Comparison of HLA-DR1-restricted T cell response induced in HLA-DR1 transgenic mice deficient for murine MHC class II and HLA-DR1 transgenic mice expressing endogenous murine MHC class II molecules

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Abstract

Transgenic mice expressing human HLA class II molecules provide a useful model for identifying HLA-restricted CD4+ epitopes. However, the influence of endogenous murine H-2-restricted T cell responses on HLA-restricted responses is not known. In the present study, we show that HLA-DR1 transgenic mice deficient for H-2 class II expression (HLA-DR1+/+/Iαβ0/0) exhibit an equivalent expression level of the transgene HLA-DR1 and a similar diversity in the TCR repertoire, but a slightly different number of CD4+ peripheral T cells, when compared to HLA-DR1 transgenic mice in which H-2 class II molecules were retained (HLA-DR1+/+/Iαβ+/+). More importantly, a strong antigen-specific HLA-DR1-restricted response was observed in nearly all HLA-DR1+/+/Iαβ0/0 mice immunized with HBV envelope protein (HBs) or capsid protein (HBc), whereas weak HBs- or HBc-specific HLA-DR1-restricted responses were detected in half of the immunized HLA-DR1+/+/Iαβ+/+ mice. Conversely, strong HBs- or HBc-specific H-2-restricted T cell responses were detected in HLA-DR1+/+/Iαβ0/0 mice but not in HLA-DR1+/+/Iαβ0/0 mice. Our results indicate that the coexpression of endogenous H-2 class II molecules reduces the intensity of HLA-DR1-restricted antigen-specific responses in transgenic mice, by favoring murine over human MHC recognition and education. Thus, HLA-DR1+/+/Iαβ0/0 mice represent a better model for identifying and characterizing HLA-DR1-restricted epitopes relevant for human disease.

Introduction

The development of protective T cell immunity against infectious pathogens involves major histocompatibility complex (MHC) class I-restricted cytotoxic CD8+ T cells (CTL) and MHC class II-restricted helper CD4+ T cells. Antigen-specific CD8+ T cell immunity primarily involves direct elimination of antigen-expressing cells (1–3) while antigen-specific CD4+ T cells promote optimal CD8+ T cell (4,5) and B cell activation (6–8). The T cell-mediated response to complex antigens involves recognition of selected peptide epitopes presented in the context of MHC molecules expressed on antigen-presenting cells (9). The identification of immunogenic epitopes among the hundreds or thousands of potential epitopes present in an antigenic protein is thus a critical step in attempts to optimize T cell-mediated immunity, and is important for the development...
of efficient vaccines. However, the identification of specific epitopes from complex antigens can be a cumbersome and difficult process, due to the multiplicity of MHC molecules encoded by distinct gene loci in both mice and humans. Moreover, using T cell clones isolated from whole antigen-stimulated cells it is not obvious to identify the specificity of the T cell epitopes and their restriction alleles. To address this problem, we and others have developed transgenic (Tg) mice expressing human leukocyte antigen (HLA) class I complexes to facilitate identification of immunogenic HLA class I-restricted CTL epitopes (10,11). This experimental animal model is also useful for in vivo studies of cellular immune responses to various candidate vaccines, and it represents an attractive vertebrate system with immunological relevance for the human immune system. However, currently available mice often fail to develop responses against CTL epitopes known to elicit HLA-class I restricted responses in humans. To increase the abundance of murine CD8+ T cells restricted by human HLA class I molecules, it is essential to eliminate endogenous expression of murine H-2 class I molecules in such a way that only the Tg HLA alleles act as restriction elements in the mice (12–14). A number of novel CTL epitopes for various antigens have already been identified with these mice, and synthetic peptides representing these epitopes have been used to prepare therapeutic vaccines to treat cancer patients. But the therapeutic effects of these vaccines are still far from optimal. One of the reasons may lies in the absence of concomitant helper T cell epitopes (T H epitopes) restricted by specific human alleles have been identified until now. We have therefore focused our efforts on developing an HLA-class II Tg animal model to facilitate identification of new immunogenic T H epitopes. In the present study, we analyzed the HLA-DR1-restricted T cell responses elicited in HLA-DR1 Tg mice deficient for H-2 class II expression (HLA-DR1+/+IA^b+), compared to those induced in ‘classic’ HLA-DR1+/+IA^b+ Tg mice. Our results demonstrate that there is competition for immunodominance between H-2 class II- and HLA class II-restricted T cell responses, and suggest that the presence of endogenous H-2 class II molecules may introduce a bias in favor of H-2 class II-restricted over HLA class II-restricted T cell responses in HLA-DR1+/+IA^b+ mice. The data presented in this report are consistent with HLA-DR1+/+IA^b+ mice, providing a more sensitive and specific model for identifying and characterizing HLA-DR1-restricted epitopes for a variety of human disease-associated Ags.

