. Autoantibody was detected with sheep anti-mouse IgG (1:2,000 dilution; Amersham Biosciences, Baie d'Urfé, Quebec, Canada) followed by enhanced chemiluminescence.

Immunofluorescence for detection of antinuclear antibodies.

HeLa cells were fixed at 4°C for 60 minutes using a Cytotfix/Cytoperm kit (BD Biosciences, San Diego, CA) and then incubated overnight at 4°C with wild-type or transgenic mouse serum (1:500 dilution in the wash buffer from the kit). After washing, the cells were reacted with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (1:1,000 dilution; Bethyl Laboratories, Montgomery, TX) overnight at 4°C. The cells were examined under a fluorescence microscope.

ELISA for anti-double-stranded DNA (anti-dsDNA) antibodies.

Ninety-six-well high-affinity ELISA microplates (Costar, Cambridge, MA) were coated with 250 µg/ml salmon sperm DNA (Invitrogen, Burlington, Ontario, Canada) in coating buffer (0.05M sodium bicarbonate solution [pH 9.5]) overnight at 4°C. After washing with PBS containing 0.05% Tween 20, the plates were incubated with blocking buffer (PBS containing 1% bovine serum albumin) for 1 hour at room temperature. Wild-type and transgenic mouse sera were diluted in blocking buffer at 1:50 and

incubated overnight in the wells at 4°C. After washing, the plates were reacted with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (1:2,000 dilution; Amersham Biosciences) for 1 hour at room temperature. Signals were revealed by addition of tetramethylbenzidine substrate (BD Biosciences), followed by 20-minute incubation at room temperature. The reaction was stopped using 50 µl 4N HCl, and the plates were read at a wavelength of 450 nm.

Detection of leukocytes, protein, and hemoglobin in urine.

Chemstrip (Roche, Laval, Quebec, Canada) was used for semiquantitative analysis of leukocytes, protein, and hemoglobin in urine.

T cell culture and apoptosis.

Spleen cells were isolated as described previously (18). In some experiments, the cells were cultured with crosslinked FasL-FLAG for 24 hours in the presence or absence of various concentrations of soluble DcR3 (0.133 mg/ml FasL-FLAG was preincubated for 24 hours at 4°C at a 1:1 ratio with 0.133 mg/ml mouse monoclonal antibody against FLAG; the final concentration of crosslinked FasL-FLAG for culture was 0.6 µg/ml). In other experiments, T cells were first activated for 48 hours with soluble anti-CD3 (2 µg/ml). The cells were washed and recultured for 48 hours in the presence of 50 units/ml interleukin-2 (IL-2); they were then washed

again, transferred to new wells coated with anti-CD3 (10 μ g/ml during coating), and coated for an additional 48 hours in the presence or absence of DcR3 at various concentrations. CD4 and CD8 cell apoptosis was analyzed by annexin V staining on CD4- or CD8-gated cells on 2-color flow cytometry (18).

RESULTS

DcR3-transgenic mice.

DcR3-transgenic mice were generated on both the C3H × C57BL/6 and the C57BL/6 backgrounds, using the human β -actin promoter. The transgenic construct pAC-DcR3 is shown in Figure 1A. Seven founders were identified by PCR of tail DNA. PCR results in line 754 (C3H × C57BL/6 background) and line 17139 (C57BL/6 background) are illustrated in Figure 1B. The results were confirmed by detection of DcR3 in serum. DcR3 was found in levels ranging from 20 ng/ml to 80 ng/ml in serum from transgenic mice, but was absent in wild-type littermates (Figure 1C). Most of the results presented herein were from line 754 (backcrossed to C57BL/6 for 5-8 generations), but line 17139 (pure C57BL/6 background) exhibited a similar phenotype, such as lymphadenopathy, pathologic findings in the kidney, liver and skin, and shortened lifespan in females (data not shown). Spleen T cells from transgenic mice were able to secrete high concentrations of DcR3 (\sim 10 ng/ml) into culture supernatants within 48 hours (Figure 1D).

DcR3-transgenic mice were fertile, with no gross anomalies observed before 4-6 months of age. In transgenic mice younger than 5-6 months of age, thymus, spleen, and lymph node weight and cellularity were in the normal range, as were cell subpopulations in these organs (data not shown). Their T cell proliferation response to solid-phase anti-CD3 alone or to anti-CD3 plus anti-CD28 was variable and did not show consistent changes in comparison with T cells from wild-type mice, but they exhibited increased secretion of IL-4 (but not interferon- γ) (data not shown).

Transgenic and wild-type mice had similar levels of IgA, IgM, IgG2A, and IgG3, but transgenic mice produced higher amounts of IgG1 and IgG2b than did wild-type mice. Transgenic mice also generated significantly higher concentrations of IgE, a typical Th2-dependent isotype, between 2 months and 6 months of age (data not shown). The IgG1 and IgE overproduction indicated that immune responses in transgenic mice were skewed toward a Th2-type response. Delayed-type hypersensitivity in transgenic mice was compromised compared with that in wild-type mice (data not shown); augmented Th2-type responses might have been responsible, at least in part, for this.

Lymphadenopathy and lifespan of DcR3-transgenic mice.

After age 4-6 months, DcR3-transgenic mice developed lymphadenopathy (defined as cervical lymph node weight >20 mg), with a higher penetrance in females (64.5%) than in males (20%) (Figures 2A and B). In 90% of cases, the lymphadenopathy was accompanied by splenomegaly (defined as spleen weight >1.5-fold the mean normal spleen weight) (Figure 2C); in contrast, the spleens of transgenic mice without lymphadenopathy did not differ in size from those of their wild-type littermates (results not shown). A large percentage of cells in the enlarged lymph nodes (51.6%) were Thy1.2⁺ T cells, among which 62.9% were CD4⁻,CD8⁻ double negative (Figure 2D). In 3 randomly selected transgenic mice, the absolute number of this double-negative population in the cervical lymph nodes was $252 \pm 85 \times 10^6$ cells (mean \pm SD), while the CD4⁺ population was $68 \pm 23 \times 10^6$ cells and the CD8⁺ population was $50 \pm 17 \times 10^6$ cells in these nodes. Unusually, a large percentage of T cells (Thy1.2⁺, CD3⁺, TCR β ⁺, CD8⁻, and predominantly CD4⁻) from the lymph nodes (57.6%) expressed a B cell marker (B220) (Figure 2E) and were CD44⁺ and CD62L^{high}. Such lymphadenopathy and the unusual phenotype of cells (B220⁺ double-negative T cells) in the enlarged lymph nodes were similar to findings in *lpr/lpr* mice, which have a defective Fas-mediated apoptosis

pathway (19), although the phenotype occurred at an older age in our DcR3-transgenic mice (>5-6 months versus 2 months).

The lifespan of DcR3-transgenic mice was shortened, especially in females and those with lymphadenopathy, compared with that of wild-type mice (Figure 2F). Among female DcR3-transgenic mice, the 14-month survival rate was 63.2%, and among male transgenic mice, it was 81.8%. Among transgenic mice that died before 14 months of age (n = 11), the majority (>80%) had lymphadenopathy. This lifespan was, however, longer than that of C57BL/6-*lpr/lpr* mice (mean \pm SD lifespan 10.1 ± 0.5 months in females [20]).

Development of an SLE-like syndrome after 6 months of age in DcR3-transgenic mice.

Various immunologic parameters were evaluated in mice >6 months old. In immunoblotting studies in which extracts from self tissues were used as antigens and self sera were used as antibodies, we evaluated the presence of serum autoantibodies against self tissue. Transgenic mice clearly expressed autoantibodies against self tissue (Figure 3A). It should be noted that in general, not all antibodies are effective in immunoblotting. Therefore, the bands shown in Figure 3A probably represent a part of tissue antigens targeted by autoantibodies. Nevertheless, comparing the

results from transgenic and wild-type mice, it is clear that abundant autoantibodies were present in the former but not the latter.

Transgenic mice also produced antinuclear antibodies (Figure 3B) and anti-dsDNA antibodies (Figure 3C), the latter being a hallmark of SLE. With positivity defined as 2 SD above the mean in serum from wild-type mice, 77% of female transgenic mice and 55% of male transgenic mice were found to be anti-dsDNA antibody positive, demonstrating a higher penetrance in females, similar to findings in human SLE.

B-1 cells are a prominent B cell population during early ontogeny ([21]). In adults, B-1 cells are abundant in celomic cavities and can be classified into B-1a and B-1b subpopulations, which are similar in their surface markers, including CD11b expression, but the former is CD5⁺ while the latter is CD5⁻ ([22]). In human SLE, the B-1a subpopulation in PBMCs is augmented ([23]). We demonstrated that the B-1a subpopulation was significantly increased in transgenic mouse PB B cells (CD19⁺) compared with wild-type mouse PB B cells (32.8% versus 11.5%) (Figure 3D). Consistent with this finding, the peritoneal B-1a, but not B-1b, subpopulation was expanded among B cells of transgenic mice (Figure 3E). There was no abnormal expansion of the total B cell population in the

PBMCs, spleen, or peritoneal cavity of transgenic mice compared with wild-type mice (data not shown).

Human SLE is associated with glomerulonephritis. Thus, renal pathology and function were studied in DcR3-transgenic mice. Beyond age 6 months, the kidneys of transgenic mice had prominently enlarged glomeruli with increased glomerular epithelial cellularity and mesangial hypertrophy, along with interstitial fibrosis, as seen with hematoxylin and eosin (H&E) staining (Figure 4A). Leakage of red blood cells into interstitia was apparent. There was also obvious IgG and C3 deposition in the glomeruli and in interstitial spaces (Figure 4A). Semiquantitative assays of urine revealed leukocyturia, proteinuria, and hematuria in transgenic, but not wild-type, mice. This phenotype was significantly more common in transgenic females (Figure 4B). In the liver of DcR3-transgenic mice >6 months of age, periarterial lymphocyte infiltration was observed (Figure 4C), similar to the liver pathology found in SLE patients ([24]).

Beyond age 12 months, DcR3-transgenic females frequently (40%) developed skin lesions, as seen in Figure 4D. Lesions in the epidermis exhibited superficial crusting and erosion. In the damaged areas, polymorphonuclear cell infiltration and hyperkeratosis of the epidermis with follicular plugging were apparent. The junction between the epidermis

and the dermis was unclear, due to the loss of epidermis pegs and the presence of cell infiltration. In the dermis, under the eroded lesion, granulomatous tissue consisting of collagen fibers, fibroblasts, and infiltrating lymphocytes was present. Mild edema and vasodilatation of the capillaries and small vessels with red blood cells were observed in the dermis, with clumps of infiltrating lymphocytes surrounding the vessels and appendages. Band-like infiltrating lymphocytes were found in some subcutaneous layers. The overall pathologic features were similar to those manifested in MRL/*lpr* mice ([25]), and were reminiscent of findings in human chronic cutaneous lupus.

In human SLE, leukocytopenia, thrombocytopenia, and anemia are common ([26]). This was also the case in DcR3-transgenic mice >6 months of age. As shown in Figure 4E, leukocytopenia occurred in 6 of 10, thrombocytopenia in 3 of 9, and anemia in 4 of 10 female transgenic mice, using the mean values minus 2 SD in wild-type mice as thresholds. Two of 9 male transgenic mice exhibited anemia, but none of the transgenic males had leukocytopenia or thrombocytopenia. The frequency of these 3 disorders in male transgenic mice did not differ significantly from that in wild-type mice, but the frequency of all 3 disorders was significantly greater in transgenic females than in wild-type mice. This again

demonstrates that transgenic females had more severe hematologic damage, similar to findings in human SLE. In transgenic mice, bone marrow did not have obvious abnormalities in cellularity as determined with H&E staining, or reduction in lineage-negative precursor cells as assessed by flow cytometry (results not shown). Furthermore, sera from transgenic mice did not contain antibodies reactive with lineage-negative precursor cells, as determined by immunofluorescence microscopy (results not shown). This suggests that the bone marrow deficiency was not the major cause of the observed hematologic damage in transgenic mice.

DcR3 promotion of survival of activated T cells.

We next investigated the mechanism by which DcR3 triggers the SLE-like syndrome in mice. In a normal immune response, T cell activation is followed by AICD, which is responsible for the shrinking of expanded T cell colonies. Abnormal T cell AICD is believed to be one of the factors in SLE pathogenesis. To assess the role of DcR3 in T cell survival after activation, we applied a typical model of *in vitro* AICD. Spleen CD4 and CD8 cells from wild-type and transgenic mice were first activated for 48 hours with soluble anti-CD3, and then expanded with IL-2 for an additional 48 hours. When these cells were further stimulated with solid-phase anti-CD3, they underwent significant apoptosis, as seen in

Figure 5A. DcR3 is capable of blocking apoptosis pathways mediated by at least 4 TNFR family members, i.e., Fas, HVEM protein, LT β R, and DR3 ([6-8]). We demonstrated that DcR3 continuously secreted by spleen cells from transgenic mice drastically inhibited CD4 cell AICD; similar protection of CD8 cells by DcR3 was also evident (Figure 5A). In these experiments, CD4 and CD8 cells from wild-type and transgenic mice were similarly activated, based on their CD25 expression (data not shown).

Experiments were then performed to investigate whether the Fas pathway could be blocked by DcR3 during T cell apoptosis. In the absence of recombinant FasL, resting transgenic or wild-type mouse CD4 cells exhibited 19.6-24.1% background apoptosis after 16-hour culture either without DcR3, with 10 μ g/ml recombinant DcR3, or with DcR3 secreted de novo from transgenic mouse T cells (Figure 5B). In the presence of FasL, which was crosslinked to enhance its efficacy ([27]), wild-type mouse CD4 cells underwent massive apoptosis (52.2%) after 16-hour culture; this is compatible with the fact that Fas is constitutively expressed in resting T cells and crosslinking of Fas results in apoptosis in various cell types ([28]). Exogenous recombinant DcR3 at 10 μ g/ml almost completely prevented such apoptosis in wild-type mouse CD4 cells (16.3% apoptosis). Although DcR3 secreted de novo by CD4 cells was sufficient to

completely protect activated CD4 cells against death in the absence of exogenous FasL, as shown in Figure 5A, it became less effective (43.7% apoptosis), but was still consistently protective, in the presence of excess exogenous FasL (600 ng/ml) (Figure 5B), likely due to the relatively high concentration of the latter. The dose-dependent protective effect of exogenous recombinant DcR3 on CD4 cells is depicted in Figure 5D. A similar dose-dependent protective effect of exogenous or de novo-secreted DcR3 could also be observed in CD8 cells (Figures 5C and D).

The above-described results demonstrate that inhibition of the Fas-mediated apoptosis pathway is at least one of the protective mechanisms used by DcR3 in promoting T cell survival. Bim, a proapoptotic BH3-only protein, has been implicated in clonal contraction due to its role in cytokine deprivation-associated cell death ([29]), and a recent study revealed that Fas signaling leads to Bim induction, linking the Fas pathway to the Bim pathway ([30]). Thus, blocking of Fas signaling by DcR3 not only directly affects the Fas pathway but can also indirectly interfere with the Bim pathway, both of which are important in clonal contraction.

In a normal immune response, T cell clonal contraction follows clonal expansion; such contraction depends on AICD. Some T cells that

underwent AICD might have had a low affinity for specific antigen in the immune response but might cross-react with self antigen; this might otherwise have evoked autoimmune responses, had AICD not been taking place. In the peripheral blood of transgenic mice >6 months of age, a significantly higher percentage of CD4 cells exhibited the CD62L^{low} (73.2%) and CD44^{high} (70.8%) phenotype, compared with CD4 cells from wild-type mice (33.4% and 22.7%, respectively) (Figure 6A). These cells represented previously but not recently activated CD4 cells, since their CD25 expression was low. As shown in Figures 6B and C, the percentages of CD44^{high},CD25⁻,CD4⁺ and CD44^{high},CD62L^{low},CD4⁺ cells in the peripheral blood of DcR3-transgenic mice were significantly elevated compared with the percentages in wild-type mice. Among transgenic mice, these percentages did not differ by sex or by presence or absence of lymphadenopathy (data not shown). These findings, plus our demonstration that DcR3 protected against AICD, suggest that DcR3 might promote the survival of activated CD4 cells in vivo, and these surviving cells might be at the core of SLE pathogenesis. Nevertheless, we cannot exclude the possibility that such a phenomenon reflects increased activation, rather than decreased apoptosis, of CD4 cells in this autoimmune model.

DISCUSSION

DcR3 is a death decoy protein that blocks multiple apoptosis pathways, including Fas/FasL. We have demonstrated that DcR3, in the form of either recombinant protein or protein secreted by transgenic leukocytes, could effectively prevent AICD of CD4 and CD8 cells. Inhibition of T cell death could disrupt clonal shrinkage after clonal expansion during an immune response, resulting in the survival of abnormal self cross-reactive T cells and, potentially, autoimmune disease. Indeed, failed T cell apoptosis is implicated in SLE pathogenesis, as shown in *lpr/lpr* and *gld/gld* mice, which in many respects manifest pathologic changes similar to those in human SLE. However, human SLE patients rarely have mutations of Fas or FasL, and thus these are not susceptibility genes in humans. Nevertheless, it is possible that some proteins (e.g., DcR3) indirectly interfering with the Fas/FasL pathway in humans might be lupus pathogenic. In support of this possibility, we have reported previously that activated T cells produce DcR3 (15). Of greater relevance, it has been demonstrated that PBMCs from SLE patients present higher levels of DcR3 transcripts than those from healthy individuals (17). We have observed augmented serum levels of DcR3 in a subpopulation of SLE patients during flares (Han B, et al: unpublished observations). Further, we

have demonstrated in this study that DcR3 overexpression resulted in a lupus-like syndrome in mice.

In theory, the interference of DcR3 in the Fas apoptosis pathway might also be involved in the autoimmune lymphoproliferative syndrome (ALPS). Although Fas, FasL, caspase 8, or caspase 10 mutations have been found in 66% of ALPS patients, the remaining 34% have no identifiable mutation (31). It would be interesting to investigate whether DcR3 overexpression occurs in this subpopulation.

It should be noted that although DcR3 can potentially block at least 3 apoptosis pathways, i.e., Fas/FasL, LIGHT/HVEM-LT β R, and TL1A/DR3, SLE pathogenesis is most likely related to its interference with the Fas/FasL pathway. No SLE phenotype has been observed in LIGHT- or DR3-null mutant mice (32,33).

We have previously shown that ~60-70% of patients with various types of tumor have elevated serum levels of DcR3, as do patients with liver cirrhosis (16). However, these patients normally do not develop SLE as a complication. It is possible that local biologically active DcR3 in lymphoid organs has pathogenic potential for the development of SLE. Indeed, our *in vitro* experiments demonstrated that DcR3 secreted by spleen cells from

transgenic mice was sufficient to protect activated T cells against death (Figure 6). Furthermore, we conducted whole-body irradiation on wild-type mice, followed by bone marrow transplantation. Six months after transplantation, 5 of 8 recipients of DcR3-transgenic mouse bone marrow developed anti-dsDNA antibodies, a hallmark of SLE; moreover, these mice had renal and liver histopathologic features similar to those in DcR3-transgenic mice. Conversely, none of the 5 recipients of wild-type mouse bone marrow developed anti-dsDNA antibodies (data not shown). Taken together, these results indicate that abnormal overexpression of DcR3 by leukocytes is sufficient to cause an SLE-like syndrome, probably due to high local levels of biologically active DcR3 in lymphoid organs.

It could be argued that the serum DcR3 levels of 20-80 ng/ml in our DcR3-transgenic mice, ~ 2 orders of magnitude higher than levels seen in SLE patients, are not physiologically or pathologically relevant. In a report by Hsu et al describing transgenic mice with phosphoglycerate kinase (PGK) promoter-driven DcR3 expression, with serum DcR3 levels of ~ 4.7 ng/ml (34), an associated SLE-like phenotype was not mentioned. It is not clear whether transgenic mice with PGK promoter-driven DcR3 expression have been studied carefully beyond the age of 6 months. If these mice do not develop an SLE-like syndrome at an older age, it is possible that either

1) very high DcR3 levels are required for SLE pathogenesis and DcR3 is thus unlikely pathogenic in most patients, or 2) human DcR3 might not interact with mouse ligands with similar affinity as it would with human ligands. It is worth mentioning that although the binding affinity of human DcR3 to its human ligands has been demonstrated by us previously (6) and human DcR3 is known to interact with mouse FasL, LIGHT, and TL1A (9), the affinity of human DcR3 with mouse ligands has not been documented.

Linkage analysis in lupus-prone mice has revealed ~ 30 lupus susceptibility loci (1). However, such studies in mice cannot identify DcR3 as an SLE susceptibility gene, because DcR3 is a human gene with no orthologous counterpart in mice. In a study of European and African Americans (35), an SLE locus was located at 20q13 (logarithm of odds 2.49) near marker D20S481, which is situated ~7.6 Mb from the DcR3 gene. However, a more recent fine mapping study (36) placed an SLE risk locus at 20Q13.1, which is a considerable distance from the DcR3 gene (~ 20 Mb). Thus, further fine mapping genetic analyses in humans are warranted to establish whether DcR3 or its expression regulators are bona fide susceptibility genes that have a pathogenic role in SLE.

Funded by:

- Canadian Institutes of Health Research for New Emerging Teams in Transplantation
- Fonds de la recherche en santé du Québec for Transfusional and Hemovigilance Medical Research
- Genome Canada/Quebec
- Canadian Institutes of Health Research; Grant Number: MOP-57697, MOP-69089
- the Kidney Foundation of Canada
- the Heart and Stroke Foundation of Quebec
- the Juvenile Diabetes Research Foundation USA; Grant Number: 1-2005-197
- J.-Louis Levesque Foundation
- Fonds de la recherche en santé du Québec National Scholar
- Canadian Institutes of Health Research; Grant Number: MOP-79565

ACKNOWLEDGEMENTS

The authors thank Mr. Ovid Da Silva and Dr. Robert Boileau (Research Support Office, Research Centre, CHUM) for editorial assistance and statistical analysis, respectively. We also thank Dr. Dasheng Xu for pathologic interpretation of skin, kidney, and liver specimens, and Mr. David La Fleur for technical assistance.

REFERENCES

- 1 Santiago-Raber ML, Laporte C, Reininger L, Izui S. Genetic basis of murine lupus. *Autoimmun Rev* 2004; 3: 33-9.
- 2 Salmon M, Gordon C. The role of apoptosis in systemic lupus erythematosus. *Rheumatology (Oxford)* 1999; 38: 1177-83.
- 3 Mysler E, Bini P, Drappa J, Ramos P, Friedman SM, Krammer PH, et al. The apoptosis-1/Fas protein in human systemic lupus erythematosus. *J Clin Invest* 1994; 93: 1029-34.
- 4 McNally J, Yoo DH, Drappa J, Chu JL, Yagita H, Friedman SM, et al. Fas ligand expression and function in systemic lupus erythematosus. *J Immunol* 1997; 159: 4628-36.
- 5 Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 1998; 396: 699-703.
- 6 Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, et al. Modulation of T-cell responses to alloantigens by TR6/DcR3. *J Clin Invest* 2001; 107: 1459-68.
- 7 Bai C, Connolly B, Metzker ML, Hilliard CA, Liu X, Sandig V, et al. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc Natl Acad Sci U S A* 2000; 97: 1230-5.
- 8 Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, et al. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 2002; 16: 479-92.
- 9 Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, et al. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem* 2006; 281: 13964-71.
- 10 Hsu TL, Chang YC, Chen SJ, Liu YJ, Chiu AW, Chio CC, et al. Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J Immunol* 2002; 168: 4846-53.
- 11 Wu Y, Han B, Luo H, Roduit R, Salcedo TW, Moore PA, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* 2003; 52: 2279-86.

