Original Article

Modulatory Role of DR4- to DQ8-restricted CD4 T-Cell Responses and Type 1 Diabetes Susceptibility

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This study addressed an important biological question, namely how certain HLA molecules modulate the disease risk conferred by other HLA molecules. The HLA molecules under investigation were HLA-DQ8 and -DR4, the two most prevalent HLA class II alleles found in Caucasian type 1 diabetic patients. A panel of human GAD (hGAD65)-specific CD4 T-cell lines and hybridomas was generated to serve as detection reagents for evaluating the peptide occupancy of DQ8 and DR4. Results indicated that DQ8 and DR4 (0401) were able to bind the same hGAD65 peptides. The coexpression of DR4 (0401) diminished DQ8-restricted T-cell responses. In addition, we also demonstrated that the diminished T-cell response varied according to the specific DRB1*04 alleles. Taken together, this study provides evidence that DR4 is able to modulate DQ8-restricted T-cell responses, possibly by competing for peptides. Given that DQ8 is a primary genetic determinant of type 1 diabetes, the decreased DQ8-restricted CD4 T-cell activity due to peptide competition may be the mechanism explaining the modulation effect of DR4 to type 1 diabetes susceptibility. *Diabetes* 55:3455–3462, 2006

Type 1 diabetes is an autoimmune disease characterized by the selective destruction of insulin-producing β-cells (1–3). It has been demonstrated that autoreactive CD4 T-cells are involved in breaking self-tolerance to β-cells (4–6). Specific HLA/major histocompatibility complex (MHC) class II molecules are responsible for the generation of autoreactive CD4 T-cells in human type 1 diabetic patients and nonobese diabetic mice (7), a murine model for spontaneous diabetes (8–11). HLA-DQ8 (DQA1*0301/DQB1*0302) is known to be a major genetic predisposing factor in the Caucasian type 1 diabetic population (12,13). However, several studies also reported that type 1 diabetes susceptibility was likely to be modulated by other HLA molecules such as HLA-DR (8,14). Results from DQ8-matched case-control studies indicated that different DQ8-DRB1*04 haplotypes were associated with variable risks of type 1 diabetes development in a hierarchical rank of DQ8-DRB1*0405 > DQ8-DRB1*0402 > DQ8-DRB1*0401 > DQ8-DRB1*0404 > DQ8-DRB1*0403 (or 0406). This rank suggested that distinct DRB1*04 alleles provided variable degrees of protection (15,16). Results from an HLA transgenic mice model demonstrated that the coexpression of DR4 (0401) with DQ8 reduced the incidence of spontaneous diabetes when the mice also transgenically expressed costimulatory molecule B7.1 on β-cells (17). However, mechanisms by which those DR4 manifest the effect are not clear. It was also demonstrated that transgenic expression of H2-E^d (murine homologue of HLA-DR) in NOD mice also decreased the incidence of diabetes (18). The degree of protection was correlated with the expression level of H2-E^d. Interestingly, mixed bone marrow transfer experiments from the same study demonstrated that autoreactive T-cells were not clonally deleted by protective H2-E^d during T-cell development, whereas the persistent presence of H2-E^d in the periphery was required to keep the animals diabetes free. Taken together, these studies suggested that DR4 or H2-E^d molecules might provide protection by modulating the activity of self-reactive T-cells in peripheral tissues.

A peptide competition model proposed that competition of key diabetogenic peptides between resistant and susceptible HLA alleles might be a mechanism of disease protection (19). A strong competitor may persistently "steal" antigenic peptides from susceptible HLA alleles, such as HLA-DQ8. This competition effect is more profound when the source antigen is rare so that the abundance of peptide-DQ8 complex is reduced. Thus, the reduced abundance of peptide-DQ8 complex can lead to a diminished activity of DQ8-restricted autoreactive T-cells. Although Nepom (19) proposed the model a decade ago, little experimental evidence has been shown to demonstrate such competition. A recent publication reported that an insulin peptide ($InsB_{5-15}$) was able to bind a type 1 diabetes–susceptible DQ*0604 and a type 1 diabetes–resistant DQ*0602 with different binding affinities (20). This binding by different alleles is an essential condition for the peptide competition model. However, peptide competition is not limited to HLA molecules encoded from the same locus; some human GAD (hGAD65) peptides are able to bind both DQ8 and DR4 (DRB1*0401) in MHC class II binding assays (21).

In this study, we performed a peptide-oriented study

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Received for publication 16 May 2006 and accepted in revised form 6 September 2006.

APC, antigen-presenting cell; B-LCL, B-lymphoblastoid cell line; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; hGAD65, human GAD; IFN-γ, γ-interferon; IL, interleukin; MHC, major histocompatibility complex; TCR, T-cell receptor.

DOI: 10.2337/db06-0680

TABLE 1
DQ8-DR4 haplotypes of human B-LCL

	WT51	FS	KT17	BM92
DQA1	0301	0301	0301	0301
DQB1	0302	0302	0302	0302
DRB1	0401	0402	0403/0406*	0404
DRB4	0101	0101	0103†	0101

*Amino acid sequences within the peptide binding site of DRB1*0403 and 0406 alleles are identical. †Amino acid sequence within the peptide binding site of DRB4*0103 is identical to the sequence of DRB4*0101.

using mouse T-cells as detection reagents to verify the role of DR4 molecules regarding their peptide competition capabilities. These T-cells were obtained from HLA-DQ8 and -DR4 transgenic mice immunized with peptides derived from hGAD65, a putative type 1 diabetes autoantigen (22,23). They do not express HLA transgenes but respond to $DQ8^+$ and/or $DR4^+$ antigen-presenting cells (APCs) in the presence of cognate peptides. We first demonstrated that DQ8 and DR4 could bind the same candidate peptides. We then found that the coexpression of DR4 (0401) with DQ8 on the same APC diminished DQ8-restricted T-cell responses. Furthermore, we also compared the T-cell responses elicited by Epstein-Barr virus–transformed human B-lymphoblastoid cell lines (B-LCLs) that expressed distinct DQ8-DRB1*04 alleles. The results indicated that the protective DRB1*0403/0406 alleles diminished DQ8-restricted T-epitope presentation greater than the susceptible DRB1*0401/0402 alleles. In addition, the effect of DR4 was blocked by a peptide that strongly bound DR4 but not DQ8. Taken together, our results support the role of peptide competition as a mechanism, which influences the variable susceptibility of type 1 diabetes observed in $DQ8^+$ populations.

