HLA-DRB1*0402 (DW10) Transgene Protects Collagen-Induced Arthritis-Susceptible H2Aq and DRB1*0401 (DW4) Transgenic Mice from Arthritis

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HLA-DRB1*0402 (DW10) Transgene Protects Collagen-Induced Arthritis-Susceptible H2Aq and DRB1*0401 (DW4) Transgenic Mice from Arthritis

Veena Taneja, Neelam Taneja, Marshall Behrens, Suchong Pan, Tad Trejo, Marie Griffiths, Harvinder Luthra, and Chella S. David

To investigate the role of HLA-DR4 in predisposition to arthritis, we generated transgenic mice carrying DRB1*0401 and DRB1*0402 genes. We have previously shown that DRB1*0401 molecule renders B10.RQB3 (H2Aq) mice susceptible to porcine and human type II collagen-induced arthritis. We report that the introduction of DRB1*0402 transgene does not lead to development of arthritis in mice when they are immunized with porcine and human type II collagen. In addition, DRB1*0402 protects B10.RQB3 mice against developing arthritis with bovine type II collagen. These data show that DRB1 can modulate the disease mediated by Aq. In vivo depletion of DRB1*0402 did not lead to induction of collagen-induced arthritis in transgenic mice. In vitro cytokine analysis shows that mice protected from collagen-induced arthritis produce lower amounts of Th1 and higher levels of Th2 type cytokines upon immunization with type II collagen. Protection of mice was also related to higher apoptosis in DW10 mice as indicated by higher amounts of BclII in response to type II collagen. On the basis of our observations in HLA transgenic mice, we hypothesize that DRB1 polymorphism can modulate disease by shaping the T cell repertoire in thymus and select autoreactive T cells. The Journal of Immunology, 2003, 171: 4431–4438.

Certain HLA-DR\DQ alleles are associated with susceptibility to autoimmune diseases such as insulin-dependent diabetes mellitus (1, 2), multiple sclerosis (3), and systemic lupus erythematosus (4). Human rheumatoid arthritis (RA)3 predisposition has been associated with expression of some HLA-DR alleles, with HLA-DR4 probably being the most extensively studied linkage (5–8). Other alleles, notably DR1, DR6, and DR10, have been reported with predisposition to RA in certain ethnic populations (9–11). Gregersen and coworkers (12) and Winchester et al. (13) formulated the ‘shared epitope’ hypothesis to account for the association of certain DR alleles with RA in various ethnic groups. According to their hypothesis, the third hypervariable region comprising residues 67–74 is shared among susceptibility alleles and is a critical region for selection of RA relevant autoreactive T cells. Importantly, the sequence motif of I/D/E/A expressed at positions 67, 70, 71, and 74 (as expressed in DRB1*0402) confers resistance to RA. Although the mechanisms by which alleles affect degrees of RA susceptibility is not understood, it is thought that the T cell selection (positive selection of potentially autoreactive T cells by susceptibility alleles or negative selection by resistant alleles) and the resultant effect on shaping T cell repertoire has significant clinical outcomes (14, 15). The shared epitope hypothesis, however, may not account for all DR alleles linked to RA. For example, HLA-DR3 and HLA-DR9, linked in some populations, have been exceptions to a shared epitope hypothesis (16, 17).

RA is a chronic inflammatory disease that is characterized by synovial inflammation and erosion of bone and cartilage leading to destruction of joints. Patients with RA have been shown to produce anti-collagen type II (CII) Abs, T cell reactivity to CII, and accumulation of CII-reactive T cells in RA synovial fluid suggesting that autoreactivity to collagen might be important in pathogenesis (18–20). Collagen-induced arthritis (CIA) in mice has served as a classical model for human RA. The advent of mouse class II knockout mice expressing human HLA-DR and HLA-DQ transgenes has significantly advanced the understanding of the role of individual HLA class II molecules in various clinical conditions including RA. We have reported that transgenic mice expressing HLA-DQA1*0301 and DQB1*0302, but lacking endogenous class II molecules, are highly susceptible to CIA (21) and can present multiple human CII peptides (22). These observations contributed to the concept that DQ may be a major contributing factor in human RA while HLA-DR may modulate RA severity. We have proposed that both the DQ and DR alleles contribute to pathogenesis of RA (23). HLA class II molecules determine the T cell repertoire in the thymus by presenting self-peptides (24). This is supported by the findings that MHC-derived peptides constitute some of the naturally processed peptides eluted from class II molecules (25, 26). Binding studies have demonstrated that RA-associated HLA-DR alleles bind fewer human CII peptides compared with HLA-DQ8 allele (22, 27), suggesting HLA-DQ molecules may be a major factor in conferring susceptibility to develop arthritis. HLA-DQ occurs in linkage disequilibrium with DR genes and is inherited en bloc as haplotypes (28). HLA-DQ8 is found in linkage disequilibrium with DRB1*0401 and in increased frequency in RA patients of some ethnic groups (9, 29, 30).

