HLA-DR*0401 expression in the NOD mice prevents the development of autoimmune diabetes by multiple alterations in the T-cell compartment

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Running title: HLA-induced obviation of autoimmune diabetes

Abstract

Several human HLA alleles have been found associated with type 1 diabetes (T1D), but their precise role is not clearly defined. Herein, we report that a human MHC class II (*HLA-DR*0401*) allele transgene that has been expressed into NOD (*H-2^{g7}I-E^{null}*) mice prone to T1D rendered the mice resistant to the disease. T1D resistance occurred in the context of multi-point T-cell alterations such as: (i) skewed CD4/CD8 T-cell ratio, (ii) decreased size of CD4⁺CD44^{high} T memory pool, (iii) aberrant TCR V β repertoire, (iv) increased neonatal number of Foxp3⁺ and TR-1⁺ regulatory cells, and (v) reduced IFN- γ inflammatory response vs. enhanced IL-10 suppressogenic response of T-cells upon polyclonal and antigen-specific stimulation. The T-cells from NOD/DR4 Tg mice were unable to induce or suppress diabetes in NOD/RAG deficient mice. This study describes a multifaceted regulatory function of the *HLA-DR*0401* allele strongly associated with the lack of T1D development in NOD mice.

Keywords: NOD humanized mice; HLA-DR*0401; Type 1 Diabetes; T-cell regulation.

1. Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease mediated by polyclonal self-reactive T-cells leading to the destruction of pancreatic insulin-secreting β -cells. The disease is strongly associated with gene polymorphism at twenty-two insulin-dependent diabetes (*Id*) loci, among which the *Id1* locus encoding for the MHC class II molecules (HLA in humans) [1-3]. The polymorphism of MHC genes accounts for 40-50% of the T1D familial aggregation [4, 5] with the highest risk linked to complementation between the DQA1*0501 and DQB1*0302 genes [5, 6].

Studies performed on a large number of families of different ethnicities suggested that the quantitative cell expression between DR4 and DQ alleles may predict the susceptibility vs. protection to T1D. Thus, higher DR4 than DQ expression would indicate low risk, whereas lower DR4 than DQ expression would indicate susceptibility [7]. This strongly suggests that the DQ8 predisposition to T1D is regulated by its closely linked DR alleles [8, 9] such as the DRB1*0401, 0403, and 0405 alleles [10].

HLA-humanized mouse models for autoimmunity offer the advantage of studying the function of individual HLA alleles. A concern of CD4 species barrier in the HLA-humanized mice [11-13] for a proper interaction between the murine CD4 and HLA molecules has been ruled out by studies demonstrating the presence of murine CD4 T-cell responses to antigen challenge in a HLA-specific fashion [14, 15]. Conversely, transgenic human CD4 molecules were able to rescue the development and function of murine CD4 T-cells in mice lacking endogenous CD4 molecules [16]. Lately, the generation of murine/human MHC class II chimeric molecules preserving the murine CD4 binding domain and the HLA antigen binding site [17, 18] has minimized the concern of CD4 species barrier and increased the value of HLA-transgenic mice for functional studies in autoimmunity.

Herein, we have generated a new transgenic (Tg) NOD mouse expressing the human MHC class II (HLA-DRA1-DRB1*0401) molecule while preserving the murine CD4 binding site in the HLA-DRA-IE^d_{α2}/HLA-DRB1*0401-IE^d_{β2} chimeric transgene. The new humanized NOD/HLA-DR4 Tg mouse was used to study the role of HLA-DR*0401 allele in T1D development in a NOD diabetogenic background. Our data revealed a complex HLA-DR4 regulatory function that can obviate the disease development through fine multi-point alterations in the T-cell compartment. Several likely mechanisms underlying the HLA-DR4-induced resistance to T1D in the NOD/HLA-DR4 Tg mice are described.

2. Methods

2.1. Mice

The humanized NOD/DR4 Tg strain was generated by inter-strain breeding. The C57BL/6 mice deficient for MHC class II molecules (H-2^{b+}, IAβ⁻/IEα⁻) and transgenic for a human/murine chimeric HLA-DR4-IE molecule (HLA-DRA-IE^d_α/HLA-DRB1*0401-IE^d_β) (Taconic, NY)[17] were backcrossed for 12 generations into the NOD diabetogenic background (IA^{g7+}, IE^d_{null}, H-2^{d+}) to generate the NOD/DR4 Tg strain (HLA-DRA-IE^d_α/HLA-DRB1*0401-IE^d_β⁺, IA^{g7+}, IE^d_{null}, H-2^{d+}). The murine CD4 binding site in the HLA-DRA-IE^d_{α2}/HLA-DRB1*0401-IE^d_{β2} chimeric transgene has been preserved in NOD/DR4 Tg mice. Full recovery of the NOD background was confirmed by microsatellite analysis. Selection of the NOD/DR4 Tg offspring was carried out by PCR using specific primers for HLA-DR*0401 (forward: GTTTCTTGAGCAGGTTAAACA; reverse: CTGCACTGTGAAGCTCTCAC). Internal control PCR primers for DNA quantification were specific for mouse IgG3 gene (forward: ACAACAGCCCC

ATCTGTCTAT; reverse: GTGGGCTACGTTGCAG ATGAC). The NOD/Rag1 KO mice used for adoptive cell transfer experiments were from Jackson Laboratories (Bar Harbor, ME). The incidence of diabetes in the NOD non Tg littermates of NOD/DR4 Tg female mice used in the experiments was 70-80%. All mice were housed in pathogen-free conditions at the USUHS/LAM facility under a care/welfare agreement with federal regulations and USUHS approved protocols.

