Expression of HLA-DR4 and human CD4 transgenes in mice determines the variable region β -chain T-cell repertoire and mediates an HLA-DR-restricted immune response

LARS FUGGER^{†‡}, SARA A. MICHIE^{§¶}, INGRID RULIFSON[†], CHRISTOPHER B. LOCK[†],
AND GRETE SØNDERSTRUP McDEVITT^{||}

Departments of [†]Microbiology and Immunology, [§]Pathology, and ^{||}Medicine, Stanford University School of Medicine, Stanford, CA 94305; and [¶]Department of Veterans Affairs, Center for Molecular Biology, Palo Alto, CA 94304

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ABSTRACT Inherited susceptibility to rheumatoid arthritis is associated with genes encoding the human major histocompatibility complex class II molecule HLA-DR4. To study the immune function of HLA-DR4 and attempt to generate a murine model of rheumatoid arthritis we have produced triple transgenic mice expressing HLA-DRA*0101, -DRB1*0401, and human CD4. The expression of the HLA transgenes is driven by the promoter of the murine major histocompatibility complex class II I-E α gene and was found on murine cells that normally display major histocompatibility complex class II molecules. The expression of the human CD4 transgene is driven by the murine CD3 δ -promoter, and therefore its gene product was found on cells that express murine CD3. In contrast to other HLA-DR and HLA-DQ transgenic mouse lines, the transgenes are functional in our mice. In H-2 I-E-negative transgenic mice, T cells expressing variable region β chain (V β) 3, 5, 6, 7, 9, 11, 12, or 13 were either absent or significantly reduced, in contrast to H-2 I-E-negative nontransgenic littermates. In addition, the peptide antigen influenza A virus hemagglutinin 307–319, which binds to the HLA-DRA*0101/-DRB1*0401 heterodimer with high affinity and induces an HLA-DR-restricted and CD4⁺ T-cell response in humans, also induced a T-cell response in the triple transgenic mice but not in nontransgenic littermates. Thus, these transgenic mice should permit extensive testing of the antigen-presentation capabilities of the HLA-DRA*0101/-DRB1*0401 molecule.

The human major histocompatibility complex, the HLA system, encodes at least three polymorphic series of HLA class II antigens, designated HLA-DR, -DQ, and -DP, which are highly polymorphic cell-surface proteins consisting of an α and a β chain. HLA class II molecules are constitutively expressed on the cell surface of B cells, monocytes, and dendritic cells in the peripheral lymphoid organs and on epithelial and dendritic cells in the thymus. The HLA class II molecules have a central role in the immune response by shaping the T-cell repertoire in the thymus and by presenting antigenic peptides to CD4⁺ T cells. This function is also thought to be the basis for the association between several autoimmune disorders and certain HLA class II alleles (1–3).

Rheumatoid arthritis (RA) is an autoimmune systemic disease affecting primarily the synovial tissue in joints. The etiology of RA is unknown but likely to be multifactorial. Family and twin studies have shown that RA aggregates in families. The concordance rate for monozygotic twins is 15–30% as compared with 10% for HLA identical siblings, suggesting a major role for environmental factors in the pathogenesis and polygenic inheritance of susceptibility to

the disease (3). The strongest genetic component identified thus far is encoded by HLA class II genes. Thus, inherited susceptibility to RA in several ethnic groups is associated with the serologically defined HLA-DR4 or HLA-DR1 groups (2). The serologically defined HLA-DR4 group is divided into at least five subtypes, of which DRB1*0401, DRB1*0404, and DRB1*0405 confer susceptibility to RA, and DRB1*0402 and DRB1*0403 do not (3). The disease-associated alleles share sequences within the third hyper-variable region of the DRB1 gene, which has led to the hypothesis that this region is, at least in part, responsible for the genetic susceptibility to RA (4). Moreover, based on the three-dimensional structure of the antigen-binding site of the HLA-DRA*0101/DRB1*0101 molecule (5) the third hyper-variable region of the β chain is found in the binding site of the class II molecule. Thus, the expression of a common RA-associated sequence may allow presentation of a putative self-antigen(s) to CD4⁺ T cells, initiating an immune response leading to disease.

We have used transgenic technology to establish mice expressing HLA-DRA*0101/-DRB1*0401 and human CD4 with the long-term goal of probing HLA-DR4 function and establishing an animal model to study the pathogenesis of RA. Here we report the initial characterization of these mice.

