Species-specific Binding of CD4 to the β2 Domain of Major Histocompatibility Complex Class II Molecules

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Summary

Exon-shuffled constructs between mouse (IAβ) and human (DR3β) class II β chains were made to study the interaction sites between CD4 and major histocompatibility complex (MHC) class II molecules, and to determine whether a species barrier is involved. The overall structure and the peptide binding groove appeared to be unaffected by the exon shuffling procedure as determined by monoclonal antibody and peptide binding assays, respectively. While purified CD4+ BALB/c T cells responded strongly in a mixed leukocyte reaction to transfectants expressing the whole IA molecule, the response to IA molecules containing a DRβ2 domain was substantially reduced. In addition, the presence of an IAβ2 domain in DR failed to restore the weak xenoreactivity to the whole DR molecule. Similar observations were made with murine HEL-specific, IAαkβ3-restricted T cell hybridomas which responded significantly stronger to the whole compared with the exon-shuffled IA molecules. The involvement of CD4 in these differential responses was confirmed by the observation that CD4 loss variants responded to both molecules comparably, and transfection of CD4 into these cells restored the parental phenotype. In contrast, CD4 loss variants transfected with human CD4 responded equally to both the whole and the exon-shuffled molecules. Taken together, these data imply the existence of a partial species barrier, and suggest that CD4 interacts with the β2 domain of MHC class II molecules, probably in addition to other contact sites. Models for the interaction of CD4 with MHC class II molecules are presented.

The interaction between CD4 and MHC class II molecules is known to be an important event in both T cell ontogeny (1) and peripheral activation of helper and cytotoxic CD4+ T cells (2–4). Several studies have suggested that CD4 binds to a nonpolymorphic region of class II molecules thereby increasing the affinity of the interaction between an antigen-presenting cell and a T cell (5–8). Direct evidence for a physical interaction between CD4 and class II molecules was first obtained by Doyle and Strominger (9) using an adhesion assay between class II+ B cells and a high CD4-expressing CV1 transfectant.

CD4 consists of four external Ig-like domains (D1–4), a transmembrane region, and a cytoplasmic tail. The two domains furthest from the cell surface (D1 and 2) have been shown to contain residues involved in binding to class II molecules (10–12). In contrast, very little is known about the interaction site on MHC class II molecules. Although there are some speculations that there may be interaction sites in the α1 (13) and β1 (14, 15) domains, no direct conclusive evidence has been provided.

Whether a species barrier exists in the interaction between CD4 and class II remains controversial. While Lamarre et al. (11) have suggested that murine CD4 does not interact with human class II HLA-DP molecules, von Hoegen et al. (16) have shown that murine CD4 loss variants, transfected with either human or mouse CD4, are functionally comparable. However, evidence for the existence of a species barrier has also come from studies with human MHC class II transgenic mice. Several studies have shown that while single DRα (17) and single DQβ (13) transgenic mice function normally in terms of their ability to delete TCR Vβ11+ cells, double DQα/β transgenics fail to do so (18). Our own experiments and data from other laboratories (17) have suggested that DRα transgenic mice, that express DRα on the surface of class II+ cells in association with IEβb, are completely comparable to IE+ mice in their ability to positively select TCR Vβ6, negatively select Vβ11, and generate a specific, DRαIEβb-restricted response.

On the basis of these observations, it seemed reasonable to suggest that such a species barrier may exist between CD4 and the human class II β chain. To determine whether such a barrier does exist and attempt to map possible interaction sites on class II molecules for CD4, two exon-shuffled constructs were made. The first encoding the β1 domain of
IAββ and the β2, transmembrane and cytoplasmic domains of DR3β (Aα/IA1/DR3β), and the second encoding the converse (DRα/DR3β/IAββ). These constructs were transfected, together with the appropriate α chain genes, into two different APC, and analyzed using two different functional systems.