RESEARCH DESIGN AND METHODS

HLA-DQ8, -DR4, and -DQ8/DR4 transgenic mice devoid of murine endogenous MHC class II were generated and fully characterized by previous studies (17,24). The animals were bred and housed under pathogen-free conditions in the animal facility of the Rangos Research Center at the Children's Hospital of Pittsburgh. The animal experiments were conducted in compliance with the animal research care and committee of the Children's Hospital of Pittsburgh.

Peptides. Peptides $hGAD65_{206\text{-}220}$ (TYEIAPVFVLLEYVT), $hGAD65_{209\text{-}217}$ (IAPVFVLLE), $hGAD65_{536\text{-}550}$ (RMMEYGTTMVSYQPL), and $hGAD65_{554\text{-}566}$ (VNFFRMVISNPAA) were synthesized by Chemicon (Temecula, CA) (21,25).

Human B-cell lines. Human B-LCLs BM92 and KT17 were obtained from International Histocompatibility Working Group (Seattle, WA). WT51 and FS were maintained at our laboratory. The DQ-DRB haplotypes of these B-LCLs are shown in Table 1.

Antibodies and fluorescence-activated cell sorter analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse T-cell receptor (TCR) Vβ panel, anti–HLA-DQ (Leu-10), anti-mouse CD86 (GL1), phycoethrin-conjugated anti-mouse CD3 (17A2), anti-mouse CD4 (GK1.5), anti–HLA-DR (L243), anti-mouse CD80 (16-10A1), APC-conjugated anti-mouse CD45R/B220 (RA3-6B2), purified anti-mouse CD16/CD32 (mouse Fc block) (2.4G2), and isotype control antibodies were purchased from BD Pharmingen (San Diego, CA). For a typical surface staining, 5×10^5 cells were stained with fluorescence-conjugated antibodies. The data were collected using a BD FACS Calibur flow cytometer (Becton Dickinson, Palo Alto, CA) and analyzed by CellQuest software (Becton Dickinson).

hGAD65 peptides specific to CD4 T-cell lines and T-hybridomas. Primary T-cell lines were generated according to previous protocols (26–28). Briefly, T-cells from peptide-primed HLA transgenic mice were maintained in a biweekly restimulation culture containing 10 μg/ml cognate peptide, 2.5–5 units/ml mouse recombinant interleukin (IL)-2 (Roche, Indianapolis, IN), and irradiated (25 Gy) $DQ8^+$ or $DR4^+$ splenocytes. After 6–8 rounds of restimulation, each T-cell culture was characterized for TCR Vβ usage and antigenic specificity. T-hybridomas were generated by fusing CD4 T-cells from day-4 restimulation cultures with $BW5147\alpha^-\beta^-$ lymphoma (provided by Dr. Willi Born from University of Colorado Health Science Center) in the presence of PEG-1500 (Sigma, St. Louis, MO). Successfully fused T-hybridomas were selected by hypoxanthine/aminopterin/thymidine supplement (Invitrogen, Carlsbad, CA) and recloned by limiting dilution according to a standard protocol (29).

RT-PCR and DNA sequencing. Total RNA was prepared from 2×10^6 T-cells with TRIzol Reagent (Invitrogen). Total RNA (1.5 μg) was used for cDNA preparation with the SuperScript II first-strand cDNA synthesis system (Invitrogen). PCR was performed with Advantage cDNA polymerase (Clontech, Palo Alto, CA). The PCR product was cloned into pDrive vector (Qiagene, Valencia, CA) and sequenced using M13/M13Reverse primers by 3130 Genetic Analyzer (Applied Biosystems, Fost City, CA). The sequences were analyzed using Sequencher software (version 4.5; Gene Codes, Ann Arbor, MI).

In vitro T-cell assay. T-cells (20,000 cells/well) from 2-week restimulation cultures were cocultured (in triplicate) with irradiated HLA transgenic mouse splenocytes (500,000 cells/well) and peptides in round-bottom 96-well plates. In some experiments, MACS microbead-conjugated anti-CD90 monoclonal antibody and MACS LS column were used to deplete T-cell subsets from spleen cells before performing the assay (Miltenyi, Auburn, CA). Supernates were harvested to determine the production of γ-interferon (IFN-γ) (72 h). For the T-hybridoma assay using human B-cell lines as APC, B-cells were inactivated by 50 μg/ml of Mitomycin C (Sigma) treatment at 37°C for 20 min. After extensive washing, B-cells (40,000 cells/well) were cocultured with T-hybridomas (20,000 cells/well) in the presence of peptide. Supernates were harvested 24 h later for IL-2 detection. Enzyme-linked immunosorbent assay (ELISA) was applied to determine the cytokine concentration with a Europium-Streptavidin detection system (Perkin Elmer). Antibodies and recombinant protein standards for IL-2 and IFN-γ ELISA application were purchased from BD Pharmingen.

Statistical analyses. Cytokine ELISA results were analyzed using Prism 4 software (GraphPad Software, San Diego, CA). Differences in T-cell responses elicited by DQ8 versus DQ8/DR4 transgenic splenocytes were assessed with the two-tailed unpaired *t* test. The results of T-hybridoma responses against a panel of B-LCLs were analyzed by one-way ANOVA. A P value <0.05 was considered statistically significant.