MATERIALS AND METHODS

Transgene Constructs. HLA-DRA*0101 and HLA-DRB1*0401 (Gly-86) cDNAs were separately cloned into the unique EcoRI site of the pDOI-5 expression vector (6). In pDOI-5, the cDNAs are each located downstream of the H-2 I-E α promoter and the rabbit β -globin intron and upstream of the β -globin polyadenylation site. The regulatory and encoding parts of the HLA-DRA*0101/pDOI-5 and the HLA-DRB1*0401/pDOI-5 constructs were assembled into one construct in the pSV5-neo expression vector (7, 8). The mouse CD3-human CD4 cDNA/genomic (mCD3-huCD4c/g) construct was provided by E. Lacy (Memorial Sloan Kettering Cancer Center, New York) and has recently been described in detail (9). In brief, the construct consists of a cDNA fragment encoding exons 2–4 and part of exon 5 of the human CD4 molecule, fused to a genomic fragment encoding the remainder of exon 5, exons 6–9, and part of exon 10 of the human CD4 molecule. The transcriptional initiation and termination signals of this construct are provided, respectively, by a fragment from the mouse CD3-delta promoter and a fragment containing the simian virus 40 poly(A) site. The assembled HLA-DRA*0101/DRB1*0401/pDOI-5 construct was released from pSV5-neo by using a Sal I/Xho I double digest, and the

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Abbreviations: RA, rheumatoid arthritis; mAb, monoclonal antibody; TCR, T-cell receptor; V β , variable region β chain; SAG, superantigen; HA, hemagglutinin.

[‡]To whom reprint requests should be addressed.

mCD3-huCD4c/g construct was released from its vector by using a *Not I* digest.

Transgenic Mice. The HLA-DRA*0101/-DRB1*0401 and mCD3-huCD4c/g constructs were comicroinjected into embryos from (DBA/1 $\times$ A.CA) F_1 matings, and viable embryos were transferred to the oviducts of pseudopregnant female (BALB/c $\times$ 129) F_1 mice for development to term (10). The DBA/1 and A.CA mice were originally purchased from The Jackson Laboratory. Transgenic founders were identified by Southern hybridization of tail DNA digested with (i) *EcoRI* to test for integration of the HLA-DRA*0101/-DRB1*0401 construct and (ii) *HindIII* to test for integration of the mCD3-huCD4c/g construct. *EcoRI* blots were made in duplicate and hybridized separately to HLA-DRA*0101 and HLA-DRB1*0401 cDNAs 32 P-labeled by random hexamer priming (11). *HindIII* blots were hybridized to a 32 P-labeled fragment from the mCD3-huCD4c/g construct.

Antibodies and Conjugates. The following monoclonal antibodies (mAbs) were grown in ascites and purified by ammonium sulfate precipitation followed by protein A chromatography (12): 10-3-6 (anti- $I-A^b$, k,r,s,u,g 7 , ref. 13), MK-D6 (anti- $I-A^b$, d, ref. 14), BP.107 (anti- $I-A^b$, d,p,q,u, ref. 15), RA3-6b2 (anti-B220, ref. 16), 33.12 (anti-IgM, ref. 17) SPVL-3 (anti-HLA-DQ, ref. 18), and L243 (anti-HLA-DR, ref. 18). Aliquots of the 10-3-6, MK-D6, anti-B220 and L243 mAbs were substituted with biotin (19). The following mAbs were purchased from Becton Dickinson Immunocytometry Systems: fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated anti-HLA-DR, and fluorescein isothiocyanate-conjugated Leu3a (anti-human CD4). The following mAbs were purchased from PharMingen: mAbs to mouse CD4, CD8, CD3, and V β T-cell receptor (TCR). Purified mAbs Leu3a, SPVL-6 (anti-HLA-DQ), and GK1.5 (anti-murine CD4) were from A. Rivas (Stanford University, Stanford, CA), W. van Schooten (Immologic Pharmaceutical Corporation, Palo Alto, CA), and I. Weissman (Stanford University).