- 12 Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin are ligands for herpesvirus entry mediator. *Immunity* 1998; 8: 21-30.
- 13 Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, et al. LIGHT, a novel ligand for lymphotoxin receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest* 1998; 102: 1142-51.
- 14 Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, et al. The lymphotoxin- receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem* 2000; 275: 14307-15.
- 15 Wan X, Shi G, Semenuk M, Zhang J, Wu J. DcR3/TR6 modulates immune cell interactions. *J Cell Biochem* 2003; 89: 603-12.
- 16 Wu Y, Han B, Sheng H, Lin M, Moore PA, Zhang Z, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 2003; 105: 724-32.
- 17 Otsuki T, Tomokuni A, Sakaguchi H, Aikoh T, Matsuki T, Isozaki Y, et al. Over-expression of the decoy receptor 3 (DcR3) gene in peripheral blood mononuclear cells (PBMC) derived from silicosis patients. *Clin Exp Immunol* 2000; 119: 323-7.
- 18 Luo H, Yu G, Tremblay J, Wu J. EphB6-null mutation results in compromised T cell function. *J Clin Invest* 2004; 114: 1762-73.
- 19 Rathmell JC, Goodnow CC. Effects of the *lpr* mutation on elimination and inactivation of self-reactive B cells. *J Immunol* 1994; 153: 2831-42.
- 20 Roths JB. Differential expression of murine autoimmunity and lymphoid hyperplasia determined by single genes. In: Kyogoku M, Wigzell H, editors. *Horizons in animal models for autoimmune diseases*. Tokyo: Academic Press; 1987. p. 21-33.
- 21 Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J Exp Med* 1985; 161: 1554-68.
- 22 Stall AM, Adams S, Herzenberg LA, Kantor AB. Characteristics and development of the murine B-1b (Ly-1 B sister) cell population. *Ann N Y Acad Sci* 1992; 651: 33-43.
- 23 Bohm I. Increased peripheral blood B-cells expressing the CD5 molecules in association to autoantibodies in patients with lupus erythematosus and evidence to selectively down-modulate

them. *Biomed Pharmacother* 2004; 58: 338-43.

24 Matsumoto T, Yoshimine T, Shimouchi K, Shiota H, Kuwabara N, Fukuda Y, et al. The liver in systemic lupus erythematosus: pathologic analysis of 52 cases and review of Japanese Autopsy Registry Data. *Hum Pathol* 1992; 23: 1151-8.

25 Furukawa F, Tanaka H, Sekita K, Nakamura T, Horiguchi Y, Hamashima Y. Dermatopathological studies on skin lesions of MRL mice. *Arch Dermatol Res* 1984; 276: 186-94.

26 Kao AH, Manzi S, Ramsey-Goldman R. Review of ACR hematologic criteria in systemic lupus erythematosus. *Lupus* 2004; 13: 865-8.

27 Connolly K, Cho YH, Duan R, Fikes J, Gregorio T, LaFleur DW, et al. In vivo inhibition of Fas ligand-mediated killing by TR6, a Fas ligand decoy receptor. *J Pharmacol Exp Ther* 2001; 298: 25-33.

28 Alderson MR, Armitage RJ, Maraskovsky E, Tough TW, Roux E, Schooley K, et al. Fas transduces activation signals in normal human T lymphocytes. *J Exp Med* 1993; 178: 2231-5.

29 Hacker G, Bauer A, Villunger A. Apoptosis in activated T cells: what are the triggers, and what the signal transducers? *Cell Cycle* 2006; 5: 2421-4.

30 Bosque A, Aguilo JI, Alava MA, Paz-Artal E, Naval J, Allende LM, et al. The induction of Bim expression in human T-cell blasts is dependent on nonapoptotic Fas/CD95 signaling. *Blood* 2007; 109: 1627-35.

31 Rao VK, Straus SE. Causes and consequences of the autoimmune lymphoproliferative syndrome. *Hematology* 2006; 11: 15-23.

32 Tamada K, Ni J, Zhu G, Fiscella M, Teng B, van Deursen JM, et al. Cutting edge: selective impairment of CD8+ T cell function in mice lacking the TNF superfamily member LIGHT. *J Immunol* 2002; 168: 4832-5.

33 Wang EC, Thern A, Denzel A, Kitson J, Farrow SN, Owen MJ. DR3 regulates negative selection during thymocyte development. *Mol Cell Biol* 2001; 21: 3451-61.

34 Hsu TL, Wu YY, Chang YC, Yang CY, Lai MZ, Su WB, et al. Attenuation of Th1 response in decoy receptor 3 transgenic mice. *J Immunol* 2005; 175: 5135-45.

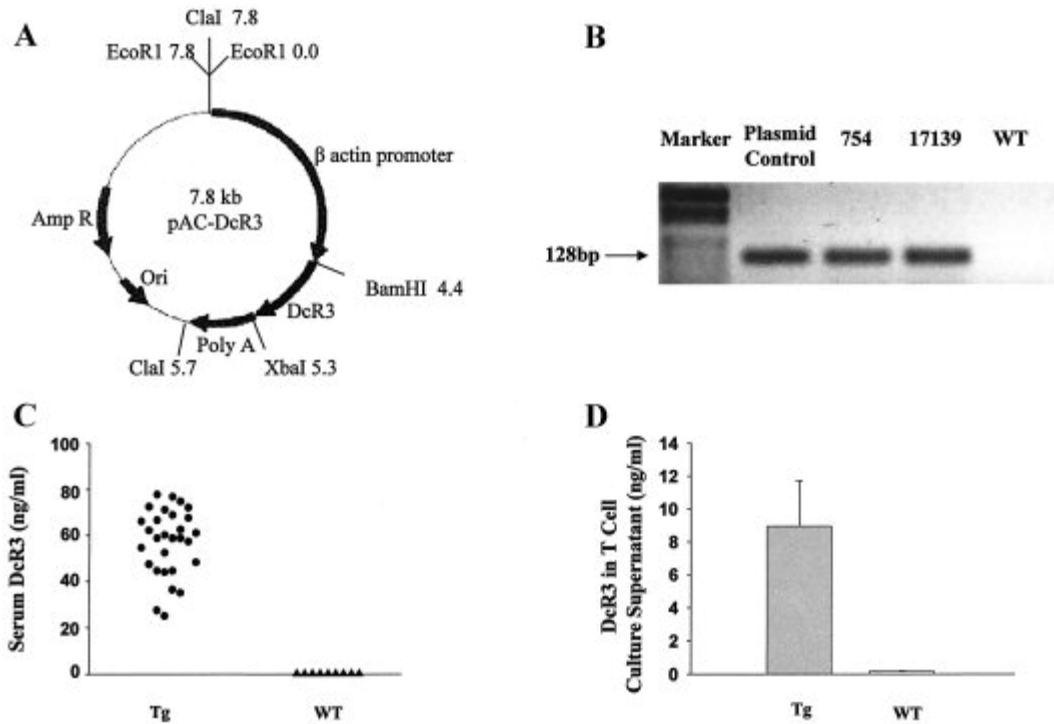
35 Moser KL, Neas BR, Salmon JE, Yu H, Gray-McGuire C, Asundi N, et al. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American

pedigrees. Proc Natl Acad Sci U S A 1998; 95: 14869-74.

36 Gaffney PM, Langefeld CD, Graham RR, Ortmann WA, Williams AH, Rodine PR, et al. Fine-mapping chromosome 20 in 230 systemic lupus erythematosus sib pair and multiplex families: evidence for genetic epistasis with chromosome 16q12. Am J Hum Genet 2006; 78: 747-58.

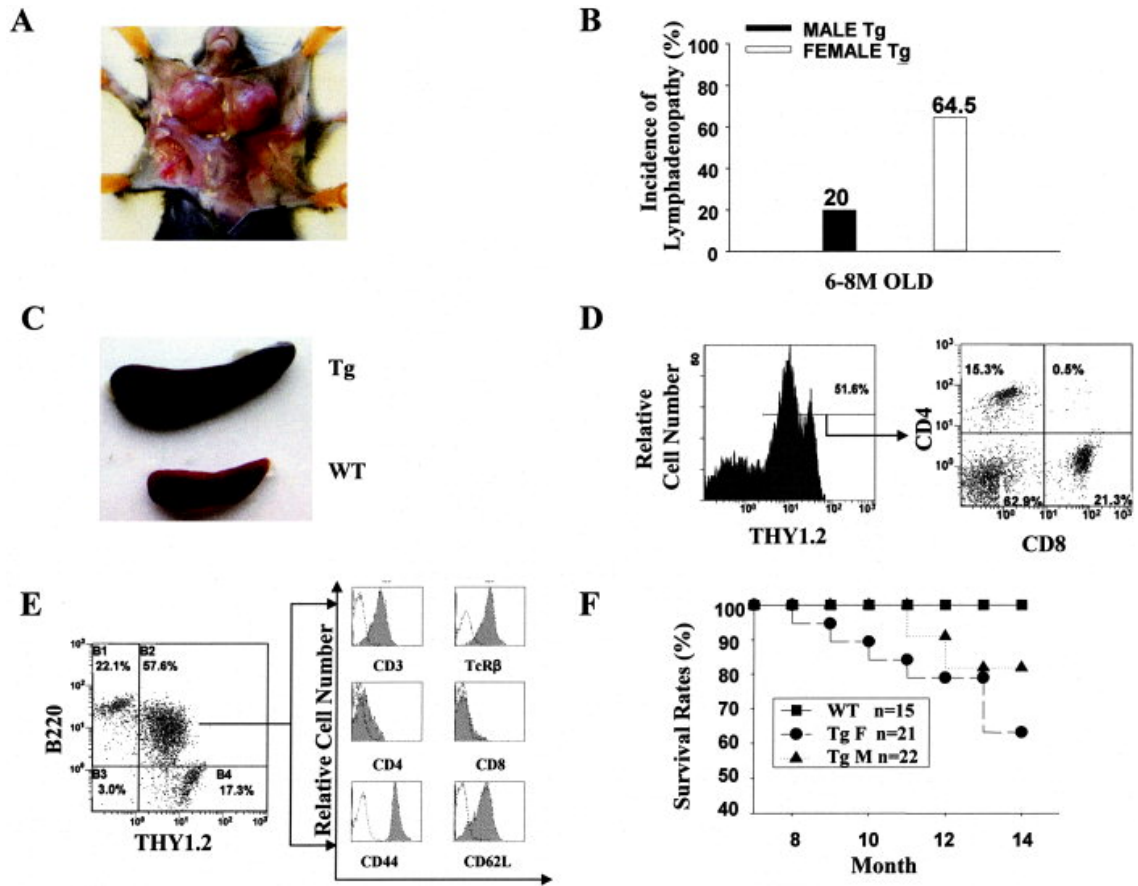
FIGURES AND LEGENDS

Figure 1. Generation and characterization of decoy receptor 3 (DcR3)-transgenic mice.



A, pAC-DcR3 construct for generation of transgenic mice. The 5.7-kb *Cla* I/*Cla* I fragment was used for microinjection. Amp R = ampicillin resistant. **B**, Polymerase chain reaction genotyping of tail DNA from DcR3 founder and wild-type (WT) mice. The 128-bp bands specific to the DcR3 transgene are indicated; pAC-DcR3 was used as a positive control. **C**, Serum DcR3 levels in 2-month-old DcR3-transgenic (Tg) and WT mice, determined by enzyme-linked immunosorbent assay (ELISA). **D**, DcR3 secreted in culture supernatants of spleen T cells from Tg or WT mice, determined by ELISA. Values are the mean and 2 SD from 2 independent experiments.

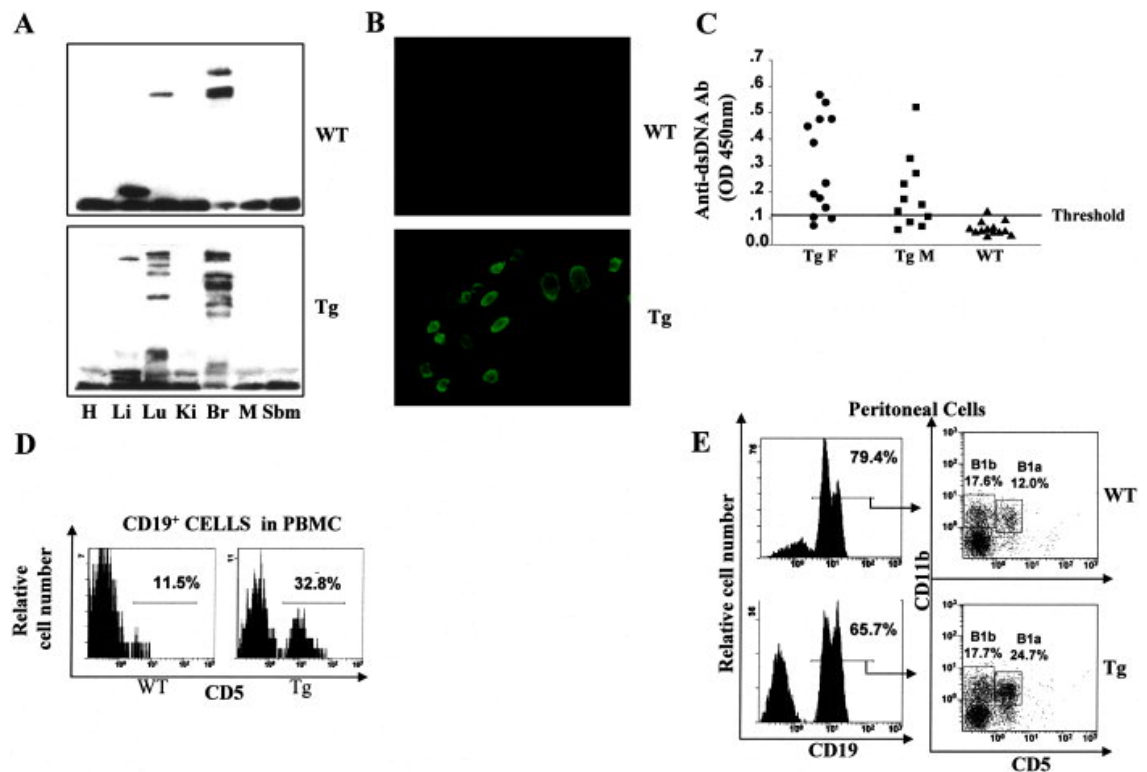
Figure 2. Lymphadenopathy and survival rates in DcR3-Tg mice.



A, Lymphadenopathy in a 7-month-old female Tg mouse. **B**, Incidence of lymphadenopathy in 6-8-month-old male and female Tg mice. **C**, Enlargement of the spleen of a 9-month-old female Tg mouse with lymphadenopathy, compared with that of a WT mouse. **D**, CD4-,CD8- double-negative cells in enlarged lymph nodes of Tg mice. Cells from enlarged lymph nodes were analyzed by 3-color flow cytometry, and Thy1.2-positive T cells were gated and further analyzed by CD4 and CD8 staining. The experiments were performed 3 times; data from a representative experiment are shown. **E**, Further phenotype analysis of cells from enlarged lymph nodes of Tg mice. Cells were stained with B220 and Th1.2, and B220+,Thy1.2+ cells were gated and further analyzed for expression of CD3, T cell receptor β (TCR β), CD4, CD8, CD44, and CD62L. Shaded areas show data on cells gated on B220+,Thy1.2+; white areas show

data on isotype controls. The experiments were performed 3 times; data from a representative experiment are shown. **F**, Survival rates in WT, female Tg, and male Tg mice. See Figure 1 for other definitions.

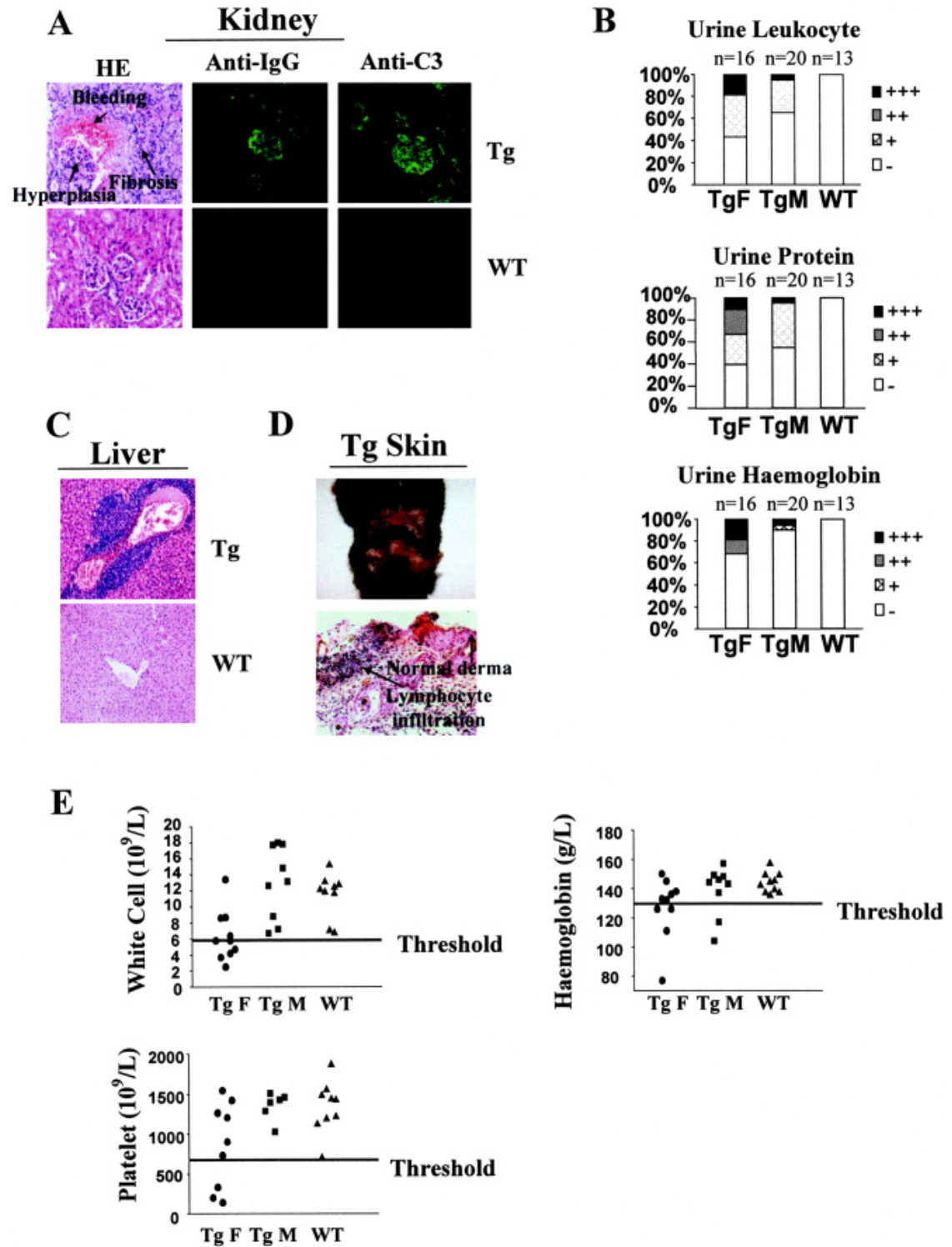
Figure 3. Autoantibodies and B-1 cells in DcR3-Tg and WT mice.



A, Presence of autoantibodies against various organs. Proteins from heart (H), liver (Li), lung (Lu), kidney (Ki), brain (Br), muscle (M), or submaxillary glands (Sbm) were blotted onto membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with sera (1:500 dilution) from a Tg mouse (7-month-old female with lymphadenopathy) and a WT littermate (female). The experiments were performed 3 times, with similar results. **B**, Presence of antinuclear antibody. Sera (1:500 dilution) from a Tg mouse (7-month-old female with lymphadenopathy) and a WT littermate were reacted with permeabilized HeLa cells, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The experiments were performed 3 times, with similar results (original magnification $\times 400$). **C**, Presence of anti-double-stranded DNA antibody (anti-dsDNA Ab). Levels of anti-dsDNA antibody in sera from >6 -month-old female Tg mice, male Tg mice, and WT littermates were determined by ELISA. The mean plus 2 SD in WT mice was used as the threshold for

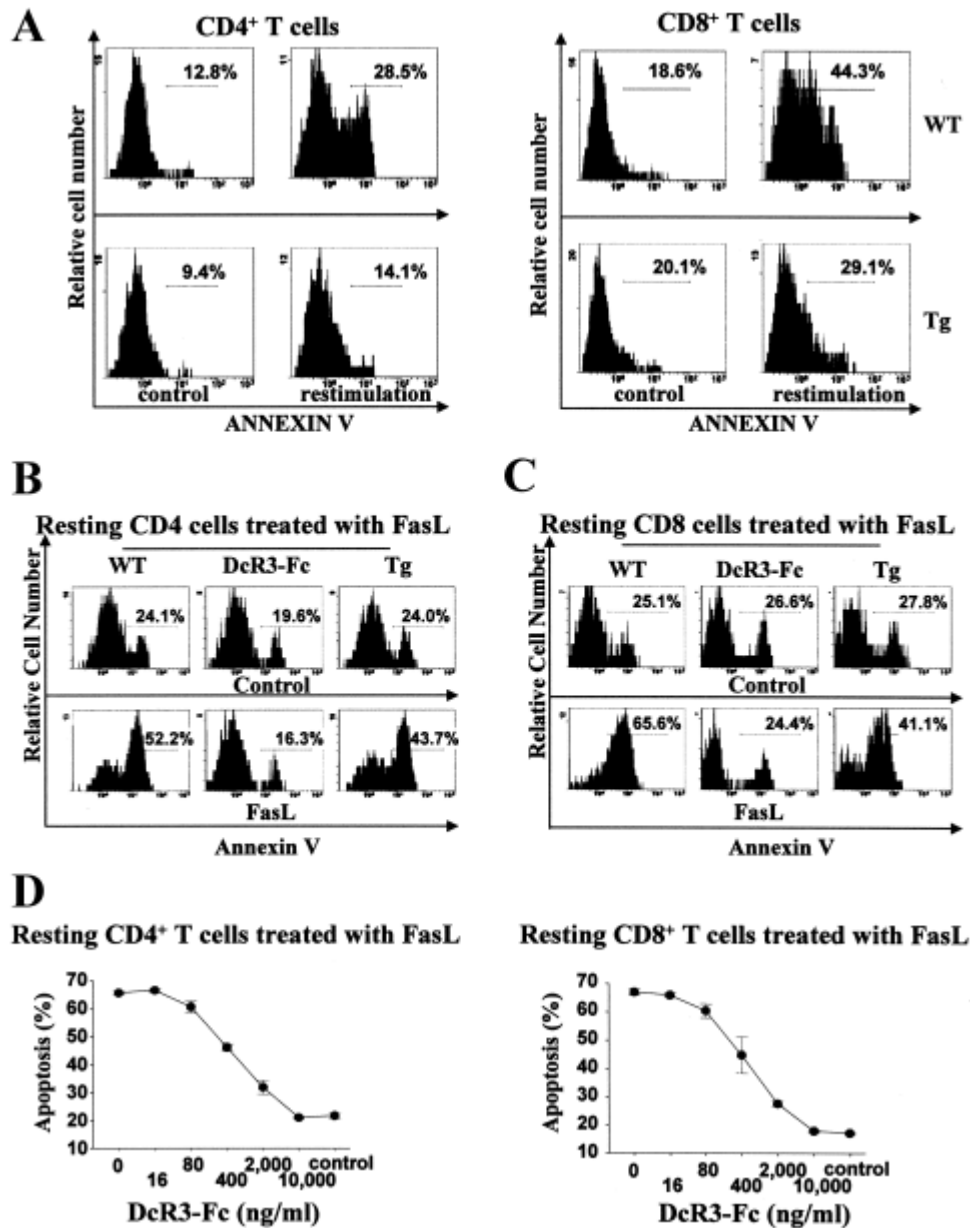
positivity. The frequency of positivity was significantly greater in female Tg mice and male Tg mice versus WT mice ($P = 0.000353$ and $P = 0.00377$, respectively, by chi-square test). OD = optical density. **D**, CD5+ B-1 cell population in peripheral blood mononuclear cells (PBMCs). CD19+ B cells from the peripheral blood of >6-month-old Tg and WT mice were gated and analyzed by 2-color flow cytometry for CD5 expression. A large CD5+ B-1 cell population was found in Tg mice. The experiments were performed >3 times, with similar results. **E**, B-1a cell percentage in peritoneal B cells. Peritoneal cells from Tg and WT mice were gated on CD19 and analyzed by flow cytometry for CD5 and CD11b expression. Percentages of B-1b (CD5-,CD11b+) and B-1a (CD5+,CD11b+) populations among CD19+ cells are indicated; the percentage of B-1a cells was increased in Tg mice. The experiments were performed twice, with similar results.