RESULTS

HLA-DQ8 and -DR4 can present the same hGAD65 peptides and generate CD4 T-cell responses. HLA-DQ8 and -DR4 (0401) transgenic mice were immunized with $hGAD65_{206\text{-}220}$ and $hGAD65_{536\text{-}550}$. One week later, the lymphocytes from draining lymph nodes and spleens of immunized mice were harvested and in vitro T-cell cultures established. Four specific CD4 T-cell lines were obtained. T206 was generated from HLA-DQ8 transgenic mice and specifically responded to $DQ8^+$ splenocytes by secreting IFN-γ in the presence of 10 μg/ml cognate peptide $hGAD65_{206\text{-}220}$ (Fig. 1*A*). T536.1 was obtained from HLA-DQ8 transgenic mice primed with $hGAD65_{536\text{-}550}$. On cognate peptide (10 μg/ml) $hGAD65_{536\text{-}550}$ recall stimulation, T536.1 produced IFN-γ (Fig. 1*B*). Neither T206 nor T536.1 had cross-reactivity to DR4. However, they responded to the splenocytes from HLA-DQ8/DR4 double-transgenic mice. Another two CD4 T-cell lines, DR4p206 and DR4p536, were obtained from $hGAD65_{206\text{-}220}$–and $hGAD65_{536\text{-}550}$–primed HLA-DR4 transgenic mice, respectively. Both secreted IFN-γ in response to cognate peptide (10 μg/ml) pulsed $DR4^+$ or $DQ8^+DR4^+$ APC (Fig. 1*C* and *D*). The data of T206 versus DR4p206 and T536.1 versus DR4p536 unambiguously demonstrated that both $hGAD65_{206\text{-}220}$ and $hGAD65_{536\text{-}550}$ could be bound by HLA-DQ8 and -DR4, yielding credence to the proposition of peptide competition.

We planned to use T206 and T536.1 as reagents to examine peptide presentation efficiency by DQ8, since IFN-γ production was correlated with the concentration of

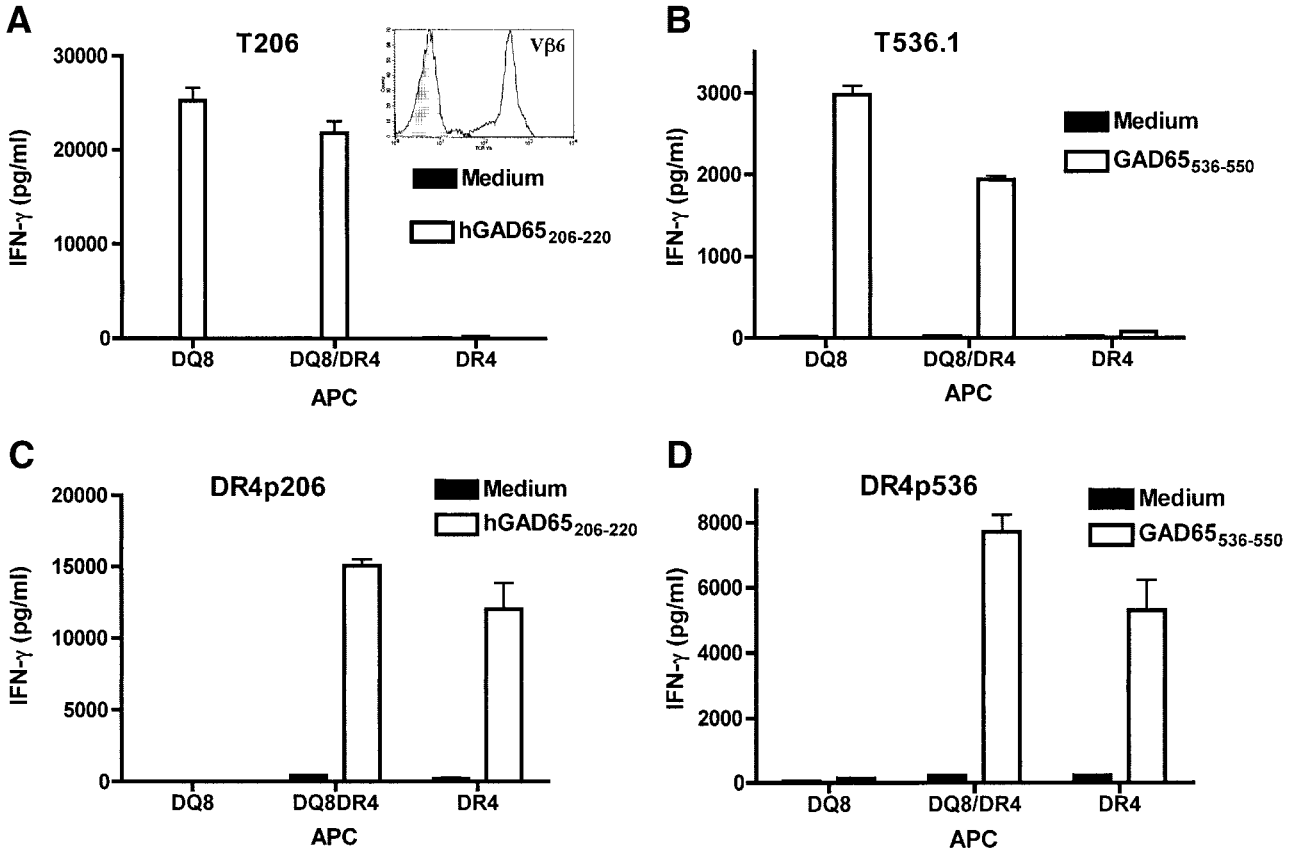

FIG. 1. hGAD65 peptide–specific CD4 T-cell lines. IFN-γ produced by CD4 T-cell lines T206 (*A*), T536.1 (*B*), DR4p206 (*C*), and DR4p536 (*D*) cocultured with irradiated splenocytes from DQ8, DQ8/DR4, and DR4 transgenic mice in the presence of 10 μg/ml cognate peptides or blank control (medium). Error bars indicate 1 SD. The inset histogram in (*A*) represents the TCR Vβ usage of T206 stained with FITC-conjugated anti-TCR Vβ6 mAb (□) and isotype control antibody (■).