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; BII, bovine CII; PII, porcine CII; HII, human CII; CB, cytochrome b; JNK, c-Jun N-terminal kinase; PARP, poly(ADP-ribose) polymerase; AICD, activation-induced cell death.
An RA-susceptible DR allele would presumably positively select an autoreactive T cell during thymic education while a non-susceptible allele would negatively select autoreactive T cells or would be neutral. To test this hypothesis we introduced DRB1*0401 and DRB1*0402 transgenes in B10.RQB3 to generate DRB1*0401.B10.RQB3 and DRB1*0402.B10.RQB3 transgenic mice. B10.RQB3 mice develop severe arthritis following immunization with bovine type II collagen (BII) but not with porcine type II collagen (PII). Introduction of DRB1*0401 gene into B10.RQB3 mice leads to development of severe arthritis in these mice following immunization with PII, indicating gene complementation of HLA-DR and H2-A is required for predisposition to develop disease (31). We report that DRB1*0402 can modulate the H2-A-restricted response to CII and protect *0402.B10.RQB3 transgenic mice from arthritis. In vivo, depletion with anti-DR Abs suggests that the autoreactive T cells are selected in thymus by DR polymorphism. We generated double transgenic mice expressing both DRB1*0401 and *0402 molecules. These mice develop milder arthritis and have a reduced incidence of CIA.

Materials and Methods
Transgenic constructs
The HLA-DRB1*0401 gene construct was made as previously described (32). DRB1*0401 gene was mutated in β2 domain (amino acids 110 and 139 DRB1*0401 (NT)) for better interaction with murine CD4. The overlap PCR method and DRB1*0401 cDNA template was used to generate the DRB1*0402 construct. The differences of the nucleotide sequence between DRB1*0401 and DRB1*0402 are: DRB1*0401, CAG AAG GAC CTC CTG GAG CAG CGG GCC; DRB1*0402, TTT AAG AAG GCC. Briefly, the 5’ and 3’ end complementary oligo primers of DRB4 (DRB1*0402) were synthesized as DW10–5’-5’-CCGGAAATTCTAGTTGTTCTGAAGTTC-3’; DW10-3’-5’-GGCCGGCTGGCCCTAGGTTCCGGTAGTTCC-3’. A pair of DRB1*0402-specific oligos were synthesized as follows: C1 (5’-AAGGACATCATCGGAGACGGCCGGC-3’); C2 (5’-GGCCGGCTGCTAGTTCGACAGTCGAAAGC-3’).

The two fragments were generated by PCR using DW10–5’ plus C2 and DW10-3’ plus C1 as primers and DRB1*0401 (NT) cDNA as template. The two fragments were purified and used in overlap extension to generate the full-length DRB1*0402 gene. This full-length gene was sequenced to exclude unexpected mutations, and then subcloned into pDOI-5 expression vector at EcoRI site downstream of the H2 Eoz promoter and rabbit β-globulin intron (33).

Transgenic mice
The DRB1*0402 β (nontransgenic)/pDOI-5 construct was double digested with NruI and XhoI to remove the plasmid sequence and microinjected into fertilized eggs from (SWR×B10)F1 mice. Viable embryos were reimplanted into oviducts of pseudopregnant foster mothers. Mice carrying the transgene were identified by Southern blot analysis using DRB1*0402 β cDNA construct as a probe. Founders were intercrossed and back-crossed to B10.RQB3 mice, which lack endogenous β2m but express Eoz intracytoplasmically. Thus the β-chain of DR molecule pairs with Eoz to express DRB1*0402.