Figure 4. Tissue histopathology and urine and hematologic findings in aged DcR-Tg and WT mice.



A, Renal histopathology. Kidney sections from 9-month-old female Tg and WT mice were stained with hematoxylin and eosin (HE), fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG, or FITC-conjugated rat anti-mouse C3. **B**, Leukocyte, protein, and hemoglobin levels in urine. Urine from Tg and WT mice was collected for a 24-hour period, and leukocytes, protein, and hemoglobin were measured semiquantitatively with Chemstrip. Severity was graded -, +, ++, or +++ (- = negative; +++ = severe). Findings in female Tg mice were significantly different from those in WT mice for all 3 parameters ($P < 0.05$ by one-way analysis of variance with Kruskal-Wallis test), whereas findings in Tg males and WT mice were not significantly different. **C**, Liver histopathology. Liver sections from a 13-month-old female Tg mouse with lymphadenopathy and a female WT littermate were stained with hematoxylin and eosin. **D**, Skin histopathology. Skin lesions from a 14-month-old female Tg mouse (pure C57BL/6 background) with lymphadenopathy are shown in the upper panel. Hematoxylin and eosin staining of a skin section from this area is depicted in the lower panel. Lymphocyte-infiltrated derma and adjacent normal derma are indicated. **E**, Leukopenia, anemia, and thrombocytopenia. Levels of white blood cells, hemoglobin, and platelets in the peripheral blood of >6-month-old female Tg mice, male Tg mice, and WT littermates were determined. The mean minus 2 SD in WT mice was used as the threshold for abnormality. Findings in female Tg mice were significantly different from those in WT mice for all 3 parameters ($P < 0.05$ by chi-square test), whereas findings in Tg males and WT mice were not significantly different. (Original magnification $\times 200$ in **A**; $\times 100$ in **C** and **D**.) See Figure 1 for other definitions.

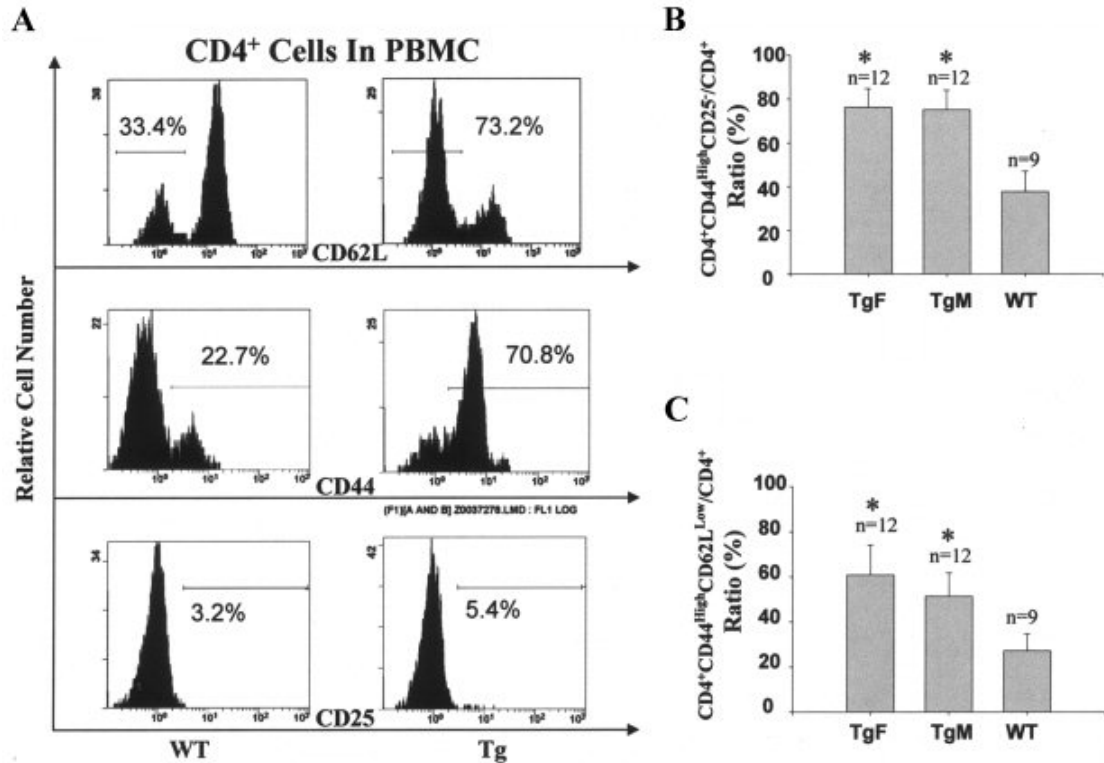
Figure 5. DcR3-induced protection of T cells against apoptosis.



A, Protection against apoptosis in activated CD4 and CD8 cells. WT and Tg mouse spleen cells were first activated for 48 hours with soluble anti-CD3, then washed and recultured for 48 hours in the presence of interleukin-2, washed again, and transferred to wells coated with anti-CD3 for an additional 48-hour culture in the absence or presence of DcR3 at various concentrations. Apoptosis of CD4 and CD8 cells was

analyzed by annexin V staining on CD4- or CD8-gated cells in 3-color flow cytometry, after propidium iodide-positive cells were gated out. The experiments were performed at least twice, and data from a representative experiment are shown. **B** and **C**, DcR3 blocking of FasL-induced apoptosis in resting T cells. WT and Tg mouse spleen cells were cultured for 16 hours in the absence or presence of crosslinked FasL, and apoptosis of CD4 and CD8 cells was analyzed by annexin V staining in 2-color flow cytometry. WT = WT mouse CD4 or CD8 cells without protection; DcR3-Fc = WT mouse CD4 or CD8 cells protected by 10 μ g/ml exogenous recombinant DcR3-Fc; Tg = DcR3-Tg mouse CD4 or CD8 cells protected by endogenous DcR3. The experiments were performed at least twice, with similar results. **D**, Dose-response of DcR3 protection. WT mouse spleen cells were incubated with crosslinked FasL in the presence of DcR3 at various concentrations. Values are the mean \pm SD. The experiments were performed twice, with similar results. See Figure 1 for other definitions.

Figure 6. Expanded CD4^{high},CD62L^{low},CD25⁻ CD4 population in peripheral blood mononuclear cells (PBMCs) of DcR3-Tg mice.



CD4 cells in the peripheral blood of 6-month-old female Tg mice or WT littermates were gated, and expression of CD62L, CD44, and CD25 was analyzed. The experiments were performed >9 times, with similar results. **A**, Histograms from a representative set of experiments. **B** and **C**, Mean and SD percentages of CD44^{high},CD25⁻ cells (**B**) and CD44^{high},CD62L^{low} cells (**C**) among total CD4 cells in the peripheral blood of female Tg, male Tg, and WT mice. * = $P < 0.01$ versus WT mice, by Student's 2-tailed *t*-test. See Figure 1 for other definitions.

Article 2.

DcR3 as a diagnostic parameter and risk factor for systemic lupus erythematosus

Bing Han, Rafael Bojalil, Luis M. Amezcua-Guerra, Rashidi Springall, Héctor Valderrama-Carvajal, Jiangping Wu and Hongyu Luo

Int Immunol. 20(8):1067-75, 2008.

Summary: In this paper, we showed high serum DcR3 levels in SLE patients. We also demonstrated that hemopoietic cell secreted DcR3 was sufficient to induce lupus-like syndromes in mouse. Our results suggest that DcR3 is a risk factor for SLE, and this finding can lead to a new parameter for clinical diagnosis of SLE.

Bing Han performed all assays and experiments, and processed and analyzed data; Rafael Bojalil, Luis M. Amezcua-Guerra, Rashidi Springall and Héctor Valderrama-Carvajal supplied sera and clinic data of SLE patients; Jiangping Wu and Hongyu Luo directed the experiment design and data analysis.

DcR3 as a diagnostic parameter and risk factor for systemic lupus erythematosus

Bing Han¹, Rafael Bojalil^{2,3}, Luis M. Amezcua-Guerra², Rashidi Springall², Héctor Valderrama-Carvajal¹, Jiangping Wu^{1,4} and Hongyu Luo¹

¹ Laboratory of Immunology, Research Centre, Centre hospitalier de l'Université de Montréal, Notre-Dame Hospital, Pavilion DeSève, Montreal, Quebec H2L 4M1, Canada

² Department of Immunology, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico

³ Department of Health Care, Universidad Autónoma Metropolitana, Mexico City, Mexico

⁴ Nephrology Service, Centre hospitalier de l'Université de Montréal, Notre-Dame Hospital, Montreal, Quebec H2L 4M1, Canada

ABSTRACT

In this study, we investigated the diagnostic value of serum death decoy receptor 3 (DcR3) for systemic lupus erythematosus (SLE). The possible pathogenic role of DcR3 in SLE was also assessed. Serum DcR3 levels of 90 SLE patients, 11 patients with rheumatic conditions and 123 healthy controls were determined by ELISA. In all, 43% of the SLE patients, 9% of patients with rheumatic conditions and 2.4% of the normal healthy individuals presented elevated serum DcR3 levels. A higher percentage of DcR3-positive SLE patients, compared with DcR3-negative SLE patients, showed abnormally high serum IgE levels, a surrogate marker of T_h2-type immune responses. To determine the cause and effect relationship of DcR3 expression and a T_h2-prone status, we studied young DcR3 transgenic (Tg) mice, whose transgene was driven by an actin promoter. These mice had IL-4 overproduction and augmented serum IgE levels, signs of dominant T_h2 immune responses. To determine possible SLE pathogenic roles of DcR3, the T-cell-depleted bone marrow of DcR3 Tg mice was transplanted into lethally irradiated syngeneic C57BL/6 female mice. The recipients developed an SLE-like syndrome. They presented anti-dsDNA and anti-nuclear antibodies, along with renal and liver pathology compatible

with that of SLE. In total, 90% of Tg bone marrow-transplanted mice, compared with 20% of wild-type bone marrow-transplanted mice, perished within 12 months after the transplantation. Our results showed that serum DcR3 could serve as an additional parameter for SLE diagnosis and that DcR3 secreted from cells of hematopoietic origin was SLE pathogenic in mice.

Keywords: DcR3, systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease inflicting damage to multiple organs. The disease prevalence is ~0.05% in the general population, with 80–90% of patients being women (1, 2). The exact etiology of SLE has not been elucidated, but it is obvious that genetics, gender, and environment are involved in its pathogenesis. Regarding genetics, SLE is under polygenic control (3). Multiple genomic loci containing SLE risk genes have been identified in humans and mice (4, 5). Certain class II MHC genes are known to contribute to SLE risk (6). Among others, molecules in the apoptosis pathways are implicated in SLE pathogenesis (7). In mice, mutations in the prototype pro-apoptotic molecules Fas or Fas ligand (FasL) lead to the occurrence of an SLE-like syndrome (8, 9), but human SLE patients rarely have mutations in Fas or FasL (10, 11). However, it is possible that molecules in the Fas/FasL pathway are SLE risk factors, but they have yet to be identified as such.

Decoy receptor 3 (DcR3) is a member of the tumor necrosis factor (TNF) receptor family. As it lacks transmembrane and intracellular sequences in its peptide, it is a secreted protein (12). It can bind to three TNF family members, i.e. FasL, LIGHT and TL1A, and interferes with the interaction of these ligands with their respective receptors, i.e. FasL with Fas, LIGHT

with HVEM and LT β R and TL1A with DR3 (12–15). Because these receptors are all capable of inducing cell death (15–17), one of DcR3's biological functions is to act as a death decoy, preventing cell death under certain circumstances (15). In total, ~50–60% of various tumors secrete DcR3 (18), which is probably a strategy evolved by tumors to gain a survival advantage over immune surveillance. Activated T cells also produce DcR3 (19). The biological significance of such DcR3 production could be (i) to modulate activation-induced cell death after clonal expansion and, hence, influence memory T-cell development (20) and (ii) to regulate T-cell migration after their activation as we have found that soluble DcR3 can inhibit T-cell chemotaxis (21).

T cells in SLE patients are abnormally activated. As activated T cells produce DcR3 (19), we examined serum DcR3 in these patients as a possible diagnostic marker. We further investigated a possible role of DcR3 in SLE pathogenesis.

METHODS

Patients and healthy controls

Sera from SLE patients, patients with other autoimmune diseases and healthy controls were collected at the Department of Immunology, Instituto Nacional de Cardiología ‘Ignacio Chávez’, Mexico City. Informed consent was obtained from serum donors, and this study was approved by the local Ethic Committee. All SLE patients (86 females and 4 males; age between 16 and 63, 37.3 ± 12.9) met the American College of Rheumatology classification criteria (22). The systemic lupus erythematosus disease activity index (SLEDAI) of each patient was obtained at the time of serum collection. All the patients were under one to four different medications, which were duly recorded and illustrated in relevant figures. Eleven patients (nine females and two males) between 20 and 59 years of age (38.8 ± 13.5) with rheumatic conditions [three with rheumatoid arthritis, one with idiopathic juvenile arthritis, one with anti-phospholipid syndrome, two with Sjögren syndrome, one with thrombophilia, two with undifferentiated connective tissue disease, one with overlap syndrome (systemic sclerosis plus polymyositis)] were included for comparison. Control sera were from healthy donors (92 females and 31 males; age between 18 and 64, 35.6 ± 10.5) approximately matched for age and gender

of SLE patients. All the patients and healthy controls were Mexican Mestizo.

DcR3 ELISA

DcR3 ELISA has been described elsewhere (18). An mAb specific to DcR3 was used for coating, and a biotinylated affinity-purified rabbit antibody against DcR3 served as detecting antibody. The sensitivity of the assay was 6 pg ml^{-1} . Human samples were tested in duplicate, and mouse samples, in triplicate. The mean + 1.64 SD (95% confidence interval, one-sided test) of the control serum DcR3 levels was used as the threshold (25 pg ml^{-1}), levels equal or above which were considered DcR3 positive.

Clinical serological tests

Serum anti-nuclear antibodies (ANAs) were detected by indirect immunofluorescence on HEp-2 cells slides (NOVA Lite, INOVA Diagnostics, San Diego, CA, USA). Anti-dsDNA antibodies were detected by indirect immunofluorescence on Crithidia luciliae substrate (NOVA Lite, INOVA Diagnostics). Anti-SSA, anti-SSB, anti-RNP and anti-SM antibodies were all detected by ELISA (QUANTA Lite, INOVA Diagnostics). All the assays were performed according to manufacturer's instructions.

Human serum IgE assay

Human serum IgE was quantified using an ELISA kit from Bethyl Laboratories (Montgomery, TX, USA), according to the manufacturer's instructions. The sensitivity of the assay was 16 ng ml^{-1} .

Cell culture

Mouse spleen cells were cultured in 96-well plates at a density of 4×10^5 cells per 200 μl per well and stimulated with phorbol myristate acetate (PMA, 10 nM) and ionomycin ($2 \mu\text{g ml}^{-1}$) for 4 h in the presence of brefeldin A ($10 \mu\text{g ml}^{-1}$) for the detection of intracellular IL-4 and IFN- γ .

Flow cytometry

Two-color flow cytometry was performed to determine the expression of CD4 and intracellular IL-4 and IFN- γ in mouse spleen T cells, as described before (23).

Measurement of Ig isotype levels in mouse sera

Mouse serum Ig isotype levels were quantified with Ig-isotyping ELISA and OptEIA kits from BD Biosciences (Mississauga, Ontario, Canada), according to the manufacturer's instructions.

Whole-body irradiation and bone marrow transplantation

C57BL/6 female mice were whole-body irradiated at 900 Rad. After 16 h,

they received intravenously (i.v.) 10 million T-cell-depleted bone marrow cells from DcR3 or wild-type (WT) mice. T-cell depletion from bone marrow cells was carried out with EasySep Mouse CD90 (Thy1.2) Positive Selection Kit according to the manufacturer's instructions (StemCell Technologies Inc., Vancouver, BC).

Immunofluorescence for the detection of ANA

HeLa cells were fixed with a BD Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA, USA) at 4°C for 60 min and then incubated overnight at 4°C with sera from mice having received WT bone marrow transplantation (BMTx) or transgenic (Tg) BMTx (1:500 dilution in wash buffer from the kit). After washing, the cells were reacted overnight at 4°C with FITC-conjugated goat anti-mouse IgG antibody (1:1000 dilution; Bethyl Laboratories). The cells were examined under fluorescence microscopy. The method was similar to that as described by Vinuesa *et al.* (24).

ELISA for anti-dsDNA

Costar 96-well high-affinity ELISA microplates were coated with 250 µg ml⁻¹ salmon sperm DNA (Invitrogen, Burlington, Ontario, Canada) in coating buffer (0.05 M sodium bicarbonate solution, pH 9.5) overnight at 4°C. After washing with PBS containing 0.05% Tween 20, the plates were incubated with blocking buffer (PBS containing 1% BSA) at room

temperature for 1 h. WT and Tg mouse sera were diluted in blocking buffer at 1:50 and incubated overnight in the wells at 4°C. After washing, the plates were reacted with HRP-conjugated sheep anti-mouse IgG antibody (1:2000 dilution; Amersham Biosciences, Piscataway, NJ, USA) for 1 h at room temperature. Signals were revealed by adding tetramethylbenzidine substrate (BD Biosciences), followed by 20-min incubation at room temperature. The reaction was stopped by 50 µl 4 N HCl, and the plates were read at a wavelength of 450 nm.

RESULTS

Elevated serum DcR3 levels in SLE patients

Sera from 90 SLE patients (86 females and 4 males) and 123 approximately age- and gender-matched healthy individuals (92 females and 31 males) were tested for DcR3. As shown in Fig. 1(A), SLE patients presented elevated serum DcR3, with 39 of them (i.e. 43.3% of total patients; 75% of males and 41.9% of females) being serum DcR3 positive, compared with only 3 out of the 123 healthy controls (2.4%). The difference was highly statistically significant ($P < 0.01$, Student's *t*-test). In these patients, who were all under medication, DcR3 levels had no obvious correlation with SLEDAI, according to linear regression analysis ($r = 0.119$, $P = 0.26$) (Fig. 1B). No significant correlation was found between age (ranging from 16 to 64 years) and serum DcR3 levels ($r = -0.1$, $P = 0.92$; Fig. 1C). Further analysis showed that serum DcR3 positiveness was not correlated to positiveness of ANA, anti-dsDNA antibody, anti-RNP antibody, anti-Sm antibody, anti-Ro/SSA antibody or anti-La/SSB antibody ($P > 0.05$, χ^2 test; Fig. 1D).

For comparison, DcR3 levels in 11 patients with rheumatic conditions were determined. Only 1 out of 12 (9%) of these patients were positive in serum DcR3. The DcR3-positive patient in this group was diagnosed with overlap syndrome (systemic sclerosis plus polymyositis). Although a large sample

size of each disease will be needed to assess the DcR3-positive incidence, our data at least suggest that DcR3-positive incidence in rheumatic conditions is not as high as that in SLE.

All SLE patients were under medication, such as cyclophosphamide (CFM), prednisone (PDN), hydroxychloroquine (HCQ), azathioprine (AZA) and/or statins, at the time of serum collection. Only 13 patients were on a single medication (11 on HCQ and 2 on PDN); all the others were treated with two to four drugs. We attempted to determine whether any particular drug would influence serum DcR3 and whether, in the absence of the said drug, there was a correlation between SLEDAI and DcR3 levels. Patients without a particular medication but on one to three other medications were compared with those taking the said medication along with zero to three other drugs. As shown in Fig. 2(A), regimens containing CFM, PDN and AZA did not seem to affect serum DcR3 levels. On the other hand, patients on regimens without HCQ appeared to have higher DcR3 levels. This was probably due to that patients taking HCQ were those with a lower SLE activity. Indeed, SLEDAI of patients with a regimen containing HCQ tended to be lower than that without HCQ (Fig. 2B), although the difference did not reach a statistically significant level. We also noticed that patients on a regimen containing statins presented higher DcR3 levels (Fig.

2A; $P < 0.004$), and they also had higher SLEDAI (Fig. 2B), albeit without reaching statistical significance. Possible explanations for such an observation are given in the Discussion. Another observation was that patients treated with a regimen containing PDN or AZA had significantly higher SLEDAI ($P = 0.03$ in both cases). This is not unexpected as these two drugs are normally administered to patients with elevated SLEDAI.

Elevated serum IgE levels in SLE patients

SLE is an autoimmune disease with an underlying T_H2 immune response, and physiological conditions such as pregnancy that boosts the T_H2 response tend to aggravate SLE (25). We wondered whether there was any correlation between serum DcR3 levels and the degree of T_H2 dominance. To test this possibility, serum IgE was employed as a surrogate marker of T_H2 response. The 90 SLE patients were divided into DcR3-positive and DcR3-negative groups, with 25 pg ml^{-1} serum DcR3 as the threshold. The results (Fig. 3) showed that DcR3-positive patients had an elevated serum IgE concentrations (with a median IgE concentration of 120 ng ml^{-1}) compared with DcR3-negative patients (with a median IgE concentration of 60 ng ml^{-1}). A higher percentage of DcR3-positive patients presented serum IgE levels above the normal range using 240 ng ml^{-1} (100 IU ml^{-1}) as the upper limit of the range (26), compared with DcR3-negative patients

($P < 0.05$; χ^2 test). These results suggest the existence of a correlation between DcR3 levels and the T_h2 phenotype.

T_h2-prone immune responses in young DcR3 Tg mice

To establish the cause and effect relationship between DcR3 expression and T_h2-prone immune responses, we generated actin promoter-driven human DcR3 Tg mice (20). The mouse does not have an orthologue of human DcR3, but human DcR3 could effectively interact with mouse FasL, LIGHT and TL1A (12, 27). Tg mice younger than 5–6 months of age, thymus, spleen and lymph node weight and cellularity were in the normal range, as were cell sub-populations in these organs (data not shown). Their T-cell proliferation in response to solid-phase anti-CD3 alone or in combination with anti-CD28 was variable and did not have consistent changes in comparison to WT T cells [data not shown; methods detailed in Luo *et al.* (23)]. However, when Tg CD4 cells experienced a short-term ionomycin and PMA stimulation, a higher percentage of them became intracellular IL-4- but not IFN- γ -positive, compared with WT CD4 cells (Fig. 4A). Because such a stimulation regimen triggered previously activated rather than resting T cells to secrete these lymphokines, this result suggests that Tg mice at this age already experienced a T_h2-dominated immune response history.

The serum antibody isotypes of these Tg mice were determined by ELISA. As illustrated in Fig. 4(B), Tg and WT mice had similar levels of IgA, IgM, IgG2A and IgG3, but Tg mice produced higher amounts of IgG1 and IgG2b than WT mice. Tg mice also generated increasingly significantly higher concentrations of IgE, a typical T_h2-dependent isotype, from 2 to 6 months of age (Fig. 4C). The overproduction of IgG1 and IgE indicated that the immune responses in Tg mice were skewed to the T_h2-type response and was consistent with increased intracellular IL-4-positive CD4 cells found in the spleens of young Tg mice. These results suggest that DcR3 over-expression is a cause of T_h2-prone immune responses.

Recipients of DcR3 Tg bone marrow developed an SLE-like syndrome

Tg mice with actin promoter-driven DcR3 expression developed an SLE-like syndrome after 5 months of age (20). They produced auto-antibodies against dsDNA and Smith antigen. The kidneys of these Tg mice showed pathological changes indicative of glomerular nephritis and IgG and C3 deposition; kidney dysfunction, such as proteuria, leukocyturia, and hemuresis, were obvious. Aged Tg mice also developed skin lesions and lymphocyte infiltration in the liver and suffered from leukopenia, anemia and thrombocytopenia. SLE-like syndrome penetrance in DcR3 Tg mice was gender dependent, with ~60% in females versus 20% in males.

These findings have been reported by us recently (20). The results suggest that DcR3 might be SLE pathogenic. In actin promoter-driven DcR3 Tg mice, DcR3 was produced almost universally by various tissues. Because activated T cells secrete DcR3 (19), we wondered whether DcR3 from such a source was sufficient to cause SLE. To explore this possibility, we irradiated C57BL/6 female mice at a lethal dosage (900 Rad) and transplanted i.v. T-cell-depleted bone marrow cells from DcR3 Tg mice. For controls, C57BL/6 female mice underwent whole-body irradiation (WBI), followed by BMTx from WT mice. Sera of Tg BMTx but not WT BMTx recipients contained human DcR3 as shown in Fig. 5A. Four months after transplantation, five of the eight Tg BMTx recipients developed anti-dsDNA antibody, a hallmark of SLE, according to ELISA, while none of the five controls receiving WT BMTx had such antibody (Fig. 5B). However, there was no correlation between the levels of DcR3 and anti-dsDNA antibody (Fig. 5C). Those Tg BMTx recipients also produced ANA (Fig. 5D), another typical laboratory finding in SLE.