2.2. *In Vivo Protocols*

2.2.1. Adoptive cell transfer experiments. Splenic T-cells ($10\text{-}50 \times 10^6$ cells) from 5-6 month-old NOD/DR4 Tg mice and hyperglycemic non transgenic NOD littermates were infused i.p. either alone or together into 3 month-old NOD Rag1 KO female recipients. Control diabetes groups were the NOD Rag1 KO recipient mice infused i.p. with 10×10^6 or 50×10^6 splenic cells from 5-6 month-old, hyperglycemic NOD mice. In some experiments, 50×10^6 splenic cells from diabetic NOD wt. mice were infused i.p. into NOD/DR4 Tg mice. The onset of diabetes in the recipient NOD Rag1 KO mice was monitored twice a week based on blood glucose levels using Accu-Check glucose meter and glucose test strips (Roche, Indianapolis, IN, USA). Mice were considered hyperglycemic after two consecutive readings higher than 200 mg/dL.

2.2.2. Cyclophosphamide treatment. Three month-old, normoglycemic NOD non Tg females and their NOD/DR4 Tg female littermates were injected i.p. 3 times with cyclophosphamide (200 mg/kg) in saline (Baxter, Deerfield, IL) at 5 days interval, and glycemia was monitored twice a week.

2.2.3. Viral immunization. Three month-old, normoglycemic NOD non Tg females and their NOD/DR4 Tg female littermates were immunized i.p. with 50 μg of UV-inactivated A/PR/8/34 influenza virus in saline (Charles River, Wilmington, MA), or with saline alone.

Three weeks later, blood serum was collected from individual mice and assessed by ELISA for PR8-specific antibodies and by Hemagglutination Inhibition Assay (HIA) for titers of anti-viral neutralizing antibodies as we previously described [19].

2.2.4. BrdU labeling. 2 month-old NOD/DR4 Tg mice and NOD non Tg littermates were injected i.p. with 0.8 mg of BrdU, and spleens were harvested 1 hr. post injection. Spleen cells were prepared for flow cytometry analysis following the manufacturer's instructions BrdU-FITC flow kit staining protocol (Cat No 559619 BD Pharmingen, San Diego, CA), and stained with an CD4 Ab-Alexa Fluor or CD8 Ab-PE conjugate for 30 min (BD Pharmingen). Cells were then fixed, permeabilized, treated with DNase, incubated with BrdU Ab-FITC conjugate, and analyzed in a BD LSR-II flow cytometer as described below.

2.3. Single-Cell Flow Cytometry

Single-cell suspension from thymus or spleen (10^6 cells) from individual mice were stained for various cell surface markers such as CD80, CD86, CD11c, CD19, B220, HLA-DR, I-E^d, CD3, CD4, CD8 and CD44 (BD Biosciences, San Jose, CA), as we previously described [20]. In some experiments, splenocytes were stained for TCR V β families and co-stained for intracellular IL-10 and transcription factor *Foxp3*. Briefly, single-cell suspension of splenocytes from individual mice in each group were incubated at 5% CO₂ and 37°C in RPMI complete media containing 10% FCS and CD3/CD28 mAbs (2 μ g/mL each/ 10^6 cells/well), and recombinant IL-2 (BD Bioscience, 100 units/ 10^6 cells). After 48 h in culture, the cells were treated with Golgi Stop containing monensin at 1.25 μ g/mL for 6 hours as recommended by the manufacturer (BD Bioscience)

instructions, and then stained with anti-IL-10 and anti-Foxp3 Ab-dye conjugates.

Some 10^4 - 10^5 cell events were acquired using a LSR II Becton-Dickinson instrument equipped with the WINLIST analysis software (Verity, Topsham, ME, USA), or with a BD FACS DIVA software (BD Biosciences).

2.4. Glucose tolerance test

Mice were fasted overnight, injected i.p. with 60 mg of glucose in saline, and the blood glucose levels were monitored at various time-points after injection using an Accu-Check glucose meter and glucose test strips (Roche).

2.5. Antibody Assays

Anti-PR8 influenza antibody response in the pre-diabetic NOD non Tg mice and NOD/DR4 Tg littermates were assessed by ELISA and Hemagglutination Inhibition Assay (HIA) as we previously described [19].

2.6. Cell isolation and cytokine assessment

CD4 T-cells were isolated from the spleen of mice by negative sorting on CD4 mouse column kits (BD Pharmingen) at higher than 95% purity according to FACS analysis. In some experiments, splenic CD3⁺ T-cells were sorted on CD3 magnetic immunobeads according to the manufacturer's instructions (Miltenyi Biotec), washed, and rested at 37°C and 5% CO₂ for 24 h in RPMI medium containing 10% FCS before use. Dead cells were removed by 10 min centrifugation at 1,000 rpm and cell viability in trypan blue staining was scored microscopically. Cell preparations with viability higher than 85% were used in the assays. Splenocytes from individual mice were incubated at

5% CO₂ and 37°C in RPMI complete media containing 10% FCS and CD3/CD28 mAbs (2 µg/mL each/10⁶ cells/well), or recombinant GAD65 protein (10 µg/10⁶ cells), or synthetic GAD65₅₅₅₋₅₆₇ peptide (20 µg/10⁶ cells) [21,22], or in medium alone. Cells were cultured in 96-well plates for 24 h to determine IL-2 secretion, and respectively 72 h to determine the IL-4, IL-10, and IFN-γ secretion. The concentration of cytokines was calculated in triplicate wells using Multiplex mouse cytokines kits and a Luminex instrument (Luminex Corporation, Austin, TX). A 5-parameter logistics model equation was used to calculate cytokines concentration according to the manufacturer's instructions (MasterplexQT software, Miraibio, San Francisco, CA).