For double transgenic mice, B10.RQB3 transgenic mice expressing *0401 and *0402 were mated to generate a line expressing both DRB1*0401 and *0402.B10.RQB3 mice. For conveniences, DRB1*0402.B10.RQB3 transgenic mice will be referred to as DW10 and DRB1*0401.B10.RQB3 transgenic mice as DW4 and double transgenic mice expressing both DR4 molecules as DW4/ DW10 in the study.

Flow cytometry
The expression of DRβ6, CD4, TCR Vβ-chains on PBLs of transgenic mice were analyzed by flow cytometry using mAbs: L227 (anti-DR), MKD6 (anti-A4), 14-4-4s (anti-Eoz), GK1.5 (anti-murine CD4), B20.6 (anti-Vβ2), KT4-10 (anti-Vβ4), MR9-4 (anti-Vβ5.1, 2), MR9-8 (anti-Vβ5.1), 44-2-1 (anti-Vβ6), TR310 (anti-Vβ7), K3-16 (anti-Vβ8.1, 2), F23.2 (anti-Vβ8.2), MR10-2 (anti-Vβ9), KT11 (anti-Vβ11), 14.2 (anti-Vβ14), KJ23a (anti-Vβ17) as previously described (34).

Induction and evaluation of CIA
Purified native BII, PII, and human type II collagen (HII) were obtained by multiple step purification (35). To induce CIA, 8- to 12-wk-old transgenic mice and negative littermates were immunized with 100 µg of CII emulsified 1:1 with CFA H37Ra (Difco Laboratories, Detroit, MI) intradermally at the base of the tail. Animals received a booster of 100 µg of CII emulsified with IFA 28 days later. DW4 transgenic mice were used as controls. Mice were monitored for the onset and progression of CIA from 3 to 12 wk postimmunization. The arthritic severity of mice was evaluated as previously described with a grading system for each paw of 0–3 (36). The mean arthritic score was determined using arthritic animals only.

Histopathology
Mice were sacrificed 10–12 wk postimmunization, and paws were decalcified and fixed. Sections were stained with H&E and examined histologically for mononuclear infiltration and bone erosion.

In vivo Ab administration
L227 (250 µg), specific for HLA-DRB1, was i.v. administered to DW10 transgenic and negative littermate mice at different time points beginning when mice were 8 wk of age. A total of 1.5 mg L227 was injected in each mouse.

Anti-CII Ab analysis
Levels of anti-bovine and anti-mouse CII IgG were detected in sera obtained 35 days following CII immunization by ELISA (37). Briefly, microtiter plates were coated overnight with CII (6 µg/well in KPOa, pH 7.6) at 4°C, washed and blocked with 1% BSA in PBS (0.05% Tween 20). Sera were added in 4-fold dilutions of 1:100, 1:400, 1:1600, and 1:6,500 (000) and incubated overnight at 4°C. The plates were washed and peroxidase-conjugated goat anti-mouse IgG (Organon Teknika, West Chester, PA) was added for another overnight incubation at 4°C. After washing, o-phenylenediamine was added, and the colorimetric change was measured at 410 nm. All assays were performed in duplicate and were quantified against a standard curve obtained with known positive sera, arbitrarily determined to equal 100 U/ml Ab units.

Serum samples of PII immunized DW10, DW4, and negative littermates obtained 35 days postimmunization were tested for Ab reactivity with cyanoanogen bromide (CB) peptides as described (31). Rheumatoid factor was measured by ELISA in transgenic mice as previously described (34).

T cell proliferation assay
Mice were immunized with 200 µg of CII emulsified 1:1 in CFA (Difco) intradermally at the base of the tail and one hind foot pad. Ten days postimmunization, draining popliteal, caudal, and lumbar lymph nodes were removed and prepared for in vitro culture. Lymph node cells (1×10⁶) were challenged with 100 µl of medium (negative control), Con A (20 µg/ml, positive control), and native collagen (50 µg/ml). For inhibition experiments, culture supernatant containing mAb (25 µg/ml) GK1.5 (anti-CD4), anti-A4 (MKD6), or Ly2 (anti-CD8) was added to the cells and challenged in vitro with CII at 50 µg/ml. The cells were incubated for 48 h at 37°C. During the last 18 h the cells were pulsed with [3H]thymidine and the tritium incorporation was determined by liquid scintillation counting. Results are calculated as the mean count per minute (Δ cpm) of triplicate cultures containing Ag mean cpm of medium.

