Dimeric MHC-peptides inserted into an immunoglobulin scaffold as new immunotherapeutic agents

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Abstract

The interactions of the T cell receptor (TCR) with cognate MHC-peptide and co-stimulatory molecules expressed at surface of antigen presenting cells (APC) leads to activation or tolerance of T cells. The development of molecular biological tools allowed for the preparation of soluble MHC-peptide molecules as surrogate for the APC. A decade ago a monomeric class II MHC molecule in which the peptide was covalently linked to β-chain of class II molecule was generated. This type of molecule had a low-binding affinity and did not cause the multimerization of TCR. The requirement of multimerization of TCR led to development of a new class of reagents, chimeric peptides covalently linked to MHC that was dimerized *via* Fc fragment of an immunoglobulin and linked to 3′ end of the β-chain of MHC class II molecule. These soluble dimerized MHC-peptide chimeric molecules display high affinity for the TCR and caused multimerization of TCR without processing by an APC. Because dimeric molecules are devoid of co-stimulatory molecules interacting with CD28, a second signal, they induce anergy rather the activation of T cells. In this review, we compare the human and murine dimerized MHC class II-peptides and their effect on CD4$^+$ T cells, particularly the generation of T regulatory cells, which make these chimeric molecules an appealing approach for the treatment of autoimmune diseases.

Keywords: dimeric class II-peptide molecules ● peptides derived from auto-antigens ● CD4 T cells ● Tr1 regulatory T cells ● interleukin-10

Introduction

Approaches to determine the frequency of antigen-specific B cells were developed several decades ago and were based on the ability of Ig receptors of a clone of naïve B cells to bind labelled antigens [1] or to score B cells producing specific antibodies in Jerne' plaque forming assay. Such approaches were useless for studying the frequency of antigen-specific T cells because while B cells recognize epitopes of the native antigen, the T cells binds only peptides derived from the processing of native antigens by the APC and the presentation to T cells in association with MHC gene products: class II for CD4$^+$ [2], class I MHC for CD8$^+$ [3] or glycolipids in association with CD1 in the case of NKT cells [4]. The first

attempt to determine the frequency of antigen-specific T cells by flow cytometry was carried out using solubilized MHC molecules from the membranes of APC [5] or recombinant MHC molecules loaded with peptide *in vitro* [6]. Abastado *et al.* [7] produced soluble single-chain class I molecule by fusing the extracellular domain of either a murine or human class I molecule to β-microglobulin which were then loaded with peptides. These molecules stimulated antigen-specific T cell hybridomas to secrete IL-2 and to develop cytotoxic lymphocyte (CTL) activity. Kozono *et al.* [8] generated a monomeric class II molecule in which a peptide derived from moth cytochrome *c* (MCC) or ovalbumin (OVA)

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was linked to the N-terminus of their β-chain. The transmembrane moiety of both α- and β-chains were deleted to allow the secretion of monomeric class II peptides. These molecules induced the production of IL-2 by T cell hybridomas bearing TCR specific for MCC or OVA peptides, respectively. However, the monovalent recombinant MHC-peptide molecules exhibited an intrinsic low affinity for TCR because the peptide was non-covalently bound to the antigen-binding groove of MHC molecules [9].

The multimerization of monovalent MHC-peptides provided a more efficient and accurate method to determine the frequency of antigen-specific T cells. Altman *et al.* [10] had made a real breakthrough in the development of recombinant-peptide-MHC tetramers as a tool to measure the frequency of antigen-specific T cells. In this attempt, the human HLA-A2 heavy chain expressing the BirA-dependent biotinylation site at the carboxyl end was tetramerized with streptavidin and was successfully used to score by fluorometry low-frequency HIV-specific T cells in HIV-infected patients. Ensuing years have seen an impressive growth of numbers of MHC class I heavy chain-peptides or β2-microglobulin-peptide tetramers used to determine the frequency of antigen-specific CD8^{+} T cells [11,12]. Tetramers made of MHC class II-self peptides or peptides derived from foreign antigens were used to score auto-reactive or T cells specific for foreign or tumour-specific antigens [13,14]. In addition, α-GalCer-CD1d tetramer was used to determine the frequency of NKT cells in human peripheral blood and in lymphoid organs of various animal species [15]. A variety of expression systems have been used to prepare MHC-peptide tetramers to overcome the inherently weak MHC-peptide TCR interaction and increase the lifetime of this interaction in order of seconds [16]. Moreover, the tetramers were never to have been used *in vivo* as immuno-modulatory agents because of a low degree of solubility, fast clearance by phagocytic systems and eventually side effects.

The progress in molecular engineering of recombinant proteins opened the door for designing novel platforms endowed with immuno-modulatory properties and led to the development of new categories of molecules, which are dimerized MHC-peptide on an immunoglobulin scaffold with the antigenic peptide covalently bound *via* a linker to β-chain of class II MHC molecule. The dimerization was accomplished by fusion of β-chain of class II molecule to a tailored immunoglobulin sequence encoding a hinge region, the CH2 and CH3 domains of Fc fragment of murine or human IgG. The disulfide bonds between two Fc fragments like in immunoglobulin molecules allowed for generation of stable and soluble dimeric molecules as surrogates of MHC-peptide molecule expressed at surface of APC [17,18]. To unify the naming of these molecules containing a variety of peptides and MHC alleles, in this review we will refer to these molecules as DEF (dimer I-E-Fc), which was the first abbreviated name of the first dimeric MHC molecule created in 1997 [17]. Figure 1 presents schematically the structure of chimeric protein made by the engineering of an MHC-peptide-Ig-dimeric molecule.

In contrast to monomer-MHC-peptide molecules that exhibit a low affinity for TCR, for, for example I-E[k]-MCC peptide is weak, with K_d's in the range of 30–90 μmol [19], DEF molecules
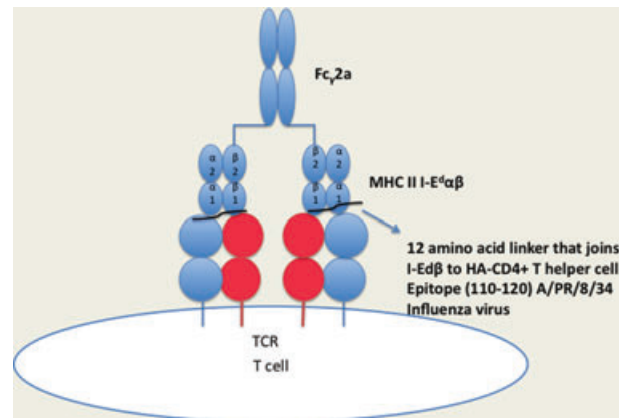