2.7. Confocal laser scanning microscopy (CLSM)

Plastic adherent cells (APCs) from the spleen of non-transgenic NOD mice and NOD/DR4 Tg littermates were co-stained for 30 min at 4°C on ice with HLA-DR4-FITC and I-A^d-Alexa Fluor conjugates (1.5 µg each/10⁶ cells) (BD Biosciences). Cells were washed twice in PBS/BSA 1% and mounted onto glass slides using Vectashield containing DAPI (Vector Laboratories Inc., Birmingham, CA). Co-localization of HLA-DR4 and I-A^{g7} molecules and their density at the single-cell level was calculated by means of fluorescence intensity for each Ab-dye conjugate captured as a 2D image with a ZEISS 710 Confocal Laser Scanning Microscope equipped with ZEISS ZEN 2009 analysis software (Thornwood, NY, USA).

2.8. Histology and Immunohistochemistry

The pancreas from individual mice was fixed overnight in 10% phosphate-buffered formalin and embedded in paraffin. 5 µm sections of pancreas were stained

with Hematoxylin-Eosin (H&E) to identify infiltrating lymphocytes and co-stained with a rabbit anti-insulin antibody (Santa Cruz Biotech, Santa Cruz, CA) revealed by a goat anti-rabbit IgG-HRP conjugate (Southern Biotechnologies, Birmingham, AL) to estimate the extent of insulin secretion and intra-islet distribution of insulin granules. Between 15 and 20 pancreatic β -islets for each pancreas were analyzed microscopically.

2.9. Biostatistics

T1D incidence in NOD non Tg mice and NOD/DR4 Tg littermates was determined within the same group and between the groups of mice under various experimental conditions by the nonparametric Kaplan-Meier test for which p^* values less than 0.05 were considered significant. Individual variations in the anti-PR8 virus-specific Ab and HIA titers in the influenza immunized mice and the intra-assay variations in the cytokine assays were calculated by Student's t -test and expressed as mean $\pm$ standard deviation (SD) at 99% interval of confidence.

3. Results

3.1. *The human HLA-DR*0401 molecules are co-expressed with the murine I-A^{g7} molecules on the APCs in NOD/DR4 Tg mice*

To find out the propensity of HLA-DR*0401 transgene on the major subsets of APCs in the NOD/DR4 Tg mice, we have carried out FACS analysis and confocal scanning microscopy at the single cell level. The HLA-DR*0401 molecules were expressed on about 30% of splenic CD11c⁺CD19⁻dendritic cells and 40% of splenic CD19⁺ B-cells in the NOD/DR4 Tg mice regardless the gender, whilst I-A^{g7} expression

was detected on $\approx 90\%$ of splenic CD11c⁺ CD19⁻ dendritic cells and 98% of CD19⁺ B-cells in both NOD/DR4 Tg mice and NOD non Tg littermates (Fig. 1A). The DR4 $\alpha\beta$ proximal domains are linked in frame with the murine I-E^d $\alpha_2\beta_2$ domains and the transgene is driven by the I-E^d promoter [17]. As the NOD mice have a deletion in the promoter region of the I-E α -chain gene [23], the cell surface expression of I-E/DR4 chimeric molecules may have been affected, since less APCs showed up positive for DR4 than I-A^{g7} in the NOD/DR4 Tg mice. A differential expression of various HLA molecules is also common in humans, as they are controlled by different gene promoters.

FACS analysis suggested that a large number of APCs may co-express both the HLA-DR4 and I-A^{g7} molecules in the NOD/DR4 Tg mice. Of note, the anti-I-A^d Ab recognizes the I-A^{g7} molecules which have only a replaced Asparagine amino acid with a Serine amino acid at position 57 in the β -chain. Indeed, single-cell CLSM analysis using enriched APCs splenic preparations from NOD/DR4 Tg mice showed that most of human and murine MHC class II molecules are co-expressed (Fig. 1B), and that the HLA-DR4 molecules have various levels of cell expression as detected by the fluorescence intensity (Fig. 1B, upper panels). Few dendritic cells ($\approx 1.5\%$) and B cells ($\approx 0.15\%$) showed single expression of human HLA-DR4 molecules (Figs. 1A and 1B, lower panels). The results indicated that the level of HLA-DR*0401 expression on NOD/DR4 APCs was relatively high and did not interfere with the level of I-A^{g7} expression when both were co-expressed on a single cell.

To find out whether the HLA-DR4 transgenic expression may have altered the expression level of B7.1 and B7.2 costimulatory molecules on APCs, spleen cells from 2 month-old NOD.DR4 Tg and non Tg NOD littermates were co-stained with CD11c/CD80, or CD11c/CD86, or B220/CD80, or B220/CD86 Ab-dye conjugates. There were no

significant differences in the expression of CD80) on B220⁺ B cells or dendritic cells CD11c⁺ dendritic cells, neither in the expression of CD86 on B220⁺ B cells or CD11c⁺ dendritic cells (Fig 2). This result indicated that the costimulatory function of major APCs was not affected by the HLA-DR 4 transgenic expression on APCs.

3.2. *The NOD/DR4 Tg mice are resistant to spontaneous and inducible diabetes*

Since the role of HLA-DR4 alleles in type 1 diabetes is still not well understood, we have next investigated the effect of HLA-DR*0401 allele on diabetes development in the NOD/DR4 Tg mice. In contrast to the NOD non Tg littermates, none of the NOD/DR4 Tg females or males (n=167) developed pancreatic lymphocyte infiltration (Fig. 3A) or hyperglycemia. In the NOD wild (wt) females, pancreatic insulitis is revealed as a lymphocyte infiltration in the β -islets by 3-5 weeks after birth, while the hyperglycemia onset is detected 4-5 months later. Consistent with the results of 32 microsatellites analyzed (data not shown), the spontaneous occurrence of diabetes in NOD non Tg littermates clearly demonstrated that the diabetogenic NOD background was fully recovered during the cross-breeding. The insulin secretory function of β -cells in NOD/DR4 Tg mice remained unaltered, whereas the pre-diabetic and diabetic NOD non Tg littermates showed a delay in restoring euglycemia upon a glucose load (Fig. 3B).