Transgenic and negative littermates were also tested for T cell response to self-DR peptide DW10 (65–79) and control peptide DW4 (65–79), synthesized and purified at Mayo Clinic Peptide Facility. The mice were primed with 200 µg of peptide and challenged in vitro with 100 µg/ml of the peptide. To determine the restriction molecule for the in vitro response, blocking studies using L227 (anti-DR) and MKD6 (anti-H2A) Abs were performed.

Measurement of cytokines
Capture ELISA was done for measuring cytokines IFN-γ, IL-2, -6, -10, -13, and TNF-α, using kits according to manufacturer’s instructions (BD PharMingen, San Diego, CA).

Immunoblotting
Spleen cells were collected by centrifugation at 200 × g for 5 min and washed with cold PBS. Whole cell lysates were prepared using cold lysis buffer (50 mM Tris, pH 7.2, 100 mM NaCl, 1 mM DTT, 1% NP-40 and protease inhibitor mixture). Lysates were centrifuged at 10,000 × g and the supernatants were analyzed for the presence of Bcl-2, Bcl-XL, c-Jun N-
terminal kinase (JNK), JNK-P, caspase-3 and, poly(ADP-ribose) polymerase (PARP) by immunoblotting using Abs against Bcl-III, Bcl-\(\chi\)- (Santa Cruz Technology, Santa Cruz, CA), caspase-3, PARP, and JNK and its phosphorlated form JNK-P (New England Biolabs, Beverly, MA).

Cell division and activation-induced cell death (AICD)

For cell division, pooled lymph node cells from two to three primed mice were collected and stained with cell division tracking dye, CFSE, and cultured in vitro for 48 h with PII. Cells were stained with CD3-PE and analyzed by FACS.

To study the sensitivity of the proliferating cells to CII-induced AICD, CD3\(^{+}\) cells were stained with annexin-V conjugated with FITC after in vitro stimulation and analyzed by FACS.

Statistical analysis

The difference in the incidence of arthritis between groups was analyzed using the chi square test. Ab levels, onset of arthritis, and mean scores for arthritic mice were compared using Student’s \(t\) test.

Results

Expression of DR and TCR V\(\beta\) in transgenic mice

Expression of DRB1*0402 molecule on the cell surface was studied by flow cytometry. All transgenic mice showed 20–40% cells positive for expression of DRB1*0402 molecule in peripheral blood. As a criterion for functional expression of DW10, we determined V\(\beta\) T cell repertoire profile. Fewer number of V\(\beta\)8.1.2- and V\(\beta\)8.2-positive T cells were selected by DW10 mice compared with negative littermates suggesting DW10 to be functional in transgenic mice (Table I). All mice showed partial deletion of V\(\beta\)5.1.2, V\(\beta\)5.1, and V\(\beta\)11 compared with B10 mice (21). DRB1*0402 protects transgenic B10.RQB3 mice from arthritis

DW4, DW10 transgenic mice, and negative littermate controls were immunized with BII to ascertain whether the introduction of DW10 molecule alters CIA susceptibility of B10.RQB3. As shown in Fig. 1A, mice expressing DW10 were protected against arthritis (\(p < 0.05\)) following immunization with BII. Introduction of DW4 in B10.RQB3 mice increases the incidence of CIA following immunization with PII (20–57%) and HII (17–60%). In contrast, DW10 did not significantly increase CIA incidence following PII or HII administration (Fig. 1B and Table II). However, DW10 expression did result in a delayed onset (40 vs 72 days, \(p < 0.005\)) following HII injection (Fig. 1C, Table II). In comparison with DW4 mice, HII-immunized DW10 mice showed significantly lower incidence (\(p < 0.05\)) and delayed onset (\(p < 0.001\)) with milder disease (\(p < 0.05\)).