Human SLE is associated with glomerulonephritis. Renal pathology was thus assessed in mice 6 months after Tg BMTx, according to hematoxylin–eosin staining (Fig. 5E, top panel). The kidneys showed mild interstitial congestion and edema, with moderate interstitial cell infiltration,

particularly around the blood vessels. Some glomeruli were extended in size, and capillary loops were markedly thickened and obliterated. Epithelial cells of Bowman's capsule proliferated focally. These pathological findings were compatible with glomerulonephritis.

In the liver of mice 6 months after Tg BMTx, periarterial lymphocyte infiltration (Fig. 5E, lower panel) was similar to the liver pathology seen in SLE patients (28).

The survival rate of WBI-BMTx mice is shown in Fig. 6. Twelve months after WBI-BMTx, 80% WT bone marrow recipients were still alive, but only 10% DcR3 Tg bone marrow recipients survived after this period. This finding was similar to that in DcR3 Tg female mice without WBI-BMTx (20).

These findings establish that DcR3 over-expression by cells of hematopoietic origin suffices to induce the SLE-like syndrome.

DISCUSSION

In this study, we demonstrated that SLE patients had elevated serum DcR3 levels, compared with healthy individuals. We further proved that DcR3 over-expressed by cells of hematopoietic origin was sufficient to induce the SLE-like syndrome in mice.

SLE has complex clinical manifestations, and additional laboratory parameters are certainly needed for more accurate diagnosis. As 43.3% of the SLE patients but only 2.4% healthy controls in our cohort were DcR3 positive, serum DcR3 could be employed as a new parameter in addition to the current ones for SLE differential diagnosis. It should be mentioned that ~9% patients with rheumatic conditions (this study), 60% of tumor patients and some liver cirrhosis patients are serum DcR3 positive (18), and these conditions should be taken into consideration if DcR3 is employed for SLE differential diagnosis.

All the patients in our cohort were treated with one to four medications. The fact that elevated serum DcR3 was detected in these patients suggests that serum DcR3 positiveness can be used for SLE diagnosis, even if they are treated with symptom-relieving medications before a final diagnosis of SLE is made.

No correlation between SLEDAI and quantitative DcR3 levels was found in our SLE cohort, but it is quite possible that the medications might suppress DcR3 production and abolish possible correlations. A prospective study on the relationship between DcR3 levels and SLEDAI in patients without medication is underway to address this question. Still, by analyzing the current data, we attempted to extract some information on the relationship between DcR3 levels and SLEDAI. We note in Fig. 2(A) that the 15 patients on a drug regimen containing no HCQ presented higher DcR3 levels. A careful look at these patients revealed that 12 of the 15 were on a regimen containing CFM and/or AZA, an indication of more severe disease activity, while 11 of the 75 patients on a regimen containing HCQ were under single-drug therapy, an indication of milder disease activity only involving skin lesions. Indeed, patients receiving a regimen containing no HCQ showed a higher SLEDAI than that with HCQ, although the difference was not statistically significant (Fig. 2B). This raises the possibility that the reduced DcR3 levels in patients treated with HCQ are not a consequence of drug administration but are due to lower disease severity in this sub-population to start with.

Statins are used to lower blood cholesterol to control cardiovascular complications in SLE (29). Patients treated with a regimen containing

statins had statistically significantly higher DcR3 levels (Fig. 2A); their SLEDAI was also higher than those on a regimen without statins, although the difference was not statistically significant (Fig. 2B). In our SLE cohort, statins were not particularly used for a selected group of patients with a severer or less severe form of disease. Statins, particularly second-generation statins, such as simvastatin and atorvastatin, have unexpected immunoregulatory functions, in addition to its cholesterol-lowering effect (30). An increasing number of cases with statin-induced lupus-like syndrome have been reported (31, 32). Taking such literature and our findings as a whole, an intriguing possibility is that statins increase DcR3 levels and aggravate SLE disease activity. Additional perspective studies including non-SLE patients taking statins will be necessary to verify this possibility.

While this article DcR3 was under review, Lee *et al.* (33) reported that serum DcR3 levels in oriental SLE patients were elevated, compared with healthy controls. The finding corroborated our report using a different ethnic population. In Lee's article, serum DcR3 levels were found to be positively correlated to SLEDAI. However, medication was not described in their patient cohort, and it will be necessary to confirm whether such

correlation only occurs in the absence of anti-inflammatory drugs or immunosuppressants.

Aberrant apoptosis has been cited as a possible cause of SLE. Several genes involved in apoptosis are considered SLE risk factors. Mice with mutations in Fas and FasL, which are in the prototype apoptosis pathway, manifest an SLE-like syndrome. Human SLE patients rarely have Fas or FasL mutations, but DcR3 could be a culprit blocking the Fas apoptosis pathway and induce SLE. Because of DcR3 elevation in SLE patients, its known roles in blocking apoptosis and the putative relationship between disease severity and DcR3 levels in HCQ- and statin-treated patients, we studied DcR3 Tg mice to assess DcR3's role in the pathogenesis of SLE. In our Tg mice, DcR3 expression was driven by an actin promoter. To avoid complications due to universal DcR3 expression from cells other than those of hematopoietic origin, we transplanted T-cell-depleted Tg bone marrow into irradiated syngeneic recipients. These mice developed an SLE-like syndrome in 4 months after BMTx. They had anti-dsDNA and ANA and presented renal and liver pathology compatible with SLE. Thus, we proved that DcR3 secreted from cells of hematopoietic origin was SLE pathogenic in an animal model.

Why do a large percentage of tumor patients (~60%) have DcR3 levels comparable to those of SLE patients (18) in the range of 30–200 pg ml⁻¹, but they rarely develop SLE? It seems that serum DcR3, at least at concentrations present in tumor patients, is not SLE pathogenic. In mice receiving WBI followed by Tg BMTx, the serum DcR3 levels were much higher at a range of 2–80 ng ml⁻¹. The biologically active local DcR3 concentrations in lymphoid organs in both patients and Tg BMTx are probably much higher than their serum levels, and the presence of such high local DcR3 concentrations is a more likely culprit.

As not all SLE patients are DcR3 positive, DcR3 is obviously only pathogenic or facilitates SLE development in a sub-population of SLE patients. The final proof of DcR3 in SLE pathogenesis will need to come from human genetic studies examining single-nucleotide polymorphisms in the DcR3 gene or genes regulating its expression in DcR3-positive SLE patients versus DcR3-negative healthy controls.

It is interesting that, in *lpr/lpr* and *gld/gld* mice (with Fas and FasL mutations, respectively) and in our actin promoter-driven DcR3 Tg mice, a large percentage (>65% of females and 20% of males) develops lymphadenopathy along with their SLE-like syndrome (20). On the other hand, none of the WBI–BMTx female mice manifested lymphadenopathy,

in spite of their SLE-like syndrome. Similar to that in our WBI-BMTx mice, SLE patients rarely develop lymphadenopathy. It appears that our WBI-BMTx model more closely resembles human SLE, compared with *lpr/lpr*, *gld/gld* and DcR3 Tg mice. All the latter three SLE mouse models, in contrast to the WBI-BMTx mice, have one thing in common, i.e. compromised Fas-mediated apoptosis in non-hematopoietic cells. This raises an intriguing possibility that the development of lymphadenopathy needs interaction between T cells and Fas pathway-defective cells of non-hematopoietic origin.

Our study has demonstrated that serum DcR3 can be considered as an additional SLE diagnostic parameter. Further genetic study in humans is needed to confirm its role in SLE pathogenesis as mice have no genes orthologous to human DcR3.

Funded by:

The Canadian Institutes of Health Research (CIHR) (MOP79565 to H.L., MOP57697, MOP69089 and PPP85159 to J.W.); the Kidney Foundation of Canada to J.W.; the Heart and Stroke Foundation of Quebec to J.W.; the Juvenile Diabetes Research Foundation USA (1-2005-197) to J.W.; the Quebec Ministry of Economic Development, Innovation and Exportation (PSR-SIIRI-069) to J.W.; the J.-Louis Levesque Foundation to J.W.; the CIHR for New Emerging Teams in Transplantation and Fonds de la recherche en santé du Québec for Transfusional and Hemovigilance Medical Research to J.W.

ACKNOWLEDGEMENTS

The authors thank Mr Ovid Da Silva and Robert Boileau, Research Support Office, Research Centre, Centre hospitalier de l'Université de Montréal, for editorial assistance and statistical analysis, respectively. They also thank Dasheng Xu for the pathological interpretation of kidney and liver sections and Real Besner for coordinating mouse irradiation.

REFERENCES

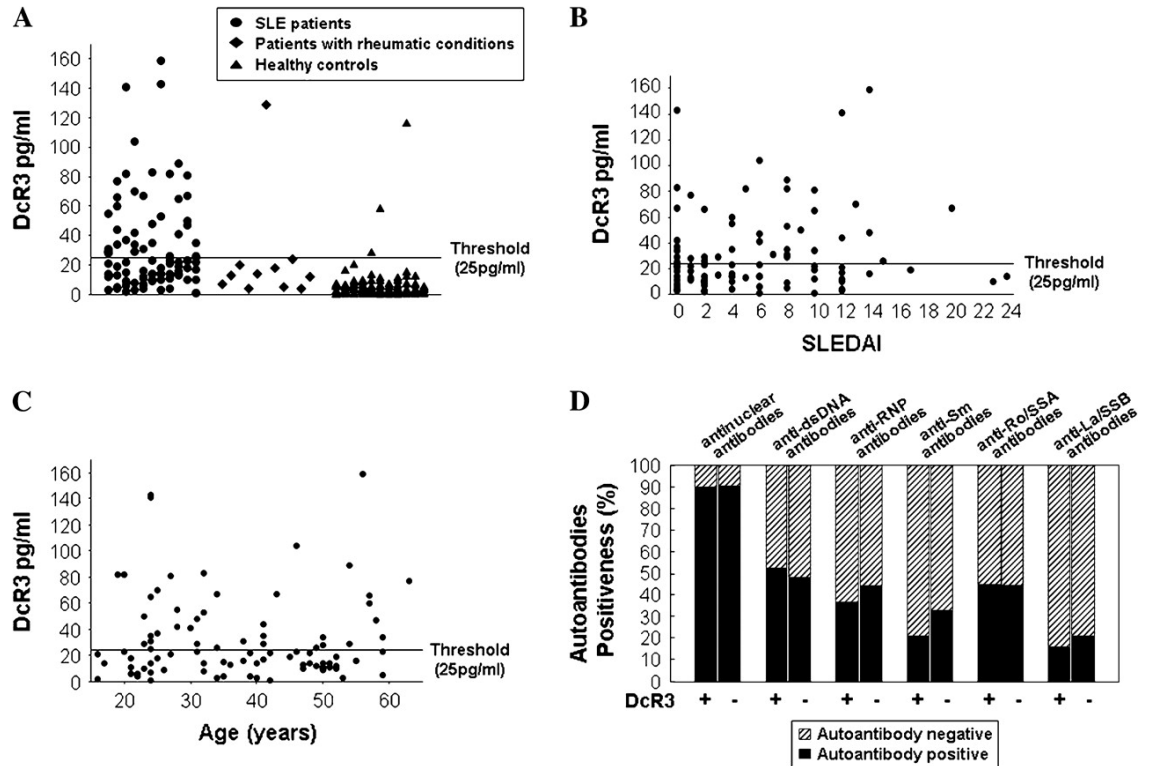
1. Lawrence RC, Helmick CG, Arnett FC, et al. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum.* (1998) 41:778.
2. Costenbader KH, Feskanich D, Stampfer MJ, Karlson EW. Reproductive and menopausal factors and risk of systemic lupus erythematosus in women. *Arthritis Rheum.* (2007) 56:1251.
3. Hochberg MC. Systemic lupus erythematosus. *Rheum. Dis. Clin. North Am.* (1990) 16:617.
4. Encinas JA, Kuchroo VK. Mapping and identification of autoimmunity genes. *Curr. Opin. Immunol.* (2000) 12:691.
5. Vyse TJ, Kotzin BL. Genetic susceptibility to systemic lupus erythematosus. *Annu. Rev. Immunol.* (1998) 16:261.
6. Shen N, Tsao BP. Current advances in the human lupus genetics. *Curr. Rheumatol. Rep.* (2004) 6:391.
7. Salmon M, Gordon C. The role of apoptosis in systemic lupus erythematosus. *Rheumatology (Oxford)* (1999) 38:1177.
8. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* (1992) 356:314.
9. Weintraub JP, Godfrey V, Wolthusen PA, Cheek RL, Eisenberg RA, Cohen PL. Immunological and pathological consequences of mutations in both Fas and Fas ligand. *Cell. Immunol.* (1998) 186:8.
10. Mysler E, Bini P, Drappa J, et al. The apoptosis-1/Fas protein in human systemic lupus erythematosus. *J. Clin. Invest.* (1994) 93:1029.
11. McNally J, Yoo DH, Drappa J, et al. Fas ligand expression and function in systemic lupus erythematosus. *J. Immunol.* (1997) 159:4628.
12. Zhang J, Salcedo TW, Wan X, et al. Modulation of T-cell responses to alloantigens by TR6/DcR3. *J. Clin. Invest.* (2001) 107:1459.
13. Pitti RM, Marsters SA, Lawrence DA, et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* (1998) 396:699.
14. Bai C, Connolly B, Metzker ML, et al. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc. Natl Acad. Sci. USA.* (2000) 97:1230.

15. Migone TS, Zhang J, Luo X, et al. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* (2002) 16:479.
16. Zhai Y, Guo R, Hsu TL, et al. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses *in vivo* tumor formation via gene transfer. *J. Clin. Invest.* (1998) 102:1142.
17. Rooney IA, Butrovich KD, Glass AA, et al. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J. Biol. Chem.* (2000) 275:14307.
18. Wu Y, Han B, Sheng H, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int. J. Cancer.* (2003) 105:724.
19. Wan X, Shi G, Semenuk M, Zhang J, Wu J. DcR3/TR6 modulates immune cell interactions. *J. Cell. Biochem.* (2003) 89:603.
20. Han B, Moore PA, Wu J, Luo H. Overexpression of human decoy receptor 3 in mice results in a systemic lupus erythematosus-like syndrome. *Arthritis Rheum.* (2007) 56:3748.
21. Shi G, Wu Y, Zhang J, Wu J. Death decoy receptor TR6/DcR3 inhibits T cell chemotaxis *in vitro* and *in vivo*. *J. Immunol.* (2003) 171:3407.
22. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* (1982) 25:1271.
23. Luo H, Yu G, Tremblay J, Wu J. EphB6-null mutation results in compromised T cell function. *J. Clin. Invest.* (2004) 114:1762.
24. Vinuesa CG, Cook MC, Angelucci C, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452.
25. Doria A, Iaccarino L, Arienti S, et al. Th2 immune deviation induced by pregnancy: the two faces of autoimmune rheumatic diseases. *Reprod. Toxicol.* (2006) 22:234.
26. Bueno EC, Vaz AJ, Machado L, Livramento JA. Total IgE detection in paired cerebrospinal fluid and serum samples from patients with neurocysticercosis. *Rev. Inst. Med. Trop. Sao Paulo.* (2000) 42:67.
27. Wu Y, Han B, Luo H, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* (2003) 52:2279.
28. Matsumoto T, Yoshimine T, Shimouchi K, et al. The liver in systemic lupus erythematosus: pathologic analysis of 52 cases and review of Japanese Autopsy Registry Data. *Hum. Pathol.* (1992) 23:1151.
29. Ferreira GA, Navarro TP, Telles RW, Andrade LE, Sato EI. Atorvastatin therapy improves endothelial-dependent vasodilation in patients with systemic lupus erythematosus: an 8 weeks controlled trial. *Rheumatology (Oxford)* (2007) 46:1560.
30. Ghayour-Mobarhan M, Lamb DJ, Vaidya N, Livingstone C, Wang T, Ferns GA. Heat shock protein antibody titers are reduced by statin therapy in dyslipidemic subjects: a pilot study. *Angiology* (2005) 56:61.

31. Hanson J, Bossingham D. Lupus-like syndrome associated with simvastatin. *Lancet* (1998) 352:1070.
32. Srivastava M, Rencic A, Diglio G, et al. Drug-induced, Ro/SSA-positive cutaneous lupus erythematosus. *Arch. Dermatol.* (2003) 139:45.
33. Lee CS, Hu CY, Tsai HF, et al. Elevated serum decoy receptor 3 with enhanced T cell activation in systemic lupus erythematosus. *Clin. Exp. Immunol.* (2008) 151:383.

FIGURES AND LENGENDS

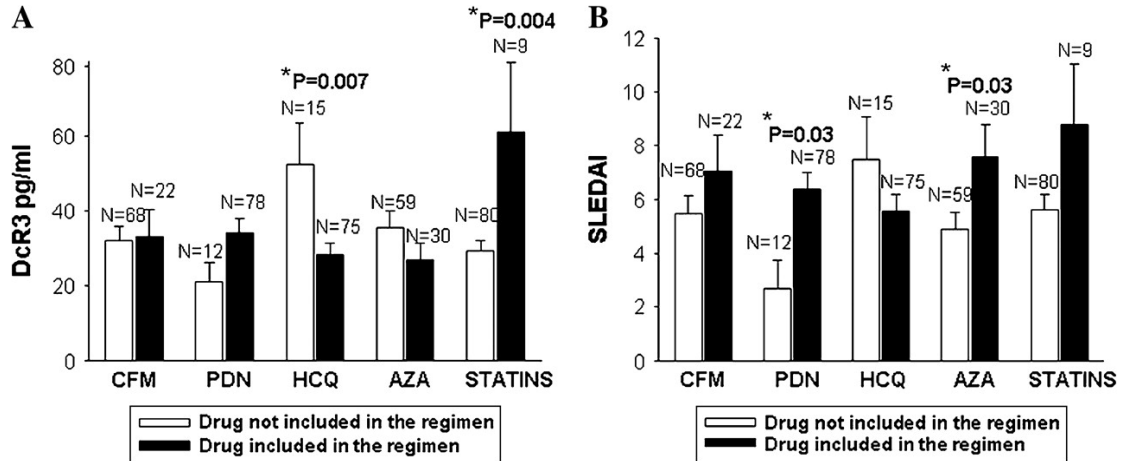
Figure 1. Serum DcR3 levels and their relationship with SLEDAI in SLE patients.



Serum DcR3 was assayed by ELISA in duplicate. DcR3 levels of 90 SLE patients (86 females and 4 males), 11 patients with rheumatic conditions (two males and nine females) and 123 healthy controls (92 females and 31 males) were measured. The threshold of DcR3 positiveness (25 pg ml^{-1}) was the mean + 1.64 SD of control serum levels. (A) SLE patients had elevated serum DcR3 levels. The serum DcR3 levels of SLE patients, patients with rheumatic conditions and healthy controls were 32 ± 32 , 22.7 ± 35.9 and $5 \pm 12 \text{ pg ml}^{-1}$, respectively (means \pm SD). The difference between SLE patients and healthy controls and patients with rheumatic conditions and healthy controls were highly significant ($P < 0.01$ for both comparisons; Student's *t*-test), although the difference between SLE and rheumatic patients were not ($P > 0.05$,

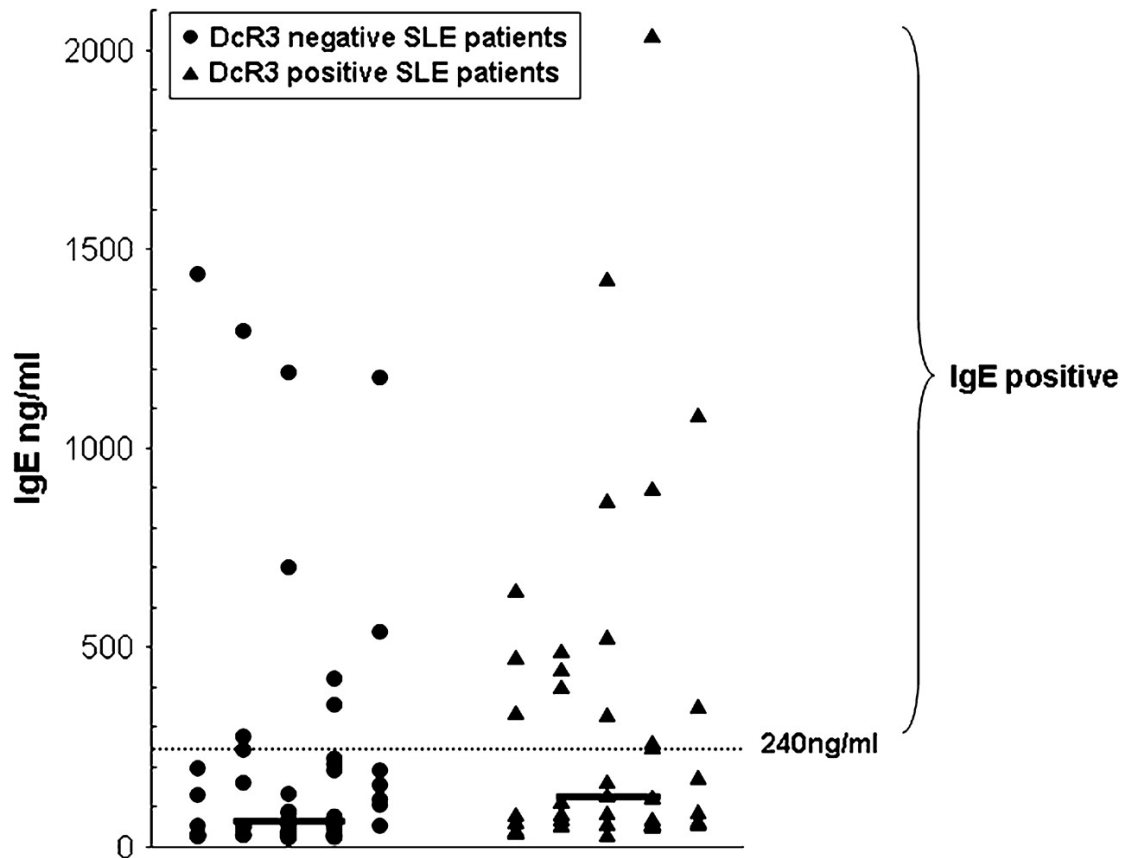
Student's *t*-test). (B) SLE patient serum DcR3 levels plotted against SLEDAI. Serum DcR3 levels of 90 SLE patients are plotted against their SLEDAI (ranging from 0 to 24). No correlation is found between these two parameters ($r = 0.119$, $P = 0.26$; Pearson correlation test). (C) SLE patient serum DcR3 levels plotted against their age. Serum DcR3 levels of 90 SLE patients are plotted against their age (16–64 years). No correlation is found between these two parameters ($r = -0.100$, $P = 0.92$; Pearson correlation test). (D) No correlation between positiveness of DcR3 and auto-antibodies in SLE patients. Serum DcR3 of SLE patients was determined as positive or negative using 25 pg ml^{-1} as a threshold (36 patients were DcR3 positive and 52 patients were DcR3 negative) and analyzed against positiveness of their serum ANA, anti-dsDNA, anti-RNP antibody, anti-Sm antibody, anti-Ro/SSA antibody and anti-La/SSB antibody, using χ^2 test. *P*-values were >0.05 in all the comparisons. The serum DcR3 positiveness was marked as '+' or '-' under each comparison. Solid black and slashes indicate serum positiveness and negativeness of a certain auto-antibody, respectively.

Figure 2. Relationship among medication, serum DcR3 levels and SLEDAI of SLE patients.