Furthermore, to find out whether diabetes can be induced in NOD/DR4 Tg mice, we treated the mice with cyclophosphamide, a cytostatic drug known to accelerate T1D onset in NOD wt mice by reduction of CD4⁺25⁺Foxp3⁺ Tregs [24]). None of the cyclophosphamide-treated NOD/DR4 Tg mice developed pancreatic insulitis or hyperglycemia during a 4-month follow-up. In contrast, 85% of non Tg littermates treated with cyclophosphamide developed hyperglycemia earlier than the untreated control

group (Fig. 3C). These results indicated that the NOD/DR4 Tg mice, but not their NOD non Tg littermates are resistant to spontaneous and inducible T1D.

3.3. T-cells from NOD/DR4 Tg mice are neither diabetogenic nor tolerogenic

We have next investigated whether the T-cells from NOD/DR4 Tg mice exhibit diabetogenic effects or interfere with the diabetogenic function of T-cells from NOD wt mice. For this, groups of NOD/Rag1 KO mice were infused i.p. with splenic T-cells from NOD/DR4 Tg mice (50×10^6 cells/mouse), or co-infused with a 1:1 or 5:1 mixture of splenic T-cells from NOD/DR4 Tg mice and hyperglycemic NOD wt mice. Control groups were the NOD/Rag1 KO mice infused with splenic T-cells from hyperglycemic NOD wt mice (10×10^6 or 50×10^6 cells/mouse) (Fig. 3D). None of the NOD/Rag1 KO recipients of T-cells from NOD/DR4 Tg mice developed hyperglycemia or pancreatic insulinitis. In contrast, the recipients of T-cells from hyperglycemic NOD wt mice or mixtures of T-cells from NOD/DR4 Tg mice and hyperglycemic NOD wt mice developed hyperglycemia within 4-6 weeks. These experiments demonstrated that the T-cells from NOD/DR4 Tg mice do not exert diabetogenic or tolerogenic effects *in vivo*.

3.4. The NOD/DR4 Tg mice and non Tg littermates express a different T-cell profile

Since autoimmune diabetes is a T-cell mediated disease, we have next analyzed the major T-cell subsets known to play a role in the development of diabetes. Several T-cell aberrations were revealed in the NOD/DR4 Tg mice. First, the frequency of CD4⁺ thymocytes was higher (* $p = 0.006$) and the frequency of CD8⁺ thymocytes was lower (* $p = 0.009$) in the NOD/DR4 Tg mice than in non Tg littermates (Fig. 4A, upper panels). Also, the frequency of CD4 mature T-cells was higher (* $p = 0.0003$) and the frequency of CD8 mature T-cells was lower in the spleen of NOD/DR4 Tg mice than in non Tg

littermates ($*p = 0.0003$) (Fig. 4A, lower panels). In spite of these alterations, the absolute number of thymocytes and splenocytes was comparable in both strains (Fig. 4B upper and lower left panels). A CD4/CD8 skewed ratio in the NOD/DR4 Tg mice (Fig. 4B upper and lower right panels) raised the question of whether altered apoptosis or homeostatic T-cell proliferation may be the underlying mechanism. Analysis of the early and late apoptosis in CD4 and CD8 mature T-cells based on Annexin V expression and respectively, DNA incorporation of propidium iodide (PI) showed no differences between the two strains (Fig. 4C). In contrast, the homeostatic proliferation of splenic CD8 T-cells but not CD4 T-cells appeared to be quite slow in the NOD/DR4 Tg mice as determined in a Bromodeoxyuridine (BrdU) experiment (Fig. 4D). Thus, an altered CD4/CD8 thymic output and homeostasis may well contribute to the CD4/CD8 skewed ratio in NOD/DR4 Tg mice.

Since the nature of MHC class II can shape the T-cell profile [25, 26], we questioned whether the CD4/CD8 skewed ratio in the NOD/DR4 Tg mice was due to the HLA-DR*0401 transgenic expression. For this, we analyzed several HLA-DR4 transgenic strains on a different genetic background in the presence or not of endogenous murine MHC class II molecules. Thus, HLA-DR*0401 expression in a C57BL/6 ($H-2^b$) background also skewed the thymic output and splenic CD4/CD8 ratio regardless the endogenous co-expression of murine MHC class II molecules (Fig. 5). These results strongly suggest that the human HLA-DR*0401 allele may exert similar regulatory effects on the CD4 and CD8 thymic output and the peripheral CD4/CD8 ratio when expressed in different genetic backgrounds.

Because the T-cell recall to self-antigens are important in T1D pathogenesis, we next compared the size of a major pool of T memory cells in NOD/DR4 Tg mice (resistant to T1D) and NOD non Tg littermates (prone to spontaneous T1D), namely the

CD44^{high} CD4⁺ and CD8⁺ T memory cells. The frequency of both CD44^{high} CD4⁺ and CD44^{high} CD8⁺ T memory cells was higher in NOD non Tg mice than in their DR4 Tg littermates ($28.9 \pm 0.87\%$ vs. $22 \pm 1.69\%$, and respectively $42.3 \pm 10.9\%$ vs. $28.86 \pm 3.79\%$) (Fig. 6), indicating a significantly reduced T memory pool. This may well account for a reduced number of diabetogenic T-memory/effector cells in the NOD/DR4 Tg mice.

3.5. The NOD/DR4 Tg mice express an aberrant TCR V β repertoire

It has been widely reported that transgenic expression of various MHC class II genes in mice leads to alterations in the TCR V β repertoire. Like in the parental C57/BL6 (Abb KO)/HLA-DRA1-IE α_2 /DRB1*0401-IE β_2 Tg mouse [17] used to generate our NOD/DR4 Tg strain, the TCR V β 5 family was poorly expressed ($*p=0.01$) and the V β 17 family was deleted (Fig. 7). The V β 6, V β 12, and V β 14 families were higher expressed in the NOD/DR4 Tg mice than in non Tg littermates ($*p=0.002$, 0.02 , and 0.003 respectively). Thus, as in the case of other human HLA transgenes expressed in mice, the HLA-DR*0401 transgenic expression in NOD mice induced quantitative alterations in the TCR V β families.