Fig. 1 DEF-HA haemaglutinin (110-120) peptide of PR8 influenza virus covalently linked at the N-terminus of I-E[d] β-chain and monomeric I-E[d] αβ complex was dimerized *via* Fc portion of murine IgG2a The genes encoding the I-E[d] and Ig-Fcg2a chain fragments were obtained by RT-PCR from total RNA isolated from 2PK3 B lymphoma cell line and 14-4-4 hybridoma cells, respectively. The gene encoding the I-E[d] a chain fragment was obtained by PCR and a pair of oligonucleotides were used for retrotranscription of I-E[d] and IgG2a mRNA, respectively. Pairs of oligonucleotides were used for PCR amplification of the leader and the first four codons of β1domain of the I-E[d] β gene (amino acids-26-14). The sequence encoding for the 11 amino acids of HA110-120 peptide and five amino acids of peptide linker containing a *Bam*HI site was introduced at the 3′ end of the oligonucleotide I-E[d] β1-R. Sequence encoding for 12 amino acids of peptide linker containing a *Bam*HI site was included at the 5′ end of I-E[d]β2-F. The sequence encoding for the amino acids 228–232 of the Fcg2a hinge region (containing a natural *Apa*I site) was included at the 3′ end of I-E[d]β2. Pairs of oligonucleotides were used for amplification of the sequence encoding for the secreted hinge, CH2 and CH3 of the IgG2a gene (amino acids 228–478). Gene fragments obtained by digestion with restriction enzymes [17].

exhibit an increased binding affinity to TCR and may exhibit immuno-modulatory properties by virtue of cross linking of TCR and interacting with T cell major co-receptors. The activation of T cells requires not only cognate recognition of MHC-peptide complex but also interaction of co-stimulatory molecules (CD28 with B7, ICOS, CTLA4) and the binding of CD4 to the MHC molecule expressed on the surface of APC [20,21,24]. A model of interaction of CD4^{+} T cells and APC is presented in Figure 2.

DEF molecules containing peptides derived from human or mouse auto-antigens and MHC class II gene products that were generated by various laboratories, their immuno-modulatory properties were thoroughly studied and they are listed as follows:

- Human DEF-MBP: Myelin basic protein (MBP)85-99 peptide was covalently linked to human DR2 β-chain. Leucine zipper domain of Fos and c-Jun was attached to both DR chains to facilitate the dimerization and Fc fragment of mouse IgG2a was placed in frame to the 3′ end of the DR-fos chain (Fig. 3A) [23].
- Human DEF-GAD65: DRB1*0401 with covalently linked human GAD65-271-285 peptide and dimerized through human Ig-Fcγ1 (Fig. 3B) [22].
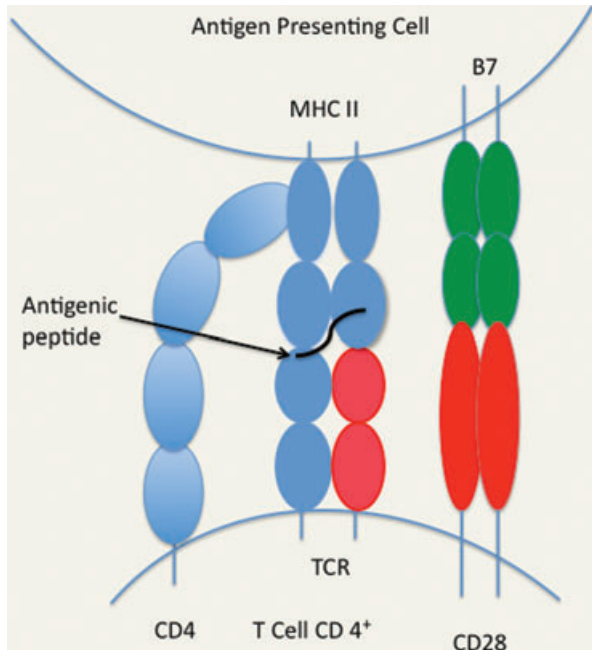
Fig. 2 Presentation of antigen to CD4$^+$ T$_{helper}$ cell. Diagram shows some of the molecules involved in the interaction between the CD4$^+$ T cell and the antigen-presenting cell (APC). The antigen a peptide is bound in a groove on the outer surface of a class II MHC glycoprotein. The MHC class II are formed from two α subunits approximately 34Kd and two β subunits approximately 29Kd. The α1 and β1 subunits contain the antigen-binding region. The MHCII binds the antigen by hydrogen bonding. The co-stimulatory molecule B7 on the APC binds to the CD28 receptor on the T cell to cause optimal clonal expansion of the T cells. The coreceptor CD4 D-1 domain binds to the β2 constant region of MHC.

Several murine DEF containing peptide derived from foreign or self-antigens were alsogenerated:

- DEF-HA: Haemagglutinin110-120 peptide of PR8 influenza virus covalently linked at the N-terminus of I-E^d β-chain and monomeric I-E^d αβ complex was dimerized *via* Fc portion of murine IgG2a (Fig. 1) [17].
- DEF MCC: Moth cytochrome *c* derived peptide linked to I-E^k and dimerized in murine immunoglobulin scaffold [24].
- NOD DEF-mimotope: Peptide mimotope1040-31 was covalently linked to I-A^{g7} β-chain and fused with Fc fragment of murine IgG2a (Fig. 4A) [25].
- DEF-interphotoreceptor retinoid peptide: [26] linked to β-chain of MHC class II I-E^r and fused with Ig^κ chain (Fig. 4B).
- NOD-DEF-GAD65: GAD65-217-230 peptide [27] was covalently linked at the N terminus of I-A^{g7} β-chain and dimerized through the mouse Fcγ2a domain.

All DEF molecules generated in various laboratories were sequenced showing that various segments composing DEF molecules were in frame and had not mutated. Table 1 illustrates the structure of DEF molecules, the functional integrity of MHC-peptide complex and Fc fragment of various DEF molecules made in various laboratories.
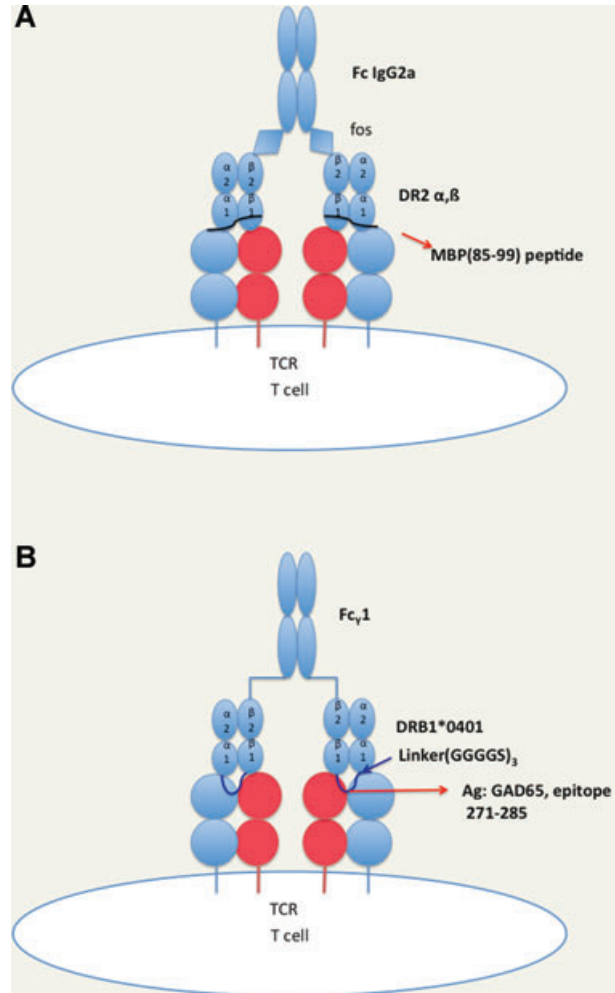

Fig. 3 (**A**) Bivalent HLA-DR peptide-mylein basic protein (MBP85-99) The Fc segment is a murine IgG2a spanning the hinge and CH2, CH3 domains were fused to DRα-Fos segment. DRα and DRβ where attached in frame to Fos and Jun, respectively. This construct was produced by overlapping PCR. The MBP(85-99) peptide was covalently linked to the N terminal of DRβ by a 16 amino acid linker [23]. (**B**) DEF-GAD65-DRB1*0401 with covalently linked human GAD65 (271-285) peptide and dimerized through human Ig-Fcg1 (hDEF-GAD65). The GAD-DEF chimera was made of the extracellular domains of HLA-DR*0401 dimerized through human Ig-Fcγ1. The human GAD65(271-285) peptide was covalently linked at the N terminus of the B1*0401 chain. Genes encoding for HLADRB1*0401 and hinge-Fcγ1 were obtained by RT-PCR from human IgG1 hybridoma, ZM-1 (ATCC) [22].