cognate peptide (data not shown). We therefore analyzed the TCR β-chain expression to examine the clonality of these DQ8-restricted T-cell lines. As revealed by flow cytometric analysis, T206 exclusively expressed Vβ6 TCR (Fig. 1*A*). T536.1 exclusively expressed Vβ1 TCR, which was identified by RT-PCR using a panel of TCR Vβ-specific primers (this panel consists of 24 specific Vβ elements, including some rare Vβ genes like *Vβ1* that does not have a corresponding monoclonal antibody) (30). To further confirm that these lines exclusively expressed a single Vβ element, the β-chain cDNAs of T206 and T536.1 were also cloned. Sequencing of 20 colonies confirmed the clonal phenotype of each cell line (GeneBank accession no. DQ421779 and DQ421780). In summary, these T-cell lines were assessed to manifest clonal-like phenotype and were stable in culture after multiple restimulation. Thus, they were considered to be reliable reagents to evaluate peptide presentation efficiency. The DR4-restricted CD4 T-cell lines DR4p206 and DR4p546 were only used to demonstrate that the same epitopes could also be presented by DR4, a requisite condition for peptide competition between DR4 and DQ8. The clonality of these lines was not critical to our main purpose of evaluating DQ8 peptide presentation efficiency.

Coexpression of DR4 with DQ8 diminished DQ8-restricted T-cell responses. Since DQ8 and DR4 could bind the same peptide, they would potentially compete for the peptide when they were coexpressed on the same APC. To test this hypothesis, we evaluated the relative peptide occupancy of DQ8 by comparing the CD4 T-cell responses elicited by $DQ8^+$ APC (in the absence of DR4 expression) with $DQ8^+DR4^+$ APC. Splenocytes from DQ8 and DQ8/DR4 transgenic mice that expressed a similar level of DQ8 were used as APC (Fig. 2*A* and *B*). In addition, no difference in expression of CD80/CD86 costimulatory molecules was observed (data not shown). T-cells were depleted from the splenocytes before assaying; thus, the activities of radiation-resistant T_{reg}-cells in the crude APC population were excluded. In the presence of DR4, IFN-γ secreted by T206 or T536.1 was decreased under different Ag concentrations (Fig. 2*C* and *D*). It indicated that DR4 coexpression diminished DQ8-restricted T-cell responses.

We also examined the T-cell responses elicited by $DQ8^+DR4^+$ mouse splenocytes and mixed APC composed of $DQ8^+$ and $DR4^+$ splenocytes in the presence of cognate peptide (2 μg/ml). IFN-γ production by T206 (Fig. 3*B*) and T536.1 (Fig. 3*C*) indicated that the presence of DR4 on separate APC only slightly reduced DQ8-restricted T-cell responses (~18% reduction for T206 and 3% reduction for T536.1), whereas the coexpression of DR4 and DQ8 on the same APC resulted in a profound decrease of T206 and T536.1 responses (72 and 47% reduction, respectively). Analysis by flow cytometry demonstrated that cell surface expression of DQ8 and DR4 was similar on the splenocytes used in this experiment (Fig. 3*A*). Taken together, these results show that DR4 modulates DQ8-restricted T-cell responses effectively only when it was coexpressed with DQ8 by the same APC.