Flow Cytometry Analysis and Immunohistochemistry. Flow cytometry analysis was done essentially as described by Hayakawa *et al.* (19). Cells were analyzed on a dual-laser FACS IV (Becton Dickinson) equipped with a logarithmic amplifier. Data were analyzed with cell cytometry desk software (Stanford University Shared FACS Facility). Cells that stained with propidium iodide were excluded from analysis. Immunohistochemistry was done as described in Gilfillan *et al.* (20).

Immunization and T-Cell Restimulation Assay. Footpads were injected with 50 μ l of the influenza A virus hemagglutinin (HA) 307–319 peptide (21, 22) at 1 mg/ml emulsified with complete Freund's adjuvant (Difco). Eight to 10 days later popliteal and inguinal lymph nodes were removed. Single-cell suspensions were prepared and resuspended at 1×10^7 cells per ml in complete medium consisting of RPMI 1640 medium (Bethesda Research Laboratories) containing 1% Nutridoma-SP (Boehringer Mannheim); 2 mM L-glutamine, penicillin at 100 units/ml, streptomycin at 100 μ g/ml, and 50 μ M 2-mercaptoethanol. Cells were plated in 96-well microtiter plates with 5×10^5 cells per well with or without soluble antigens. After 72 hr, the cells were pulsed with 0.5 μ Ci of [*methyl*- 3 H]thymidine (1 Ci = 37 GBq; DuPont/New England Nuclear) and harvested 16 hr later; radioactivity was counted in a scintillation counter. mAbs used for blocking T-cell responses were added to the cell cultures to give final concentrations of 4 mg/ml, 2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml.

RESULTS

Production of Transgenic Mice. Out of 600 offspring, 6 mice were identified that had integrated the HLA-DRA*0101/

-DRB1*0401 construct. Five of these mice had also integrated the mCD3-huCD4c/g construct. No mouse had integrated only the mCD3-huCD4c/g construct. Two-color cell cytometry analysis with anti-human CD4 and anti-HLA-DR mAbs demonstrated that all of the triple transgenic mice expressed both HLA-DR and human CD4 on the cell surface of peripheral blood mononuclear cells. The HLA-DRA*0101/-DRB1*0401 transgenic mouse, lacking human CD4, did not express HLA-DR on the cell surface. Copy number of integrated transgenes varied from ≈ 2 to 20. All transgenic founders, now >1 yr old, and their first litters are healthy. One founder and its offspring were chosen for further analysis. This transgenic line has integrated into its genome approximately five copies of the HLA-DRA*0101/-DRB1*0401 construct and two to three copies of the human CD4 construct. The three transgenes in this line are transmitted in a simple Mendelian fashion and are inherited together, suggesting that they are in strong linkage disequilibrium and most likely have integrated in tandem. Mice homozygous for all three transgenes have been bred, are ≈ 1 yr old and healthy, indicating that the transgenes have not disrupted the function of essential genes.

Characterization of Transgenic Mice. Peripheral blood mononuclear cells from transgenic mice and nontransgenic littermates were analyzed for expression of the transgenes. Transgenic mice expressed HLA-DR and human CD4, whereas nontransgenic mice did not (Fig. 1). In the cell cytometry histogram, HLA-DR $^+$ cells are represented by one population and constitute 20% of peripheral blood mononuclear cells. Human CD4 $^+$ cells are represented by two populations, one with low fluorescence intensity (human CD4 low), constituting 20% of peripheral blood mononuclear cells, and one with high fluorescence intensity (human CD4 high), constituting 70% of peripheral blood mononuclear cells.

In the spleen, the HLA-DR $^+$ cells constituted 40% of the cells and were included in the I-A $^+$ cell population, constituting $\approx 70\%$ of the I-A $^+$ cells (Fig. 2A). The level of HLA-DR expression was similar to that of endogenous I-A expression. The majority of HLA-DR $^+$ cells were B220 $^+$ and $\approx 65\%$ of the B220 $^+$ cells were HLA-DR $^+$ (Fig. 2B). The HLA-DR $^+$ cells were totally included in the IgM $^+$ cells and $\approx 70\%$ of the IgM $^+$ cells were HLA-DR $^+$. A small percentage of HLA-DR $^+$ cells expressed CD11b (Mac-1), whereas no coexpression of CD3 and HLA-DR was detected (data not shown). In the thymus there was a direct and absolute correlation between expression of endogenous I-A and HLA-DR molecules.