In vitro activation of antigen specific T cells by DEF containing peptides derived from foreign antigens

In the late 1990s we generated DEF-HA molecule and demonstrated its ability to activate *in vitro* and *in vivo* HA110-120
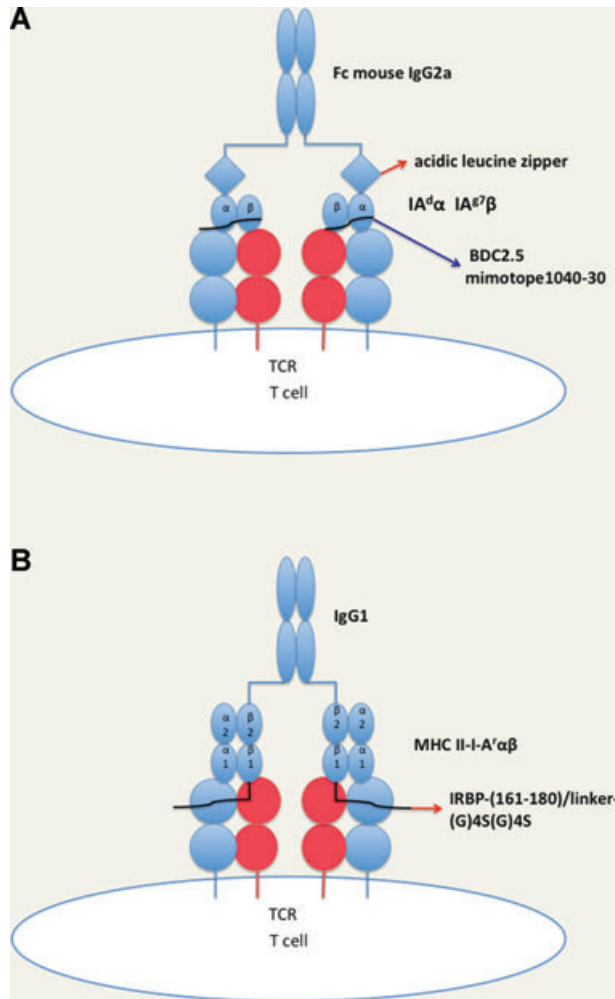
Fig. 4 (**A**) NOD DEF-mimotope. The construct encoding I-A^{g7} β-chains fused to acidic/basic leucine zipper sequences with the I-A^{d} α and further was fused to the Fc portion of mouse IgG2a. The template was used for introducing DNA sequences encoding the BDC2.5 mimotope peptide 1040-31 using overlapping PCR. For the empty I-A^{g7} dimer, the cDNA encoding the signal peptide and extracellular domain of I-A^{g7} β were cloned by PCR from a plasmid construct and inserted upstream of the basic leucine zipper [25]. (**B**) DEF-interphotoreceptor retinoid peptide (IRBP3) Peptide 161-180 encodes a dominant pathogenic IRBP epitope for the H-2^{r} haplotype. The MHC class II I-A^{r}-α and I-E^{r}-β cDNAs were obtained RT-PCR of mRNA derived from splenocytes of B10.RIII mice. The truncated versions of class II I-A^{r} and gene was subsequently generated by PCR using primers designed to introduce a cloning site. The gene was truncated just before the transmembrane region and nucleotide sequences encoding the chimeric Ig protein linkers were introduced and the mature mouse IgG1 heavy chain constant regions and GSL at the 3′ end of the class II I-A^{r}-α and constant region of Igk light chain. The DNA sequence for the uveitogenic peptide 161-180 linked to a flexible spacer (G)4S(G)4S was inserted between the native chain leader and the IA^{r}-1 domain (first external domain of the mature IA-β protein) by combination of an overlap extension PCR and cassette-cloning strategy [26].

specific T cells [17]. An important question that was addressed in further studies dealt with the biological mechanism of DEF–TCR interactions leading to the activation of T cells. Three models were proposed for the activation of T cells *via* TCR: the aggregation, dimerization or multimerization of TCR [28,29,30]. We found that DEF–HA was 88 times more potent that HA peptide to stimulate HA-specific T cells and did not require the presentation *via* the Fc receptor nor processing by APC. Indeed, the pre-treatment of APC with anti-Fc receptor monoclonal antibody (MAb) or chloroquine had no effect on DEF–HA induced proliferation. It is noteworthy to point out that DEF was able not only to stimulate *in vitro* T cell activation but also to prime *in vivo* HA110-120 specific T cells, which proliferated vigorously upon *in vitro* exposure to peptide. This was probably related to increased density of TCR on activated T cells and to higher avidity of DEF–HA compared to monomeric MHC-peptide or peptide itself [31]. It has been well documented that the T cells bind *via* TCR, not only to MHC-peptide complexes present on the surface of APC but also to co-receptors such as CD4 [32,33], and CD28, which binds to B7, ICOS and CTLA4 [20]. Although the binding of CD4 to MHC class II molecules has an extremely low affinity $<10^{-4}$ M^{-1} [33], we observed an early phosphorylation of p56lck and ZAP-70, occurring at 5 min. after incubation of T cells with DEF–HA. *In vitro* activation of HA110-120 specific T cell by DEF led to increased synthesis of IL-2, IL-4 and IL-10 but not of IFN-γ. This corresponds to lack of phosphorylation of STAT4 and hyperphosphorylation of STAT6 [34]. Thus, DEF–HA molecule induced the proliferation of HA110-120 specific T cells and polarized their differentiation towards TH2 and eventually Tr1 cells, based on the pattern of lymphokine secretion. In another study, Hamad [24] showed a potent T cell activation of T cell hybridomas bearing a TCR recognizing a peptide derived from MCC presented to the T cell by an Ig-I-E^{k} chimera. In this study, the DEF-like molecule I-E^{k}-MCC peptide in an immunoglobulin scaffold was 5- to 10-fold more potent than bivalent anti-CD3 antibody in stimulating T cell hybridomas with respect to magnitude of proliferation and IL-2 production. In this case, the DEF–MCC–CD4 interaction enhanced the activation of T cells but did not contribute to stability of its binding to TCR.