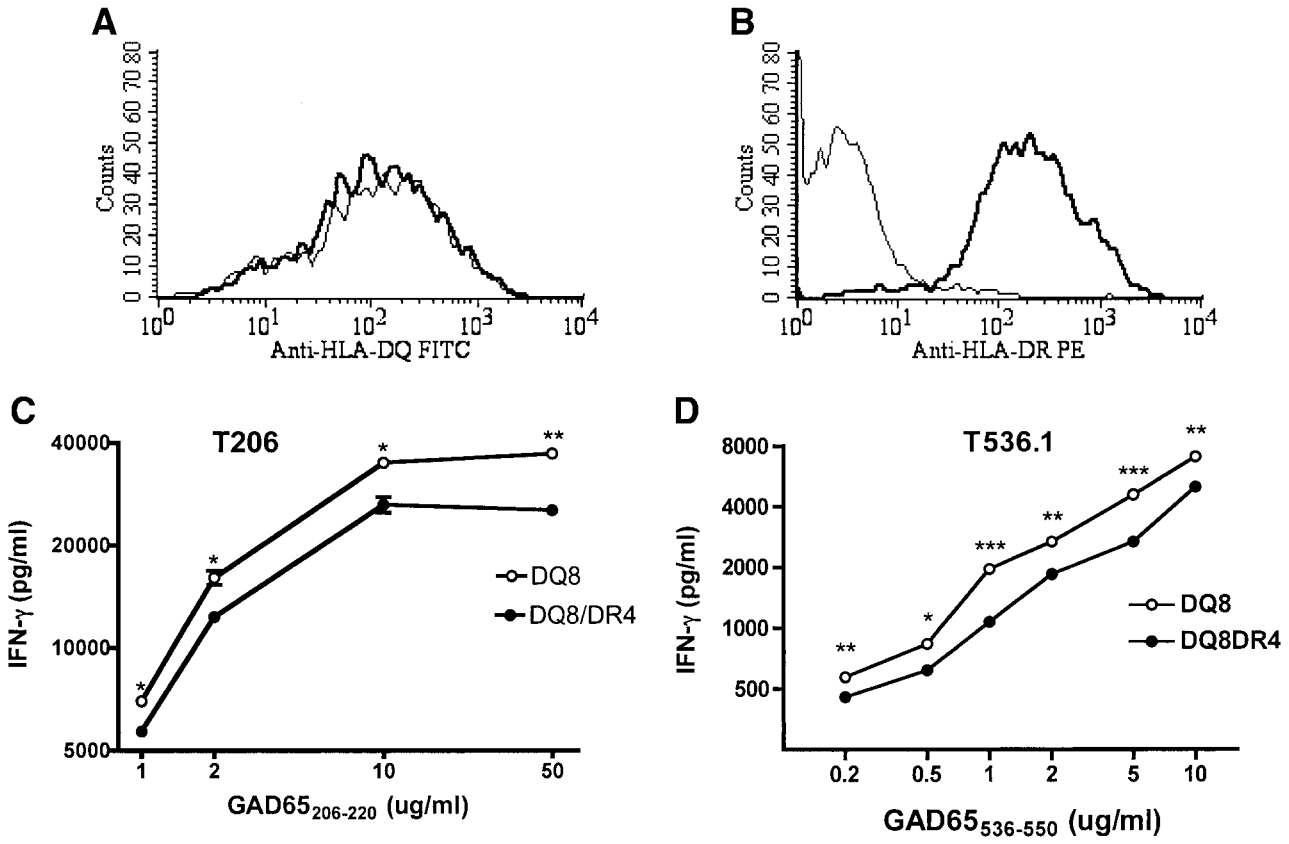

FIG. 2. The antigen presentation of DQ8$^+$ APCs compared with DQ8$^+$DR4$^+$ APCs. Histograms represent the FITC-conjugated anti–HLA-DQ mAb staining results (*A*) and phycoethrin-conjugated anti–HLA-DR mAb staining results (*B*) of T-cell–depleted DQ8 splenocytes (thin line) and DQ8/DR4 splenocytes (thick line). IFN-γ produced by T206 (*C*) and T536.1 (*D*) cocultured with T-cell–depleted irradiated splenocytes from DQ8 and DQ8/DR4 transgenic mice in the presence of indicated amount of peptide. X- and Y-axis are plotted in a log2 scale. The results are representative of three different experiments. Error bars indicate 1 SD. *P = 0.05; **P = 0.005; ***P = 0.001.

Different DR4 subtypes (DRB1*0401, 0402, 0403, 0404, and 0406) manifested different levels of modulation of DQ8-restricted CD4 T-cell responses. We next examined the effects of other DR4 subtypes (DRB1*0401, 0402, 0403, 0404, and 0406) on DQ8-restricted CD4 T-cell responses. It is known that DQ8-DRB1*04 haplotypes carrying specific DRB1*04 alleles are associated with variable susceptibility to type 1 diabetes (15,16). The sequence alignment indicated that these DRB1*04 alleles differed from each other mainly at the β86, β74, β71, β70, and β57 positions (Fig. 4*A*). These residues are responsible for the size and polarity of the P1, P4, P6, and P9 pockets (31). This implies that distinct DR4 subtypes bind peptides with different affinities and manifest variable peptide competition potentials.

Since transgenic mice expressing other DRB1*04 alleles have not been developed, the most available sources of antigen-presenting cells are Epstein-Barr virus–transformed human B-cells. B-LCLs, including WT51, FS, BM92, and KT17, homozygously express identical DQ8 and DRB4 but different DRB1*04 alleles (Table 1). As the only exception, KT17 heterozygously expresses DRB1*0403 and 0406 at the DRB1 locus. However, since the amino acid sequence within the DR peptide–binding groove is identical between 0403 and 0406, it is presumed that DRA1*0101/DRB1*0403 and DRA1*0101/DRB1*0406 heterodimers manifest an identical affinity for peptide binding. Considering human B-LCLs that did not express murine costimulatory molecules, we used T-hybridomas TH206 and TH536.1 instead of primary T-cell lines as detection reagents, since T-hybridoma responses were less dependent on costimulation than primary T-cells (32). These T-hybridomas were generated by fusing primary T-cell lines, T206 and T536.1, with CD3/TCR-deficient BW5147$\alpha^-\beta^-$ lymphoma (33). They expressed identical TCRs as their parental T-cell clones and responded to their cognate peptide recall by secreting IL-2. TH206, however, responded to a short truncated peptide hGAD65$_{209\text{-}217}$ better than hGAD65$_{206\text{-}220}$ (data not shown).

All DQ8$^+$ human B-cell lines were able to stimulate TH206 and TH536.1 responses (Fig. 4*B* and *C*). However, the magnitude of hybridoma responses elicited by human B-LCLs was variable and ranked from high to low in the order of FS > WT51 > BM9 > KT17. This was not simply due to the level of DQ8 expression because the weak T-cell responses induced by high-DQ–expressing KT17 and BM92 were in contrast to the strong T-cell responses triggered by relatively low DQ expression of WT51 and FS (Table 2). Although the B-LCLs utilized for this purpose may display variable amounts of DQ and DR molecules so that the relative amount of DR4 may also be involved, we found that the results obtained were suggestive of a consistently decreasing peptide presenting efficiency of DQ8 in the presence of distinct DRB1*04 alleles. The DRB1*0403/0406$^+$ KT17 always required 5- to 10-fold more peptide to achieve an equivalent T-cell response in comparison with DRB1*0402$^+$ FS and DRB1*0401$^+$ WT5. These results are consistent with the interpretation that DRB1*0403/0406 and DRB1*0404 alleles may have a stron-