Materials and Methods

Plasmids and Constructs. Two exon-shuffled constructs were produced for these studies. The first, pD10.4, encodes the β1 domain of IAββ and the β2, transmembrane, and cytoplasmic domains of DR3β (see Fig. 1a). The second, pD12.2, is the converse encoding the β1 domain of DR3β and the β2, transmembrane and cytoplasmic domains of IAββ. The cosmid Cos4.1 containing the entire DR3β gene (generously provided by Bernard Mach, University of Geneva, Geneva, Switzerland) and the entire IAββ gene in pBR322 were used to make the constructs. Production of a detailed restriction map of the DR3β gene revealed a unique EcoRV site in the second intron, and appropriate restriction sites within IAββ and the B2, transmembrane, and cytoplasmic domains of the 151 domain of DR3β and the β2, transmembrane and cytoplasmic domains of IAββ. The cosmids Cos4.1 containing the entire DR3β gene were deduced from the published sequence. It is important to note that nonhomologous class II genes were used to produce the exon-shuffled constructs. This was due to the availability of the IAββ and DR3β genes and information concerning appropriate cleavage sites. To our knowledge, no study has yet shown that the α2 and β2 domains play a role in class II pairing. pD10.4 was produced by ligating a 5.7-kb PvuII fragment containing the promoter and first two exons of the IAββ gene, and an EcoRI/PvuII fragment of pBR322, with a 8-kb EcoRV fragment containing exons 3-6 of the DR3β gene (see Fig. 1).
pD12.2 was produced in four stages. First, a 2.7-kb Dral fragment containing exons 3-6 of the IAββ gene was subcloned into the Smal site of pUC19 (pD5.1). To obtain the entire 3' region of the gene, a 2.4-kb BamHI fragment from the IAββ gene was subcloned into pD5.1 cut with BamHI (pD6.6). In a separate subcloning event, a 15.5-kb Sall/EcoRV fragment containing the promoter and first two exons of the DR3β gene was cloned into a Sall/Smal cut pSP72 plasmid vector (Promega Corp., Madison, WI) to produce pD11.5. Finally, a 3.8-kb KpnI/SphI blunt fragment containing the entire insert from pD6.6 was cloned into the EcoRV site in pD11.5 (pD12.2). CD4 expression vectors were produced by inserting either mouse (kindly provided by Jane Parnes, Stanford University, Palo Alto, CA) or human (IA), 25-9-17 (IAββ), 17/227 (IAββ), 53-6-7 (CD8), and 19/178 (CD4). CD4+ T cells were assayed for purity and routinely found to be <1% IA+, <1% CD8+ and >93% CD4+.

Flow Cytometry and Peptide Binding Assay. Cells were prepared for flow cytometric analysis by staining with the relevant biotinylated or unconjugated antibody at saturating concentrations for 30 min on ice in staining buffer (PBS containing 0.05% NaN3 and 5% FCS). A20.J cells were also stained in the presence of 5% normal rabbit serum to block Fc receptor binding. Cells were washed twice and then stained with either streptavidin-PE, rabbit anti-mouse γ chain-PE or rabbit anti-rat-PE (Southern Biotechnology Associates, Birmingham, AL), as described above. Finally, cells were washed twice and resuspended in staining buffer containing 0.5 μg/ml propidium iodide to facilitate the gating out of dead cells during flow cytometric analysis (FACScan®; Becton Dickinson & Co., Mountain View, CA).

For the peptide binding assay, cells were incubated in the presence of HEL 46-61 (rat mAb [26]) at 4°C for 4 h. Cells were subsequently washed and stained with streptavidin-PE as described above.

Transfection Procedures. Two transfection procedures were used. Calcium phosphate precipitation for Ltk− L cells and electroporation for the A20.J B cells and the CD4 loss variant T cell hybridoma A167.6N (Vignali, D., J. Moreno, D. Schiller, and G. Hämmerling, manuscript submitted for publication) as previously described. A20.J cells were transfected with either the IAαb gene plus IAββ or IAβ1:DRβ2, or the DRαβ gene plus DRββ or DRβ1:αβ2.

Individual clones derived from either transfection method were obtained by staining with the relevant antibody and sorting at one cell/well into 96-well microtitre plates using a Coulter Autoclone system linked to an Epics V cell sorter (Coulter Corp., Hialeah, FL). Clones were routinely tested for homogeneous levels of MHC class II expression by flow cytometric analysis using a FACScan® (Becton Dickinson & Co.). All T cell hybridomas were further selected and routinely tested for their comparable expression of TCR, CD3, CD2, and LFA-1, and comparable responses to immobilized anti-TCR and anti-CD3. Briefly, flat-bottomed 96-well microtitre plates were coated with purified anti-TCR (H57,157) or anti-CD3ε (500.A2) in PBS overnight at 37°C. After extensive washing, T cell variants were added at 5 × 10⁴ in 200 μl. Supernatants (100 μl) were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2-dependent T cell line CTLL-2 (10⁴; 100 μl). After 20 h, cells were pulsed for 8 h with 1 μCi [3H]thymidine before liquid scintillation counting. SE was, on average, <5%.