In vivo DW10 depletion does not lead to development of arthritis

DW10 mice were treated with anti-DR Ab to deplete DW10 in vivo (Fig. 2A). Expression of HLA-DR was determined by flow cytometry before collagen administration and at different time points thereafter (Fig. 2B). Mice exhibiting at least 40–50% decrease in HLA-DR expression were immunized with BII following Ab treatment. Only two of seven mice (28%) developed one swollen digit in one hind paw after 7 wk of immunization, consistent with a requirement for T cell selection.

HLA-DR molecules do not determine the specificity of anti-collagen Abs

All arthritic mice developed anti-collagen Abs to heterologous and self-collagen. A comparison of levels of anti-CII Abs showed that DW10 mice, negative littermates, and DW4 (positive control) mice produced an equal amount of anti-PII and anti-mouse CII Abs (Fig. 3A). In addition, arthritic mice primed with BII or HII also produced high levels of anti-CII and self-CII Abs (data not shown). We analyzed sera for reactivity to PII CB-digested fragments (CB 9/7, 8, 10, 11, 12). Expression of either DW4 or DW10 molecules did not significantly alter the pattern of Ab binding measured against PII CB polypeptides. Sera from both transgene positive and negative littermates showed a similar heterogeneous pattern of reactivity against major CB 12, CB 11, and CB 9/7 fragments (Fig. 3B). No difference was observed in reactivity against CB fragments in CIA\(^{+}\) vs CIA\(^{-}\) transgenic mice.

Transgenic mice can mount in vitro response to CII but are tolerant to self-peptide

DW10 mice and negative littermates were immunized with 200 \(\mu\)g of PII, HII, or BII and draining lymph node cells challenged in

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**Table I. Percentage of V\(\beta\) T cell repertoire in DW10 and DW4 transgenic mice and transgene-negative B10.RQB3 mice**

<table>
<thead>
<tr>
<th></th>
<th>5.1.2</th>
<th>6</th>
<th>8.1.2.3</th>
<th>8.1.2</th>
<th>8.2</th>
<th>11</th>
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<tr>
<td>DW10</td>
<td>2.1</td>
<td>19.5</td>
<td>17.4</td>
<td>7.8</td>
<td>3.9</td>
<td>2.5</td>
</tr>
<tr>
<td>DW4</td>
<td>1.2</td>
<td>16.3</td>
<td>24.3</td>
<td>31.2</td>
<td>17.1</td>
<td>0.4</td>
</tr>
<tr>
<td>B10.RQB3</td>
<td>2.4</td>
<td>17.9</td>
<td>32.2</td>
<td>24.2</td>
<td>19.8</td>
<td>3.6</td>
</tr>
</tbody>
</table>

\(^{a}\) PBLs were pooled from three mice per strain and V\(\beta\) profile was analyzed by flow cytometry using specific Abs as described in Materials and Methods. Experiment was repeated three times.
Table II. Incidence, onset, and severity of collagen-induced arthritis in DW10 transgenic mice and controls

<table>
<thead>
<tr>
<th>Mice</th>
<th>Incidence (%)</th>
<th>Onset ± SE</th>
<th>Severity ± SE</th>
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</thead>
<tbody>
<tr>
<td>BII</td>
<td>7/30 b,c (23)</td>
<td>54 ± 5d</td>
<td>2 ± 0.8c</td>
</tr>
<tr>
<td>DW10</td>
<td>5/20 b,c (25)</td>
<td>56 ± 4d,e</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>DW4</td>
<td>5/10 (62.5)</td>
<td>39 ± 8</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td>B10.RQB3</td>
<td>6/10 (60)</td>
<td>36 ± 5</td>
<td>4 ± 1.3</td>
</tr>
</tbody>
</table>

a Mice were immunized with various CII and monitored for arthritis. Arthritis score for each mouse was calculated as sum of arthritic severity of all four paws. Mean arthritic severity was calculated at the termination of the experiment at 12 wk postimmunization using arthritic mice only.

b and c DW10 vs DW4. d and e DW10 vs B10.RQB3. b,c p < 0.05; d p < 0.005; e p < 0.001.