In vitro activation of auto-reactive T cells by DEF containing peptides derived from self-antigens

The DEF-like molecule containing peptides derived from self-antigens may represent an attractive approach to determine the frequency and activation or unresponsiveness of auto-reactive T cells as well as the possibility for the development of new category of therapeutics for autoimmune diseases. CD4^{+} T cells play a key role in autoimmune diseases either initiating the destruction of

Table 1 Functional integrity of DEF molecules

Dimer	MHC	Ingerity of dimeric MHC-peptides				Intergrity of Fc		
		Peptide inserted	Staining T cells	Proliferation of T cells	Secretion of interleukins	Effect of anti-CD4	Binding to complement	Binding to Fc receptor
DEF- HA	I-E^d	HA 110-120	+	+	IL-4, IL-10	Inhibition	Yes	Yes
DEF- GAD65	I-A^{g7}	217–230	+	+	IL-10	Inhibition	Yes	Yes
DEF-λ	I-A^{g7}	112–124	−*	−*	ND†	ND	ND	ND
DEF- mimotope	I-A^{g7}	Decapeptide	+	+	IL-4, IL-10	ND	ND	ND
DEF- lysozyme	I-A^{g7}	11–25	−	−	INF-γ	ND	ND	ND

*Does not induced the proliferation of diabetogenic T cells.
†ND: not done.

cells bearing auto-antigens in T cell mediated diseases or providing help to B cells producing pathogenic autoantibodies in organ-specific autoimmune diseases [35].

Multiple sclerosis is a human autoimmune disease mediated by T cells, which recognizes peptides derived from myelin basic protein (MBP) or proteolipid protein-1 in association with class II MHC such as HLA-DR2 or HLA-DQ1 in humans [36]. The DEF-MBP chimeric protein was formed from the MBP85-99 peptide that was covalently linked to DR2 β-chain and the dimerization was carried out by fusion with the Fc fragment of mouse IgG2a [23,37]. The binding between soluble TCR of a clone specific for MBP peptide and DEF–MBP molecule, examined by surface plasmon resonance, was specific with a half-time for dissociation of 2.9 and 43.1 min., respectively. DEF–MBP stimulated MBP-specific T cell clone at >100-fold lower concentration than anti-CD3 antibodies. The stimulation was specific because DEF–MBP did not stimulate T cell clones specific for peptides derived from tetanus toxin (830–843) or desmoglein (190–204) associated with DR2 (Fig. 3A).

Type-1-diabetes (T1D) is an autoimmune disease characterized by hyperglycaemia resulting from pancreatic β-islet cells being targeted and destroyed, leading to the loss of insulin production. Examinations of the islet region in the pancreatic tissue from diabetic patients revealed the predominance of CD4$^+$ and CD8$^+$ T cells along with the presence of B cells and macrophages in the infiltrates. It is believed that IFN-γ-producing Th1 cells are important in the initiation of inflammation in the islet region and ultimately the destruction of β-islet cells is achieved by CD8$^+$ T cells [38,39]. In contrast, Th2 cells may be protective in T1D because transgenic non-obese diabetic (Tg NOD) mice expressing IL-4 in β-islet cells under rat insulin promoter (RIP) remain euglycaemic. In humans HLA-DRB1*0401, DRB1*0301 DQB180302 and DQA1*0301 confer high-risk susceptibility indicating that the peptides derived from pancreatic antigens must be associated with class II antigens to be recognized by diabetogenic T cells [41]. We recently constructed a human DEF-molecule composed of DRB1*0401 with covalently linked human GAD65-271-285 and dimerized through human IgG1 (hDEF-GAD65) [22] (Fig. 3B). The human DEF–GAD65 molecule was used to score the frequency of GAD-specific T cells in the peripheral blood of diabetic patients and its capacity to activate

in vitro GAD65-specific T cells [22]. FACS analysis using hDEF-GAD65 and anti-CD3 antibody showed that GAD-specific T cells were detected in the blood of 19 of 30 patients with overt 1–17 years of T1D, and in six of seven their relatives. However, the GAD65-specific T cells were not detected in seven patients at the clinical onset of T1D suggesting that they migrated to the pancreas. Lack of detection of GAD65-specific T cell in the blood of some patients might be related to T cells displaying low avidity TCR in the blood because they did not encounter the antigen in the pancreas. It is known that T cells exposed to antigens exhibit 20- to 50-fold increase of the avidity due to avidity maturation [42,43]. Indeed, a sharp increase of number of cells binding hDEF-GAD65 was observed upon a short in vitro stimulation with GAD65 peptide of peripheral blood cells and reanalysed by FACS. Using this method we found 40–600/10^5, as the frequency of high avidity GAD65-specific T cells in T1D patients.

DEF–GAD65 stimulates in vitro secretion of IL-10 but not IL-2, INF-γ, IL-4, TGF-β or TNF-α by peripheral blood mononuclear cells (PBMC) from patients with T1D, first-degree relative and unrelated controls expressing DRB1*0401 molecules. hDEF-GAD65 stimulation of CD4$^+$ T cells from diabetic patients induces an IL-10 dominant response. IL-10 secreted by hDEF-GAD65 stimulated T cells from diabetic patients exhibited a suppressive effect on tetanus toxoid specific T cells when T cells from PBMC of diabetic patients are stimulated with hDEF-GAD65 and were co-incubated with tetanus toxoid stimulated T cells. These observations strongly suggest that hDEF-GAD65 stimulate T regulatory-Tr1 cells, which may regulate auto-reactive GAD65 diabetogenic T cells [22].

A DEF like molecule was also used to determine the frequency of CD4$^+$ T cell mediated experimental uveitis (EV) in mice, which is a model for human autoimmune uveitis. EV maybe induced in B10-RIII mice by injection of interphotoreceptor retinoid binding protein bearing auto-immunogenic dominant epitope161-180. A DEF like molecule composed of peptide (161-180) covalently linked to I-A^r was dimerized by addition of mouse Ig^k chain [25]. This molecule stained 90% of a peptide-specific T cell line and increased its proliferation in presence of anti-CD28 antibody.

In summary, DEF molecules containing epitopes derived from foreign antigens or self-antigens were successfully used to

determine the frequency of naïve antigen-specific T cells and they exhibit immuno-modulatory properties *in vitro*.

In vivo effect of DEF chimeras on prevention and reversal of autoimmune diseases

In vivo effect of DEF-like molecules was studied mainly in mice spontaneously developing T1D caused by autoreactive T cells specific for various β islet cells pancreatic antigens. The presence of Th1 cells specific for GAD65 present in pancreas correlates with the occurrence of insulitis [44] and this disease is transferred by stem cells, splenic cells and most importantly by T cell clones isolated from islets of NOD mice [44–47]. The effect of DEF molecules in T1D was tested in double transgenic (dTg) and NOD mice. The major difference between these two animal models consists in the monoclonal nature of diabetogenic T cells in dTg mice and polyclonal nature in NOD mice.