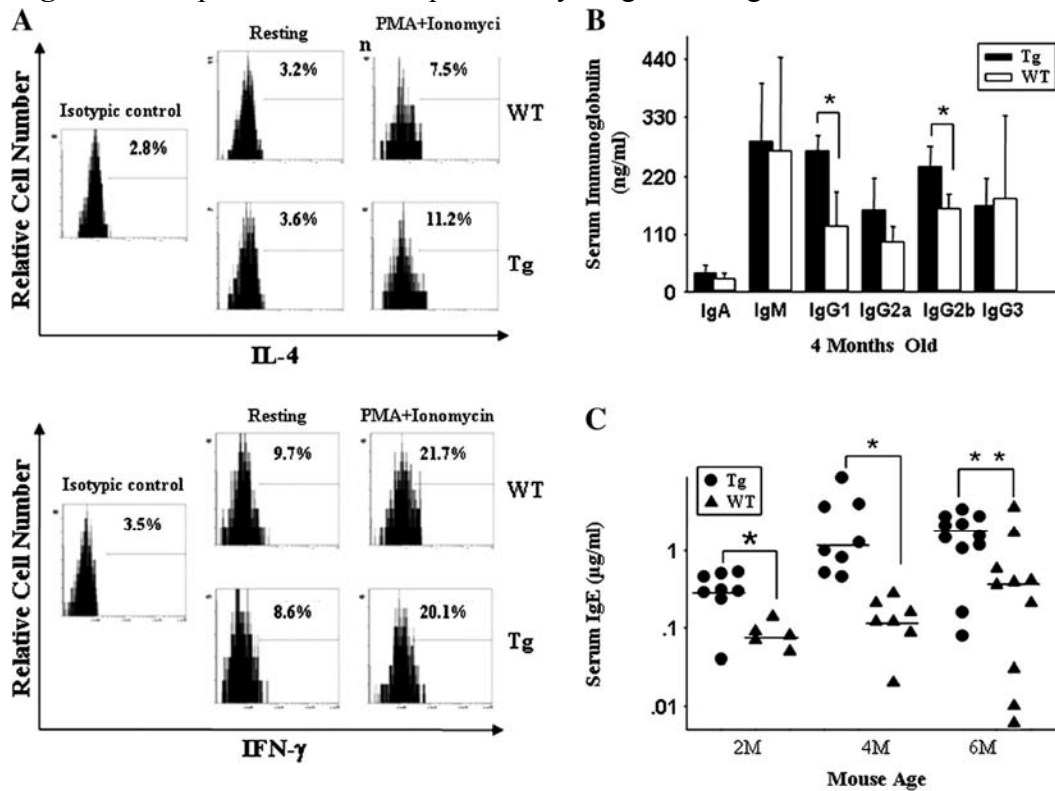
All 90 SLE patients were under medication, and 77 of them were under two to four drug treatments. Serum DcR3 levels of patients without a particular drug but with one to four other medications were compared with those taking that particular drug along with zero to three other medications. Patient number in each group and *P*-value (Student's *t*-test) are shown. (A) Serum DcR3 levels of SLE patients treated with a regimen containing or not containing a certain drug. SLE patients treated with a drug regimen containing HCQ or statins had higher serum DcR3 levels ($P = 0.007$ and $P = 0.004$, respectively; Student's *t*-test). (B) SLEDAI of SLE patients treated with a regimen containing or not containing a certain drug. SLE patients treated with a drug regimen containing PDN or statins had higher SLEDAI ($P = 0.03$ in both cases; Student's *t*-test).

Figure 3. Elevated serum IgE levels in SLE patients.



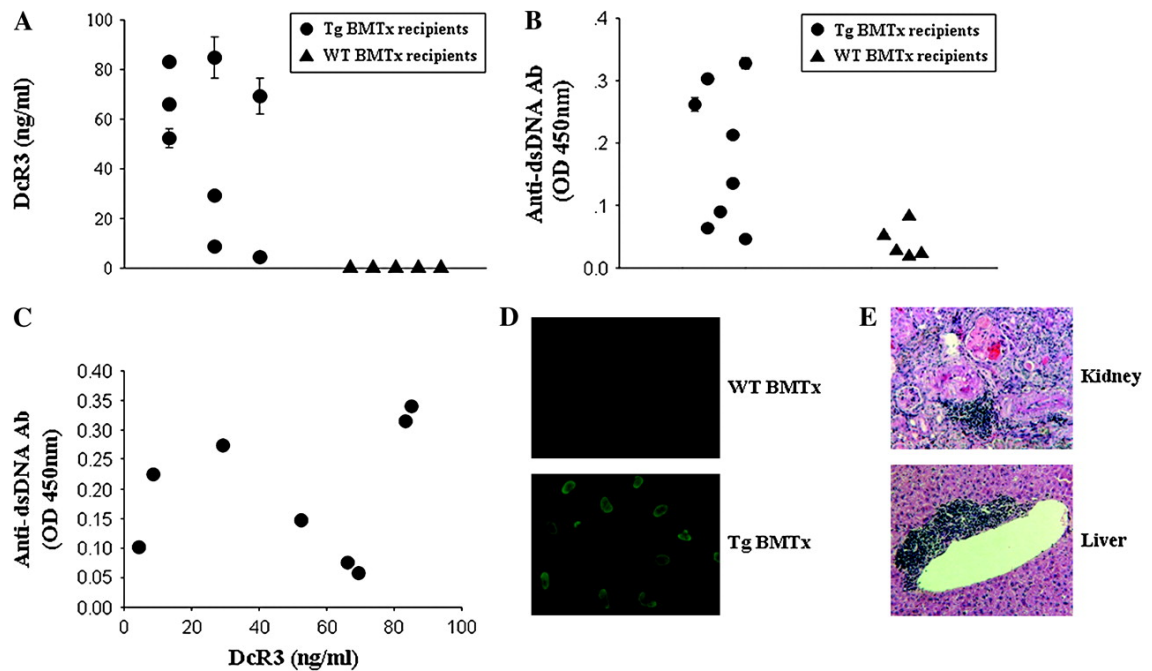
The serum IgE of 90 SLE patients were determined by ELISA, for which sera were tested in duplicate. The median IgE level in DcR3-negative patients was 60 ng ml^{-1} (short horizontal bar) and that in DcR3-positive patients was 120 ng ml^{-1} (short horizontal bar). Using 240 ng ml^{-1} serum IgE concentration {the upper limit of normal serum IgE levels of healthy individuals [Bueno et al. (26)]} as a threshold, a significantly higher percentage of DcR3-positive SLE patients presented elevated serum IgE levels (41.0%), compared with that of DcR3-negative SLE patients (19.6%; $P < 0.05$, χ^2 test).

Figure 4. T_h2-prone immune responses in young DcR3 Tg mice.



The experiments were conducted in 2- to 4-month-old DcR3 Tg and WT littermates. (A) Increased intracellular IL-4 but not IFN- γ in Tg CD4 cells. Spleen cells were either not stimulated (resting) or stimulated with PMA (10 nM) and ionomycin (2 $\mu\text{g ml}^{-1}$) for 4 h. Intracellular IL-4 and IFN- γ were analyzed by two-color flow cytometry (CD4/IL-4 or CD4/IFN- γ). The experiments were repeated two times and data from a representative experiment are shown. (B) Serum IgM, IgA and IgG levels in 4-month-old Tg and WT littermates. Sera from DcR3 Tg and WT littermates ($n = 5$ pairs) were assayed by ELISA. Asterisks over IgG1 and IgG2b indicate statistically significant differences ($P < 0.05$, two-tailed Student's t -test). (C) Increased serum IgE levels in Tg mice. Serum IgE levels of DcR3 Tg and WT littermates at ages of 2, 4 and 6 months were assayed with ELISA. Median levels are indicated by horizontal bars. Each dot or triangle represents an individual mouse. Tg mice at all age groups present higher IgE levels than their littermates ($P < 0.05$, two-tailed Student's t -test).

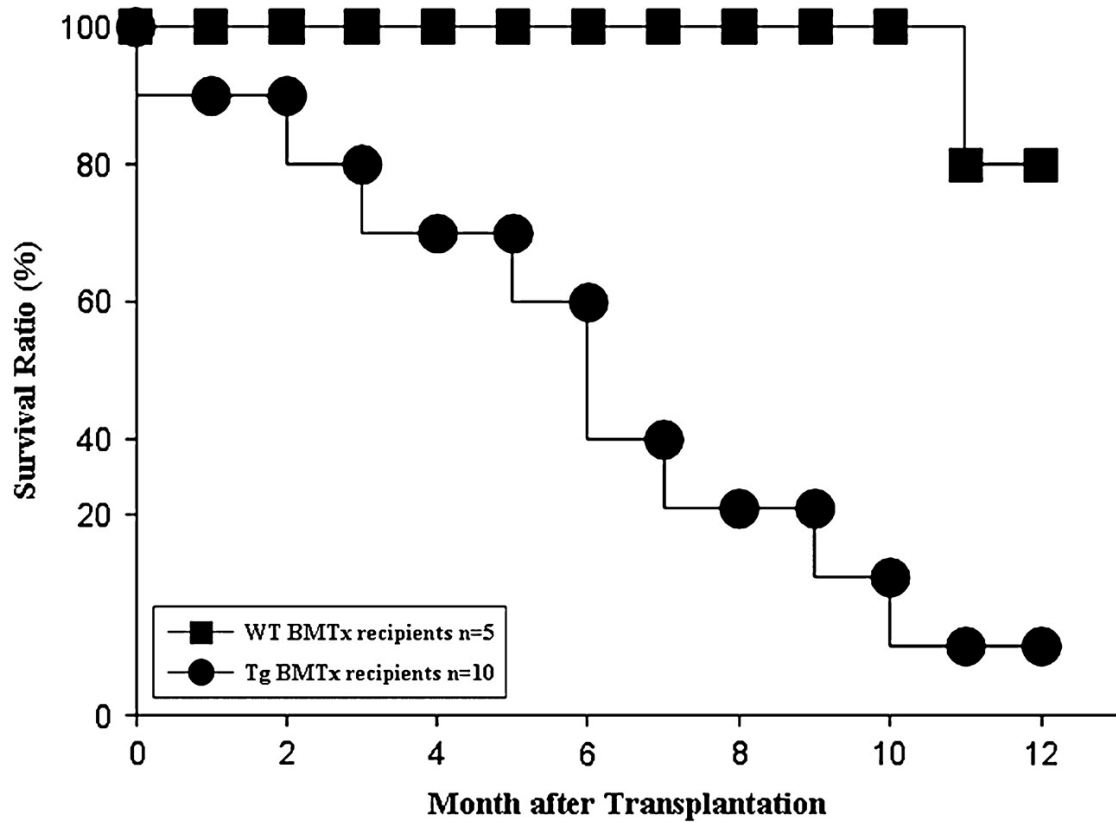
Figure 5. Development of an SLE-like syndrome in WBI–BMTx mice.



WBI female C57BL/6 mice received T-cell-depleted BMTx from DcR3 Tg or WT mice. Four months after BMTx, their serum DcR3, anti-dsDNA antibody and ANA were assessed. Tg BMTx, recipients of Tg BMTx; WT BMTx, recipients of WT BMTx. (A) Serum DcR3 levels of WBI–BMTx mice. Serum DcR3 levels of Tg BMTx or WT BMTx recipients 6 months after BMTx are shown. The latter had no detectable DcR3. (B) Anti-dsDNA antibody in Tg BMTx recipient sera according to ELISA. Sera from Tg BMTx or WT BMTx recipients were measured for anti-dsDNA 6 months after WBI–BMTx. The positive threshold was determined according to the mean + 2 SD of serum DcR3 levels of WT BMTx recipients. The DcR3 level difference between Tg BMTx and WT BMTx recipients is highly significant ($P < 0.01$, Student's *t*-test). (C) No correlation between serum DcR3 and anti-dsDNA levels in Tg BMTx recipients. For Tg BMTx recipients, their serum DcR3 levels were plotted against anti-dsDNA levels. No obvious correlation was observed. (D) ANA in Tg BMTx recipient sera

according to immunofluorescence. Sera from Tg and WT BMTx recipients 6 months after WBI-BMTx were reacted against permeabilized HeLa cells, and the cells were examined by immunofluorescence microscopy. (E) Kidney and liver pathology of Tg BMTx recipients. Hematoxylin–eosin staining of kidney and liver sections from 6-month-old Tg BMTx recipients. Original magnification: x100.

Figure 6. Reduced survival rate in Tg BMTx recipients.



WBI female C57BL/6 mice received Tg or WT BMTx. The survival rate of these recipients within 12 months after BMTx is plotted. The difference between these two groups is highly significant ($P < 0.01$; Student's t -test).

Article 3.

DcR3 protects islet β -cells from apoptosis through modulating *Adcyap1* and *Bank1* expression

Bing Han and Jiangping Wu

Under revision after submission to J. Immunol.

Summary: In this paper, we investigated the mechanism of DcR3 in protecting islets. We found that: 1) the TL1A/DR3 pathway and the LIGHT/HVEM-LT β R pathway mediated islet apoptosis; 2) DcR3 transgenic islets were resistant to apoptosis; 3) two novel downstream mediators of DcR3's effect, *Adcyap1* and *Bank1*, were discovered.

Bing Han performed all assays and experiments, and processed and analyzed data; Jiangping Wu directed the experiment design and data analysis.

DcR3 protects islet β -cells from apoptosis through modulating *Adcyap1* and *Bank1* expression¹

*Bing Han and *⁺Jiangping Wu

From *the Laboratory of Immunology and ⁺the Nephrology Service, Centre hospitalier de l'Université de Montréal (CHUM), Notre-Dame Hospital, Montreal, Quebec, Canada

Running title: Running title: DcR3 protects islets via *Adcyap1* and *Bank1*

Key words: DcR3, islet apoptosis, TL1A, LIGHT, *Adcyap1*, *Bank1*

Footnotes

¹This work was supported by grants from the Canadian Institutes of Health Research (MOP57697, MOP69089, and PPP86159), the Kidney Foundation of Canada, the Heart and Stroke Foundation of Quebec, the Juvenile Diabetes Research Foundation USA (1-2005-197), and the J.-Louis Levesque Foundation to J.W. It was also supported by group grants from Fonds de la recherche en santé du Québec (FRSQ) for Transfusional and Hemovigilance Medical Research, and Genome Canada/Quebec.

ABSTRACT

The islet primary non-function (PNF) is a serious problem in islet transplantation. In this study, we investigated whether DcR3-secreting transgenic (Tg) islets could reduce PNF. We generated transgenic mice expressing human DcR3. The transgenically expressed DcR3 protected islets from IFN- γ plus IL-1 β , or TNF- α plus IL-1 β -induced dysfunction and apoptosis in vitro. The Tg islets presented significantly reduced PNF after transplantation. Mechanistically, in addition to the known FasL apoptotic pathway, components of two other apoptosis pathways, i.e., HVEM/LT β R for the LIGHT pathway, and DR3 for the TL1A pathway, were found to be expressed in islets. Recombinant LIGHT and TL1A induced islet apoptosis in the absence of the FasL/Fas pathway, and DcR3 could block such induction. These results for the first time demonstrated that, LIGHT and TL1A were capable of inducing islet apoptosis in addition to FasL, while DcR3 protected the islets by blocking all the three apoptosis pathways. By DNA microarray analysis, we discovered that Adcyap was upregulated more than 700 folds and Bank1 was downregulated 50 folds in the cytokine-assaulted Tg islets, compared to WT islets. Forced overexpression of Adcyap1 by plasmid transfection or knockdown of Bank1 expression by

siRNA in insulinoma NIT-1 cells protected them from cytokine-triggered apoptosis, indicating that indeed DcR3 protects β -cells via the action of these two downstream molecules. This study has revealed novel mechanisms by which DcR3 protects islet survival, and identified new therapeutic targets of diabetes.

INTRODUCTION

Islet transplantation is an effective treatment for diabetes. Islet primary non-function (PNF) is a condition defined as the loss of islet function after transplantation for reasons other than graft rejection. It is one of the major obstacles limiting islet transplantation efficacy. PNF frequency can be very high in islet transplant models (1). In humans, pancreas transplantation produces better results than islet transplantation in reversing diabetic status (2), suggesting the occurrence of serious PNF in islet transplantation. With the best islet transplantation protocol, the Edmonton Protocol (3), 8×10^5 islets on average (from two or more donors) are needed to achieve insulin independence (3, 4), and it is known that 3×10^5 islets in the pancreas are sufficient for insulin independence (5). This suggests that PNF is still a serious problem.

Isolated islets experience dramatic stress from enzyme digestion, mechanical shear, and deprivation of obligatory trophic support (6,7,8). When these islets are transplanted into diabetic patients, they immediately face unfavorable conditions, such as inflammatory cytokines either released by local inflammatory responses or due to underlying diabetic conditions. These in vitro and in vivo factors result in islet dysfunction and apoptosis,

and consequently PNF. PNF is thus likely one of the major culprits in low islet transplantation efficiency. If PNF can be overcome, the efficacy of islet transplantation can be significantly increased. This would be equivalent to augmenting the donor supply.

Human DcR3 belongs to the TNFR family (9), but lacks transmembrane and cytoplasmic domains in its sequence. It is thus a secreted protein (10). DcR3 can bind to TNF family members FasL (9), LIGHT (10,11) and TL1A (12), and block their interaction with their respective receptors, i.e., Fas, HVEM and DR3 (10,11,12). The mouse does not have an orthologous counterpart of human DcR3 gene according to a genome-wide computer search. We and others have established that human DcR3 can bind to mouse FasL, LIGHT and TL1A (9,10,12,13,14), allowing human DcR3 to function in mouse models both in vitro and in vivo.

The role of DcR3 in apoptosis is obvious. FasL is a well-known molecule involved in apoptosis. LIGHT is a ligand for HVEM and LT β R, in addition to being a ligand for DcR3 (13). LIGHT can induce apoptosis in cells expressing both HVEM and LT β R15, or LT β R alone (14,15). TL1A, a member of the TNF family, can evoke apoptosis via its receptor DR3 (12). Consequently, the interaction of DcR3 with FasL, LIGHT, and TL1A blocks

apoptosis mediated by Fas, HVEM, LT β R and DR3.

In this study, we generated transgenic (Tg) mice with actin promoter-driven human DcR3 expression. The minute amount of DcR3 secreted by these Tg islets rendered protection against PNF *in vivo*. We also discovered that LIGHT and TL1A mediated cytokine-triggered islet apoptosis. Through DNA microarray, we identified *Adcyap1* and *Bank1* as two critical downstream molecules mediating DcR3's protective effect on islets. The essential roles of *Adcyap1* and *Bank1* in the case was confirmed by the fact that modulating their expression could indeed reduce β -cell apoptosis.

MATERIALS AND METHODS

DcR3 ELISA

DcR3 ELISA has been described elsewhere (16). A mAb and a biotinylated affinity-purified rabbit Ab specific to DcR3 were used for coating and detection, respectively. The sensitivity of the assay was 6 pg/ml.

Islet isolation

The islet isolation procedure has been detailed in our earlier publication (17).

Flow cytometry

Flow cytometry was performed to quantify the apoptosis of islet cells. The islets were first dispersed by trypsin digestion, and then stained with FITC-annexin V, followed by flow cytometry (18).

Reverse transcription/Real-time PCR (RT/qPCR)

Tg and wild type islets were isolated and cultured in the absence or presence of IFN- γ (0.5 μ g/ml) plus IL-1 β (0.5 ng/ml), or TNF- α (100 ng/ml) plus IL-1 β (0.5 ng/ml). The islets were then harvested, and their total RNA was extracted with Trizol (Invitrogen, Carlsbad CA). RNA was

reverse-transcribed into cDNA by M-MLV Reverse Transcriptase Kit. QIAGEN Quanti-Test SYBR Green PCR Kits were employed for all real-time PCR amplification of cDNA templates. The PCR amplification program was: 95°C, 15 min, 1 cycle; 95°C, 10 sec, 56°C, 15 sec, 72°C, 20 sec, 45 cycles. The primers for LIGHT, TL1A, HVEM, LT β R and DR3 mRNA quantification are listed in Table I-A. The primers for DNA microarray data verification on IFN- γ /IL-1 β - and TNF- α /IL-1 β -treated islets are listed in Table I-B and Table I-C, respectively. In addition to melting curve analysis, the PCR products were also resolved by agarose gel electrophoresis to ascertain that the expected band-size was obtained.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay

Apoptosis of islet cells treated with recombinant mouse LIGHT (R&D Systems, Minneapolis, MN) and TL1A (R&D Systems) were analyzed by a fluorescent TUNEL assay, using the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) followed by flow cytometry, according to manufacturer's instructions.

Insulin release assay

The assay was conducted as reported elsewhere (17).

DNA microarray analysis

Three independent batches of islet isolation were carried out. For each batch, islets were obtained from 4 Tg and 4 WT mice, and pooled respectively. The pooled islets (Tg or WT) were then divided into 4 groups: 2 were cultured in the presence of IFN- γ (0.5 $\mu\text{g/ml}$) plus IL-1 β (0.5 ng/ml) with 1 harvested at 24 h and 1 harvested at 48 h; 2 were cultured in the presence of TNF- α (100 ng/ml) plus IL-1 β (0.5 ng/ml) with 1 harvested at 24 h and 1 harvested at 48 h. Total RNA was extracted from Tg and WT islets with QIAGEN RNeasy Micro Kits. After reverse transcription, cDNA served as a template for PCR-based cRNA amplification. cRNA was then reverse-transcribed to produce second-generation cDNA, which was employed to generate biotin-labelled cRNA probes. The probes were hybridized on the GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), which contains 39,000 transcripts. DNA microarray analysis was performed at the McGill University Genome Quebec Innovation Centre. Each treatment employed 3 chips using RNA from 3 different batches of islet isolation. For each treatment, genes with a mean signal strength difference above 2-fold between Tg and WT islets were selected for reversePCR confirmation. Details of the experimental protocols and data analysis procedure can be found in <http://genomequebec.mcgill.ca/centre.php> and

http://www.affymetrix.com/corporate/outreach/lesson_plan/downloads/manufacturing_teachernotes.pdf.

Islet transplantation

Diabetes in mice were chemically induced by injecting C57BL/6 male mice (6-10 weeks old) with streptozocin (STZ, 90 mg/kg) 3 times q.2.d. Three weeks after STZ injection, blood glucose was monitored every day with an Ascensia Contour glucose meter until it was above 20 mM for 2 consecutive days. Two hundred islets from Tg or WT mice were injected i.p. in diabetic recipients. Blood glucose was measured once every day for the first 7 days after transplantation and then once every 3 days.

Plasmid construction and transfection

Full-length (2113 bp) mouse Adcyap1 cDNA (clone 6829765; Open Biosystems, Huntsville, AL) was cloned into a mammalian cell expression vector pCEP4 (Invitrogen, Carlsbad, CA) at Kpn1 and Not1 sites downstream of the CMV promoter. The resulting plasmid is named pCEP4-Adcyap1. The plasmid was transfected into NIT-1 insulinoma cells using X-tremeGENE Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction (2 µg/ml plasmid for 0.2×10^5 NIT-1 cells/0.2 ml/well in 48-well plates). The mRNA

overexpression of Adcyap1 in the transfected cells was confirmed by RT/qPCR after 48 h. The cells were treated with or without IFN- γ (3 μ g/ml) plus IL-1 β (3 ng/ml) 24 h after the transfection. Cell apoptosis was assessed by annexin V staining after additional 48 h with flow cytometry.

Bank1 siRNA transfection

Two pairs of mouse Bank1 siRNA were synthesized (Integrated DNA Technologies, Coralville, IA). The sequences of the first pair were rArGrArArArCrArArCrArCrCrUrUrArCr and rUrUrGrArGrCrUrArUrGrUrArArGrGrUr, and the second pair, rGrGrArCrUrUrArCrUrArArArUrGrUrCr and rUrGrArGrArGrArArArGrArCrArUrUrUr. Scrambled Negative Control Duplex siRNA was used as controls. The sequences of the controls were rCrUrUrCrCrUrCrUrCrUrUrUrCrUrCrUrCrCrUrUrGrUGA, and rUrCrArCrArArGrGrGrArGrArGrArArArGrArGrArGrGrArArGrGrA. The paired siRNA was first heated to 94°C for 2 min and then cooled to the room temperature for annealing. The 2 pairs of annealed Bank1 siRNA were mixed at a 1:1 ratio and transfected into NIT-1 cells by X-tremeGENE transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) at the final concentration of 0.1 μ M siRNA for 0.2 x 10⁵ NIT-1 cells/0.2 ml/well in 48-well plates. Bank1 mRNA knockdown was confirmed by RT/qPCR 48 h

after the transfection. The primers used for this purpose were TTATAAACAGACCACCGGCTCCCA and TCACCATCAGATTGTCTTCGGGCT. The cells were treated with or without IFN- γ (3 μ g/ml) plus IL-1 β (3 ng/ml) 24 h after the transfection. Cell apoptosis was assessed by annexin V staining after additional 48 h with flow cytometry.