Methods

Mice

The mice used in this study were bred and maintained in pathogen free conditions in the animal facilities of the Pasteur Institute, Paris. The HLA-DR1 IA^b+ transgenic mice were obtained at the Pasteur Institute of Lille by back-crossing the original HLA-DR1 transgenic mice (19) with A^b+ mice (B6-IA^b+ mice) (20).

Peptides

The HLA-DR1 binding peptides, HBs179-194 QAQFLLLTRL-TIPQS, HBC111-125 GRETVEYLVFSGW, HIV-1 Gag263-277 KRWILGLNKVRMY, the H-2 IA^b binding peptides HBs126-138 RGLYFPAGGGSSG and HBC128-140 TPPAYRPNPAIL were synthesized by Primm (Milano, Italy) and dissolved in PBS–10% DMSO at a concentration of 1 mg/ml.

FACS analysis of peripheral B and T lymphocytes

B lymphocytes were purified on a mini-MACS column (Miltenyi Biotec, Bergish Gladbach, Germany) by positive selection. The expression of HLA-DR1 and H-2 IA^b molecules was analyzed by immunofluorescence using FITC-labeled anti-HLA-DR (L243) and PE-labeled anti-IA^b (AF6-120.1) (BD Biosciences, San Diego, CA). The percentage of single CD4+ and CD8+ T lymphocytes was determined by double staining, using PE-labeled anti-mouse CD4 and FITC-labeled anti-mouse CD8 (Caltag, South San Francisco, CA). The expression of the different BV TCR was analyzed using PE-labeled anti-mouse CD4 and the following biotinylated specific antibodies: BV 2 (B20.6), BV 3 (KJ25), BV 4 (KT10.4), BV 5.1.2 (MR.9.4), BV 6 (44.22), BV 7 (TR 130), BV 8.1.2.3 (F23.1), BV 9 (MR.10.2), BV 10 (B.21.5), BV 11 (RR.3.15), BV 12 (MR11.1), BV 13 (MR.12.4), BV14 (1412) and BV 17 (KJ 23.1). Cells were incubated for 30 min on ice with the first antibody and, following two washes, incubated with the second antibody (4°C, 30 min), washed twice, fixd with paraformaldehyde and analyzed by a FACSCalibur (Becton Dickinson, Bedford, MA).

Immunoscope analysis

The TCR repertoire of CD4+ T cells from naïve transgenic mice was analyzed for BV usage using oligonucleotides specific for each family of BV segments, as described elsewhere (21). In brief, spleen CD4+ T cells were positively selected on auto MACS (Miltenyi Biotec, Bergish Gladbach, Germany). RNA was prepared using the RNA easy Kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using oligo (dT) and SuperScript II (Invitrogen, Carlsbad, CA). Half of the reaction product was then amplified by 40 cycles of PCR (annealing at 60°C) in 25 μl, in the presence of BV-specific primers and subjected to an elongation reaction (run-off) with internal BC or BJ fluorescent-tagged primers. The labeled products were then loaded on a 6% acrylamide/8 M urea gel and separated by a run of 7 h at 35 W on a 373 DNA sequencer (Applied Biosystems). Data were analyzed using immunoscope software (21) designed for this purpose.

Immunization with HBc and HBs antigen

The HBc antigen used in this study was produced in Escherichia coli and purchased at Diasorin (Balugia, Italia). The recombinant preS2/HBs antigen (ayw HBV subtype) was derived from transfected mammalian cells (CHO) (22). For HBc immunization (4 μg of HBc in 50 μl alum adjuvant; Serva, Heidelberg, Germany), mice were injected twice at the base of the tail. Similarly, for HBs immunization, 2 μg of HBs was injected and the mice were also immunized twice. Before immunization, all mice were anesthetized intraperitoneally...
(i.p.) with 75 mg/kg of pentobarbital (Ceva, Santé Animale, Libourne, France).