The first model consists of dTg mice obtained by crossing of TCR-HA, Tg mice expressing the 14.3 TCR specific for the haemagglutinin110-120 CD4$^+$ immuno-dominant epitope of A/PR/8/34 influenza virus with RIP-HA-Tg mice expressing the HA protein of A/PR/8/34 influenza virus under RIP in the pancreatic β cells [48]. The dTg mice developed a juvenile T1D [49]. The DEF–HA molecule was used to study *in vivo* effects on T1D in these dTg mice. Treatment of pre-diabetic dTg mice with DEF–HA prevented diabetes and restored normo-glycaemia in mice with recent onset of diabetes. DEF–HA induced protection resulted from anergy of autoreactive CD4$^+$ T cells and the stimulation of Tr1 regulatory cell secreting IL-10 cytokine. The anergy induced by DEF was antigen specific and associated with negative regulation of ZAP-70 and p56^{lck} kinases, which are critical in early signalling events of T cell activation. DEF-induced anergy was reversible by IL-2. The concept of DEF–HA effect on Tr1 was supported by several findings including high IL-10 secretion of pancreatic T cells and by inhibition of suppressive effect on diabetogenic CD4$^+$ T cells by anti-IL-10 antibodies. DEF stimulation enhances the expression of CD62L, which plays an important role in the migration of T cells including Tr1 cells into pancreas where they exert the suppressive effect on diabetogenic cells [50].

A number of studies have attempted to reverse autoimmune destruction of insulin secreting β islet cells in newly diagnosed patients with the use of non-specific immuno-suppression. These efforts have only been partially successful and there are substantial risks associated with non-specific immuno-suppression. With the failure of prevention, exogenous insulin administration is currently the most common and effective therapy in T1D. The generation of syngeneic insulin-producing β islet cells from embryonic stem cells, haematopoietic stem cells, pancreatic ductal cells and other stem cell sources offers the possibility of obtaining sufficient numbers of syngeneic β islet cells for treatment of T1D. However,

despite a large number of islets used for transplantation, only as few as <30% survival long-term grafts [51]. Apparently, the early massive death of transplanted islets is mostly due to the re-activation of autoreactive T cells. Because we showed that DEF–HA polarized the differentiation of T cells *versus* Th2 phenotype and activate Tr1 cells suppressing the diabetogenic T cells we tested the ability of DEF–HA in protecting transplanted syngeneic islets against the re-emerging autoimmune response. Purified islet cells were transplanted under the renal capsule of overtly diabetic dTg mice and mice were injected daily with 2 μg of DEF starting 5 days before transplantation. The recurrent administration of DEF to diabetic mice transplanted with pancreatic islets cells resulted in prolonged graft survival. Of the 12 mice in this group, seven mice showed reversal from hyperglycaemia and a prolonged euglycaemia for up to 70 days and two mice exhibited a reversal of hyperglycaemia for 20 and 65 days after the transplant. All control mice consisting of diabetic mice transplanted with the same number of islets but non-treated with DEF became diabetic a few weeks after transplantation (Figs. 5A). The data illustrated in Figure 5B shows the persistence of synthesis of insulin in islets transplanted into the capsular region of kidney. The long-lasting euglycaemic status observed in mice transplanted and treated with DEF was attributed to the long-term survival of grafts, because a relapse in hyperglycaemia occurred shortly (1–2 days) after nephrectomy of the kidney bearing islets (Fig. 5C). To determine the mechanism by which DEF prolonged the graft survival, we determined the phenotype and Th1 and Th2 cytokines pattern of splenocytes isolated from mice transplanted with β islets cells and treated with DEF. Although splenic TCR-HA CD4$^+$ T cells from both DEF-treated and non-treated mice stimulated with antigenic HA110-120 peptide *in vitro* showed comparable proliferative capacities, the CD4$^+$ T cells from DEF-treated islet recipients produced higher levels of IL-4 and IL-10 compared with those from untreated control recipients [52]. These results demonstrated that DEF–HA prolonged the survival of grafted islets a phenomenon related to polarization of T cells response towards Th2 and Tr1 endowed with capacity of suppressing Th1 diabetogenic T cells.

An important question addressed in the studies of DEF it was related to the effect on diabetes in NOD mice, an inbred strain that represent a T1D animal model resembling to human T1D except for the late onset in adult life. Among various pancreatic antigens, GAD65 may be the earliest initiator of disease development, because GAD65-specific T cell responses can be detected as early as 4 weeks of age in the NOD mouse [44]. The unusual structure of I-A^{g7} may well contribute to the priming of autoreactive CD4$^+$ T cells in NOD mice since it exhibits low affinity binding to several foreign and self-derived peptides that do not usually bind to other MHC II molecules [53]. Low affinity binding self-peptides may also favour the survival of autoreactive CD4$^+$ T cells instead of being eliminated in thymus by negative selection [54].