**Low TNF-α production in DW10 transgenic mice in response to CII**

Draining lymph nodes from primed mice were cultured for 48 h in vitro in presence or absence of CII. At which time culture supernatants were harvested and assessed for the presence of IFN-γ and TNF-α (Th1), IL-13 and IL-10 (Th2), and IL-6 (regulatory) cytokines. DW10 transgenic mice produced high amounts of IFN-γ, IL-10, and IL-13 but undetectable amounts of TNF when immunized with PII (Fig. 4). Negative littermates produced similar amounts of IFN-γ in response to PII. Transgenic mice immunized with BII produced lower amounts of IFN-γ and undetectable levels of IL-10 and IL-13 (data not shown). Lymph node cells harvested from DW10 mice after 12 wk of immunization with BII or PII produced IL-6 and IL-10 with barely detectable IFN-γ and TNF-α to PII while lower amounts of IL-10 were produced. Both strains of transgenic mice and negative littersmates primed mice produce IL-6 (Fig. 6A).

Protected mice exhibit increased apoptosis

Mice primed with PII were sacrificed after 2 wk or 8–10 wk of immunization and spleen cells were collected. Whole cell extracts were prepared and analyzed for the expression of Bcl/II. Levels of Bcl/II were similar among the transgenic mice, negative littersmates and positive controls 2 wk after immunization (data not shown). Lysates prepared following 8–10 wk of immunization clearly showed lower levels of Bcl/II and Bcl-xL in cells from DW10 mice compared with those from DW4 transgenic mice while similar levels were observed in negative littersmates (Fig. 5, A and B). To further define the role of apoptosis in disease development in all three strains we also studied apoptosis markers, caspase-3 and PARP. There was an increased activation of PARP and caspase-3, as apparent by enhanced cleavage of both proteins in resistant mice compared with DW4 (Fig. 5, C and D). A stress-activated kinase, JNK, involved in apoptosis and proliferation is known to be induced by TNF-α. Because our results showed that TNF-α levels were undetectable in DW10 mice, we decided to determine the levels of JNK and its phosphorylation. Lysates from splenic cells showed similar amounts of JNK (Fig. 5E) but lower levels of its phosphorylation in DW10 mice compared with DW4 mice following immunization (Fig. 5F).

**DW10 transgene protects DW4/DW10 double transgenic mice from arthritis**

To determine whether DW10 can protect DW4 transgenic mice from arthritis, we generated double transgenic mice expressing both DR4 molecules, DW4/DW10. Cell characterization demonstrated that T and B cell profiles were similar in all mice (Table III). For CIA studies, single and double transgenic mice were immunized with PII and monitored for development of arthritis. A delayed onset with lower incidence of arthritis was observed in DW4/DW10 mice compared with DW4 mice, p < 0.05 (Table IV). Because mice with human transgenes are known to produce rheumatoid factor in CIA model (34), we analyzed rheumatoid factor in all transgenic mice. All the DW4 and 40% of DW4/DW10 mice produced rheumatoid factor, although no significant difference was observed in level of autoantibody. None of the DW10 mice produced rheumatoid factor (data not shown).
To investigate whether the modulation of disease was due to changes in cytokine profile, inflammatory cytokines IL-2, IL-6, IL-18, and TNF-α were measured by ELISA from the supernatants of splenic cells cultured in the presence of PII. Lower amounts of IL-2 and IL-18 were produced by DW10 and DW4/DW10 mice compared with DW4 mice (Fig. 6A). To study the kinetics of cell division and proliferation, lymph node cells CD3+/H11001 cells were isolated from primed mice and challenged in vitro with PII after staining them with CFSE. Higher number of dividing CD3+ T cells were observed in DW4 compared with DW10 and DW4/DW10 mice after 48 h in response to PII (Fig. 6B). We further investigated whether increased apoptosis was observed in double transgenic mice. Splenic cells were isolated from all the primed transgenic mice and cultured in the presence of PII for 72 h and then stained with annexin-V. Equal percent of CD3+ cells were found to be annexin-positive in DW10 (42%) and B10.RQB3 mice (38%) while higher numbers of apoptotic cells were observed in DW4/DW10 mice (52%) compared with that of DW4 mice (25%), (Fig. 6C).