In the spleen, 70% of the cells were human CD4 $^+$, and similar to the peripheral blood, two populations of human CD4 $^+$ spleen cells could be identified on the basis of their difference in fluorescence intensity. The human CD4 low and CD4 high positive populations constituted 40% and 30%, re-

FIG. 1. Histogram of single-color cell cytometry analysis of HLA-DR (A) and human CD4 (B) cell-surface expression on peripheral blood mononuclear cells from an HLA-DRA*0101, HLA-DRB1*0401, and human CD4 triple transgenic mouse (—) and a nontransgenic littermate (---).

FIG. 2. Contour plots of two-color flow cytometry analysis of HLA-DR and I-A (A) and HLA-DR and B220 (B) cell-surface expression on splenocytes from an HLA-DRA*0101, HLA-DRB1*0401, and human CD4 triple transgenic mouse.

spectively, of the spleen cells. All human CD4^{low} positive spleen cells also expressed HLA-DR, B220, and IgM, whereas the human CD4^{high} positive population expressed none of these antigens but was CD3⁺ (data not shown). In popliteal and inguinal lymph nodes $\approx 5\%$ of the cells were CD4^{low} positive and 70% of the cells were CD4^{high} positive. About 75% of the CD4^{high} positive cells were also murine CD4⁺, whereas the remaining 25% were CD8⁺ (Fig. 3). All the CD4^{high} positive cells were CD3⁺ but HLA-DR and B220 negative (data not shown). The CD4^{low} positive cells were all HLA-DR-, B220- and IgM-positive and murine CD4-, CD8-, and CD3-negative (data not shown). The ratio between murine CD4⁺ and CD8⁺ cells was $\approx 3:1$ in transgenic mice compared with $\approx 2:1$ in nontransgenic littermates.

Further analysis of single-cell suspensions from thymus demonstrated that both CD4 and CD8 single-positive as well as double-positive thymocytes were human CD4^{high} positive (data not shown). The percentages and ratios of the CD4⁺CD8⁻ double-negative (5%), CD4⁺CD8⁺ double-positive (70%), CD4⁻CD8⁺ single-positive (5%), and CD4⁺CD8⁻ single-positive thymocytes (20%) in transgenic mice were similar to that found in nontransgenic littermates.

Immunohistochemical staining of mesenteric lymph nodes from transgenic and nontransgenic mice showed normal architecture, with well defined cortex, paracortex, and medulla. The majority of the cells in the paracortex, as well as a few cells in the cortex, expressed mouse CD4 in both transgenic and nontransgenic mice and human CD4 in transgenic mice only. In the mesenteric lymph nodes from the transgenic mice, HLA-DR was expressed diffusely in the cortex, by scattered dendritic cells in the paracortex and by most of the cells in the medulla, analogous to the expression of I-A antigens. There was no staining of nontransgenic mice mesenteric lymph nodes with anti-HLA-DR mAb. In the

FIG. 3. Contour plots of two-color flow cytometry analysis of human CD4 and murine CD4 (A) and human CD4 and CD8 (B) cell-surface expression on single-cell suspensions from lymph nodes from an HLA-DRA*0101, HLA-DRB1*0401, and human CD4 triple transgenic mouse.

Table 1. TCR V β expression in CD4⁺ T cells

V β	Transgenic, %	Nontransgenic, %
3	0.1	0.6
5	0.5	1.8
6	0.1	2.4
7	0.4	1.7
8	20.5	15.6
9	0.0	0.6
10	4.5	2.2
11	0.6	3.3
12	0.1	1.7
13	1.1	2.3
14	8.8	4.6
17	0.6	0.2

TCR V β region expression in peripheral T cells. Numbers represent the percentage of T cells in the CD4⁺ population expressing a particular V β and are the mean of results from two or three mice.

thymus, most cells in the cortex and medulla from transgenic and nontransgenic mice expressed mouse CD4. Human CD4 was expressed in an identical pattern in the transgenic thymus. HLA-DR expression by dendritic cells in the cortex and medulla, analogous to the expression of I-A antigens, was seen in the transgenic thymus but not in the nontransgenic thymus. No histologic abnormalities were noted in examination of routine sections of formalin-fixed, paraffin-embedded lymphoid tissues from transgenic and nontransgenic mice.