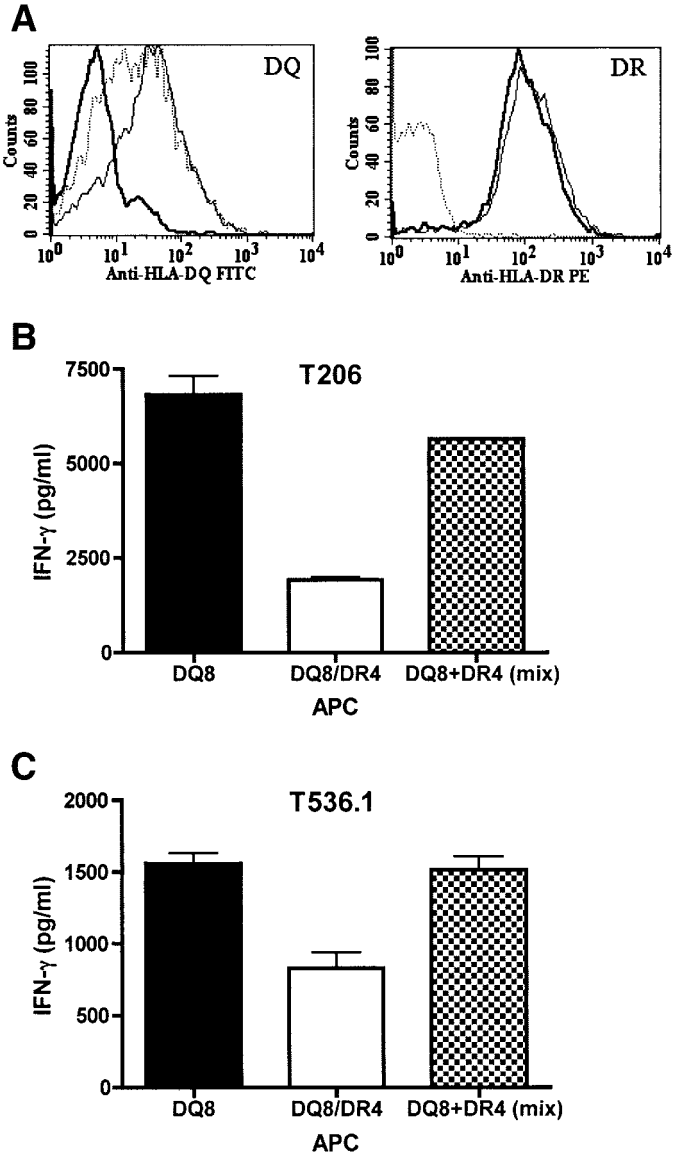

FIG. 3. DQ8-restricted CD4 T-cell responses induced by DQ8$^+$DR4$^+$ APC and DQ8$^+$ APC mixed with DR4$^+$ APC. *A*: Histograms represent the anti–HLA-DQ (*left panel*) and anti–HLA-DR (*right panel*) mAb staining results of splenocytes from HLA-DQ8 (dotted line), HLA-DQ8/DR4 (thin line), and HLA-DR4 (thick line) transgenic mice. In the presence of 2 μg/ml cognate peptide, IFN-γ procuded by T206 (*B*) and T536.1 (*C*) cocultured with splenocytes from DQ8 and DQ8/DR4 transgenic mice or mixed splenocytes from DQ8 and DR4 transgenic mice. Error bars indicate 1 SD.

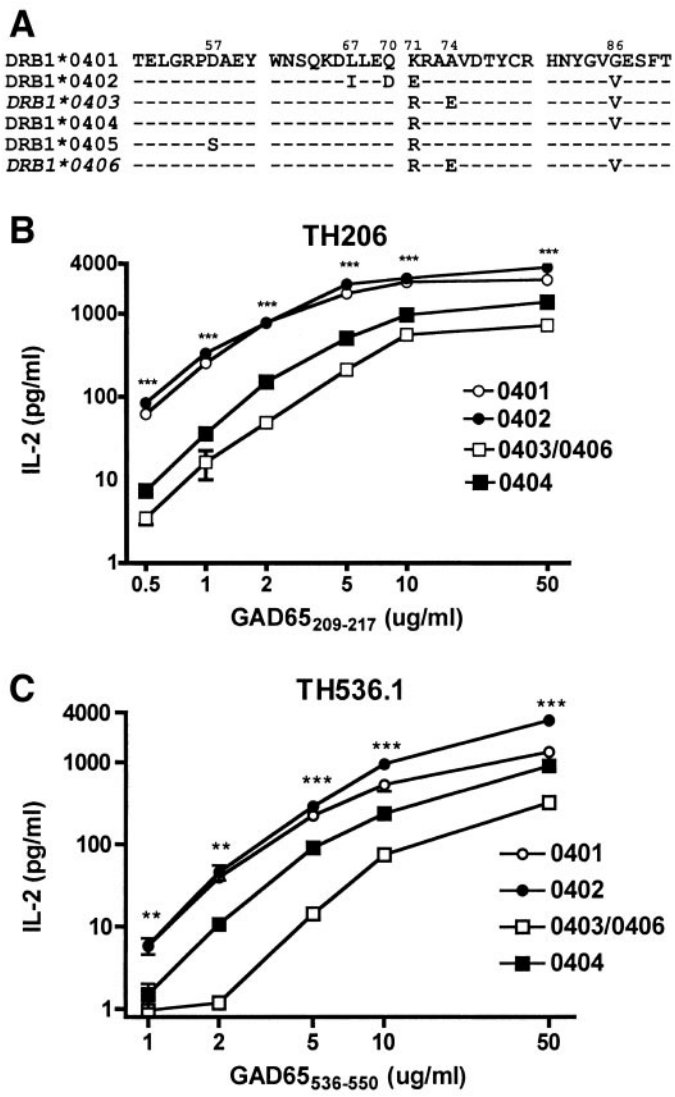

FIG. 4. The antigen presentation of a panel of human B-cell lines that expressed distinct HLA-DQ8-DRB1*04 haplotypes. *A*: The amino acid sequences of DRB1*0401, 0402, 0403, 0404, 0405, and 0406 peptide binding region were aligned to DRB1*0401. The dashes represent amino acid residues identical to the DRB1*0401 reference. IL-2 produced by T-hybridomas TH206 (*B*) and TH536.1 (*C*) cocultured with mitomycin C–treated human B-cell line WT51 (0401), FS (0402), KT17 (0403/0406), and BM92 (0404) in the presence of variable amounts of cognate peptide. *x*- and *y*-axis are plotted in a log2 scale. Error bars indicate 1 SD. **$P = 0.005$; ***$P = 0.001$.

ger competition for these peptides than DRB1*0402 or DRB1*0401.