Two DEF-like molecules containing peptide covalently linked with β-chain of I-A^{g7} were previously generated and tested into NOD mice or NOD BDC2.5 Tg mice. BDC2.5 TCR Tg mice were generated using the TCR from a T cell clone isolated from NOD mice. This TCR is specific for an antigen borne by only NOD
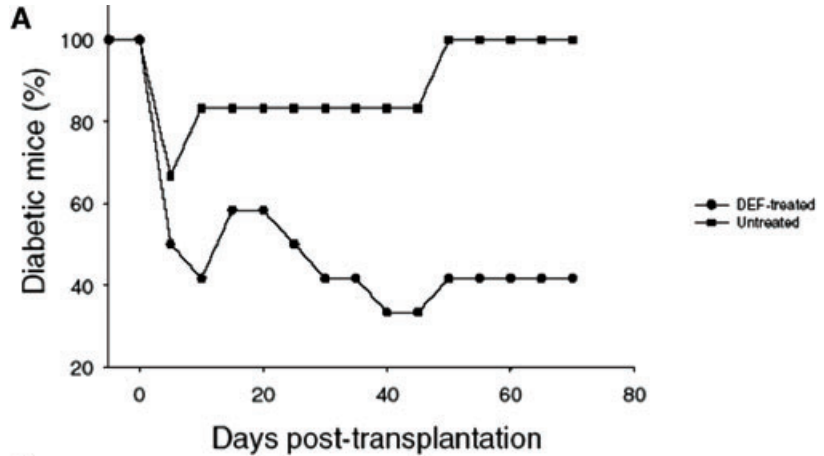
Fig. 5 (**A**) Transplanted islet survival of DEF-treated mice: incidence of diabetes. The effect of DEF on glycaemia levels and islet survival in islet transplanted mice. Mice where transplanted with synergistic islets and given 2 μg DEF daily (**A**) or untreated (**B**). The data expressed is the incidence of diabetes in treated and untreated mice during the 70-day trial. This figure is published with the permission of Wolters Kluwer, Lippincott Publishing [51]. (**B**) Histology of grafted islets of mice treated with DEF. Nephrectomized kidneys with engrafted islet cells under the capsular region stained with haematoxylin–eosin (left). Anti-insulin antibody shows presence of insulin secreting cells in the kidney capsular region. This figure is published with the permission of Wolters Kluwer, Lippincott Publishing [51]. (**C**) Glycaemia in mice prior and following nephrectomy. Five mice were grafted with islets under the kidney capsule from donors treated with DEF and nephrectomized 3 days after grafting the islets. Glycaemia levels were measured during a period of 60 days. Four of five became hyperglycemic 3 days after nephrectomy and one 55 days post nephrectomy. This figure is published with the permission of Wolters Kluwer, Lippincott Publishing [51].
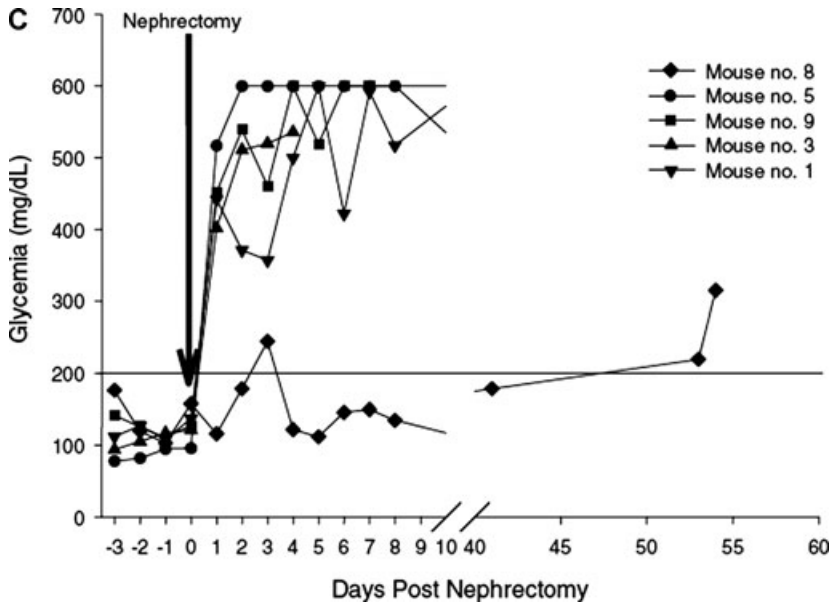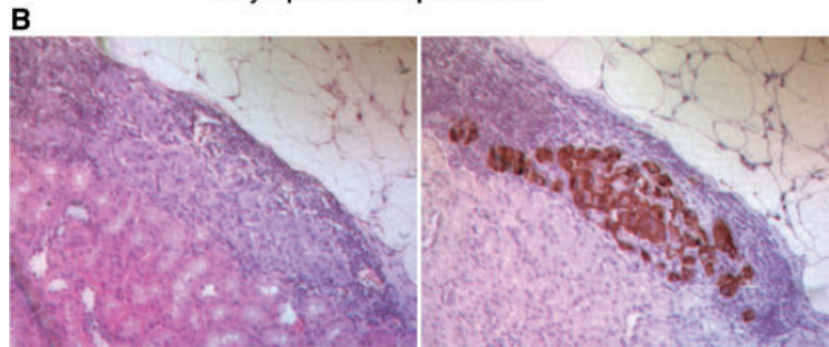
pancreatic β-cells and recognized in association with I-A^{g7} [55]. By screening peptide libraries, a decapeptide was identified that was able to stimulate the T cells from BDC2.5 Tg and NOD BDC2.5 Tg mice [25]. In spite that NOD BDC2.5 Tg mice exhibit a very extensive cellular infiltration of islets they are free of overt diabetes for a long period of time. A DEF-like molecule composed of I-A^{g7} β-chain fused with Fc fragment of murine IgG2a and the peptide mimotope1040-31 was introduced at 5′ end of I-A^{g7} β-chain [25]. *In vitro* treatment of T cells from BDC2.5 Tg mice with the mimotope-peptide transferred into TCR-deficient NOD mice

Table 2 Comparative effect of dimer Class II peptides on prevention and reversal of type I diabetes

A. Prevention of diabetes					
Mice	**Age**	**Treatment**	**Duration of treatment**	**Euglycaemic mice**	**Mediated by**
dTg	15 days	2 μg/5 days	4.5 months	10/12	Tr1-IL-10
NOD	15 days	5 μg/week	20 weeks	6/8	Tr1- IL-10
BDC2.5*	Adults	10 mg	Once	6/6[†]	Tr1- IL-10
B. Reversal of diabetes					
Dimeric molecules	**Mice**	**Glycaemia mg/Dl**	**Treatment**	**Duration of euglycaemia**	
DEF-HA 110-120	dTg	275± 44	2 μg	8 days in 9 of 17 mice	
DEF-GAD 217230	NOD	250–300	5 μg	5 days in 3 of 7 mice	
DEF-GAD 217-230	NOD	250–300	5 μg × 4	48 days in 8 of 12 mice	

*Diabetes was induced by *in vitro* injection of activated T cells.
[†]Mice were euglycaemic only 21 days after injection of DEF.

caused rapid development (6–8 days after transfer) of T1D. In sharp contrast, a significant delay of 21 days of occurrence of hyperglycaemia was observed when the recipient mice were injected once with DEF-like molecule. The effect was specific for diabetogenic T cells because the injection of peptide, empty I-A[g7] dimer or a DEF-like molecule containing a peptide derived from HEL did not prevent the early occurrence of overt T1D. Such treatment generated the IL-10 producing Tr1 cells and pre-treatment with anti-IL-10R antibody reversed the tolerogenic effect of dimer therapy. The FACS analysis of mimotope-reactive CD4^{+} T cells in wild NOD mice showed that their frequency was very low (0.04%) and was significantly increased in mice injected with mimotope peptide (0.34%). Low frequency of mimotope-reactive CD4^{+} T cells in wild NOD mice explain failure to prevent or reverse the T1D in NOD mice treated with DEF-like molecule [25].

Unlike the transgenic BDC2.5 Tg animal model in which monoclonal population of auto-reactive T cells mediates the T1D, polyclonal auto-reactive T cells cause T1D in wild NOD mice as well as in humans. Therefore, it was important to evaluate a potential therapeutic effect of DEF molecule on T cells specific for a dominant islet antigen such as GAD65 and the bystander effect on T cells specific for other minor islets antigens. GAD65 bears several epitopes, some recognized by diabetogenic CD4 or CD8 cells and others able to stimulate Treg cell. For this reason we created a new molecule called herein NOD–DEF. The GAD65-217-230 is known to activate Treg cells and as control we created a DEF containing phage λ12-24 peptide. The peptide was covalently linked at the N terminus of I-A[g7] β-chain and dimerized through the mouse Fc-IgG2a domain [27]. The preventive effect of dimeric therapy was studied in a group of 120-day-old NOD normo-glycaemia females injected i.v. with four doses of 2 μg of DEF–GAD65 ($n = 7$) or DEF-λ control chimera ($n = 7$). All mice treated with DEF–GAD65 chimera had a significant delay in hyperglycaemia onset of 72 days after the last injection, one mouse injected with DEF–GAD65 developed hyperglycaemia 82 days later and never returned to a normoglycaemic status. Two other mice in this group did not develop hyperglycaemia for 140 days so far. In contrast, mice injected with DEF-λ control were not prevented from hyperglycaemia development. Furthermore, in the reversal experiments, a single 5 μg administration of DEF–GAD65 in overtly diabetic NOD mice (glycaemia 250–350 mg/dl) restored the normoglycaemia within 2 days in five of seven mice. When the hyperglycaemic NOD mice at the early onset of disease were injected with four doses of 5 μg of DEF–GAD65, the hyperglycaemia was reversed and the normoglycaemic status persisted for more than 2 months after interruption of treatment. The pancreas of normoglycaemic mice treated with DEF–GAD65 showed a reduced degree of insulitis and high number of insulin-secreting β-islet cells as compared with those treated with DEF-λ control.