Function of Transgenes. To determine whether the transgenes influenced the T-cell repertoire peripheral lymph node cells were analyzed for the coexpression of murine CD4 and a number of TCR V β chains (Table 1). All TCR V β s tested were expressed in the CD4⁺ T-cell compartment in the nontransgenic mice except for V β 17a. In the transgenic mice, however, T cells coexpressing murine CD4 and either V β 3, -5, -6, -7, -9, -11, -12, or -13 were either absent or significantly reduced, whereas T cells coexpressing murine CD4 and V β 10 and murine CD4 and V β 14 were increased by a factor of two, compared with the nontransgenic mice. As in the nontransgenic mice, no murine CD4⁺ T cells coexpressing V β 17a were detected.

To test whether the transgenes were capable of mediating an HLA-DRA*0101/-DRB1*0401-restricted T-cell response, transgenic and nontransgenic mice were immunized with the HLA-DRA*0101/-DRB1*0401-restricted peptide HA 307-319 (22). The HLA-DRA*0101/-DRB1*0401 and human CD4 transgenic mice responded to the HA 307-319 peptide, whereas the nontransgenic mice did not (Fig. 4A). Furthermore the immune response could be blocked by an anti-HLA-DR mAb but was unaffected by anti-I-A^f, -I-A^q, anti-

FIG. 4. (A) T-cell proliferative response of an HLA-DRA*0101, HLA-DRB1*0401, and human CD4 triple transgenic mouse (upper line) and a nontransgenic littermate (lower line) to the HA 307-319 peptide antigen. (B) Blocking effect of various mAbs on the HA 307-319 *in vitro* T-cell response from a triple transgenic mouse. Cont., control; α -Mo-CD4, α -mouse-CD4.

murine CD4, and anti-HLA-DQ mAbs (Fig. 4B). Unexpectedly, the HLA-DRA*0101/-DRB1*0401-restricted immune response could not be blocked by the anti-human CD4 mAb Leu3a, which had a mitogenic effect on cells from transgenic mice but no effect on cells from nontransgenic mice (data not shown).

DISCUSSION

Production of HLA transgenic mice, in which the transgenes are functional and expressed in a tissue-specific fashion, has proven difficult (R. Tisch and H. O. McDevitt, personal communication). Possible explanations for this difficulty are that murine CD4 cannot interact productively with HLA class II molecules (23–25), and that human genomic constructs may not contain the regulatory sequences that allow for tissue-specific expression in mouse. To facilitate the interaction between murine T cells and the HLA-DRA*0101/HLA-DRB1*0401 molecule, we have produced mice expressing both the HLA-DR heterodimer as well as the human CD4 molecule. To avoid the formation of hybrid HLA-DR α /H-2 I-E β molecules as described by Lawrance *et al.* (26), our transgenes were injected into fertilized eggs from (DBA/1 $\times$ A.CA) F_1 mice that carry the H-2 a and H-2 f haplotypes, respectively. Both of these haplotypes are H-2 I-E negative because they fail to synthesize both the I-E α and I-E β chains (27, 28).

Expression of transgene-encoded HLA-DR molecules was coregulated with expression of the endogenous I-A molecules in peripheral blood, spleen, lymph node, and thymus. This appropriate expression pattern was obtained by placing the HLA-DRA*0101 and HLA-DRB1*0401 cDNAs under the transcriptional control of the H-2 I-E α promoter in the plasmid vector pDOI-5 (6).

The human CD4 promoter by itself is not capable of driving the appropriate subclass-specific expression of the human CD4 gene in transgenic mice but requires two additional sets of sequences (9, 29). However, at the time we started to produce triple transgenic mice, these regulatory elements were unidentified. We therefore decided to use a hybrid CD4 transgene construct in which the mouse CD3- δ promoter and enhancer replaces the human CD4 promoter and drives the expression of the human CD4 molecule. Consistent with this, the human CD4 molecule is expressed on all CD3 $^+$ thymocytes and peripheral blood T cells in the transgenic mice. Unexpectedly, the human CD4 molecule is also expressed on the cell surface of B cells in the transgenic mice, however, at a lower level than that seen in T cells. This inappropriate expression is in accordance with a recent study (9) and most likely reflects that the mCD3-hCD4c/g construct lacks the human CD4 regulatory sequences that presumably preclude expression of CD4 in B cells.