3.6. IFN- γ and IL-10 responses are altered in the NOD/DR4 Tg mice

Since the IFN- γ pro-inflammatory T-cell responses to β -cell antigens can damage the pancreatic islets and the IL-10 responses can suppress inflammatory processes [27, 28], we compared the IFN- γ and IL-10 responses in the NOD/DR4 Tg mice that do not develop diabetes and their NOD non-Tg littermates that develop the disease. The splenic T-cells from the NOD/DR4 Tg mice showed a significantly reduced IFN- γ response and increased IL-10 response to polyclonal stimulation with CD3/CD28 Abs

(* $p=0.028$) (Fig. 8, left panel). Similarly, the IFN- γ response to recombinant GAD65 protein (major autoantigen in T1D [25, 26]) was significantly low in the NOD/DR4 Tg mice as compared with their non Tg littermates (* $p=0.0065$) (Fig. 8, middle panel). Furthermore, stimulation of NOD/DR4 spleen cells with a major diabetogenic peptide (GAD65/67₅₅₅₋₅₆₇) failed to induce IL-2 secretion (Fig. 8, right panel). There was no significant alteration in the IL-4 response to the CD3/CD28 polyclonal stimulation or rGAD65 protein between the two groups of mice.

These results revealed first, a deficiency in the IFN- γ inflammatory response whilst a higher IL-10 suppressogenic response to polyclonal T-cell stimulation in the NOD/DR4 Tg mice. Secondly, the T-cell response to a major GAD65/67₅₅₅₋₅₆₇ diabetogenic epitope was absent in the NOD/DR4 Tg mouse.

3.7. The NOD/DR4 Tg newborns have a stronger Treg compartment than the non Tg littermates

The anti-diabetogenic roles of Foxp3 T regulatory cells (Treg) and IL-10-secreting T-cells in T1D have been widely demonstrated [27, 28, 29-31]. We thus analyzed and compared these Treg cell subsets in the NOD/Dr4 Tg mice and their age-matched NOD non Tg littermates early after birth and in the adulthood. The NOD/DR4 Tg newborns showed a significantly higher number of Foxp3⁺ T-cells as compared with their age-matched, non Tg littermates (* $p=0.035$), though the number of Foxp3⁺ T-cells in the adulthood was comparable to that of non Tg littermates (Fig. 9A). These data revealed that the expression of *HLA-DR*0401* allele in the NOD background favored the development of a high number of Foxp3⁺ Treg cells in the neonatal stage, which coincides with a sensitive time-window of lymphocyte infiltration in the pancreas.

In contrast to the differential expansion of Foxp3⁺ Treg cells in NOD/DR4 Tg neonates, the T-cells from both groups of mice showed comparable IL-10 synthesis in the neonatal and adult stages (Fig. 9B). Although the IL-10⁺ T-cell frequency was comparable in both groups of mice, the polyclonal IL-10 response was higher in NOD/DR4 Tg mice.

We have reported that the splenic CD3⁺4⁻8⁻ double negative (DNCD3) T-cells from NOD wt mice is a unique T-cell population that can delay the onset of diabetes in NOD/Scid mice infused with diabetogenic T-cells by a mechanism of differentiation into IL-10-secreting T-cells in pancreas [32]. To find out if the DNCD3 peripheral T-cells may play a role in the resistance of NOD/DR4 Tg mice to autoimmune diabetes, we further analyzed and compared this cell subset in both groups of mice. Analysis of DNCD3 T-cells in NOD/DR4 Tg mice and their non Tg littermates showed no quantitative difference (Fig. S1), which ruled out a role of these regulatory cells in the resistance of NOD/DR4 Tg mice to T1D.

3.8. The NOD/DR4 Tg mice have unaltered antigen-specific B-cell responses

Since the NOD/DR4 Tg mice showed a number of quantitative and qualitative T-cell aberrations critical for the development of diabetes, we questioned whether the B-cell responses are also altered in these mice. Both groups of mice were immunized with influenza viral antigens (UV-inactivated A/PR8/34 influenza virus), and 21 days later the virus-specific IgG antibodies and their ability to neutralize the virus were determined by ELISA, and respectively Hemagglutination Inhibition Assay (HIA). The amount of total IgG before immunization and titers of virus neutralizing antibodies in sera were comparable in both groups of mice (Fig. S2). Thus, in contrast to the phenotypic and

functional alterations in the T-cell compartment in the NOD/DR4 Tg mice, the antigen-specific B-cell responses were less if not at all affected by the HLA-DR4 expression.

4. Discussion

This study reveals multi-point alterations of the T-cell compartment in NOD mice upon transgenic expression of human MHC class II HLA-DR*0401 allele that have been strongly associated with the resistance to autoimmune diabetes.

The HLA-DR*0401 molecules were co-expressed with the murine I-A^{g7} molecules on the APCs in NOD/DR4 Tg mice. A differential cell expression of human HLA-DR4 versus murine I-A^{g7} molecules in the NOD/HLA-DR4 Tg mice that may “dilute” the diabetogenic effect of I-A^{g7} peptide presentation to T-cells, which could be responsible for the T1D resistance has been ruled out by FACS and CLSM. Both assays showed no “dilution” of the I-A^{g7} expression on B-cells and dendritic cells when co-expressed with HLA-DR4, and the frequency of these cells expressing I-A^{g7} molecules has not been altered in the NOD/DR4 Tg mouse.