MLR and Adhesion Assay. CD4+ T cells were purified from BALB/c spleens using a negative selection panning procedure as previously described (33). MHC class II+ and CD8+ were removed using the following mAbs; P7/7 (IA), 25-9-17 (IAββ), 17/227 (IAββ), 53-6-7 (CD8), and 19/178 (CD4). CD4+ T cells were assayed for purity and routine and found to be <1% IA+, <1% CD8+ and >93% CD4+.

A primary MLR was performed by culturing purified CD4+ T cells (5 × 10⁵; 100 μl) as responders with either syngeneic BALB/c or allogeneic C57BL/6 irradiated splenocytes (3,000 rads; 2.5 × 10⁶; 100 μl), or irradiated A20.J (BALB/c origin) transfectants (10,000 rad; 2 × 10⁴; 100 μl) as stimulators. After 3 d, 100 μl supernatant was removed for estimation of IL-2 using CTLL-2.
cells as described above. The degree of adhesion/clustering was evaluated visually after 3 d before removal of the supernatant. Wells were scored as follows: (−) no significant clustering (<5 cells/cluster), (+) low clustering (5–50 cells/cluster), (+++) moderate clustering (50–100 cells/cluster), (++++) extensive clustering (>100 cells/cluster).

Murine T Cell Hybridoma Experiments. The hybridomas A167, A167.N, and A481, and the antigen presentation procedure have been described elsewhere (34, 35; Vignali, D., et al., manuscript submitted for publication). T cell variants were continually monitored for cell surface expression of important markers, responsiveness to immobilized anti-TCR, and screened for mycoplasma to maximize reproducibility. In addition, hybridomas were passed frequently and kept in culture for no more than 4–6 wk, thereby limiting phenotypic change. T cell hybridomas (5 × 10⁴; 100 ml) were cultured with 2.5 × 10⁴ (100 μl) A20 transfectant and various molar concentrations of either HEL (Sigma Chemical Co., St. Louis, MO) or the synthetic peptide 46–61. After 24 h, IL-2 secretion was determined as described above.

Results and Discussion

To study the interaction between CD4 and MHC class II molecules, two exon-shuffled constructs were made. One encoded the β1 domain of IAα and the β2, transmembrane, and cytoplasmic domains of the nonhomologous human class II molecule, DR3, and the converse encoded the β1 domain of DR3 and the β2, transmembrane, and cytoplasmic domains of IAα (Fig. 1 A). These constructs and the native genes were subsequently transfected, together with their appropriate α chains (IAα or DRα) into L cell fibroblasts (MHC class II negative) and A20.J B cells (IA4, IE4). The hybrid glycoproteins are referred to here as IAα/IAβ1:DRβ2 and DRα/DRβ1:IAβ2, respectively. Positive clones were isolated by FACS® with mAbs specific for the transfected gene products. The four heterodimer combinations together with the nomenclature used here are depicted in Fig. 1 B.

The first question to answer was whether the exon-shuffling
procedure had altered the conformation of these molecules. This was assessed by using both antibody and peptide binding assays. The ability of eight anti-IA mAbs to recognize Aα/Aβ and Aα/Aβ1:DRβ2, and 14 anti-DR mAbs to recognize DRα/DRβ and DRα/DRβ1:Αβ2 was determined using flow cytometry (data not shown). The degree of binding of all mAbs between the exon-shuffled and native molecules was indistinguishable, demonstrating that the epitopes recognized by these mAbs were not destroyed or grossly altered.