The function of the transgenes in the thymus was assessed by their ability to influence the shaping of the murine T-cell repertoire and in the periphery by their ability to mediate an HLA-DR-restricted immune response. Mouse mammary tumor virus-encoded superantigens (SAGs) play an important role in the shaping of the murine T-cell repertoire. In the thymus, the SAGs bind to major histocompatibility complex class II molecules and mediate clonal deletion of maturing T cells that bear particular V β segments (30, 31). The deletion of the V β 6-, V β 7-, and V β 9-bearing T cells in the triple transgenic mice, which are H-2 I-E negative, is identical to findings in H-2 I-E $^+$ and Mls-1 a mouse strains (32). The deletion of V β 3-, V β 5-, and V β 11-bearing T cells is most likely due to the presence of other SAGs in the (DBA/1 $\times$ A.CA) F_1 strain. Thus, the Mtv-9-encoded SAG Ect-1 in the context of H-2 I-E deletes V β 5.1-, V β 5.2-, and V β 11-bearing T cells, whereas the Mtv-6-encoded SAG Mls-3 a , also in the context of H-2 I-E, deletes V β 3- and V β 5-bearing T cells (32).

Whether these SAGs or possibly other genetic elements are responsible for the deletion pattern seen in our transgenic mice is currently unknown because a characterization of SAGs in the A.CA strain has yet to be reported. Likewise, an unidentified determinant has been reported to mediate the deletion of V β 12-bearing T cells in H-2 I-E $^+$ mice and therefore may also be involved in deletion of V β 12-bearing T cells in the transgenic mice.

No SAG responsible for the deletion of V β 13 has been described to our knowledge. This report thus provides evidence for the existence of such a genetic element, presumably in A.CA mice. The increased percentage of T cells bearing V β 10 and V β 14 chains may be due to HLA-DR-mediated positive selection of certain helper T-cell subsets. This is also reflected by the increased number of murine CD4 $^+$ cells in the transgenic mice compared with their nontransgenic littermates. Thus, HLA-DR, most likely in association with human CD4 (23–25), presents [similar to H-2 I-E in association with murine CD4 (33)] retrovirally encoded SAGs in the triple transgenic mice, thereby shaping the murine T-cell repertoire. This apparent evolutionary conservation of the major histocompatibility complex class II gene-encoded structures involved in the binding of SAGs is compatible with the idea that the human T-cell repertoire is shaped by analogous mechanisms. A recent *in vitro* study in which human T cells proliferate efficiently in response to Mls-1 a presented by the HLA-DRA*0101/HLA-DRB1*0101 molecule (34) is also consistent with this idea. The fact that SAGs encoded within the human genome have yet to be identified is an important caveat to this hypothesis. However, this does not rule out the existence of these genetic elements.

The peptide antigen HA-(307–319) binds to the HLA-DRA*0101/-DRB1*0401 heterodimer with high affinity (21) and induces an HLA-DR-restricted CD4 $^+$ T-cell response in humans (22). This peptide also induced a T-cell response in the triple transgenic mice but not in nontransgenic littermates. The HA-(307–319) response could be blocked with an anti-HLA-DR mAb but was unaffected by anti-I-A f , anti-I-A g , and anti-HLA-DQ mAbs, proving that the response is indeed mediated by the HLA-DRA*0101/-DRB1*0401 heterodimer in the transgenic mice. In accordance with the fact that murine CD4 cannot interact productively with human major histocompatibility complex class II molecules (23), the anti-murine CD4 mAb had no effect on the HA-(307–319) T-cell response. It was, however, not possible to demonstrate the potential blocking effect of the anti-human CD4 mAb, Leu3a, because this mAb unexpectedly had a mitogenic effect on lymph node cells from the transgenic mice. The reason for the mitogenic effect of Leu3a is unclear, but may be Fc receptor-mediated.

The triple transgenic mice reported here should allow a detailed characterization of HLA-DRA*0101/HLA-DRB1*0401-restricted normal immune responses to a number of well-defined peptides and to delineate the role of this HLA class II molecule in immune responses possibly related to RA. Moreover, because our transgenic mice do not spontaneously develop arthritis or any other autoimmune disease, they may also be useful in the identification of environmental agents that are of potential significance in the pathogenesis of RA, such as Epstein-Barr virus, human T-lymphotropic virus type I, and mycobacteria.

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