A peptide-blocking DR4 peptide binding site is able to partially recover DQ8-restrcited T-cell responses. To confirm the effect of DR4 on DQ8-restricted T-epitope presentation, we thus investigated the hybridoma responses in the presence of a blocking peptide, hGAD65$_{554-566}$, which was previously shown to bind DR4 (DRA1*0101/DRB1*0401) with a high affinity but only weakly to DQ8 (21). DQ8-DRB1*0401–expressing WT51 was used as APC. Results showed that responses of TH206 and TH536.1 were increased in the presence of as low as 2 μg/ml peptide blockade (Fig. 5*A* and *B*). When the blocking peptide was increased, T-hybridoma responses were increased accordingly. This dose-dependent increase of the T-hybridoma response indicated that the reduced response without blocking peptide was related to the availability of the DR4 peptide–binding groove.

DISCUSSION

The rationale of studying DR4 peptide competition is to have a better understanding of DQ8, DR4, and autoimmune diabetes. Coexpression of specific DRB1*04 alleles with type 1 diabetes–suspceptible DQ8 in humans modulates the risk of type 1 diabetes development, while the mechanisms have not been fully unveiled. Our study compared the DQ8-restricted T-cell responses elicited by different DQ8$^+$ APC (HLA transgenic mice splenocytes and human B-cells). The major variances were DR4 alleles. The diminished Th1 responses (Fig. 2*C* and *D*) not only

TABLE 2
Relative abundance of HLA-DQ and HLA-DR on the surface of B-LCLs (revealed by FACS analysis)

	HLA-DQ*	HLA-DR†
WT51 (0401)	446.09	1,012.41
FS (0402)	313	536.8
KT17 (0403/0406)	801.6	2,158.5
BM92 (0404)	606.9	2,698.8

*Determined by the mean fluorescence of FITC-conjugated anti–HLA-DQ mAb staining. †Determined by the mean fluorescence of phycoethrin-conjugated anti–HLA-DR mAb staining. FACS, fluorescence-activated cell sorter.

demonstrated the reduced DQ8 peptide presentation in the presence of DRB1*0401 but also implicated more profound biological significance regarding the role of Th1 cells in the progression of diabetes. It has been well acknowledged that IFN-γ has multiple impacts on the process of β-cell destruction, such as recruiting pathogenic CD4/CD8 T-cells to the islets and sensitizing β-cells via upregulating antigen processing/presentation machinery (34,35). Therefore, the decreased IFN-γ production from Th1 cells due to peptide competition partially contributes to the reduced CD4/CD8 T-cell infiltration observed in the islets of HLA-DQ8/DR4 transgenic mice compared with HLA-DQ8 transgenic mice (both HLA transgenic lines are in the *mII*$^{-}$*/RIP-B7.1*-C57BL/6 background) (17). By examining T-hybridoma responses triggered by human B-LCLs, we tentatively concluded that specific DRB1*04 alleles were associated with variable competition potential in the order of DRB1*0402 < DRB1*0401 < DRB1*0404 < DRB1*0403/0406. This order is consistent with the degrees of protection conferred by these DRB1*04 alleles. It has been broadly discussed that the variable degrees of protection are correlated with the polymorphisms in the DR peptide binding site (36). However, no evidence has been shown that the protective DRB1*0403 is more capable of deleting autoreactive CD4 T-cells or selecting regulatory T-cells in the thymus than susceptible DRB1*0401 alleles. This lack of evidence thus favors peripheral tolerance mechanisms such as the peptide competition model. By using our data, we now suggest that the DQ8-DRB1*0403 haplotype is more protective than the DQ8-DRB1*0401 haplotype because the DRB1*0403 allele can compete for diabetogenic peptides better than the DRB1*0401 allele. We are further exploring the effect of protective DRB1*04 alleles in comparison with other susceptible alleles in alternative systems because the variable expression levels of DQ8 and DR4 in those B-LCLs leave the question that the weak T-hybridoma response observed in KT17 is not only due to the competition by DRB1*0403/0406 alleles for the antigens but also to higher relative abundance of DR over DQ molecules per cell.

The results that compared T-cell responses elicited by DQ8^{+}DR4^{+} APC and DQ8^{+} APC mixed with DR4^{+} APC showed that the variation of T-cell activity could not be due to the binding by DR4 on the APC surface. These data actually helped us better understand to what extent extra- and intracellular peptide competition contributes to the decreased T-cell responses. Extracellular peptide loading occurs by peptide replacement or direct loading into an empty peptide-binding groove. Intracellular loading, mediated by endocytosis and H2-M (or HLA-DM in human)