T cell proliferation assay

Twelve days after immunization, the spleen was removed from animals and placed in RPMI serum-free medium. Splenocytes were RBC-depleted and submitted to a Ficoll gradient, then cultured in RPMI supplemented with 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, $5 \times 10^{-5}$ M 2-mercaptoethanol. The spleen suspension was then adjusted to $10 \times 10^6$ cells/ml ($5 \times 10^5$ cells/well) and incubated with $20 \mu$g/ml, $6 \mu$g/ml or $2 \mu$g/ml of peptide. The cell suspension was incubated in 3% FCS in complete medium for 72 h at 37°C in 5% CO$_2$. One µCi of $[^{3}H]$thymidine was added to each well 16 h before the cells were harvested on a TOMTEC collector and the incorporated radioactivity was measured on a micro beta counter (Perkin Elmer, Courtaboeuf, France). Results are shown as SI (stimulation index) = (c.p.m. peptide)/(c.p.m. medium).

IL4 secretion assay

Twelve days after the last immunization with HBC Ag, the spleen suspension was stimulated with 20 µg/ml of HBC111–125 or irrelevant HIV-1 Gag263–277 peptide. After 4 h of incubation, IL4 secretion was determined by use of a cytokine secretion capture assay, following the protocol supplied by the manufacturer (Miltenyi Biotec, Bergish Gladbach, Germany) and adapted from Brosterhus et al. (23). Cells were then labeled with IL4 catch antibody and PE-detection antibody and counterstained with FITC-labelled CD4 antibody. After additional labeling with anti-FITC microbeads, the CD4+ cells were sorted and analyzed by FACS for IL4 secretion.

Results

Expression of HLA-DR1 molecules and size of the peripheral CD4+ T cell pool in HLA-DR1+/+/IAβ0/0 and HLA-DR1+/+/IAβ+/+ Tg mice

The cell surface expression of HLA-DR1 and H-2-IAβ molecules, and the percentage of peripheral CD4+ T cells, was evaluated by flow cytometry analysis on B cell (B220+)-enriched and T cell-enriched splenocytes. As illustrated in Fig. 1(A), a similar level of HLA-DR1 expression was observed in HLA-DR1+/+/IAβ0/0 and HLA-DR1+/+/IAβ+/+ Tg mice, but not in non-Tg B6-IAβ0/0 mice. As expected, a high level of H-2-IAβ expression is observed exclusively in HLA-DR1+/+/IAβ+/+ transgenic mice. CD4+ T cells represent 0.9% of the splenic cell population in B6-IAβ0/0 mice as reported by Cosgrove et al. (20), 17% in HLA-DR1+/+/IAβ+/+ and 14% in HLA-DR1+/+/IAβ0/0 Tg mice (Fig. 1B).

Peripheral CD4+ T cells in HLA-DR1+/+/IAβ0/0 and HLA-DR1+/+/IAβ+/+ Tg mice display similar TCR BV chain usage and CDR3 diversity

Expression of the various BV families was studied on purified CD4+ T cells by flow cytometry analysis using each of the 14 BV specific mAb. The percentage of CD4+ T cells expressing a given TCR BV is shown in Fig. 2(A) for HLA-DR1+/+/IAβ0/0, HLA-DR1+/+/IAβ+/+ Tg mice and wild-type mice. Although

Fig. 1. (A) Surface expression of transgenic DR1 and endogenous IAβ0 molecules on purified B220+ B cells from B6, DR1+/+ IAβ0/0 and DR1+/+ IAβ+/+ mice. B cells were stained with L243 (anti DR)-FITC or AF61201 (anti-IA)-PE antibody and analyzed by flow cytometry. (B) Restitution of the peripheral pool of CD4+ T lymphocytes in DR1+/+ IAβ0/0 mice. Double staining was performed with PE labeled anti-CD4 (y-axis, log scale) and FITC-labeled anti-CD8.
some differences were observed, they were not significant. Thus, all BV families and subfamilies are represented similarly in the three strains of mice. The CDR3 length distribution for each BV chain was also analyzed, using the RT–PCR-based immunoscope assay, as described in the Methods. Again, no significant differences between the three strains of mice were detected (Fig. 2B).

While numerous HLA class I-restricted CTL epitopes from various antigens are known, only a limited number of Th epitopes restricted by HLA-DR1 have been reported to date. In order to compare the two types of HLA-DR1-expressing mice, we thus took advantage of the fact that HLA-DR1- and H-2-IA-restricted epitopes have been identified for the hepatitis virus capsid (HBc) and envelope (HBs) proteins, by following the T cell responses induced by immunization with these antigens in HLA-DR1+/+/IA^β_0/0 Tg mice and comparing them with those elicited in HLA-DR1+/+/IA^β_+/+ mice.