The structural integrity of the exon-shuffled Aα/Aβ1:DRβ2 molecules was further assessed using a direct peptide binding assay. A20 transfected and untransfected cells were labeled with various concentrations of biotinylated HEL 46-61 followed by a streptavidin-PE conjugate and subsequently analyzed by flow cytometry (Fig. 2). Staining of two Aα/Aβ and Aα/Aβ1:DRβ2 transfectants with anti-IA mAb confirmed that comparable levels of class II were present on the cell surface amongst the different clones. Only low level binding of the biotinylated peptide was observed to the IA4 and IEα1 molecules on untransfected A20.J cells, and peptide bound to Aα/Aβ (IAα/β2) heterodimers in a high affinity, dose-dependent manner with detectable fluorescence down to 1 nM peptide. Similar results were obtained with L cell transfectants (data not shown). Taken together, these data clearly show that the peptide and antibody binding characteristics of all the Aα/Aβ and Aα/Aβ1:DRβ2 clones tested were indistinguishable, suggesting that the exon shuffling procedure did not alter the ability of these molecules to bind immunogenic peptides. However, we cannot rule out the remote possibility that the peptide still binds to the same quantitative extent, but in a conformation that reduces effective T cell recognition.

To assess the functional consequences of the exon shuffling procedure on CD4 binding and whether a species barrier existed in this interaction, two different antigen presentation systems were used. First, a primary MLR using murine CD4+ T cells as responders against the A20 transfectants, and second, a classical antigen presentation system using murine HEL-specific, Iαα/β2-restricted T cell hybridomas. Purified CD4+ BALB/c spleenocytes were used as responders in a primary MLR against all four transfectants. A20.J cells express IAα and IEβ and are of BALB/c origin, thus

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**Figure 2.** The peptide binding groove of class II was not affected by the exon shuffling procedure. Two different Aα/Aβ (IAα/β2) and Aα/Aβ1:DRβ2 A20.J transfectants together with untransfected A20.J (IAβ) cells were incubated with biotinylated HEL 46-61, H116.32 (IAβ), and 25.9.17 (IAβ2), followed by streptavidin-PE. Stained cells were analyzed by flow cytometry. Data presented are representative of four experiments, and are expressed as log10 mean fluorescence of individual samples.

**Figure 3.** Loss of reactivity in a primary MLR to MHC class II molecules containing a foreign β2 domain. CD4+ BALB/c spleen cells were used as responders against the following stimulators: BALB/c splenocytes (syngeneic), B6 splenocytes (allogeneic), untransfected A20.J B cells (BALB/c origin), and the Aα/Aβ, Aα/Aβ1:DRβ2, DRα/DRβ1:Aβ2, and DRα/DRβ A20.J transfectants. Supernatants were analyzed for IL2 using a CTLL assay. Adhesion was determined visually. Wells were scored as follows: (−) no significant clustering was observed (<5 cells/cluster); (+) low clustering (5-50 cells/cluster); (+ +) moderate clustering (50-100 cells/cluster); (+ + +) extensive clustering (>100 cells/cluster). Data presented are representative of five experiments and are expressed as the mean of two transfectants analyzed in triplicate. Standard deviations were typically below 10% of the total.

**Figure 4.** Ability of Iαα/β2-restricted T cell hybridomas to respond to HEL peptide 46-61 by Aα/Aβ or Aα/Aβ1:DRβ2 transfected A20.J cells. Two hybridomas were analyzed, A167 and A481 and response determined by IL2 release. Data presented are representative of five experiments and are expressed as the mean of two transfectants analyzed in triplicate. Data presented are representative of seven experiments and are expressed as the mean of triplets. Standard deviations were typically below 10% of the total.
constitutively expressed class II. In addition, it has been demonstrated that the IAcββ chains do not pair with the IAβ chains (36). However, it is probable that the DR chains do pair with the IAβ chains, thus contributing to any observed response. IL-2 release, as determined by stimulation of an IL-2-dependent T cell line CTLL-2, and adhesion, as determined visually by clustering, were used to determine the ability of the T cells to recognize both allodeneic and xenodeneic MHC class II molecules. Minimal levels of IL-2 release and adhesion were observed in response to either syngeneic, irradiated BALB/c splenocytes, or the untransfected BALB/c-derived A20.J cells (Fig. 3). In contrast, a strong response, in terms of IL-2 release and adhesion, was seen towards allogeneic B6 splenocytes and the Aαβ A20.J transfectants. As expected, only a weak but positive xenogenic response was observed towards the DRAβ/DRβ transfectant. It is striking that both adhesion and T cell function responses to Aαβ1:DRβ2 were substantially reduced, but not ablated, as a result of the inclusion of a foreign β2 domain in IA. This inferred the presence of a species barrier in the interaction between mouse CD4 and the β2 domain of human class II molecules. However, the fact that some response still remains suggests that several interaction sites between these molecules may exist and that some, but not all, are species specific. It is interesting that the presence of an IAβ2 domain in DR (DRAβ/DRβ1:IAβ2) failed to restore the defect. There are three possible explanations. First, binding has only been partially ablated because of the involvement of the β1 domain; second, two CD4 molecules bind to the same class II molecules, and binding of one of these to the α chain is unaffected; or third, CD4 serves a novel, and as yet undescribed function that does not require CD4 binding to class II. In support of the latter, we have recently shown that CD4 interacts with the TCR and may help to maintain the fidelity of T cell specificity (Vignali, D., et al., manuscript submitted for publication). Alternatively, the residual response seen could be due to a small percentage of CD4 independent T cells present in the heterogeneous pool of alloreactive cells.