**Discussion**

DRB1*0402 (DW10) has been associated with protection from RA in human. In this study we report that DW10 can protect CIA susceptible mice against development of arthritis. B10.RQB3 mice develop severe arthritis following BII immunization but exhibit a
milder and a lower incidence following PII challenge. However, introduction of a DW4 transgene renders them susceptible to PII-induced arthritis (31). No increase in incidence and severity of arthritis was observed in DW10 mice compared with parental mice after immunization with PII. Double transgenic mice expressing both DW4 and DW10 molecules had a lower incidence of CIA with PII compared with DW4 mice. DW10 mice are also protected from developing arthritis following immunization with BII. The present and previous data suggest that development of arthritis in B10.RQB3 mice is modulated by DR transgenes. Primed mice were capable of mounting a T cell response to PII in vitro and producing Abs to self and heterologous CII. However, the fine specificity of the Abs as measured against CB polypeptides was not different among the mouse groups in this study suggesting that the anti-collagen Ab response was primarily determined by H2-A molecule. These results confirm our previous studies with HLA-DR/DQ and DR/H2-A\(^*\) transgenic mice suggesting that complementation between DR and DQ/H2-A genes is required for the development of arthritis (31, 38).

MHC molecules have been shown to be important in selection of T cell repertoire in thymus and various V\(\beta\) TCRs (39). Our results are consistent with this hypothesis in that a difference in V\(\beta\) repertoire was observed between the two transgenic strains. Expression of DW10 transgene leads to negative selection of V\(\beta\)8.2 T cell population. Previous studies have shown that V\(\beta\)8.2 is important in induction of CIA in H2A\(^*\) mice. Absence of V\(\beta\)8.2 TCR population in FVB mice is one of the reasons for nonsusceptibility to CIA (40). In vivo depletion of DW10 did not make the transgenic mice susceptible to arthritis suggesting that negative/positive selection of autoreactive cells occur in thymus and is probably influenced by polymorphism in MHC. Contribution of DRB1 molecule toward T cell selection is further supported by the fact that DW10 mice mount milder response to CII immunization than DW4 mice and negative littermates although the major response is restricted by A\(^*\) molecule. These findings are reminiscent of the MHC linked protection described in diabetes model (41). According to this model, MHC molecules provide dominant resistance to a given autoimmune disease by deleting the most pathogenic autoreactive T cells rather than all autoreactive T cells. Thus the reason that RA patients carrying DRB1*0402 are rare might be explained by the fact that most of the pathogenic autoreactive cells have been deleted in these individuals and even if the other

### Table III. Cell characterization in DRB1*04 single- and double-transgenic mice\(^a\)

<table>
<thead>
<tr>
<th>Marker</th>
<th>B10.RQB3</th>
<th>DW4</th>
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<tbody>
<tr>
<td>CD3</td>
<td>25 ± 2</td>
<td>20 ± 1</td>
<td>25 ± 2</td>
<td>23 ± 1</td>
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<td>CD4</td>
<td>55 ± 3</td>
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<tr>
<td>B220</td>
<td>65 ± 3</td>
<td>74 ± 1</td>
<td>65 ± 5</td>
<td>67 ± 4</td>
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</tbody>
</table>

\(^a\) Splenic cells isolated from naive transgenic and control mice were harvested and stained with conjugated Abs CD3-PE, CD4-Percp, CD8-APC, and B220-FITC and analyzed by FACS. Numbers indicate the percent of the CD3 and B cells in each strain. CD3\(^{+}\) cells were gated for analyzing CD4 and CD8 cells. The experiment was done three times with two to three spleens pooled at each time from each strain.

### Table IV. Incidence and severity of arthritis in single- and double-transgenic mice following immunization with porcine CII\(^a\)

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<tr>
<th></th>
<th>Incidence (%)</th>
<th>Severity Mean ± SE</th>
<th>Day of Onset Mean ± SE (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW4/DW10</td>
<td>6/19 (32)(^b)</td>
<td>3.7 ± 0.9</td>
<td>8.3 ± 2.3(^c)</td>
</tr>
<tr>
<td>DW4</td>
<td>7/10 (70)(^b)</td>
<td>5.6 ± 2.6</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>Dw10</td>
<td>0/4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mice with one or both transgenes were immunized with porcine CII and monitored for development of arthritis. Arthritic severity was calculated as sum of all the arthritic paws. A delayed onset with lower incidence of arthritis was observed in DW4/DW10 mice compared to DW4 transgenics.