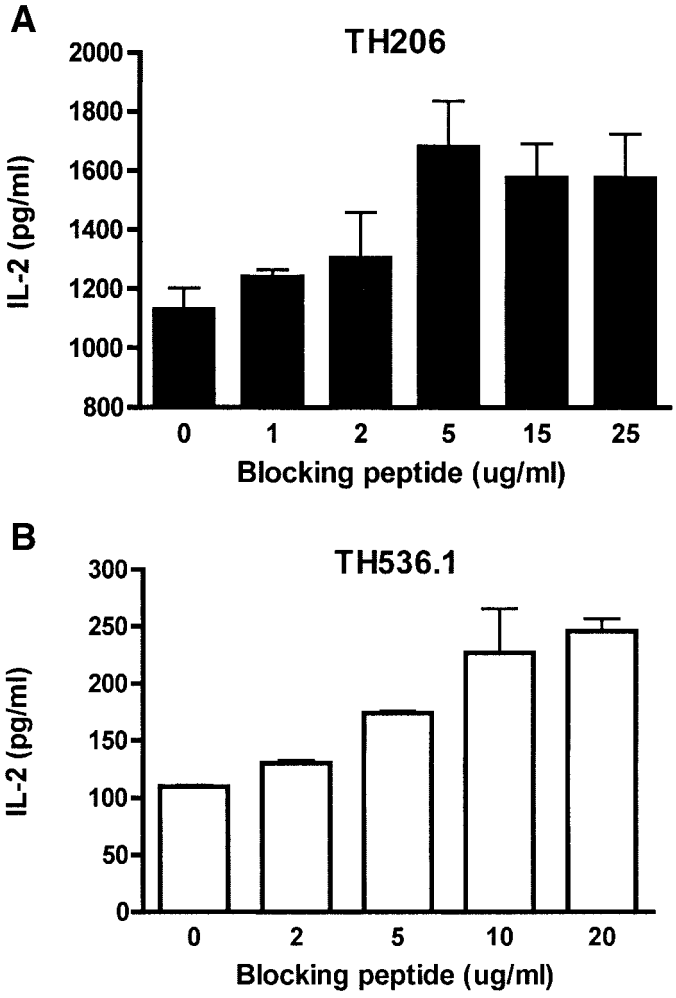

FIG. 5. The presentation of DQ8-restricted T-epitopes in the presence of DR4 blockade. IL-2 produced by T-hybirdomas TH206 (*A*) and TH536.1 (*B*) cocultured with human B-cell line WT51 (DQ8-DRB1*0401) in the presence of cognate peptide (10 μg/ml hGAD65$_{209\text{-}217}$ for TH206 and 2 μg/ml hGAD65$_{536\text{-}550}$ for TH536.1) and variable amounts of hGAD65$_{554\text{-}566}$ blockade. Error bars indicate 1 SD.

(37–39), is not only applied to whole protein antigen but also to short peptides (40). Whether the extra- or intracellular process prevails may be related to the specific peptide (40). In our cases, DR4 can certainly compete for both extracellular peptides and engulfed peptides since it is intrinsically able to bind the peptides (Fig. 1). However, the competition for the extracellular peptides is insufficient to affect DQ8-restricted T-cell responses dramatically. Because excessive amounts of peptide were added into the medium to ensure a detectable T-cell response, the amount of peptide "stolen" by DR4 on the cell surface is probably insignificant compared with what is left in the medium. This notion was confirmed by the results that DR4 expressed on separate APCs was less capable of diminishing DQ8-restricted T-cell responses (Fig. 3). In contrast, coexpression of DR4 with DQ8 on the same APC had a very different effect (Fig. 3). These results indicated that the surface peptide loading of DR4 (at about pH 7) does not contribute to the reduction of DQ8-restricted T-cell responses. It thus suggested that the diminished T-cell responses due to DR4 coexpression was mainly caused by peptide competition within intracellular compartments where the pH environment was low (about pH

5), and the quantity of peptide was limited by the small volume of internalized medium.

An alternative explanation for the diminished DQ8-restricted T-cell responses is that the presence of DR4 interferes with the mobility of peptide-DQ8 complexes to form immunological synapses. This explanation also requires the coexpression of HLA-DQ8 and -DR4 on the same cell surface. However, the data from our DR4 blockade experiment revealed that the availability of DR4 binding site, instead of DR4 itself, was relevant to the variation of T-cell responses (Fig. 5). Therefore, our study does not support this possibility.

It is noteworthy that our results were obtained from in vitro T-cell assays using activated/effector T-cells to evaluate peptide occupancy. The decreased T-cell responses caused by peptide competition implies a more profound biological significance under in vivo situations, where the autoantigen resource is limited, the frequency of autoreactive T-cells is low, and most T-cells express naïve phenotypes. Mature/effector T-cells and naïve/immature T-cells have been known to be different regarding the requirements for activation. For the naïve T-cells, the quantity of TCR-peptide-DQ8 engagement had to reach a threshold (~8,000 TCR/cell) to obtain activation (41,42); otherwise, these autoreactive T-cells are "silenced" or anergic. Although costimulation lowers the threshold (~2,000 TCR/cell), the number of available self-peptide–DQ8 complexes on the APC is still a crucial parameter to direct naïve autoreactive T-cells to differentiate into harmful effector Th1 cells or to become nonresponsive. It is more critical for the cells that naturally have lower TCR surface density. They might require an unattainably high concentration of antigen to engage enough TCRs for a successful activation. Indeed, T-cells that escaped thymic selection with potentially autoreactive TCRs often intrinsically had a lower TCR density on their surface (43).

Many previous studies have addressed the peptide competition issue in different ways. The evidence of peptide elution studies suggested that there was no competition between different MHC alleles (44). However, those peptides eluted from different MHC molecules represent the most abundant self-proteins and may not be diabetogenic. In addition, since only strongly bound peptides can be copurified with MHC before analysis, the pools of eluted peptide actually exclude the weakly bound peptides that are more likely the real candidates of type 1 diabetes–relevant peptide competition. Examining the affinity between synthesized peptides and purified MHC class II molecules is another useful approach to provide chemical evidence to show that different MHC molecules can bind to the same peptide (21). However, different detergent concentrations and reference peptides were used to stabilize DQ8 and DR4 heterodimers in the solution. The approach of directly comparing binding affinity is hence less capable of revealing the outcome of competition.

Our observations provided in vitro evidence for peptide competition as an explanation of the modulating role of DR4 in the T-cell response to diabetes-related autoantigens. Our T-cell response data are consistent to the hierarchical association of different DQ8-DRB1*04 haplotypes with type 1 diabetes susceptibility and bridges protein structure studies with genetic analysis. It thus refined our understanding toward the role of DR4 to the risk of type 1 diabetes in the context of DQ8-conferred primary susceptibility. There is no doubt that DR4 expression directed positive selection for TCRs with novel specificities (45). Some of those might potentially recognized islet antigen–derived peptides. However, this experimental model did not address whether and how these DR4-restricted T-cells contribute to the progression of diabetes.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes of Health Grants AIO56374 and RO1 DK024021 (to M.T.).

We are grateful to Dr. Willi Born for the generous gift of the BW5147$\alpha^-\beta^-$ T lymphoma.

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