The second experimental system employed the use of a panel of murine IAcββ-restricted T cell hybridomas specific for the peptide 46–61 of HEL. Initially, eight individually cloned hybridomas with the same specificity and restriction element were assayed for their ability to recognize both the whole protein HEL and the peptide 46–61 presented by either Aαβ or Aαβ1:DRβ2 A20.J transfectants. Fig. 4 shows representative data of the response of two of those hybridomas, A167 and A481, to HEL 46–61. All the hybridomas tested clearly responded better to the Aαβ than the Aαβ1:DRβ2 transfectants. Similar results were obtained when either responses to the whole HEL molecule were analyzed, or L cell transfectants were used as presenters (data not shown). One hybridoma, A167, was selected for further study.

If the results above truly indicate that CD4 binds to the β2 domain of class II molecules in a species-specific manner, one should be able to make two predictions. First, CD4 loss variants of A167 should respond comparably to Aαβ and Aαβ1:DRβ2; and second, reexpression of CD4 by these hybridomas by transfection should restore the parental phenotype. Both predictions were found to be correct.

CD4 loss variants of A167 were isolated by FACs. One clone, A167.N, was chosen for further study because of its possessing comparable levels of TCR, CD3, CD2, and LFA-1 expression to A167, and comparable responses to immobilized anti-TCR and -CD3 (Vignali, D., et al., manuscript submitted for publication). Although, not surprisingly, A167 responded far better than A167.N, detectable levels of IL-2 release were still observed at 3-μM peptide (Fig. 5 A). In contrast to A167, which reacted noticeably better to Aαβ than did Aαβ1:DRβ2, the response to these transfectants

![Figure 5](image-url)
by A167.N was indistinguishable. These data further support the notion that the exon shuffling procedure had not altered the ability of the TCR to recognize its peptide/MHC combination. When A167.N was transfected with the mouse CD4 cDNA under the control of a human β actin promoter, several clones were isolated that had levels of CD4 comparable to A167 (data not shown). As expected, reexpression of CD4 by the A167.N.mCD4 transfected hybridomas restored the original parental phenotype exhibited by A167 (Fig. 5A). These data clearly demonstrate that the reduced responsiveness to Aα/β1:DRβ2 is due to inefficient binding of CD4 to MHC class II molecules. It is interesting that the response of A167 to Aα/β1:DRβ2 was not completely reduced down to the level of A167.N. This may suggest either that CD4 binds to another part of the IA molecule other than the β2 domain, or that CD4 is performing another function that does not require a high degree of binding to MHC.

The final question addressed was whether the transfection of the CD4 loss variant A167.N with human CD4 could restore reactivity to Aα/β1:DRβ2. Both A167.N.mCD4 and A167.N.hCD4 expressed comparable levels of CD4 as determined by flow cytometry. In contrast to the observations with A167.N.mCD4, the response to A167.N.hCD4 of the two transfectants was identical not only to each other but also to the response to A167.N.mCD4 of Aα/β1:DRβ2 (Fig. 5B). These data may suggest that hCD4 cannot fully replace mCD4, and that a similar barrier exists between hCD4 and mouse class II. However, this conclusion would contradict the observations of von Hoegen et al. (16) who found that mouse and human CD4were comparable in their ability to enhance T cell responses in their system. Three potential differences may account for these discrepancies: first, the hybridomas used in the present study are ~5 times more sensitive; second, our studies were primarily based on observations using peptides rather than whole proteins; and third, all our clones were tested for their comparable ability to produce IL-2 in response to immobilized anti-TCR and -CD3, controls which were not indicated in their studies. Alternatively, the reduced responses could be due to lower levels of hCD4 compared with mCD4 that could not be discerned by FACS® analysis. If this conclusion were correct, our data would imply that hCD4 can bind to both mouse and human class II, corroborating the conclusions of von Hoegen et al. (16).