The NOD/DR4 Tg mice showed a skewed CD4/CD8 T-cell ratio at the expense of CD8 T-cells due to deficient CD8 thymic output and homeostasis. This may well account for the lack of pancreatic insulinitis and later development of T1D in the NOD/DR4 Tg mice, since the CD8 T cells are the main executioners of the pancreatic β -cells. Skewed CD4/CD8 T-cell ratio has been also reported in some mouse strains [33, 34], rats [35] and humans [36]. A suggested mechanism refers to the MHC polymorphism in several discrete regions encoded by the exon 3 of human DR*0401 or murine I-E α chains [37, 38]. This mechanism has been ruled out in our NOD/DR4 Tg mice, since the HLA-DRA1-DRB1*0401/I-E^d _{α 2 β 2} chimeric transgene does not incorporate the human and

411 murine exon 3. It has been also suggested that the TCR specificity [39, 40] and the
412 nature of HLA haplotype [33-35] can dramatically change the CD4/CD8 T-cell ratio. This
413 seems to be more likely the case of T1D resistance in the NOD/DR4 Tg mice. Newly-
414 generated TCR specificities through a mechanism of HLA-DR4/murine I-A^{g7} α/β
415 hybridization in the NOD/DR4 Tg mice is unlikely to occur, since no such miss pairing
416 events have been detected in the parental C57BL/6 (Abb KO), HLA-DRA1-DRB1*0401/I-
417 E^d _{$\alpha 2\beta 2$} Tg strain [17]) used to generate our NOD/DR4 Tg mouse. Instead, as previously
418 suggested by studies in mice expressing various HLA alleles [26, 41], new TCR
419 specificities may have been generated in the NOD/DR4 Tg thymus by HLA-DR4
420 presentation of a different set of peptides than those presented by the murine
421 endogenous MHC class II molecules (I-A^{g7}). On the other hand, some TCR specificities
422 could be lost, as we found in the NOD/DR4 Tg mouse lacking a CD4 T-cell response to
423 the GAD65₅₅₅₋₅₆₇ diabetogenic peptide. This could be an additional event contributing to
424 the resistance to T1D in these mice.

425 The NOD/DR4 Tg mice expressed an aberrant TCR V β repertoire, as reported
426 for some HLA transgenic mice including the parental C57BL/6 (Abb KO), HLA-DRA1-
427 DRB1*0401/I-E^d _{$\alpha 2\beta 2$} Tg mouse [17] used to generate our NOD/DR4 Tg mouse. Such
428 aberrations were linked to endogenous mouse mammary tumor viruses (MMTV)
429 harbored in the C57BL/6 mouse (MMTV-8, -9, and -17) [42], that could engage the IE
430 constant domains and delete certain TCR V β families [43]. So far, correlates between
431 T1D development and TCR V β families have not been clearly defined in the NOD mouse
432 or humans, making the MMTV hypothesis less accountable for the T1D resistance in
433 NOD/DR4 Tg mice. The MMTV hypothesis can neither explain those preferential TCR

V β aberrations only in the CD4 T-cells but not in the IL-10⁺ and Foxp3⁺ Treg subsets in the NOD/DR4 Tg mouse.

The NOD/DR4 T-cells were not anergic, as they responded to CD3/CD28 polyclonal stimulation. However, the IFN- γ response was lower whereas the IL-10 response was higher than in non Tg littermates, indicating a dual shift towards a less inflammatory and a more suppressogenic T-cell response. Low IFN- γ response can be attributed to lower number of IFN- γ ⁺ CD8 T-cells as a consequence of a low CD8 thymic output, whereas increased IL-10 secretion may account for an increased number of CD4 T-cells in NOD/DR4 Tg mice. These two sets of functional alterations may have been also contributed to the T1D resistance in NOD/DR4 Tg mouse. The T-cells from NOD/DR4 Tg mice were neither diabetogenic, nor suppressogenic, since they were unable to induce or suppress diabetes when transferred alone, or respectively co-transferred with diabetogenic T-cells in NOD/RAG deficient mice. Failure to induce diabetes in NOD/DR4 Tg mice by treatment with cyclophosphamide strongly suggests that only a deficiency in CD4⁺25⁺Foxp3⁺ Treg pool does not suffice for T1D development.

In contrast to these multi-point T-cell alterations, the antigen-specific B-cell response in the NOD/DR4 Tg mice such as to influenza virus remained unaltered. This was somehow expected, since the primary anti-flu B-cell response occurs mainly in a T-cell independent manner [44]. In summary, this study revealed a complex regulatory function of human *HLA-DR*0401* allele on several T-cell subsets with critical role in T1D development. Using a different system, namely C56Bl/6 mice deficient for endogenous MHC class II molecules and at the same time triple transgenic for human DQ8 and DR4 expressed on APCs, and B7 expressed under the insulin promoter (DQ8DR4/RIP-B7 mice), Li et al. showed that DR4 molecules seem to downregulate the DQ8 diabetogenicity. The authors suggest that a Th2-like protective immune response might

459 be the DR4 regulatory mechanism in C57Bl/6 background [45]. The regulatory effect of
460 DR4 over the D88 diabetogenicity was also suggested in humans [7], though the
461 underlying mechanism remains to be investigated.

462 Interesting enough, we have recently reported that over-costimulation of T-cells in
463 NOD/DR4 mice by expression of a B7 transgene in pancreas (NOD/DR4/B7 double
464 transgenic mouse) was able to break the HLA-DR4-induced tolerance to T1D, since 80%
465 of these mice developed the disease regardless the gender [46]. This is in agreement
466 with previous data in C57BL/6 transgenic mice lacking endogenous mouse MHC class II
467 molecules and expressing HLA-DQ8 and/or DR4 molecules at the same time with the
468 B7.1 costimulatory molecules on pancreatic beta cells [45]. It thus appears that
469 enhanced costimulation of T-cells in pancreas is more beneficial for the effector T-cells
470 than regulatory T-cells. **In conclusion, this study describes a multifaceted regulatory**
471 **function of the *HLA-DR*0401* allele strongly associated with the lack of T1D**
472 **development in NOD mice. It would be of clinical interest to find out whether some or all**
473 **T-cell features found in the NOD/DR4 Tg mice may correlate with resistance to**
474 **autoimmune diabetes in humans, particularly in siblings showing disparities in terms of**
475 **disease occurrence.**