It is important to point out that the naïve NOD splenocytes first stimulated *in vitro* with DEF–GAD65 chimera, secreted IL-10, whereas the amounts of secreted IFN-γ, IL-2 and IL-4 were insignificant. Sorted CD4^{+} T cells from NOD splenocytes produced the IL-10 upon *in vitro* stimulation with DEF–GAD65 and not in CD3^{+} T cell depleted cultures solely stimulation with DEF–GAD65. The interaction of DEF–GAD65 with CD4 co-receptor was critical for stimulating specific T cells towards IL-10 secretion because the pre-treatment of naïve NOD splenocytes with anti-CD4 antibody inhibited the DEF–GAD65 induced IL-10 secretion.

A critical mechanism leading to the long-term reversing hyperglycaemia in diabetic NOD mice treated with DEF–GAD65 was most likely the stabilization of pancreatic insulitis that may inhibit epitope spreading that occurs during a progressive insulitis due to the suppressive effects of IL-10, which allowed insulin production by the residual β islet cells. The IL-10 secreting CD4^{+} T cells from the spleen of DEF–GAD65 treated animals could migrate in the pancreas and proliferate in the presence of GAD65 antigen, and/or alternatively they could proliferate in the spleen upon DEF–GAD65 stimulation followed by migration into the pancreas [27]. Table 2 summarizes the results of experiments preventing or reversal of T1D in TCR–HA double transgenic mice, BCD2.5 transgenic or NOD mice treated with DEF–HA, DEF mimotope or DEF NOD GAD65.

Perspectives for the utilization of DEF molecules as a new modality in the treatment of autoimmune diseases

Recent improvements in the generation of soluble dimeric molecules in which the peptide is covalently linked to MHC class II molecules fused to an Ig scaffold was extended to the production of recombinant molecules composed of peptide recognized by T cells from different antigen and allelic variants of MHC.

The activation of the T cells requires the recognition by the TCR of a peptide generated from the processing and presentation of antigen by APC in association with an MHC gene product and interaction of co-stimulatory molecules. Recent development of molecular biology allowed for generation of soluble dimeric MHC-peptide molecules, which may function as MHC peptide surrogates. These molecules are devoid of second co-stimulatory signal, which rather induces peripheral tolerance rather than activation of T cells.

A variety of peptides and various allelic variants of MHC were made in various laboratories. In these molecules, the peptide was covalently linked to the MHC. These DEF molecules are stable with a longer half-life and devoid of side effects. The ability of DEF molecules to bind to TCR and CD4 molecules circumvent the need for the uptake and processing by the APC.

There is the possibility of many applications in clinical immunology and medicine for these new classes of compounds. The dimeric molecule labelled with a fluorescent dye may be use to score the frequency of diabetogenic antigen specific T cells instead of tetramers. The increased affinity by virtue of covalently bound peptide may allow the detection of T cells bearing a low affinity TCR and stabilizes the binding to interaction with co-receptors and reduces the membrane clustering of TCR. The dimeric compounds can be utilized for staining with antibodies specific for other molecules allowing for defining the phenotype of antigen-specific T cells.

DEF molecules stimulate regulatory cells Tr1, which exerts their suppressor effect on antigen-specific T cells and induces IL-10-mediated peripheral tolerance. In a majority of the cases discussed in this review, the suppressor activity of the T regulatory cells induced by DEF was inhibited by either anti-IL-10 or anti-IL-10 receptor monoclonal antibodies suggesting that various DEF chimeras stimulate Tr1 regulatory cells.

The induction of Tr1 may be related to lack of co-stimulatory molecules which tolerize T cells.

Several DEF-like molecules containing peptides derived from self-antigens have been generated during the past decade for the treatment of autoimmune disease mediated by pathogenic T cells. In addition, they can be used in organ-specific autoimmune diseases by inactivating CD4 T cells, which delivered the second signal to autoantibody specific B cells.

We believe that during the coming years the dimeric soluble MHC-peptide expressed in an Ig scaffold will open ways for new immunotherapeutic approaches for preventing disease for subjects with high risk or treatment autoimmune disease.

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Davie JM, Paul WE.** Antigen-binding receptors on lymphocytes. *Contemp Top Immunobiol.* 1974; 3: 171–92.
2. **Babbitt BP, Allen PM, Matsueda G,** *et al.* Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 1985; 317: 359–61.
3. **Zinkernagel RM, Doherty PC.** Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 1974; 248: 701–2.
4. **Brigl M, Brenner MB.** CD1: antigen presentation and T cell function. *Annu Rev Immunol.* 2004; 22: 817–90.
5. **Sharma SD, Nag B, Su X,** *et al.* Antigen-specific therapy of experimental allergic encephalomyelitis by soluble class II major histocompatibility complex-peptide complexes. *Proc Natl Acad Sci USA.* 1991; 88: 11465–9.
6. **Godeau F, Luescher IF, Ojcius DM,** *et al.* Purification and ligand binding of a soluble class I major histocompatibility complex molecule consisting of the first three domains of H-2K^d fused to beta 2-microglobulin expressed in the baculovirus-insect cell system. *J Biol Chem.* 1992; 267: 24223–9.
7. **Abastado JP, Lone YC, Casrouge A,** *et al.* Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. *J Exp Med.* 1995; 182: 439–47.
8. **Kozono H, White J, Clements J,** *et al.* Production of soluble MHC class II proteins with covalently bound single peptides. *Nature* 1994; 369: 151–4.
9. **Weber S, Traunecker A, Oliveri F,** *et al.* Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature* 1992; 356: 793–6.
10. **Altman JD, Moss PA, Goulder PJ,** *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996; 274: 94–6.
11. **Hansen TH, Lybarger L.** Exciting applications of single chain trimers of MHC-I molecules. *Cancer Immunol Immunother.* 2006; 55: 235–6.
12. **Greten TF, Korangy F, Neumann G,** *et al.* Peptide-beta 2 microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-I complexes. *J Immunol Methods.* 2002; 271: 125–35.
13. **Nepom, GT, Erlich H.** MHC class-II molecules and autoimmunity. *Annu Rev Immunol.* 1991; 9: 493–525.
14. **Durai M, Krueger C, Ye Z,** *et al. In vivo* functional efficacy of tumor-specific T cells expanded using HLA-Ig based artificial

antigen presenting cells (aAPC). *Cancer Immunol Immunother.* 2009; 58: 209–20.

15. **Godfrey DI, MacDonald HR, Kronenberg M, *et al.*** NKT cells: what's in a name? *Nat Rev Immunol.* 2004; 4: 231–7.

16. **Vollers SS, Stern LJ.** Class II major histocompatibility complex tetramer staining: progress, problems, and prospects. *Immunology* 2008; 123: 305–13.

17. **Casares S, Bona CA, Brumeanu TD.** Engineering and characterization of a murine MHC class II-immunoglobulin chimera expressing an immunodominant CD4 T viral epitope. *Protein Eng.* 1997; 10: 1295–301.

18. **Lebowitz MS, O'Herrin SM, Hamad AR, *et al.*** Soluble, high-affinity dimers of T-cell receptors and class II major histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. *Cell Immunol.* 1999; 192: 175–84.

19. **Davis MM, Boniface JJ, Reich Z, et al.** Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol.* 1999; 16: 523–44.