RESULTS

DcR3 Tg islets secrete DcR3

To continuously supply biologically active DcR3 to islets, DcR3 Tg mice were generated in both the C3H x C57BL/6 and C57BL/6 backgrounds, using the human β -actin promoter, as described in our previous publication (19). Two Tg mouse lines were generated. Line 754, originally in the C3H x C57BL/6 background, was backcrossed to the C57BL/6 background for 8 generations. Line 17139 was generated in the C57BL/6 background. Both lines have a similar phenotype. They are fertile and present no gross anomalies before 5 months of age (19). After 5 months, these mice develop a systemic lupus erythematosus-like syndrome (19). For this study, only 2- to 5 month-old mice were used. We demonstrated that DcR3 Tg islet cells from both lines were able to secrete DcR3 into culture supernatants at 8-15 ng/ml/200 islets within 24 h, as expected (Fig. 1). The remaining results in this study were from line 754 islets, but line 17139 islets behaved similarly.

DcR3 Tg islets are resistant to apoptosis and preserve better function in vitro

DcR3 could block multiple apoptosis pathways, as reviewed in the

Introduction. We have demonstrated previously that recombinant DcR3 could reduce FasL plus IFN- γ -induced islet cell apoptosis. Here, we showed that after 4 days of culture, Tg islet cells showed decreased apoptosis in plain medium (control), in medium containing IFN- γ /IL-1 β , and in medium containing TNF- α /IL-1 β , compared to WT islet cells (Fig. 2A). Such an effect was attributed to DcR3 produced by Tg islets themselves, since no exogenous DcR3 was added.

To pin-point the different degrees of islet apoptosis to β -cells, we evaluated the function of Tg islets by insulin-release assay (Fig. 2B). The Tg and WT islets were cultured in the absence or presence of the above-described cytokines for 48 h before the insulin-release assay. IFN- γ /IL-1 β - and TNF- α /IL-1 β -treated WT islets had significantly reduced insulin-release capability, while Tg islets retained their insulin-secretion function. This suggests that the apoptosis in islet cells, as seen in Fig. 2A, was attributed to β -cells.

DcR3 inhibits islet apoptosis by interfering with the TL1A/DR3 and LIGHT/HVEM-LT β R pathways

DcR3 binds to FasL and thus blocks the FasL/Fas-mediated apoptosis pathway (20). We have proved previously that this pathway is critical in islet apoptosis, and the presence of recombinant DcR3 reduces islet apoptosis

triggered by FasL (17). As DcR3 could interfere with 2 additional death pathways (i.e., TL1A acting through DR3, and LIGHT acting through LT β R/HVEM), we investigated whether these two additional pathways functioned in islets and whether blocking them was a mechanism for DcR3's protective effect on islets,

We first analyzed the expression of ligands and receptors involved in these 2 pathways. As shown in Figs. 3A and 3B (first rows), TL1A mRNA was detectable in islets cultured in medium (empty columns), and its expression was upregulated in the presence of IFN- γ /IL-1 β , or TNF- α /IL-1 β (black bars). LIGHT expression was not detectable in islets without stimulation; after 6-h IFN- γ /IL-1 β or TNF- α /IL-1 β stimulation, there was a transient and drastic induction of this gene expression (Figs. 3A and 3B; upper left panels, black bars). mRNA of the receptors, i.e., DR3, LT β R and HVEM, was all detectable in cultured islets, and they were moderately upregulated after the culture (Figs. 3A and 3B; second and third rows; empty bars; 6h versus 24h); the cytokines did not further induce their expression (Figs. 3A and 3B; second row; empty versus black bars), with the exception of HVEM, which was induced by IFN- γ /IL-1 β , and by TNF- α /IL-1 β (Figs. 3A and 3B; third row; empty versus black bars). As a matter of fact, IFN- γ /IL-1 β actually moderately suppressed the spontaneous DR3 upregulation, and

TNF- α /IL-1 β moderately hindered the spontaneous DR3 and LT β R upregulation after culture (Figs. 3A and 3B, second row; empty columns versus black columns). In summary, the components of LIGHT and TL1A death pathways were all present in islets, and this provided a basis for them to be functional there.

We next investigated whether these pathways were actively involved in islet death, and whether DcR3 could protect islets by interfering with these pathways. Islets from *lpr/lpr* mice, whose Fas is not functional due to a spontaneous mutation in the gene were used, so that the involvement of Fas/FasL could be excluded. Freshly isolated islets had little apoptosis (Fig. 4A). After 96 h culture in medium, 15.9% of the islet cells became apoptotic (Figs. 4B and D, first panel). Recombinant DcR3 could not suppress such spontaneous apoptosis (Fig. C. first panel). These islets were assaulted with recombinant mouse LIGHT (Figs. B and C) or TL1A (Fig. D). In the case of LIGHT, IFN- γ /IL-1 β (Fig. 4B,) and TNF- α /IL-1 β (Fig. 4C) were added to the culture. It is to be mentioned that in this experiment, these cytokines were used at suboptimal concentrations, about 1/10th of that used for apoptosis induction (Fig. 2). As a result, after 96 h culture, islets cultured in the presence of cytokines (Figs. 4B and 4C, second panels) were not much different from that cultured in medium (Figs. 4B and D, first panels). In the

case of TL1A (Fig. 4D), no cytokines were added to the culture. The decision whether to add cytokines into the culture was made empirically, because our preliminary results showed that LIGHT but not TL1A needed a low level of cytokine priming to trigger islet apoptosis. Thus, exogenous LIGHT was added to the cytokine-treated islets, and exogenous TL1A was added to islets without cytokine treatment.

The purpose of the addition of exogenous LIGHT and TL1A, in spite of the existence of endogenous LIGHT and TL1A in islets, to trigger apoptosis was 2-fold, 1) If these molecules induced islet apoptosis, we will be able to identify these molecules as inducers of islet apoptosis, or as synergistic factors for apoptosis induction for islets; 2) we can use DcR3 to neutralize their apoptosis-inducing effect, to ascertain the specificity.

Indeed, both LIGHT (Figs. 4B and 4C, third panels compared to second panels) and TL1A (Fig. 4D, second panel compared to first panel) augmented islet apoptosis after 96 h. The exogenous recombinant DcR3 blocked such an effect (Figs. 4B-D, last panels), proving the specificity of the action of these 2 recombinant proteins. Therefore, for the first time, we demonstrated that the LIGHT/HVEM-LT β R pathway as well as the TL1A/DR3 pathway were involved in islet apoptosis, while DcR3 protected

islets by antagonizing their pro-apoptotic effect.

Reduced PNF in transplanted Tg islets

To test whether the presence of long-term local DcR3 could reduce PNF, we transplanted a suboptimal number (200 islets per recipient) of Tg and WT islets into syngeneic diabetic recipients. With such a number, only 10-38% of WT islet recipients could transiently achieve euglycemic status (blood glucose <12 mM) at a given time point in the first 16 days after transplantation, and all of them reversed to diabetic thereafter (Fig. 5A). On the other hand, 100% of Tg islets recipients became transiently euglycemic in the first 16 days, and 50% of them achieved long-term euglycemia (after 23 days until the end of the experiment at day 32 post-transplantation). The Tg islet recipient groups also presented significantly lower mean blood glucose levels throughout the test period, i.e., from day 1 until day 32 post-transplantation (Fig. 5B). This result indicated that local DcR3 presence, even at a very low level, rendered islet transplantation much more efficient, because of PNF reduction.

Downstream genes involved in the DcR3 protective effect on islet apoptosis

We conducted DNA microarray analysis to discover downstream molecules involved in the DcR3's protective effect on islet apoptosis. Islets were

cultured in the presence of IFN- γ /IL-1 β for 24h, and their mRNA was analyzed by DNA microarray. A total of 34 genes presented over 2-fold expression difference between Tg and WT islets according to the microarray. The difference in 5 out of these 34 genes could be confirmed by real-time PCR, and the list is shown in Table 2. Notably, among these 5 genes, the adenylate cyclase activating polypeptide 1 (*Adcyap1*) expression level in Tg islets was 779-fold higher than in WT islets, and *Bank1* expression was 50 times lower in Tg than in WT islets, after 24-h culture in the presence of the cytokines.

For TNF- α /IL-1 β -treated Tg and WT islets, expression levels of 52 genes differed more than 2 fold according to DNA microarray analysis, and 7 genes presented consistent differences after real-time PCR verification. Among these 7 genes, in Tg islets, *Ms4a4c* showed 4-fold increase, and *Ttyh1*, a 7-fold decrease, with WT islets as references.

DcR3 acts through Adcyap1 and Bank1 for islet protection

We found that DcR3 Tg islets presented 779-fold higher *Adcyap1* expression and 50-fold lower *Bank1* expression compared to WT islets upon IFN- γ /IL-1 β assault (Table 2). We next investigated whether the modulation of these two genes was causative to islet survival, or just parallel events not

relevant to the protective effect of DcR3. Unstimulated Tg islet had about 10-fold higher *Adcyap1* expression than WT islets; upon IFN- γ /IL-1 β assault, Tg islets maintained their high *Adcyap1* levels, but WT islet drastically downregulated the expression (about 100 fold) (Fig. 6A). To understand whether the high *Adcyap1* levels was protective to islets, we generated a mammalian cell expression construct of *Adcyap1* named pCEP4-*Adcyap1* (Fig. 6B), and transfected it into NIT-1 insulinoma cells. *Adcyap1* mRNA overexpression driven by the CMV promoter in NIT-1 cells 48 h after transfection was confirmed by RT-qPCR (Fig. 6C). When the empty vector-transfected NIT-1 cells were assaulted by IFN- γ /IL-1 β , 42.5% of them underwent apoptosis, as expected. However, *Adcyap1* overexpression protected NIT-1 cells from such cytokine-induced apoptosis; only 24.7% cells became apoptotic (Fig. 6D). A summary of data from 3 independent experiments is shown in Fig. 6E. This result indicates that the upregulation of *Adcyap1* is beneficial to β -cell survival. It further implies that DcR3 protects β -cells against IFN- γ /IL-1 β through enhancing *Adcyap1* expression.

In WT islets, IFN- γ /IL-1 β stimulation caused 10-fold augmentation of *Bank1*; the presence of DcR3 suppressed such augmentation and *Bank1* levels remained unchanged (Fig. 7A). We wondered whether this *Bank1* suppression was another DcR3's protective mechanism for islets. To verify

this hypothesis, we transfected Bank1 siRNA into NIT-1 cells and Bank1 mRNA knockdown was confirmed by RT/qPCR (Fig. 7B). We demonstrated that Bank1 siRNA but not control siRNA transfection resulted in reduced NIT-1 cell apoptosis from 36.2% to 26% upon IFN- γ /IL-1 β stimulation (Fig. 7C). A summary of data from 3 independent experiments is illustrated in Fig. 7D. This demonstrates that repressed Bank1 expression protects β cells from IFN- γ /IL-1 β insults, and this is another mechanism through which DcR3 exerts its beneficial effect on β -cell survival.

DISCUSSION

The novel findings of this study are as follows. 1) For the first time, the TL1A/DR3 pathway and the LIGHT/HVEM-LT β R pathway were found operative in islets and mediating islet apoptosis. 2) We demonstrated that DcR3 secreted by the islets in minute amounts, about 3 orders of magnitude less than that we administered i.p. in our previous study (17), was sufficient to protect islets from apoptosis. 3) Two novel downstream mediators of DcR3's effect, Adcyap1 and Bank1, were discovered by DNA microarray and confirmed by functional studies for their protective and detrimental roles, respectively, in β -cells apoptosis.

In our previous study, we identified the FasL/Fas pathway as a culprit in causing islet PNF (17), and reported that DcR3 protected islets by blocking that pathway. As DcR3 acts on several additional pathways, we proved in this study that the TL1A and LIGHT pathways were also responsible for islet apoptosis. This new mechanistic finding is useful for designing methods to reduce PNF, because now we know that all these three pathways will need to be blocked to achieve maximal protective effects. Our findings are also important in understanding the pathogenesis of diabetes, because IFN- γ /IL-1 β and TNF- α /IL-1 β are present in the milieu of type I diabetes

(21,22), and TNF- α /IL-1 β , in type II diabetes (23,24). We have shown that these cytokines were able to induce LIGHT and its receptor HVEM in islets, to make the LIGHT apoptosis pathway operational. Although in the islets the receptors of LIGHT (i.e., HVEM and LT β R) expressed constitutively, albeit at low levels (Figs. 3A and 3B), we found that exogenous recombinant LIGHT alone could not induce islet apoptosis (data not shown), unless the islets were primed with low-dose cytokines (i.e., IFN- γ /IL-1 β or TNF- α /IL-1 β). This suggests a 2-hit *modus operandi*: the LIGHT-triggered signalling needs to interact with the cytokine-triggered signalling to achieve islet apoptosis. On the other hand, TL1A signalling alone seemed sufficient to cause islets apoptosis, in the absence of cytokine priming. Therefore, one hit seemed to be enough for TL1A.

In our previous study (17), we administered recombinant DcR3 to mice to reduce PNF. The effective peak dosage was about 15 μ g/ml body volume. In the current study, with continuous secretion of biologically active DcR3 from islets, we estimated that DcR3 at a level of several ng per ml body volume (about 1000-fold lower than the exogenous recombinant DcR3 employed before; ref. 17) was sufficient to protect the islets, as 100 Tg islets secreted about 8 ng DcR3 in 24 h and we transplanted 200 islets into one recipients. The effective DcR3 concentration at such a low level makes

clinical application feasible: isolated islets could be infected with DcR3-expressing virus vectors in islet transplantation to render themselves resistant to PNF. Such a measure will reduce the islet number required to attain recipient insulin independence. This is equivalent to an increase in the available donor pool, currently a limiting factor in islet transplantation.

To gain mechanistic insights, we employed DNA microarrays to discover downstream molecules affected by the protective effect of DcR3, which acted on the Fas HVEM/LT β R and DR3 pathways in islets. Tg and WT islets were assaulted by IFN- γ /IL-1 β to mimic the post-transplantation environment they would experience in type I diabetes recipients, and by TFN- α /IL-1 β , in type II diabetes recipients. Five genes in the former and 7 genes in the latter environment manifested expression differences between Tg and WT islets. It is surprising that these two treatments did not yield overlapping gene expression patterns as there was no common gene in Tables 2 and 3, although DcR3 acted in both cases by blocking the Fas, HVEM/LT β R and DR3 pathways. This reminds us of the interesting theory of the “butterfly effect”, which claims that a minor difference in the beginning of an event could lead to drastically different consequences in a complex world full of multiple interacting components. In our case, the beginning of the event, i.e., islets were treated by two different sets of

cytokines, was indeed different, and signalling molecules inside islet cells are indeed complex and highly interactive. For the time being, we do not clearly understand how such an initial difference travelled through the Fas, HVEM/LT β R and DR3 pathways and interfered by DcR3 ended up in totally different gene expression patterns. Nevertheless, the genes discovered could serve a practical purpose as putative target molecules to promote islet survival.

Among the 12 genes identified by DNA microarray, we selected two that were drastically modulated for further investigation. We found that in normal islets, upon inflammatory cytokine stimulation, there was a 2-order magnitude decrease of Adcyap1 expression. On the other hand, DcR3 Tg islets had about a 10-fold higher basal Adcyap1 level compared to WT islets, and this level did not decrease upon the cytokine stimulation. So the end result was that there was more than 700-fold differences in Adcyap1 expression between Tg and WT islets. The active Adcyap1 is a short secreted peptide (27 or 38 amino acid residues, derived from the same precursor peptide) belonging to the vasoactive intestinal polypeptide superfamily, and is also a neurotransmitter (25). It is secreted by neurons and many other tissues including β -cells (26). It has profound effects in various tissue and organs. It is involved in circadian rhythm (27,28), cerebellum development

(29,30), energy homeostasis and regulation of body weight (31,32), embryo implantation (33), inhibition of inflammation (34), promotion of regulatory T cell generation (35), maintenance of pulmonary vascular tone (36), to name a few of its functions. The following Adcyap1 functions are probably directly related to the findings in this study. 1) Adycap1 is anti-apoptotic for neurons experienced ischemia (37). 2) It is present in the nerve fibers innervating the islets (38), and is produced by β -cells (26). 3) Adcyap1 can stimulate insulin release (38) by β -cells. 4) Transgenic Adcyap1 overexpression in islet β -cells protects β -cells from STZ-induced apoptosis and renders the Tg mice resistant to STZ-induced diabetes. (39). These anti-apoptotic and islet protective effects are in consistent with our finding that: 1) Adcyap1 overexpression protects β -cells from inflammatory cytokine-induced apoptosis, 2) Tg islets maintained high levels of Adcyap1 expression while WT islets drastically reduced Adcyap1 expression upon cytokine assault, and 3) high Adcyap1 expression in Tg islets was associated with reduced apoptosis. It seems that DcR3 not only directly protected islets by interfering with Fas, Hvem and DR3 death pathways, but also hijacked an additional β -cell trophic molecule Adcyap1 to achieve its beneficial effect on islets.

Bank1 is the other molecule that showed major difference in expression

between Tg and WT islets. WT islets upregulated Bank1 expression 50 times upon cytokine stimulation, while its expression remained almost unchanged in Tg islets. Bank1 is an adaptor protein and a substrate of Lyn tyrosine kinase (40). It forms tri-molecule complex with Lyn and IP3R; its overexpression leads to enhanced calcium mobilization from intracellular stores in B cells upon B cell receptor activation. In islet β -cells, it is known that calcium fluxes are required for IL-1 β -and glucose-induced apoptosis (41). Thus, a plausible additional mechanism for DcR3 to protect β -cells is to inhibit cytokine-induced Bank1 upregulation, the truncation of which in turn represses the pro-apoptotic calcium fluxes in the cells.

How the interference of Fas, Hvem and DR3 signaling by DcR3 leads to the prevention of Adcyap1 downregulation and Bank1 upregulation in β -cells remains to be investigated. Obviously, the DcR3 presence also affects the expression of multiple genes, some of which might also be true mediators of DcR3's protective effect. Further functional relevance verification of these identified genes is necessary. The two verified mediator, Adcyap1 and Bank1, and other verified genes can serve as therapeutic targets to promote islet survival in the future.

ACKNOWLEDGEMENTS

The authors thank Mr. Ovid Da Silva, Research Support Office, Research Centre, CHUM, for his editorial assistance.

REFERENCES

-
- 1 Kenmochi T, Miyamoto M, Mullen Y. Protection of mouse islet isografts from nonspecific inflammatory damage by recipient treatment with nicotinamide and 15-deoxyspergualin. *Cell Transplant* 1996 Jan;5(1):41-7.
 - 2 Gruessner AC, Sutherland DE. Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004. *Clin Transplant* 2005 Aug;19(4):433-55.
 - 3 Witkowski P, Zakai SB, Rana A, Sledzinski Z, Hardy MA. Pancreatic islet transplantation, what has been achieved since Edmonton break-through. *Ann Transplant* 2006;11(2):5-13.
 - 4 Rosenberg L. Clinical islet cell transplantation. Are we there yet? *Int J Pancreatol* 1998 Dec;24(3):145-68.
 - 5 Wahoff DC, Papalois BE, Najarian JS, Kendall DM, Farney AC, Leone JP, et al. Autologous islet transplantation to prevent diabetes after pancreatic resection. *Ann Surg* 1995 Oct;222(4):562-75.
 - 6 Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 1999 Aug;126(2):393-8.
 - 7 Thomas FT, Contreras JL, Bilbao G, Ricordi C, Curiel D, Thomas JM. Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery* 1999 Aug;126(2):299-304.
 - 8 Lupi R, Dotta F, Marselli L, Del GS, Masini M, Santangelo C, et al. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* 2002 May;51(5):1437-42.

-
- 9 Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 1998 Dec 17;396(6712):699-703.
- 10 Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, et al. Modulation of T-cell responses to alloantigens by TR6/DcR3. *J Clin Invest* 2001 Jun;107(11):1459-68.
- 11 Bai C, Connolly B, Metzker ML, Hilliard CA, Liu X, Sandig V, et al. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc Natl Acad Sci U S A* 2000 Feb 1;97(3):1230-5.
- 12 Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, et al. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 2002 Mar;16(3):479-92.
- 13 Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* 1998 Jan;8(1):21-30.
- 14 Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, et al. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem* 2000 May 12;275(19):14307-15.
- 15 Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, et al. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest* 1998 Sep 15;102(6):1142-51.
- 16 Wu Y, Han B, Sheng H, Lin M, Moore PA, Zhang J, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 2003 Jul 10;105(5):724-32.
- 17 Wu Y, Han B, Luo H, Roduit R, Salcedo TW, Moore PA, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* 2003 Sep;52(9):2279-86.
- 18 Luo H, Yu G, Tremblay J, Wu J. EphB6-null mutation results in compromised T cell function. *J Clin Invest* 2004 Dec;114(12):1762-73.
- 19 Han B, Paul A. Moore, Jiangping Wu, Hongyu Luo. Human DcR3 overexpression in mice results in a systemic lupus erythematosus-like syndrome. *ARTHRITIS & RHEUMATISM* 2007 Nov;56(11):3648-3758.
- 20 Roth W, Isenmann S, Nakamura M, Platten M, Wick W, Kleihues P, et al. Soluble decoy receptor 3 is expressed by malignant gliomas and suppresses CD95 ligand-induced apoptosis and chemotaxis.

Cancer Res 2001 Mar 15;61(6):2759-65.

21 Pavlovic D, Chen MC, Gysemans CA, Mathieu C, Eizirik DL. The role of interferon regulatory factor-1 in cytokine-induced mRNA expression and cell death in murine pancreatic beta-cells. *Eur Cytokine Netw* 1999 Sep;10(3):403-12.

22 Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC, Power RF. IFN-gamma gene expression in pancreatic islet-infiltrating mononuclear cells correlates with autoimmune diabetes in nonobese diabetic mice. *J Immunol* 1995 May 1;154(9):4874-82.

23 Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, et al. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 2003 Mar;52(3):812-7.

24 Giulietti A, van EE, Overbergh L, Stoffels K, Bouillon R, Mathieu C. Monocytes from type 2 diabetic patients have a pro-inflammatory profile. 1,25-Dihydroxyvitamin D(3) works as anti-inflammatory. *Diabetes Res Clin Pract* 2007 Jul;77(1):47-57.

25 Heindel, J. J., J. Sneeden, C. J. Powell, B. Davis, and M. D. Culler. 1996. A novel hypothalamic peptide, pituitary adenylate cyclase-activating peptide, regulates the function of rat granulosa cells in vitro. *Biol. Reprod.* 54:523.

26 Yada, T., M. Sakurada, H. Ishihara, M. Nakata, S. Shioda, K. Yaekura, N. Hamakawa, K. Yanagida, M. Kikuchi, and Y. Oka. 1997. Pituitary adenylate cyclase-activating polypeptide (PACAP) is an islet substance serving as an intra-islet amplifier of glucose-induced insulin secretion in rats. *J. Physiol* 505 (Pt 2):319.

27 Hatanaka, M., M. Tanida, N. Shintani, Y. Isojima, C. Kawaguchi, H. Hashimoto, M. Kakuda, R. Haba, K. Nagai, and A. Baba. 2008. Lack of light-induced elevation of renal sympathetic nerve activity and plasma corticosterone levels in PACAP-deficient mice. *Neurosci. Lett.* 444:153.

28 Kawaguchi, C., K. Tanaka, Y. Isojima, N. Shintani, H. Hashimoto, A. Baba, and K. Nagai. 2003. Changes in light-induced phase shift of circadian rhythm in mice lacking PACAP. *Biochem. Biophys. Res. Commun.* 310:169.