\(^b\) and \(^c\) \(p < 0.05\).
haplotype has susceptible alleles, resistance is dominant. Our re-
sults with double transgenic DW4/DW10 mice are in agreement
with this hypothesis. In these mice we observed lower incidence
and delayed onset of arthritis with only 40% of mice producing RF
compared with 100% of DW4 mice tested. H2-A9 mice patho-
genic response to CII has been mapped to CBII region of CII
with amino acid 260–270 being the immunodominant region (42).
Interestingly, both transgenic strains, DW10 and DW4, mount re-
sponse to peptide 254–270 of human type II collagen although a
lower response was observed in DW10 mice (Ref. 31 and data not
shown). Both DW4 and DW10 have been shown to be able to bind
this peptide, albeit with different anchors in P1 pocket (43). Pre-
sentation of this peptide in double transgenic mice might lead to
increase in proliferation as observed with CFSE staining after in
vitro culture with PII thus reaching a threshold for autoreactivity
and development of arthritis.

Both transgenic strains produce high amounts of IFN-γ and
IL-10 in vitro when challenged with PII. IL-10 has been associated
with protection from arthritis in most of the experimental models
studied. IL-10 deficiency in a CIA resistant mouse leads to severe
disease (44). IL-10 has been shown to be produced by regulatory
cells that have become anergic in vivo (45) or by CD4+ T cells in
response to autoantigen presented by B cells (46). DW4 mice pro-
duce higher amounts of proinflammatory cytokines, IL-18 and
TNF-α, than DW10 and DW4/DW10 mice thus indicating that a
balance of Th1/Th2 cytokines is required for development of dis-
b ease. IL-18 is known to be induced by TNF-α and has recently
been shown to be important in CIA (47). Inflammatory cytokines
TNF-α and IL-1 have been shown to induce activation of JNK, a
stress activated kinase. Using JNK inhibitor and JNK knockout
mice, it has been shown that it is required for the expression of
metalloproteinase and joint destruction in inflammatory arthritis
(48). JNK has also been implicated in signaling cell survival (49).
We observed increased phosphorylation of JNK in cells of DW4
mice compared with DW10 mice.

Our data using annexin support the previously described results.
Double transgenic and DW10 mice had higher number of CD3
cells positive for annexin. Increased apoptosis in DW10 mice was
confirmed by enhanced cleavage of PARP and caspase-3. From
our results of apoptotic proteins, it appears that early on there is no
difference in apoptosis after immunization in DW4 and DW10
mice. However, a longer duration following immunization resulted in
a different pattern in transgenic mice. A comparison of various
proteins associated with apoptosis after 8–10 wk of immunization
showed decreased Bcl/II and Bcl-xL levels in DW10 transgenic
mice compared with DW4 transgenic mice. Increased levels of
Bcl/III and phosphorylation of JNK in DW4 mice might contribute
to decreased cell death and to clonal expansion in these mice.
Decreased proliferation with increased apoptosis might result in
protecting the DW10 expressing mice from developing arthritis.
CFSE staining of cultured lymph node cells confirmed decreased
cell division in DW10 mice compared with both DW4 and DW4/
DW10. This might explain stronger in vitro response to CIA
immunization and development of arthritis in DW4 mice and not in
DW10 mice. Recent studies have shown oligoclonal expansion of
T cells in periphery and joints of RA patients, which is thought to
be driven in part by CIA, suggesting that T cells reactive to CIA may
be mediators of RA pathogenesis (50, 51). In double transgenic
mouse resistant allele is able to modulate the development of arth-
ritis by lower amounts of inflammatory cytokines and increased ap-
optosis leading to decreased incidence. Thus even though our re-
sults suggest that the major response to immunizing collagen in
vivo is H2-A9 restricted, it is apparent that cytokine milieu pro-
duced by T cells selected in thymus lead to pathogenesis.

Thus on the basis of our observations, following mechanisms occurring alone or in combination can be suggested: 1) DRB1 polymorphism leads to selection of TCR in thymus thus control-
ling the selection of autoreactive cells available in periphery, 2)
presentation of a peptide by DR molecule might enhance the au-
toantigenic immune response, and 3) variability in DRB1 loci is
associated with differential AICD or apoptosis thus leading to
protection/susceptibility.

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