20. **Jenkins MK, Schwartz RH.** Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo. J Exp Med.*1987; 165: 302–19.

21. **Powell JD, Ragheb JA, Kitagawa-Sakakida S, *et al.*** Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy. *Immunol Rev.*1998; 165: 287–300.

22. **Preda I, McEvoy RC, Lin M, *et al.*** Soluble, dimeric HLA DR4-peptide chimeras: an approach for detection and immunoregulation of human type-1 diabetes. *Eur J Immunol.* 2005; 35: 2762–75.

23. **Wucherpfennig KW, Yu B, Bhol K, *et al.*** Structural basis for major histocompatibility complex (MHC)-linked susceptibility to autoimmunity: charged residues of a single MHC binding pocket confer selective presentation of self-peptides in pemphigus vulgaris. *Proc Natl Acad Sci USA.* 1995; 92: 11935–9.

24. **Hamad AR, O'Herrin SM, Lebowitz MS, *et al.*** Potent T cell activation with dimeric peptide-major histocompatibility complex class II ligand: the role of CD4 co-receptor. *J Exp Med.* 1998; 188: 1633–40.

25. **Masteller EL, Warren MR, Ferlin W, *et al.*** Peptide-MHC class II dimers as therapeutics to modulate antigen-specific T cell responses in autoimmune diabetes. *J Immunol.* 2003; 171: 5587–95.

26. **Karabekian Z, Lytton SD, Silver PB, *et al.*** Antigen/MHC class II/Ig dimers for study of uveitogenic T cells: IRBP p161-180 presented by both IA and IE molecules. *Invest Ophthalmol Vis Sci.* 2005; 46: 3769–76.

27. **Lin M, Stoica-Nazarov C, Surls J, *et al.*** Reversal of type 1 diabetes by a new MHC II-peptide chimera: "single-epitope mediated suppression" to stabilize a polyclonal autoimmune T-cell process. *Eur J Immunol.* 2010; 40: 2277–88.

28. **Ratcliffe MJ, Coggeshall KM, Newell MK, *et al.*** T cell receptor aggregation, but not dimerization, induces increased cytosolic calcium concentrations and reveals a lack of stable association between CD4 and the T cell receptor. *J Immunol* 1992; 148: 1643–51.

29. **Reich Z, Boniface JJ, Lyons DS, *et al.*** Ligand-specific oligomerization of T-cell receptor molecules. *Nature* 1997; 387: 617–20.

30. **Viola A, Lanzavecchia A.** T cell activation determined by T cell receptor number and tunable thresholds. *Science* 1996; 273: 104–6.

31. **Schneck JP.** Monitoring antigen-specific T cells using MHC-Ig dimers. *Immunol Invest.* 2000; 29: 63–9.

32. **Doyle C, Strominger JL.** Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* 1987; 330: 256–9.

33. **Gay D, Maddon P, Sekaly R, *et al.*** Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* 1987; 328: 626–9.

34. **Casares S, Zong CS, Radu DL, *et al.*** Antigen-specific signaling by a soluble, dimeric peptide/major histocompatibility complex class II/Fc chimera leading to T helper cell type 2 differentiation. *J Exp Med.* 1999; 190: 543–53.

35. **Bona C, Stevenson F.** B cells producing pathogenic autoantibodies. In: Alt F, Honjo T, Neuberger M, editors. Molecular biology of B cells. The Netherlands: Elsevier; 2004. p. 381–401.

36. **Nicholson MJ, Hahn M, Wucherpfennig KW.** Unusual features of self- peptide/MHC binding by autoimmune T cell receptors. *Immunity* 2005; 23: 351–60.

37. **Kaladadze AM, Galleno M, Foncerrada JL, *et al.*** Expression of recombinant HLA-DR2 molecules: replacememt of hydrophobic transmembrane region by a leucine zipper dimerization motif allow as the assemblay and secretion of soluble

DRab heterodimers. *J Bio Chem.* 1996; 271: 20156–62.

38. **Bradley LM, Asensio VC, Schoitz LK, *et al.*** Islet-specific Th1, but not Th2, cells secrete multiple chemokines and promote rapid induction of autoimmune diabetis. *J Immunol.* 1999; 192: 2511–20.

39. **Vizler C, Bercovic N, Cornet A, *et al.*** Role of autoreactive CD8+ T cells in organ specific autoimmune diseases: insight in transgenic mice *Imunnol Rev.* 1999; 169: 81–92.

40. **Gallichan WS, Balasa B, Davies JD, *et al.*** Pancreatic IL-4 expression results in islet-reactive Th2 cells that inhibit diabetogenic lymphocytes in the nonobese diabetic mouse. *J Immunol.* 1999; 163: 1696–703.

41. **Todd JA, Wicker LS.** Genetic protection from the inflammatory disease type 1 diabetes in humans and animal models. *Immunity* 2001; 15: 387–95.

42. **Fahmy TM, Bieler JG, Edidin M, *et al.*** Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity* 2001; 14: 135–43.

43. **Amrani A, Verdaguer J, Serra P, *et al.*** Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 2000; 406: 739–42.

44. **Tisch R, Yang XD, Singer SM, *et al.*** Immune response to glutamic acid decarboxylase correlates with insulitis in nonobese diabetic mice. *Nature* 1993; 366: 72–5.

45. **Serreze DV, Johnson EA, Chapman HD, *et al.*** Autoreactive diabetogenic T-cells in NOD mice can efficiently expand from a greatly reduced precursor pool. *Diabetes* 2001; 50: 1992–2000.

46. **Wicker LS, Miller BJ, Mullen Y.** Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes* 1986; 35: 855–60.

47. **Peterson JD, Pike B, McDuffie M, *et al.*** Islet-specific T cell clones transfer diabetes to nonobese diabetic (NOD) F1 mice. *J Immunol.* 1994; 153: 2800–6.

48. **Kirberg J, Baron A, Jakob S, *et al.*** Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. *J Exp Med.* 1994; 180: 25–34.

49. **Radu DL, Brumeanu TD, McEvoy RC, *et al.*** Escape from self-tolerance leads to neonatal insulin-dependent diabetes mellitus. *Autoimmunity* 1999; 30: 199–20.

50. **Casares S, Hurtado A, McEvoy RC, *et al.*** Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC

class II chimera. *Nature Immunol.* 2002; 3: 383–91.

51. **Barshes NR, Lee T, Goodpasture S, *et al.*** Achievement of insulin independence *via* pancreatic islet transplantation using a remote isolation center: a first-year review. *Transplant Proc.* 2004; 36: 1127–9.

52. **Casares S, Lin M, Zhang N, *et al.*** A peptide-major Histocompatibility complex II chimera favors survival of pancreatic (beta islets grafted in type 1 diabetic mice. *Transplantation* 2008; 285: 1717–27.

53. **Kanagawa O, Martin SM, Vaupel BA, *et al.*** Autoreactivity of T cells from nonobese diabetic mice: an I-A^{g7}-dependent reaction. *Proc Natl Acad Sci USA.* 1998; 95: 1721–4.

54. **Kaufman DL, Clare-Salzler M, Tian J, *et al.*** Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993; 366: 69–72.

55. **Haskins K, McDuffie M.** Acceleration of diabetes in young NOD mice with a CD4^{+} islet-specific T cell clone. *Science* 1990; 249: 1433–6.