Comparative analysis of HBc- and HBs-specific CD4+ T cell responses in HLA-DR1+/+/IA^β_0/0 Tg mice, compared with HLA-DR1+/+/IA^β_+/+ Tg mice

HLA-DR1+/+/IA^β_0/0 and HLA-DR1+/+/IA^β_+/+ Tg mice were immunized subcutaneously with HBc or HBs antigen, as described in the Methods. The splenocytes derived from the primed mice were then stimulated in vitro with HLA-DR1-restricted peptides (HBc_{111-125}, HBs_{179-194} or Gag_{263-277}) or with H-2-IA-restricted epitopes (HBc_{128-140} or HBs_{126-138}). In the HLA-DR1+/+/IA^β_0/0 Tg mice, a stronger proliferative response directed against the HLA-DR1-restricted peptides (HBc_{111-125}, Fig. 3A; or HBs_{179-194}, Fig. 4A) was observed, while the H-2-IA-restricted peptides were inefficient (Figs 3A and 4A). In contrast, in the HLA-DR1+/+/IA^β_+/+ Tg mice, the H-2-IA-restricted anti-HBc or anti-HBs responses were predominant (Figs 3B and 4B), and an additional in vitro recall with the HBc_{111-125} (Fig. 3C) or HBs_{179-194} (data not shown).
peptides was necessary to detect a weak HLA-DR1-restricted response. As expected, no responses were induced by the Gag263–277 peptide in either type of Tg mouse. Thus, expression of the HLA-DR1 Tg molecule in the context of endogenous IA molecules diminishes HLA-DR1-restricted Ag-specific T cell proliferative responses.

**Frequency of anti-HBc-specific HLA-DR1-restricted CD4+ T cells**

The number of HLA-DR1-restricted HBc111–125-specific CD4+ T cells was measured in the Tg mice immunized with HBc antigen, using an IL-4 secretion assay from Miltenyi Biotec. Figure 5 shows a representative experiment from three experiments that were performed. Thus, 1.27% and 0.78% HBc111–125-specific HLA-DR1-restricted, IL4-secreting CD4+ T cells, were observed in HLA-DR1+/+/IAβ0/0 and HLA-DR1+/+/IAβ+/+ mice, respectively. The splenocytes used in these experiments were from the mice shown in Figs 3(A) and (B). The HLA-DR1+/+IAβ0/0 mice displayed strong HLA-DR1-restricted HBc-specific responses, while the HLA-DR1+/+IAβ+/+ mice responded to the same antigen only weakly.

Simultaneously, an IFNγ secretion assay was performed on these splenocytes, and no HBc111–125-HLA-DR1-restricted, IFNγ-secreting CD4+ T cells were observed (data not shown). Thus, the CD4+ T cells that proliferated after stimulation with the HBc-derived peptide had the TH2 phenotype.

**Discussion**

We have previously described the improved capacity of HLA-A2-transgenic mice deficient for H-2 class I expression (HLA-A2+/+β2m0/0) to mount HLA-A2-restricted CTL responses, as compared with HLA-A2 transgenic mice that still express their endogenous murine H-2 class I molecules (HLA-A2+/+β2m+/+). Recent studies have revealed a major role for CD4+ TH lymphocytes in both the initiation and maintenance of CD8+ immune responses. However, only a limited number of helper T helper cell epitopes restricted by specific human alleles have been identified to date (25,26).
HLA class II transgenic mice were first designed in order to study autoimmune disease (19, 27, 28). More recently, HLA class II Tg animals that express HLA class II molecules in the absence of murine class II molecules (Ab00) have been generated and were rapidly used for screening of epitopes for human vaccinology (29, 30). In the present study, we evaluated the influence of endogenous H-2 class II restricted T cell responses on the HLA-DR1-restricted T cell responses in human HLA class II Tg mice. We therefore compared the HLA-DR1-restricted T cell responses in HLA-DR1 Tg mice deficient for H-2 class II expression (HLA-DR1+/+/IAβ0/0) where all CD4+ T cells are restricted by the HLA molecules, and in HLA-DR1+/+/IAβ+/+ mice.