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Figure Legends

Figure 1. HLA-DR4 expression by APCs in the NOD/DR4 Tg mice. *Panel A*, FACS analysis for HLA-DR4 and I-A^{g7} expression on splenic CD11c⁺ dendritic cells and CD19⁺ B-cells from NOD non Tg mice (n=4) and their NOD/DR4 Tg littermates (n=5). Cells were triple-stained with anti-mouse I-A^d-Alexa Fluor, anti-human HLA-DR-FITC, and anti-mouse CD11c-PE or anti-mouse CD19-PE conjugates. 2x10⁵ cell events were acquired for individual mice in each group. Shown is the % mean ± SD values for positive cells in each group of mice. *Panel B*, CLSM single-cell analysis of I-A^{g7} and HLA-DR*0401 expression on enriched population of splenic APCs collected from 2 month-old NOD/DR4 Tg mice and NOD non Tg littermates; *upper left panel*, single-color stained APCs for nuclei with DAPI (blue color), anti-I-A^d Ab-Alexa Fluor 647 conjugate (red), anti-HLA-DR Ab-FITC conjugate (green). The I-A^{g7} and HLA-DR*0401 co-expression by double staining with anti-I-A^d and anti-HLA-DR Ab-dye conjugates (merged orange color, yellow arrow) is shown; *upper right panel*, MFI calculations based on the average color intensity of I-A^{g7} and HLA-DR4 stained molecules (n=3 mice per group) among 200 scored positive cells per mouse and using a ZEISS ZEN 2009 analysis software. Two representative cells are shown (yellow arrows, right lower panel) from one mouse in each group expressing different cell density of I-A^{g7} (red color histogram) and HLA-DR4 molecules (green color histogram). MFI absolute values for each color are shown in each quadrant, where X axis indicates the fluorescence intensity, and Y axis indicates the absolute frequency of pixels; *middle left panel*, group of splenocytes from a NOD non Tg mouse expressing only I-A^{g7} (red color), and *middle right panel*, group of splenocytes from a NOD/DR4 Tg littermate expressing both I-A^{g7} and HLA-DR4 molecules (merged, orange color). Of note, some cells staining only in blue (DAPI) in each group of mice indicates the lack of I-A^{g7} or HLA-DR4 expression on the surface; *lower panels*, large

number of APCs stained with the same Ab-dye conjugates like in upper panels for a representative NOD non Tg mouse (*lower left panel*) and a NOD/DR4 Tg mouse. Of note, very few APCs (2-3%) express only HLA-DR4 molecules in the NOD/DR4 Tg mouse (*lower right panel*, green color).

Fig 2. Effect of HLA-DR4 transgene on the expression level of CD80 and CD86 costimulatory molecules on APCs. 10^6 splenic cells from 2 month-old individual NOD/DR4 Tg mice (n=4) and non Tg NOD littermates (n=4) were triple-stained with anti-mouse CD80-FITC and B220-PerCP and CD11c-PE, or with CD86-FITC, B220-PerCP and CD11c-PE (BD Pharmingen, San Jose, CA). 2×10^5 cell events were acquired for individual mice in each group. Shown is the % mean $\pm$ SD values for positive cells in each group of mice. *Panel A*, level of CD80 expression on CD11c⁺/B220⁻ dendritic cells as indicated by the MFI values; *Panel B*, level of CD80 expression on CD11c⁻/B220⁺ B cells; *Panel C*, level of CD86 expression on CD11c⁺/B220⁻ dendritic cells; *Panel D*, level of CD86 expression on CD11c⁻/B220⁺ B cells. Bar graphs in each panel represent the MFI values for the corresponding NOD non-Tg mice and NOD/DR4 Tg mice for one of two representative experiments.

Figure 3. Resistance of NOD/DR4 Tg mice to T1D. *Panel A*, a representative pancreatic β -islet from a 5 month-old NOD/DR4 Tg mouse and a 5 month-old normoglycemic non Tg littermate after staining with a rabbit anti-Insulin Ab-HRP conjugate to reveal the intra-islet granules of insulin (brown color), and over stained with HE to reveal the lymphocyte infiltration (blue color). Two different degrees of lymphocytes infiltration of the pancreatic islets in a 5 month-old hyperglycemic non Tg NOD mice is shown in the lower panels. *Panel B*, glucose tolerance test for adult NOD/DR4 Tg mice and NOD non Tg littermates females and males as described.

Glycemia was monitored every other hour after glucose load. Red arrow indicates the time of glucose load, and the red dotted line indicates the upper level of normoglycemia established in a cohort of 2-6 month-old NOD non Tg mice (n=25 mice). *Panel C*, 2 month-old NOD non Tg mice (n=7) and NOD/DR4 Tg littermates (n=7) were treated with Cyclophosphamide (CycloPh) as described, and the cumulative incidence of hyperglycemia (Y-axis) was monitored weekly after the last injection. Red arrow (X-axis) indicates the time of last CycloPh injection. *Panel D*, 2 month-old NOD/Rag1 KO mice (n=7 mice/group) received i.p. splenic T-cells from hyperglycemic NOD wt mice in a mixture or not with splenic T-cells from a pool of 4 month-old euglycemic NOD/DR4 Tg mice, and glycemia was monitored on a weekly basis. Red arrow on X-axis indicates the time of cell infusion or co-infusion.

Figure 4. Frequency and phenotype of thymic and splenic CD4 and CD8 T-cells populations and their homeostatic rates in NOD/DR4 Tg mice and non Tg littermates.