29 Shintani, N., H. Hashimoto, K. Tanaka, N. Kawagishi, C. Kawaguchi, M. Hatanaka, Y. Ago, T. Matsuda, and A. Baba. 2006. Serotonergic inhibition of intense jumping behavior in mice lacking PACAP (*Adcyap1*^{-/-}). *Ann. N. Y. Acad. Sci.* 1070:545.

-
- 30 Allais, A., D. Burel, E. R. Isaac, S. L. Gray, M. Basille, A. Ravni, N. M. Sherwood, H. Vaudry, and B. J. Gonzalez. 2007. Altered cerebellar development in mice lacking pituitary adenylate cyclase-activating polypeptide. *Eur. J. Neurosci.* 25:2604.
- 31 Tomimoto, S., T. Ojika, N. Shintani, H. Hashimoto, K. Hamagami, K. Ikeda, M. Nakata, T. Yada, Y. Sakurai, T. Shimada, Y. Morita, C. Ishida, and A. Baba. 2008. Markedly reduced white adipose tissue and increased insulin sensitivity in adcyap1-deficient mice. *J. Pharmacol. Sci.* 107:41.
- 32 Adams, B. A., S. L. Gray, E. R. Isaac, A. C. Bianco, A. J. Vidal-Puig, and N. M. Sherwood. 2008. Feeding and metabolism in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 149:1571.
- 33 Isaac, E. R., and N. M. Sherwood. 2008. Pituitary adenylate cyclase-activating polypeptide (PACAP) is important for embryo implantation in mice. *Mol. Cell Endocrinol.* 280:13.
- 34 Nemetz, N., C. Abad, G. Lawson, H. Nobuta, S. Chhith, L. Duong, G. Tse, J. Braun, and J. A. Waschek. 2008. Induction of colitis and rapid development of colorectal tumors in mice deficient in the neuropeptide PACAP. *Int. J. Cancer* 122:1803.
- 35 Tan, Y. V., C. Abad, R. Lopez, H. Dong, S. Liu, A. Lee, R. P. Gomariz, J. Leceta, and J. A. Waschek. 2009. Pituitary adenylyl cyclase-activating polypeptide is an intrinsic regulator of Treg abundance and protects against experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A* 106:2012.
- 36 Otto, C., L. Hein, M. Brede, R. Jahns, S. Engelhardt, H. J. Grone, and G. Schutz. 2004. Pulmonary hypertension and right heart failure in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. *Circulation* 110:3245.
- .
- 37 Ohtaki, H., T. Nakamachi, K. Dohi, Y. Aizawa, A. Takaki, K. Hodoyama, S. Yofu, H. Hashimoto, N. Shintani, A. Baba, M. Kopf, Y. Iwakura, K. Matsuda, A. Arimura, and S. Shioda. 2006. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. *Proc. Natl. Acad. Sci. U. S. A* 103:7488.
- 38 Yada, T., M. Sakurada, K. Ihida, M. Nakata, F. Murata, A. Arimura, and M. Kikuchi. 1994. Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet beta-cells. *J. Biol. Chem.* 269:1290.
- 39 Yamamoto, K., H. Hashimoto, S. Tomimoto, N. Shintani, J. Miyazaki, F. Tashiro, H. Aihara, T. Nammo, M. Li, K. Yamagata, J. Miyagawa, Y. Matsuzawa, Y. Kawabata, Y. Fukuyama, K. Koga, W. Mori, K. Tanaka, T. Matsuda, and A. Baba. 2003. Overexpression of PACAP in transgenic mouse

pancreatic beta-cells enhances insulin secretion and ameliorates streptozotocin-induced diabetes. *Diabetes* 52:1155.

40 Yokoyama, K., I. Su, I. T. Tezuka, T. Yasuda, K. Mikoshiba, A. Tarakhovsky, and T. Yamamoto. 2002. BANK regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP(3) receptor. *EMBO J.* 21:83.

41 Fei, H., B. Zhao, S. Zhao, and Q. Wang. 2008. Requirements of calcium fluxes and ERK kinase activation for glucose- and interleukin-1beta-induced beta-cell apoptosis. *Mol. Cell Biochem.* 315:75.

TABLES

Table 1-A. Real-time PCR primers used to quantify mRNA levels of *LIGHT*, *TL1A*, *HVEM*, *LTβR* and *DR3*.

Gene Symbol	Forward Primer	Reverse Primer	Product Size
LIGHT	AGACTGCATCAACGTCTTGG	TTGGCTCCTGTAAGATGTGC	128
TL1A	GATCTGAGCCTTCACCACAG	AATTGTCAGGTGTGCTCTCG	68
HVEM	CAGGGCAGAGGGTAGAGAAG	CCAAGTGAGAAGGTCCCTGT	82
LTβR	TCTCTGCAGAAAGCTGGGTA	AGACATGGGTAGGAGTGGCT	124
DR3	TCTAGGAGTCGCGTTCCTTT	AGAGATGGGCAGTCTGTGGT	131

Table 1-B. Real-time PCR primers to quantify mRNA levels of microarray-identified genes from *IFN-γ* plus *IL-1β*-treated *Tg* versus *WT* islets.

Gene Symbol	Forward Primer	Reverse Primer	Product Size
Adcyap1	GCCCCGCTGTCCTACTTAGTC	AGGTGAACAGGAGACACTGCT	117
Bank1	CAGACCTGCTGCATATTGCT	CTTGCTTGCTATTTCTGCCA	99
Cryab	GATTGACACCGGACTCTCAG	AGAGAAGTGCTTCACGTCCA	76
Cyp7b1	TTTCCTGCAGTCAACAGGTC	TCTCGGATGATGCTGGAGTA	141
Gap43	CCAAGCTGAGGAGGAGAAAG	TCAGGCATGTTCTTGGTCAG	106

Table 1-C. Real-time PCR primers to quantifying mRNA levels of microarray-identified genes from *TNF-α* plus *IL-1β*-treated *Tg* versus *WT* islets.

Gene Symbol	Forward Primer	Reverse Primer	Product Size
Gngt2	AGATTTCTCAGCTGATGAGGCGCT	TCACTTCCTTCTTCAGCTGCTCCA	178
Ms4a4c	AATGGGTGTAGTCAGTGTGGCTGT	ACCTCGCTGGAGTTACAACAGGAA	171
Plat	TACAATGCAAGGAGGCCAAATGCC	AGACATAGCACCAGGGCTTCAAGT	97
Scand1	ACCAAGGAGCAGATCGTGGAGAT	ATTCAGGGTAGGGTGTGAGTCCAA	156
Slc28a2	GAAGGTTGCCTGGTTCCTGCAAAT	TGCTATGGTAGCAAAGCCTCCAGT	178
Tmem45b	TGCTCTCTGCATCGTAGCCATCAA	TGCTTGATGTCACTCCTCATCCGA	163
Ttyh1	ACCTTTCAACCCTCAGGAATCCA	AGCAGGTTGCCATCCTAAGTGAGA	198

Table 2. Genes identified by DNA microarray and verified by real-time PCR from IFN- γ plus IL-1 β -treated Tg versus WT islets

Gene Symbol	Category	Tg/WT signal ratio	
		at 24 h	at 48 h
Adcyap1	Islet survival and function	779.15	470.70
Bank1	Cell survival	0.02	0.30
Cryab	Apoptosis, insulin secretion	2.22	2.83
Cyp7b1	Immune modulator	0.46	0.29
Gap43	Proliferation	1.74	3.14

Thirty-four gene transcripts were found modulated above 2-fold in Tg islets compared with WT islets after IFN- γ plus IL-1 β treatment, according to DNA microarray. These 34 transcripts were verified using real-time PCR, and 5 of them were proven to be modulated above 2 fold at either 24 h or 48 h after the cytokine treatment in 2 independent experiments. The mean signal ratios of Tg versus WT islets from a representative experiment at 24 h and 48 h are shown.

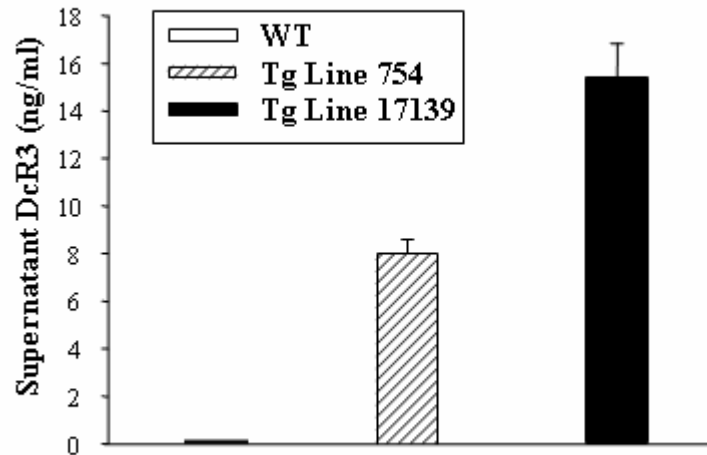
Table 3. Genes identified by DNA microarray and verified by real-time PCR from *TNF- α* plus *IL-1 β* -treated Tg versus WT islets

Gene Symbol	Category	Tg/WT signal ratio	
		at 24 h	at 48 h
Gngt2	Signal transducer activity	0.83	2.25
Ms4a4c	Signal transduction	4.37	4.83
Plat	Inflammation, apoptosis	0.48	1.45
Scand1	Regulation of transcription	2.68	1.85
Slc28a2	Sodium-dependent nucleoside transporter	0.35	0.82
Tmem45b	Unknown	0.45	1.27
Ttyh1	Cell-cell, cell-substrate adhesion	0.14	0.72

Fifty-two gene transcripts were found modulated above 2-fold in Tg islets compared with WT islets after IFN- γ plus IL-1 β treatment, according to DNA microarray. These 52 transcripts were verified using real-time PCR, and 7 of them were proven to be modulated above 2 fold at either 24 h or 48 h after the cytokine treatment in 2 independent experiments. The mean signal ratios of Tg versus WT islets from a representative experiments at 24 h and 48 h are shown.

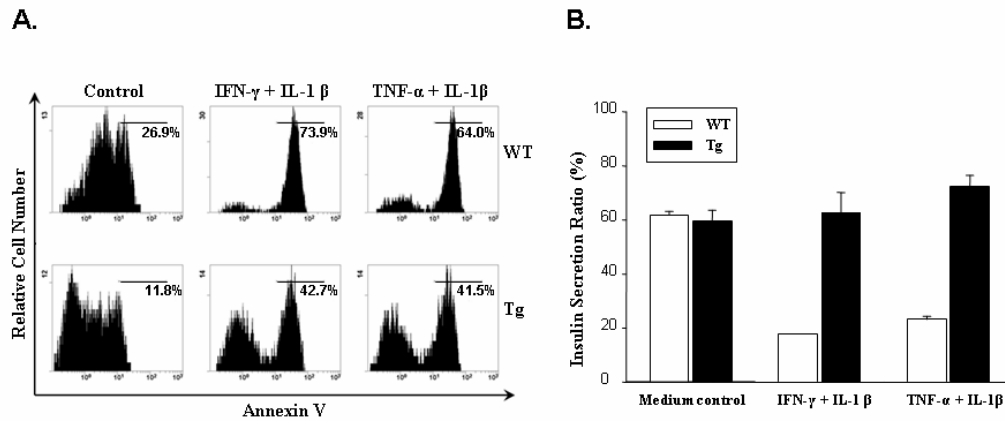
FIGURES AND LENGENDS

Figure 1. DcR3 secretion by Tg islets



Islets from Tg lines 754 and 17139 and from WT mice were cultured for 24 h (100 islets/0.5 ml/well in 24-well plates). The supernatants were harvested and analyzed by ELISA for DcR3 concentration. Samples were in triplicate. The experiments were repeated at least twice, and similar results were obtained. Data from a representative experiment are shown.

Figure 2. Tg islets present reduced apoptosis and maintain their function under cytokines assault



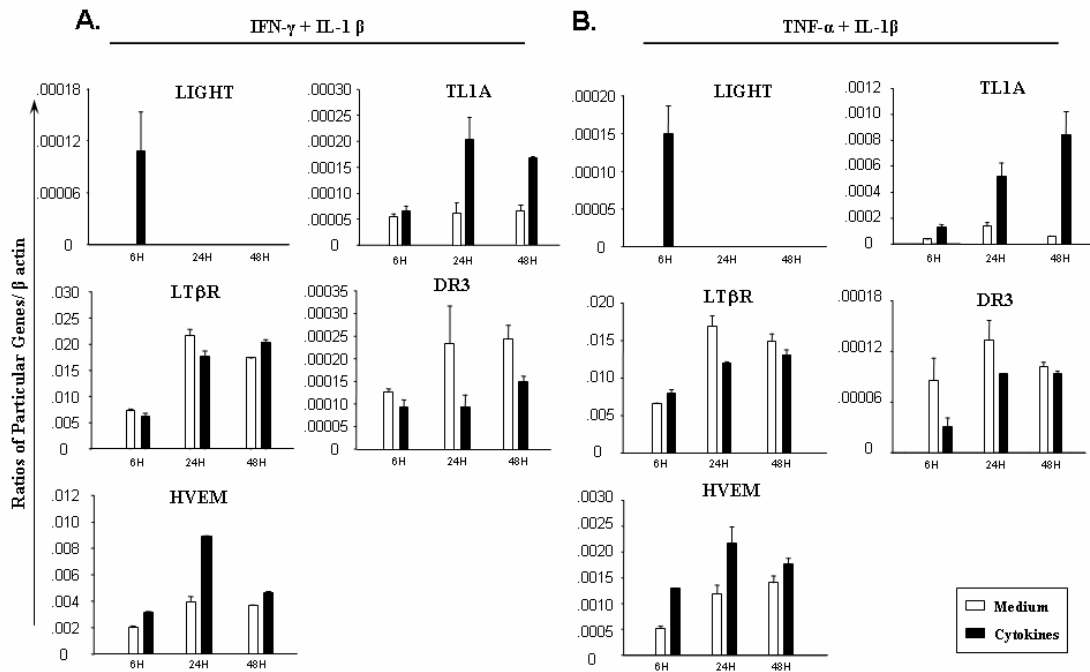
A. Tg and WT islets were cultured in the absence or presence of IFN- γ (0.5 μ g/ml) plus IL-1 β (0.5 ng/ml), or TNF- α (100 ng/ml) plus IL-1 β (0.5 ng/ml) for 4 days. They were harvested and then dispersed by trypsin-digestion. Their apoptosis were analyzed by annexin V staining, followed by flow cytometry. The percentage of annexin V-positive islet cells is indicated.

B. Tg and WT islets were cultured as described in A. After 48 h, 10 islets were hand-picked from each treatment, and transferred to 12-well plates. The islets were sequentially incubated with 2.8 mM and 16.7 mM glucose. Insulin concentrations in the supernatants after each treatment were analyzed by ELISA. Samples were in triplicate. The insulin secretion ratio was calculated as follows:

Insulin secretion ratio = insulin concentration after 16.7 mM glucose treatment/insulin concentration after 2.8 mM glucose treatment.

The experiments were repeated 3 times, and data from a representative experiment are shown.

Figure 3. Expression of TL1A and LIGHT pathway components in islets according to real-time PCR



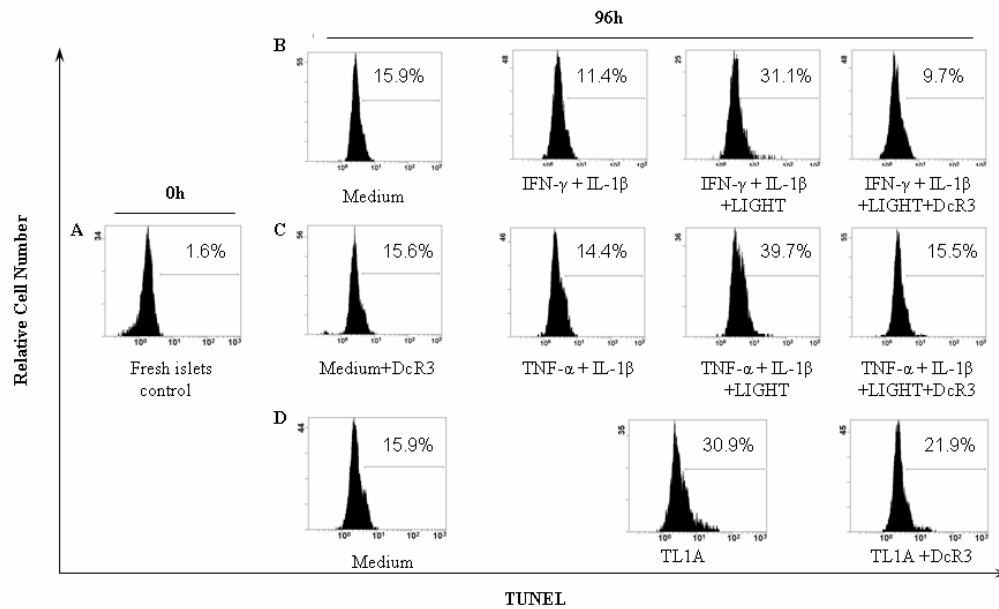
WT islets were cultured in the absence (medium) or presence of IFN- γ (0.5 μ g/ml) plus IL-1 β (0.5 ng/ml) or TNF- α (100 ng/ml) plus IL-1 β (0.5 ng/ml). They were harvested at 6 h, 24 h, and 48 h after culture. Their TL1A, LIGHT, DR3, LT β R and HVEM mRNA levels were assessed by real-time PCR after reverse transcription.

A. Islets treated with IFN- γ plus IL-1 β

B. Islets treated with TNF- α plus IL-1 β

The experiments were repeated twice, and data from a representative experiment are shown.

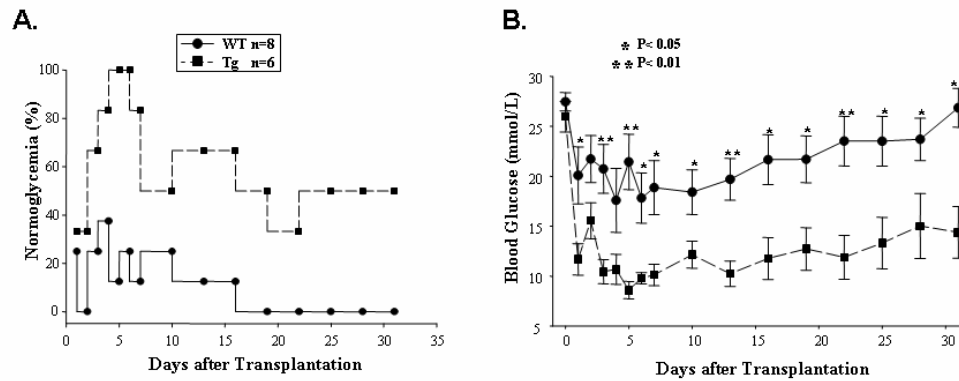
Figure 4. Recombinant mouse LIGHT and TL1A induced *lpr/lpr* islet cell apoptosis



Islets from *lpr/lpr* mice were cultured in the absence or presence of IFN- γ (0.05 μ g/ml) plus IL-1 β (0.05 ng/ml), or TNF- α (10 ng/ml) plus IL-1 β (0.05 ng/ml), as shown. In some experiments, recombinant mouse LIGHT (5 μ g/ml) and TL1A (100 ng/ml), and recombinant human DcR3 (5 μ g/ml) were added, as indicated. The histogram in the first column is from freshly isolated islets; all other histograms were from islets cultured for 96 h. The islets were dispersed and stained by fluorescent TUNEL, and were analyzed by flow cytometry.

The experiments were repeated 2-4 times, and data from a representative experiment are shown.

Figure 5. Reduced islet PNF in transplantation using DcR3 Tg islets



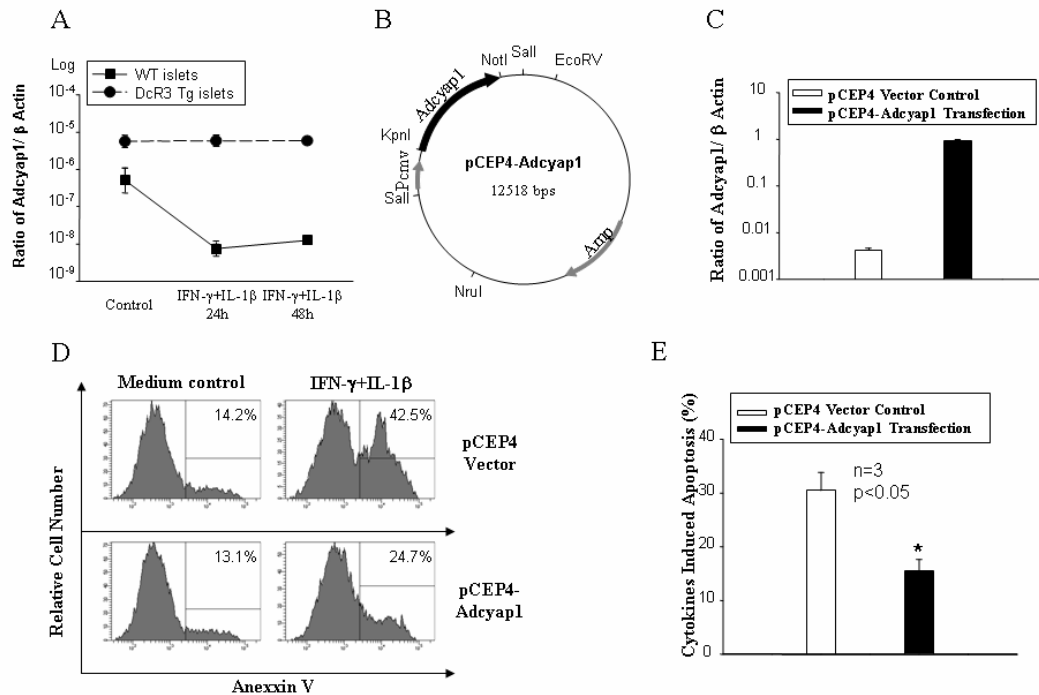
Suboptimal number of Tg or WT islets were transplanted i.p. into syngeneic C57BL/6 mice with STZ-induced diabetes (200 islets/mouse). Recipient blood glucose was monitored everyday for the first 7 days, and once every other day from days 8 to 30 after transplantation.

A. Percentage of mice with normoglycemia (below 12 mM) after transplantation

B. Blood glucose concentration after transplantation

The data were analyzed with Student's t test. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

Figure 6. *Adcyap1* overexpression protected β -cells from cytokine-induced apoptosis



A. *Adcyap1* mRNA expression in Tg and WT islets upon cytokine-stimulation

Tg and WT islets were stimulated with IFN- γ (0.5 μ g/ml) plus IL-1 β (0.5 ng/ml) for 24 and 48 h, and their *Adcyap1* mRNA levels were assessed by RT/qPCR. The ratios (mean + SD) of *Adcyap1* versus β -actin signals were expressed in a log scale.

B. *Adcyap1* expression construct

Full-length (2113 bp) mouse *Adcyap1* cDNA was cloned into a mammalian cell expression vector pCEP4 at KpnI and NotI sites downstream of the CMV promoter. The resulting plasmid is named pCEP4-*Adcyap1*, and was used for NIT-1 cell transfection.

C. *Adcyap1* overexpression in pCEP4-*Adcyap1*-transfected NIT-1 cells

Plasmid pCEP4-*Adcyap1* was transfected into NIT-1 insulinoma cells. *Adcyap1* mRNA

in the transfected cells was measured by RT/qPCR after 48 h. The ratios (mean + SD) of Bank1 versus β -actin signals are shown.

D and E. Adcyap1 overexpression in NIT-1 cells reduced cytokine-induced apoptosis

NIT-1 cells were transfected with pCEP4-Adcyap1 or the empty vector pCEP4. The cells were treated with or without IFN- γ (3 μ g/ml) plus IL-1 β (3 ng/ml) 24 h after the transfection. Cell apoptosis was assessed by annexin V staining after additional 48 h with flow cytometry. A representative set of histograms are shown in panel D, and a summary of data from 3 independent experiments are illustrated in panel E. The difference between pCEP4- and pCEP4Adcyap1-transfected cells is highly significant ($p < 0.05$, Student's t test).