One prominent feature of this present study was that the reduction in response of both mouse and human CD4 + T cell hybridomas to the exon-shuffled molecules was only one third of that seen with the CD4 + hybridomas. Two possible explanations present themselves: (a) binding is not completely abrogated because CD4 also binds to other class II domains and/or the species barrier is partial; or (b) binding is completely ablated and the partial response observed is due to the existence of a novel function for CD4. Evidence for both of these possibilities exists.

Evidence for the former notion has come from several studies that have speculated that CD4 may bind to either the α1 or β1 domains on class II (13–15). Furthermore, two groups have shown that between three and four contact sites for class II exist spanning over two or three CD4 domains, implying the presence of a large contact area (10–12). However, these studies have also shown that the removal of just one of these was sufficient to completely abrogate binding and functional activity. This would tend to suggest that even if there were more than one domain involved in binding, the removal of just one binding residue would result in no significant binding between CD4 and class II. Alternatively, binding may not be completely lost if CD4 bound to both the α and β chains. Evidence that two CD4 molecules may associate with the same TCR/CD3 complex has come from the studies of Anderson et al. (34), raising the possibility that CD4 may bind to both the α and β chains of class II. This scenario could explain differences between our findings and those of others (14, 15, 17) that CD4 binds to the β chain, and the suggestions of Zhou et al., (13) that CD4 may bind to the α1 domain. It is possible that when a foreign β2 domain is introduced, one CD4 molecule can no longer bind to the β chain, and binding of the other to the α chain is unaffected. This may explain why the responses of A167.N.mCD4 to Aα/β1:DRβ2, and A167.N.hCD4 to both Aα/ββ and Aα/β1:DRβ2 were all identical, yet were all stronger than the response seen with A167.N.

The second possible explanation which may, at least in part, explain why some of these responses are not reduced down to the level of the CD4 loss variant, comes from data we have recently obtained that suggests that CD4 may help to maintain the fidelity of the TCR (Vignali, D., et al., manuscript submitted for publication). These studies imply that the presence of CD4 can be important in allowing the TCR to effectively recognize its class II/peptide ligand. Consequently, in the absence of CD4, the TCR would recognize the class II/peptide complex less effectively.

In conclusion, our experiments imply the existence of a species barrier in the interaction between CD4 and the class II β2 domain. Furthermore, the evidence presented here is consistent with the suggestions of others (10–15) that CD4 may also bind to the class IIα and/or β1 domains. In addition, the data imply that the species barrier may only be partial. However, it is also possible that the apparent species barrier is indirect, with various portions of the class II molecule interfering with the binding of murine CD4 to a conserved site that itself is not species specific.

In an attempt to produce a model for CD4/MHC class II interaction, two observations are worthy of note. First, Anderson et al., (34) have shown that 10% of surface CD4 is internalized after CD3 modulation, and only 5% of CD3 is internalized after CD4 modulation, implying that two CD4 molecules may associate with the same TCR/CD3 complex. Second, a number of studies have shown that several residues on opposing sides of the CD4 molecule are involved in binding class II molecules (10–12). On the basis of these observations and the data presented here, it is tempting to speculate that two CD4 molecules may bind to the same CD3/TCR complex, not as a dimer, but as separate molecules, with one side of one CD4 molecule binding to the α chain, and the other side of the second CD4 molecule binding to the β chain.
It is hoped that the data presented here and this model will assist in the elucidation of the exact contact residues between CD4 and class II MHC molecules. Furthermore, the observation of a species barrier in the interaction between CD4 and DR molecules is relevant to the establishment of models for autoimmune diseases in human class II transgenic mice.

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