Panel A, single-cell suspensions from the thymus or spleen of individual 2 month-old, female NOD/DR4 Tg mice (n=6) and NOD non Tg mice (n=6) double stained with CD4 Ab-PE and CD8 Ab-FITC conjugates (InVitrogen) analyzed by FACS. *Panel B*, Total number of splenocytes and thymocytes were comparable in both groups of mice (n=6) (left panel), but the CD4 and CD8 total number (middle panel) and CD4/CD8 T cell ratios (right panel) were significantly higher in the NOD/DR4 Tg mice as compared to their NOD non Tg littermates. *Panel C*, spleen cells from the same mice in panel A were co-stained with CD4 Ab-PercP-Cy5.5 or CD8 Ab-Alexa Fluor 647, and over stained with Annexin V-FITC (Abcam) and Propidium Iodide (PI). Shown are gated CD4 and CD8 cells undergoing early apoptosis (low-right quadrant in each histogram) and apoptotic cells (upper-right quadrant in each histogram). *Panel D*, single-cell suspensions from the spleen of a 2 month-old, NOD/DR4 Tg female and a NOD non Tg female, 1 hour after

BrdU injection as analyzed for the rate of proliferation. Cells collected post-injection were stained with CD4 Ab-PerCP-Cy5.5 or CD8 Ab-Alexa Fluor 647 and analyzed in individual mice in each group for the percent of cells that uptake BrdU.

Figure 4. Effect of HLA-DR4 transgenic expression on the CD4 and CD8 T-cell

frequency in the thymus and spleen of $H-2^{g7}$ and $H-2^b$ mice. Single-cell suspensions (10^6 cells) from the thymus and spleen of 2 month-old C57BL/6 ($H-2^b$, *HLA-DR4*) Tg mice, or C57BL/6 (*Abb^{-/-}*, *HLA-DR4*) Tg mice, or NOD ($H-2^{g7}$, *HLA-DR4*) Tg mice, or NOD ($H-2^{g7}$) non Tg mice, or NOD ($H-2^{g7}$)/C57BL/6 ($H-2^b$) F1 hybrid mice (4-6 mice/group) were individually co-stained with CD3 Ab-PE and CD4 Ab-PerCP (or CD8 Ab-FITC) conjugates. The significant differences in the CD4/CD8 ratio in both thymus and spleen ($p < 0.005$, t-test) between the groups of mice analyzed is indicated. Of note, the HLA-DR4 expression altered significantly the CD4/CD8 ratio in the $H-2^{g7}$ and $H-2^b$ backgrounds.

Figure 6. Frequency of T memory cells in NOD/DR4 Tg mice and non Tg

littermates. Single-cell suspensions from the spleen of individual 2 month-old, female NOD/DR4 Tg mice ($n=4$) and NOD non Tg mice ($n=4$) were triple stained with CD4 Ab-PerCP-Cy5.5, CD8 Ab-Alexa Fluor 647, and CD44 Ab-PE. Quadrants marking the 10^3 - 10^4 fluorescence intensity for CD44 staining show the mean percent $\pm$ SD of memory ($CD44^{high}$) $CD4^+$ and $CD8^+$ T-cells.

Figure 7. TCR V β repertoire in CD4 T-cells from NOD/DR4 Tg mice and non Tg

littermates. Single-cell suspensions of splenocytes from 2 month-old NOD non Tg mice ($n=5$) and NOD/DR4 Tg littermates ($n=5$) were co-stained with CD3-PE Ab, CD4 Ab-PerCP-Cy5.5, and TCR V β s Ab-FITC conjugates (Bioscience, Palo Alta, CA), and analyzed by FACS for the mean percent $\pm$ SD for each TCR V β family in individual mice

from each group. Stars indicate significant differences ($*p \leq 0.05$) between the group of mice. Of note, V β -17a family has not been detected in NOD/DR4 Tg mice and their non Tg littermates.

Figure 8. T-cell responses in NOD/DR4 Tg mice and NOD non Tg littermates. 10^6 splenocytes from individual 2 month-old NOD/DR4 Tg mice and their NOD non Tg littermates (n=4/group) were stimulated *in vitro* with CD3 and CD28 mAbs (2 μ g mAb each) (*left panel*), or recombinant mouse GAD65 protein (10 μ g/ 10^6 cells, *middle panel*), or synthetic GAD65₅₅₅₋₅₆₇ diabetogenic peptide (20 μ g/ 10^6 cells, *right panel*), and the concentration of cytokines secreted in cell culture supernatants were determined by Luminex. Stimulation assays for determining IL-2 concentrations were carried out for 24 h and for other cytokines for 72 h. Shown are the significant $*p$ values between the group of mice (IFN- γ in the left panel ($*p=0.03$) and in the middle panel ($*p=0.02$), and for IL-10 in the left panel ($*p=0.041$).

Figure 9. Frequency of Foxp3⁺ and IL-10⁺ T-cells in the NOD/DR4 Tg mice and NOD non Tg littermates. *Panel A*, splenocytes (10^6 cells) from 2 and 8 week-old NOD/DR4 Tg mice (n=7) and NOD non Tg littermates (n=5) were co-stained with CD3-PerCP and Foxp3 Ab-FITC conjugates, and cell frequency of Foxp3 expressing cells was determined by FACS. Shown is a significant difference between the mouse groups at 2 weeks of age ($*p=0.027$). *Panel B*, splenocytes (10^6 cells) from individual 2 and 8 week-old NOD non Tg mice (n=4) and NOD/DR4 Tg littermates (n=5) were first stimulated *in vitro* for 2 days with CD3/CD28 mAbs (2.5 μ g mAb each/ 10^6 cells). Stimulated cells were treated for the last 4 h with monensin (Golgi Stop, # 554715) according to the manufacturer instructions (BD Biosciences, San Diego, CA), and then intracellularly

stained with IL-10 Ab-APC conjugate. The mean percentage $\pm$ SD of IL-10 positive T-cells analyzed by FACS in individual mice from each group.

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