The expression level of HLA-DR1 molecules was equivalent in both lineages (0.8 log increase over the control) and the HLA-DR1 transgene was fully functional in thymic T cell education, since 14% of CD4+ peripheral T cells were detected in HLA-DR1+/+/IAβ0/0 mice, compared to the residual 0.9% found in IAβ0/0 mice. While we observed a minor difference in the number of mobilizable CD4+ peripheral T cells in HLA-DR1+/+/IAβ0/0 and HLA-DR1+/+IAβ+/+ mice, the Vβ gene segment usage was similar. The small increase in the overall number of CD4+ T cells in HLA-DR1+/+/IAβ0/0 mice could be due to the higher cell surface expression of endogenous IAβ molecules and thus enhanced T cell thymic education in these mice. In addition, no significant difference in the CDR3 length distribution for each BV chain usage was observed between the three strains of mice, demonstrating that the transgenic complex HLA-DR1 is fully functional in the selection process, and suggesting that BV usage is determined mainly by intracellular processes (e.g., recombination events, promoter strength). We then examined and compared the antigen-specific HLA-DR1-restricted responses induced by immunization with HBV capsid (HBc) or envelope (HBs) protein. A high stimulation index was induced by either HLA-DR1-restricted HBc or HBs peptides in nearly all immunized HLA-DR1+/+/IAβ0/0 mice (Table 1). The same peptides elicited weak responses in half of the HLA-DR1+/+IAβ+/+ immunized mice; interestingly, all the mice exhibited strong H-2-restricted T cell responses (Table 1). These findings indicate that coexpression of endogenous

![Fig. 4.](image_url) (A and B) T cell proliferation to HBs antigenic peptides in HLA-DR1+/+IAβ0/0 and HLA-DR1+/+IAβ+/+ mice. Mice were immunized twice with 2 µg of HBs protein in alum at the base of the tail. Splenocytes from primed transgenic mice were rechallenged in vitro with 20 µg/ml (grey histogram), 6 µg/ml (dark histogram) or 2 µg/ml (white histogram) of each peptide. Control samples were cultured in medium alone or with an irrelevant peptide. Proliferation was determined by [3H]thymidine incorporation and represented as the SI means of duplicate cultures of two representative experiments.

![Fig. 5.](image_url) Cytokine production after peptide stimulation. Splenocytes from DR1+/+IAβ0/0 or DR1+/+IAβ+/+ HBc immunized mice were analyzed on day 12. Cells were stimulated for 4 h with 20 µg/ml of HBc111-125 or irrelevant peptide. IL4 secretion was measured by flow cytometry. The percentages represent the percent of total cells in each quadrant.
H-2 class II molecules interfere with the capacity of mice to respond efficiently to HLA-DR1-restricted antigenic peptides. The proliferative index (Fig. 3) correlated with the number of antigen-specific HLA-DR1-restricted T cells present in the immunized mice (Fig. 5), suggesting that the recruitment of antigen-specific HLA-DR1-restricted peripheral T cells in HLA-DR1+/+ mice was reduced compared to HLA-DR1+/+ mice. In the case of immunization with HBC Ag, we showed that the frequency of HBC111–125 specific HLA-DR1-restricted CD4+ T cells was higher in HLA-DR1+/+ mice than in HLA-DR1+/+ mice, and that the cells secrete IL4 but not IFNγ. This is not surprising, as it was reported that immunization with an antigen having alum as adjuvant generally drives the response toward a TH2 phenotype (31). The fact that there was no difference between both transgenic mice in Vβ gene segment or CDR3 length distribution suggests that thymic education proceeds normally in both backgrounds. Nevertheless, in HLA-DR1+/+ mice, competition between HLA-DR1 and IAβ molecules likely begins at the thymic educational stage. The higher level of expression of endogenous IAβ and the more efficient interaction of mouse CD4+ molecules with IAβ than with HLA-DR1 may both favor IAβ-over DR1-restricted precursor T cells in HLA-DR1+/+ mice during the lymphocyte life cycle: thymic education, peripheral maintenance and antigen-driven final mobilization (32,33). In addition, as shown in vivo, peripheral competition between T lymphocyte populations each expressing monoclonoally distinct TCR regulates the final size of these populations in lymphoid tissues (34). This competition for immunodominance between HLA-DR1- and H-2 class II-educated lymphocytes could provide a plausible explanation for the reduced size of the mobilized HLA-DR1 population in HLA-DR1+/+ mice compared to HLA-DR1+/+ mice. Given the abundance of peptide vaccine candidates, there is a growing need to evaluate their immunogenicity and their efficacy at inducing desired T-cell responses related to human diseases. For this purpose, the HLA-DR1+/+ mice provide a more sensitive and specific screening model than the HLA-DR1+/+ mice. The ‘humanized’ class II Tg mice thus represent a time-saving model for the identification and development of more promising vaccination strategies with relevance for human disease.

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Abbreviations

BC β constant
BJ β junction
BV β variable
CDR complementarity determining region
CTL cytotoxic T lymphocyte
Th helper T lymphocyte
HBC hepatitis B virus capsid protein
HBs hepatitis B virus surface protein

References

a chimeric human-mouse class I major histocompatibility complex.