Figure 7. Inhibition of Bank1 expression decreased cytokine-induced apoptosis in NIT-1 cells

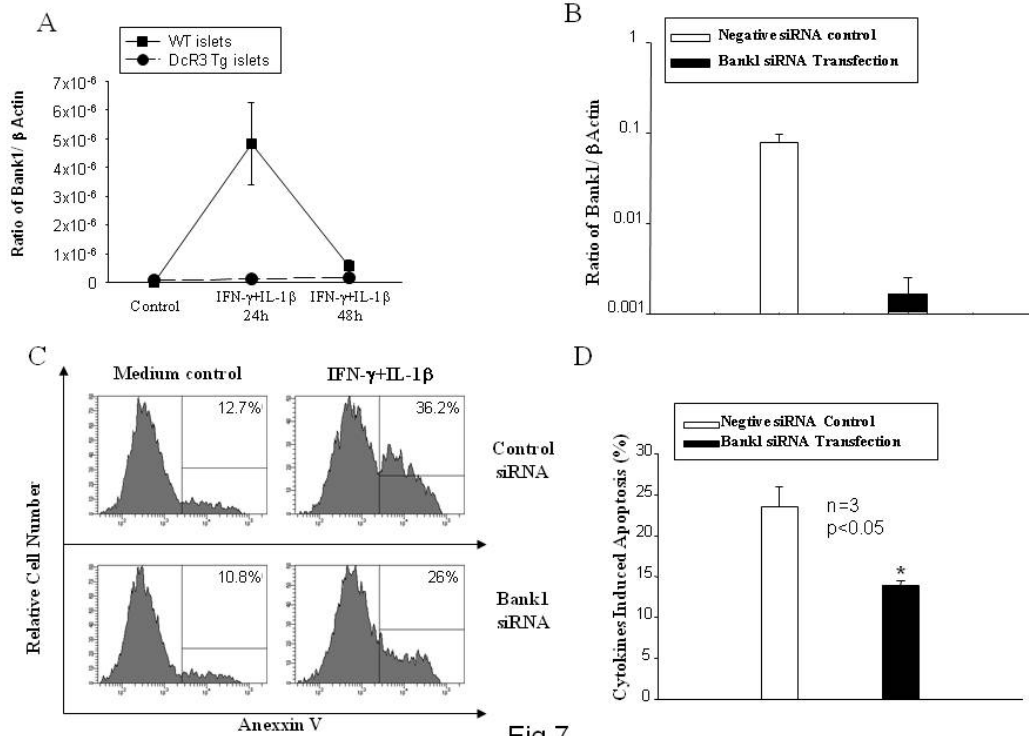


Fig.7

A. Bank1 mRNA expression in Tg and WT islets upon cytokine-stimulation

Tg and WT islets were stimulated with IFN-γ (0.5 μg/ml) plus IL-1β (0.5 ng/ml) for 24 and 48 h, and their Bank1 mRNA levels were assessed by RT/qPCR. The ratios (mean + SD) of Bank1 versus β-actin signals are shown.

B. Bank1 mRNA knockdown in Bank1 siRNA-transfected NIT-1 cells

Bank1 siRNA or control siRNA was transfected into NIT-1 insulinoma cells. Bank1 mRNA in the transfected cells was measured by RT/qPCR after 48 h. The ratios (mean + SD) of Bank1 versus β-actin signals are shown.

C and D. Bank1 knockdown in NIT-1 cells reduced cytokine-induced apoptosis

NIT-1 cells were transfected with Bank1 or control siRNA. The cells were treated with or without IFN- γ (3 μ g/ml) plus IL-1 β (3 ng/ml) 24 h after the transfection. Cell apoptosis was assessed by annexin V staining after additional 48 h with flow cytometry. A representative set of histograms are shown in panel C, and a summary of data from 3 independent experiments are illustrated in panel D. The difference between Bank1- and control siRNA-transfected cells is highly significant ($p < 0.05$, Student's t test).

III. Discussion

In our studies, we investigated the relationship between DcR3 and the pathogenesis of the SLE. Elevated serum DcR3 levels were frequently found in human SLE, and DcR3 overexpression induced a lupus-like syndrome in mouse. We also exploited the mechanisms of DcR3 in islet protection and found that LIGHT and TL1A were involved in islet apoptosis. Furthermore, we discovered that Adcyap1 and Bank1 were downstream molecules mediating DcR3's effect. Several issues related to our findings are discussed below.

1. DcR3 and the pathogenesis of SLE

The defect of Fas or FasL causes lupus like syndromes in mouse models. However, in humans, simple mutations in Fas or FasL do not lead to typical SLE, and SLE patients do not have genetic abnormality of Fas/FasL. Moreover, apoptosis of peripheral lymphocytes in SLE patients is increased both in vivo and in vitro. These conflicting findings about the relationship between Fas-mediated apoptosis signaling pathway and SLE pathogenesis are puzzling.

Here we found that DcR3, a blocker for multiple apoptosis pathways, was present at high levels in human SLE patients, and DcR3 overexpression induced a lupus-like syndrome in a mouse model. We further demonstrated that hematopoietic cell-restricted overexpression of DcR3 was sufficient

for the pathogenesis of an SLE-like disease in mice.

Our finding reveals several possible mechanisms of SLE pathogenesis involving the Fas apoptosis pathway. They are discussed as follows.

1-1 . The DcR3 overexpression in self-reactive lymphocyte interferes with their anergy.

In our investigation, we found that elevated DcR3 serum levels occur in healthy population more frequently (>2%) than the prevalence of SLE (~0.05-0.1%). We do not know whether the higher expression of DcR3 in this “healthy population” is due to undetected underlying tumors or other disease conditions, but it is not due to acute inflammation (1). Also, serum levels of DcR3 in many malignant tumor patients (1) were similar to those in SLE patients. Although autoantibodies (including anti-dsDNA antibodies) were found in such tumor patients (2), they do not have increased SLE incidence. These suggest that high level serum DcR3 alone is not sufficient for SLE pathogenesis. However, DcR3 produced by lymphoid cells seems to be more relevant to SLE pathogenesis. DcR3 is overexpressed in a variety of lymphoma cells (3) and in PBMC of SLE patients (4), SLE patients have increased incidence of lymphomas (5,6). It is possible that only high levels of DcR3 in the micro-environment around lymphocytes, especially auto-reactive lymphocytes, is responsible for breaking self-tolerance.

According to this hypothesis, DcR3 can be up-regulated in various cells and tissues due to genetic susceptibilities and environmental factors. Under most conditions, such up-regulation only causes increased risk of certain cancers. However, when this occurs in or near auto-reactive immune cells, SLE is initiated.

To take the Epstein-Barr virus (EBV) infection as an example, it has been related to increased incidence of both SLE and lymphoma (7,8). EBV also elevates DcR3 expression through a Rta-dependent pathway. It implies causality among EBV infection, DcR3 expression, SLE, and lymphoma. The EBV infection of B lymphocytes may be the initial event in the pathological process: it induces DcR3 overexpression in B lymphocytes. Elevated DcR3 will increase the risk of malignant change of EBV-infected B lymphocytes. When this infection occurs in auto-reactive B lymphocytes or near auto-reactive T cells, increased DcR3 will not only elevate the risk of lymphomas, but also boost these auto-reactive lymphocytes to break self-tolerance and trigger SLE pathogenesis.

The high local DcR3 concentration in or near auto-reactive lymphocytes interferes with self-tolerance by blocking the Fas/FasL apoptosis pathway of these cells, but the elements of the Fas pathway are not compromised during this process. Thus, the Fas signaling pathway works normally

everywhere else except in the micro-environment.

This hypothesis can explain why the apoptosis of peripheral lymphocyte increase in SLE patients. While a small proportion of auto-reactive lymphocytes are protected by an in situ high concentration of DcR3, most of DcR3 negative lymphocytes are still susceptible to apoptosis. Anti-lymphocyte autoantibodies and disordered cytokines in SLE patients will lead to increased apoptosis of those normal lymphocytes, which are not essential for SLE generation.

1-2. DcR3 impairs the clearance of apoptotic cells and increases autoantigen presentation.

It has been reported that clearance of apoptotic debris by phagocytes is impaired in SLE patients (9). Impaired removal of apoptotic cells and debris may lead to the exposure of autoantigens to the immune system. In SLE patients, apoptotic debris accumulates in germinal centers (GC) (10), bone marrow (11), and attached to the surfaces of follicular dendritic cells (FDC) (12). These exposed cell components may break self-tolerance (13). DcR3 can significantly suppress the expression of CD14, CD16 (FcγRIII), and HLA-DR on the macrophage (14). DcR3 also inhibits chemotaxis and phagocytic activity of macrophages (14, 15). Thus, clearance of apoptotic cells/debris by macrophages in the presence of

DcR3 may be compromised. This may be one of the pathogenic mechanisms of DcR3-induced SLE.

1-3 . DcR3 increases the memory T cell pool and enhances the secondary immune response to autoantigens

T cells can be divided into naïve T cells and memory T cells based on previous exposure to antigens. When naïve T cells are exposed to antigens in lymphoid tissues, some of them will differentiate into effector cells and then undergo activation-induced cell death (AICD). Others will differentiate into memory T cells, and circulate throughout the body. Memory T cells can be further categorized into central memory T cells (TCM) and effector memory T cells (TEM), according to the expression of cell surface markers. TCM circulate in peripheral blood and migrate into T-cell areas of secondary lymphoid organs (16). They display a capacity for self-renewal (17) and IL-2 production. TEM exist mainly in local tissue or peripheral blood; they can react immediately after exposure to antigens again, and produce effector cytokines like IFN- γ and IL-4. In SLE patients, the ratio of naïve T cells/ memory T cells is decreased (18,19) and the number of activated TEM is increased as well (20).

In our DcR3 Tg mouse model, we demonstrated that the DcR3 could block AICD of activated naïve T cells via inhibiting the Fas-FasL and probably

other apoptosis pathways, and probably by doing so, drives more activated cells into memory T cells, especially TEM. The increased TEM would more easily react to autoantigens in local tissue and cause severe inflammation.

1-4 . DcR3 disturbs B-cell homeostasis and tolerance.

SLE is an autoimmune disease dependent on both T- and B-cells. B cell hyperactivity has been identified as a central feature in SLE patients (21). Among B-cell populations, there is one subset called B1a cells, which expresses the T cell surface marker CD5. These cells are present in the peripheral blood and coelomic cavity, and produce low affinity IgM polyreactive antibodies, some of which can recognize autoantigens. Besides secretion of low affinity autoantibodies, B1a cells also produce high levels of IL-10, and enhance their own capacity for antigen presentation (22,23). B1a cells constitutively express FasL (24) and express Fas upon activation (25).

Expanded B1a subset is observed in both human SLE and murine lupus models (22,26). This phenomenon was also found in our DcR3 Tg mouse model. Such expansion may be related to the defect of Fas-induced apoptosis of B cells, because this expansion is also present in human ALPS, an autoimmune syndrome caused by Fas gene mutation (27). In the mouse

lupus model, autoantibody producing B1a cells have low Fas expression and resistance to anti-Fas Ab induced apoptosis (28). This implies that the Fas/FasL-induced apoptosis is important to the homeostasis and self-tolerance of B cells. DcR3 may inhibit B1a apoptosis and favor autoantibody production through blocking Fas pathway.

1-5 . DcR3 favors Th2 development

In contrast to the MRL/lpr mouse model, which inclines to Th1 responses (29), human SLE shows more Th2 responses (30). Th2 cytokines lead to B cell hyperactivity and autoantibody production in SLE (31). Our DcR3 Tg lupus mouse model also exhibited a Th2-prone immune response, including increased IL-4 secreting CD4⁺ T cells and enhanced Th2 type immunoglobulins. Such a bias is observed in human lupus as well: IgE production is elevated in DcR3 positive SLE patients.

Such an effect of DcR3 might be achieved through dendritic cells (DCs). Hsu et. al. showed that DcR3 up-regulates CD86/B7.2 expression and suppresses CD54/ICAM-1 expression in human DCs (32). DcR3-treated DCs enhance IL-4 production of naïve CD4⁺ T cells.

We have listed above several possible mechanisms by which DcR3 could trigger SLE pathogenesis. We noted that only about 50% of SLE patients

are serum DcR3 positive at a given time point. For this subgroup, DcR3 could be used as a therapeutic target. Neutralizing or inactivating DcR3 might increase elimination of auto-reactive lymphocytes and reestablish immune tolerance to self-antigens, and stop SLE progression.

It remains to be investigated whether DcR3 negative patients are negative during the full course of SLE. If so, it will indicate that DcR3 is only responsible for SLE pathogenesis in a subgroup of SLE patients. This assumption is compatible with the fact that SLE is a polygenic disease.

2. The mechanisms by which DcR3 protects pancreatic islets

Our group previously used recombinant DcR3 to protect islet grafts from PNF for the first time. Recombinant DcR3 effectively blocked Fas/FasL-mediated islets apoptosis, which was induced by multiple cytokines (33).

Thus, we employed DcR3 transgenic mouse islets in the study of PNF prevention, and its underlying mechanisms. Several points are worth discussing.

2-1. DcR3 Tg islet graft resisted PNF and presented long term normoglycemia in syngeneic diabetes mouse receiving a suboptimal dose of islets.

DcR3 Tg islets showed high expression of endogenous DcR3. In the

syngeneic islet transplantation model, all diabetic recipient mice which received only a suboptimal amount of DcR3 Tg islets reached normoglycemia in 5 days after transplantation. Half of them achieved long term normoglycemia. Compared to the recipients of wild type islets, in which none had normoglycemia after 2 weeks post-transplant, the recipients of DcR3 Tg islets performed favourably. Of note, the DcR3 Tg islets only produce minute amounts of DcR3. Our result implies that enhanced local DcR3 concentration near the islets is sufficient to protect them.

2-2. DcR3 acts through the TL1A and LIGHT pathways.

In our earlier study, we already showed that DcR3 could protect islets through blocking the Fas/FasL signal. Here we identified that this protection also the result of interference of the LIGHT and TL1A apoptosis pathway linked to DcR3. We also reported expression of LIGHT, TL1A and their receptors in pancreas islets. Although LIGHT and TL1A are not strong apoptosis inducers of islet β -cell like FasL (~15% vs. 30% inducible apoptosis), they may work synergistically with FasL to reduce β -cell survival under inflammatory conditions.

2-3. Using DNA microarray to identify downstream molecules of DcR3 action.

We compared the mRNA expression patterns of DcR3 Tg and WT islets at different time points after cytokine treatment. Twelve genes showed significant difference in their expression, especially Adcyap1 (adenylate cyclase activating polypeptide 1) and Bank1 (B-cell scaffold protein with ankyrin repeats 1). Adcyap1 is also called PACAP (pituitary adenylate cyclase-activating polypeptide). Its expression was maintained at high levels in DcR3 Tg islets, while in WT islets, its expression was reduced drastically (2 orders of magnitude) upon cytokine stimulation. According to our result, Bank1 was significantly upregulated in WT but not in Tg islets after cytokine stimulation. We overexpressed Adcyap1 or silenced Bank1 expression in NIT-1 insulinoma cells. These manipulations ameliorated the survival of NIT-1 cells upon cytokine assaults.

Base on the present studies, Adcyap1, a short secreted peptide, stimulates insulin secretion from islets (34), increases islet mass, and protect islets from STZ-triggered apoptosis (35). Moreover, the human Adcyap1 gene is located in a T2DM susceptibility locus (35). Thus, this gene is closely related to islet function and survival. Its mechanism of action is still unclear. It might affect the regulation of intracellular calcium levels in β -cells (36). Bank1 is an adaptor protein, and is also a regulator of

intracellular calcium levels (37). DcR3 may prevent β -cell calcium flux via Adcyap1 and Bank1 upon cytokine stimulation. The two newly found intermediators may become potential novel breakthrough for promoting islet survival.

The success of islet transplantation, the most promising potential cure for diabetes, is limited by the low efficacy caused by PNF. Currently, more than one donor is needed for a successful transplant. According to our studies, DcR3 is a potential protector for the islets. Adding soluble DcR3 during islet isolation and transplantation, or genetically modifying graft islets to overexpress DcR3, might increase islet resistance to apoptosis caused by inflammation during or after transplantation. This would prolong islet survival, and reduce required effective doses of islet. This could be another therapeutic advance in islet transplantation.

Conclusion and Future Perspectives

The major findings from my Ph.D. program are as follows.

- a. We discovered the DcR3 is a causative factor of SLE in a mouse model, and is overexpressed in human SLE;
- b. Serum DcR3 levels can be used a diagnostic parameter in clinical practice;
- c. DcR3 can reduce islet primary nonfunction after transplantation and such effect is mediated by Adcyap1 and Bank1 pathway in islets.

These findings can be translated into clinical application in SLE and islet transplantation patients.

Some interesting questions remain to be addressed in guture investigations.

- a. Can antagonists of DcR3 be employed to treat SLE?
- b. Can small molecule agonists or antagonists to Adcyap1 and Bank1 be developed to treat diabetes and to improve islet transplantation efficiency?

Reference

- 1 Wu Y, Han B, Sheng H, Lin M, Moore PA, Zhang J, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 2003 Jul 10;105(5):724-32.
- 2 Toubi E, Shoenfeld Y. Protective autoimmunity in cancer (review). *Oncol Rep* 2007 Jan;17(1):245-51.
- 3 Ohshima K, Haraoka S, Sugihara M, Suzumiya J, Kawasaki C, Kanda M, et al. Amplification and expression of a decoy receptor for fas ligand (DcR3) in virus (EBV or HTLV-I) associated lymphomas. *Cancer Lett* 2000 Nov 10;160(1):89-97.
- 4 Otsuki T, Tomokuni A, Sakaguchi H, Aikoh T, Matsuki T, Isozaki Y, et al. Over-expression of the decoy receptor 3 (DcR3) gene in peripheral blood mononuclear cells (PBMC) derived from silicosis patients. *Clin Exp Immunol* 2000 Feb;119(2):323-7.
- 5 Bernatsky S, Ramsey-Goldman R, Isenberg D, Rahman A, Dooley MA, Sibley J, et al. Hodgkin's lymphoma in systemic lupus erythematosus. *Rheumatology (Oxford)* 2007 May;46(5):830-2.
- 6 Bernatsky S, Ramsey-Goldman R, Clarke AE. Revisiting the issue of malignancy risk in systemic lupus erythematosus. *Curr Rheumatol Rep* 2005 Dec;7(6):476-81.
- 7 Toussirot E, Roudier J. Epstein-Barr virus in autoimmune diseases. *Best Pract Res Clin Rheumatol* 2008 Oct;22(5):883-96.
- 8 Cohen JI, Bollard CM, Khanna R, Pittaluga S. Current understanding of the role of Epstein-Barr virus in lymphomagenesis and therapeutic approaches to EBV-associated lymphomas. *Leuk Lymphoma* 2008;49 Suppl 1:27-34.
- 9 Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum* 1998 Jul;41(7):1241-50.
- 10 Baumann I, Kolowos W, Voll RE, Manger B, Gaipl U, Neuhuber WL, et al. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum* 2002 Jan;46(1):191-201.
- 11 Hepburn AL, Lampert IA, Boyle JJ, Horncastle D, Ng WF, Layton M, et al. In vivo evidence for apoptosis in the bone marrow in systemic lupus erythematosus. *Ann Rheum Dis* 2007 Aug;66(8):1106-9.
- 12 Kuenkele S, Beyer TD, Voll RE, Kalden JR, Herrmann M. Impaired clearance of apoptotic cells in systemic lupus erythematosus: challenge of T and B cell tolerance. *Curr Rheumatol Rep* 2003 Jun;5(3):175-7.
- 13 Grossmayer GE, Munoz LE, Gaipl US, Franz S, Sheriff A, Voll RE, et al. Removal of dying cells and systemic lupus erythematosus. *Mod Rheumatol* 2005;15(6):383-90.
- 14 Chang YC, Hsu TL, Lin HH, Chio CC, Chiu AW, Chen NJ, et al. Modulation of macrophage differentiation and activation by decoy receptor 3. *J Leukoc Biol* 2004 Mar;75(3):486-94.

-
- 15 Ka SM, Sytwu HK, Chang DM, Hsieh SL, Tsai PY, Chen A. Decoy receptor 3 ameliorates an autoimmune crescentic glomerulonephritis model in mice. *J Am Soc Nephrol* 2007 Sep;18(9):2473-85.
- 16 Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999 Oct 1;99(1):23-33.
- 17 Willinger T, Freeman T, Hasegawa H, McMichael AJ, Callan MF. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol* 2005 Nov 1;175(9):5895-903.
- 18 Sato K, Miyasaka N, Yamaoka K, Okuda M, Yata J, Nishioka K. Quantitative defect of CD4+2H4+ cells in systemic lupus erythematosus and Sjogren's syndrome. *Arthritis Rheum* 1987 Dec;30(12):1407-11.
- 19 Raziuddin S, Nur MA, Alwabel AA. Selective loss of the CD4+ inducers of suppressor T cell subsets (2H4+) in active systemic lupus erythematosus. *J Rheumatol* 1989 Oct;16(10):1315-9.
- 20 Watanabe T, Suzuki J, Mitsuo A, Nakano S, Tamayama Y, Katagiri A, et al. Striking alteration of some populations of T/B cells in systemic lupus erythematosus: relationship to expression of CD62L or some chemokine receptors. *Lupus* 2008;17(1):26-33.
- 21 Kind P, Lipsky PE, Sontheimer RD. Circulating T- and B-cell abnormalities in cutaneous lupus erythematosus. *J Invest Dermatol* 1986 Mar;86(3):235-9.
- 22 Mohan C, Morel L, Yang P, Wakeland EK. Accumulation of splenic B1a cells with potent antigen-presenting capability in NZM2410 lupus-prone mice. *Arthritis Rheum* 1998 Sep;41(9):1652-62.
- 23 O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* 1992 Mar;22(3):711-7.
- 24 Lundy SK, Boros DL. Fas ligand-expressing B-1a lymphocytes mediate CD4(+)-T-cell apoptosis during schistosomal infection: induction by interleukin 4 (IL-4) and IL-10. *Infect Immun* 2002 Feb;70(2):812-9.
- 25 Mandik L, Nguyen KA, Erikson J. Fas receptor expression on B-lineage cells. *Eur J Immunol* 1995 Nov;25(11):3148-54.
- 26 Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood* 2005 Jun 1;105(11):4390-8.
- 27 Poppema S, Maggio E, van den Berg A. Development of lymphoma in Autoimmune Lymphoproliferative Syndrome (ALPS) and its relationship to Fas gene mutations. *Leuk Lymphoma* 2004 Mar;45(3):423-31.
- 28 Hirose S, Yan K, Abe M, Jiang Y, Hamano Y, Tsurui H, et al. Precursor B cells for autoantibody production in genomically Fas-intact autoimmune disease are not subject to Fas-mediated immune elimination. *Proc Natl Acad Sci U S A* 1997 Aug 19;94(17):9291-5.
- 29 Takahashi S, Fossati L, Iwamoto M, Merino R, Motta R, Kobayakawa T, et al. Imbalance

towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J Clin Invest* 1996 Apr 1;97(7):1597-604.

30 Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol Res* 1999;20(2):147-61.

31 Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol* 2003 Jul;56(7):481-90.

32 Hsu TL, Chang YC, Chen SJ, Liu YJ, Chiu AW, Chio CC, et al. Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J Immunol* 2002 May 15;168(10):4846-53.

33 Wu Y, Han B, Luo H, Roduit R, Salcedo TW, Moore PA, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* 2003 Sep;52(9):2279-86.

34 Filipsson K, Kvist-Reimer M, Ahren B. The neuropeptide pituitary adenylate cyclase-activating polypeptide and islet function. *Diabetes* 2001 Sep;50(9):1959-69.

35 Yamamoto K, Hashimoto H, Tomimoto S, Shintani N, Miyazaki J, Tashiro F, et al. Overexpression of PACAP in transgenic mouse pancreatic beta-cells enhances insulin secretion and ameliorates streptozotocin-induced diabetes. *Diabetes* 2003 May;52(5):1155-62.

36 Yanagida K, Yaekura K, Arima T, Yada T. Glucose-insensitivity induced by Ca²⁺ toxicity in islet beta-cells and its prevention by PACAP. *Peptides* 2002 Jan;23(1):135-42.

37 Yokoyama K, Su IH, Tezuka T, Yasuda T, Mikoshiba K, Tarakhovsky A, et al. BANK regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP(3) receptor. *EMBO J* 2002 Jan 15;21(1-2):83-92.