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Immunology

Identification of HLA class II-restricted determinants of *Mycobacterium tuberculosis*-derived proteins by using HLA-transgenic, class II-deficient mice

ANNEMIEKE GELUK[†‡§¶], VEENA TANEJA[‡¶], KRISTA E. VAN MEIJGAARDEN[†], ERIC ZANELLI[†], CHRISTIANE ABOU-ZEID[‖], JELLE E. R. THOLE[**], RENÉ R. P. DE VRIES[†], CHELLA S. DAVID[‡], AND TOM H. M. OTTENHOFF[†]

[†]Department of Immunohematology and Blood Bank, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; [‡]Department of Immunology, Mayo Clinic, Rochester, MN 55905; [‖]Department of Infectious Diseases and Microbiology, Imperial School of Medicine, London W2 1PG, United Kingdom; and [**]Division of Immunological and Infectious Diseases, TNO-PG, 2333 CK Leiden, The Netherlands

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ABSTRACT **T helper 1 cells play a major role in protective immunity against mycobacterial pathogens. Since the antigen (Ag) specificity of CD4$^+$ human T cells is strongly controlled by HLA class II polymorphism, the immunogenic potential of candidate Ags needs to be defined in the context of HLA polymorphism. We have taken advantage of class II-deficient (Ab^0) mice, transgenic for either HLA-DRA/B1*0301 (DR3) or HLA-DQB1*0302/DQA*0301 (DQ8) alleles. In these animals, all CD4$^+$ T cells are restricted by the HLA molecule. We reported previously that human DR3-restricted T cells frequently recognize heat shock protein (hsp)65 of *Mycobacterium tuberculosis*, and only a single hsp65 epitope, p1–20. DR3.Ab^0 mice, immunized with bacillus Calmette–Guérin or hsp65, developed T cell responses to *M. tuberculosis*, and recognized the same hsp65 epitope, p1–20. Hsp65-immunized DQ8.Ab^0 mice mounted a strong response to bacillus Calmette–Guérin but not to p1–20. Instead, we identified three new DQ8-restricted T cell epitopes in the regions 171–200, 311–340, and 411–440. DR3.Ab^0 mice immunized with a second major *M. tuberculosis* protein, Ag85 (composed of 85A, 85B, and 85C), also developed T cell responses against only one determinant, 85B p51–70, that was identified in this study. Importantly, subsequent analysis of human T cell responses revealed that HLA-DR3+, Ag85-reactive individuals recognize exactly the same peptide epitope as DR3.Ab^0 mice. Strikingly, both DR3-restricted T cell epitopes represent the best DR3-binding sequences in hsp65 and 85B, revealing a strong association between peptide-immunodominance and HLA binding affinity. Immunization of DR3.Ab^0 with the immunodominant peptides p1–20 and p51–70 induced T cell reactivity to *M. tuberculosis*. Thus, for two different Ags, T cells from DR3.Ab^0 mice and HLA-DR3+ humans recognize the same immunodominant determinants. Our data support the use of HLA-transgenic mice in identifying human T cell determinants for the design of new vaccines.**

Mycobacterial pathogens are major causes of morbidity and mortality worldwide: one-third of the world population is infected with *Mycobacterium tuberculosis.* Each year, eight million new active pulmonary tuberculosis cases arise and approximately three million patients die of the disease, more than of any other infectious disease (1). The incidence of tuberculosis is rising also in Western countries, and multidrug-resistant strains are emerging. The widely used tuberculosis vaccine strain *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) remains among the most controversial vaccines today, because its protective efficacy against tuberculosis varies widely in different trials (2). There is a general consent that novel strategies are needed for the prevention of tuberculosis, including the design of more effective vaccines.

Animals lacking functional genes encoding H2-Ab (class II −/−, CD4$^+$ T cell-deficient), interferon (IFN)-γ, IFN-γ receptor, tumor necrosis factor-α receptor, or interleukin (IL)-12p40, as well as animals treated with neutralizing tumor necrosis factor-α or IL-12 antibodies, are much more susceptible to infections with *M. bovis* BCG and *M. tuberculosis* than are control littermates (3–7). Similarly, defects in CD4$^+$ T helper (Th)1 immunity, type 1-cytokine production, or type 1-cytokine receptor signaling are strongly associated with progressive infection and bacterial dissemination in human beings (8–10). Thus, protective immunity to mycobacteria is strongly dependent on CD4$^+$, major histocompatibility complex (MHC) class II-restricted Th cells, and type 1 cytokines (11). An essential requirement for effective vaccines in tuberculosis, therefore, is the induction of efficient CD4$^+$ Th 1 immunity. Recent studies in mice have shown that vaccination with individual subunit antigens (Ags), delivered as protein or DNA vaccines, can indeed induce protective immunity in the context of murine MHC (12–14).

Our previous work has shown that HLA-DRB1 polymorphism plays a major role in dictating the specificity, type, and magnitude of the human CD4$^+$ T cell response to mycobacterial Ags (15). HLA-DRA/B1*0301 (DR3) is a major class II allele that is present in >20% of the human population. HLA-DR3 is associated with high-responder (tuberculoid) leprosy and with strong T cell activity to mycobacterial Ags, *in vitro* and *in vivo* (16). Importantly, HLA-DR3-restricted, *M. tuberculosis*-reactive Th 1 cells frequently respond to the immunodominant heat shock protein (hsp)65 and 85A, 85B, and 85C proteins (17–20). Hsp65 contains only one HLA-DR3-restricted epitope, notably p1–20 (21), while HLA-DR3-restricted T cell epitopes of 85A, 85B, or 85C have not yet been determined.

Thus, since HLA polymorphism controls human T cell responsiveness to mycobacteria, the immunogenicity and protective efficacy of candidate vaccine Ags need to be defined in the context of HLA polymorphism. To start addressing this issue, we have used recently generated DR3.Ab^0 (DRA/DRB1*0301) and DQ8.Ab^0 (DQA*0301/DQB1*0302) transgenic (tg) mice (in which Ab^0 indicates class II-deficient). A major advantage of these animals is that they are devoid of any murine class II molecules expressed at the cell surface, such that all CD4$^+$ T cells are restricted by the human class II molecule. Here we show that

Abbreviations: DR3, HLA-DRB1*0301; DQ8, DQB1*0302/DQA*0301; Ag, antigen; Ab^0, class II-deficient; hsp, heat shock protein; LNC, lymph node cell; BCG, bacillus Calmette–Guérin; tg, transgenic; MHC, major histocompatibility complex; IFA, incomplete Freund's adjuvant; Il, interleukin; IFN, interferon; Th, T helper.

[§]To whom reprint requests should be addressed at: Department of Immunohematology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. e-mail: Geluk@Rullf2.Medfac.Leidenuniv.nl.

[¶]A.G. and V.T. made equal contributions to this study.

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HLA class II transgene-restricted T cells recognize the same immunodominant Ags and peptide epitopes of *M. tuberculosis* as human T cells. Moreover, peptide immunization leads to efficient T cell responses to *M. tuberculosis*. Thus, HLA-tg Ab^0 mice provide an immunologically relevant model system for the development of immune intervention strategies, such as the design of subunit vaccines.

MATERIALS AND METHODS

HLA-Tg Mice. DRB1*0301/DRA tg, murine class II-deficient ($DR3.Ab^0$) mice were generated as detailed elsewhere (22, 23). Briefly, cosmids carrying HLA-DRA and HLA-DRB1*0301 genes were coinjected into $(C57BL/6 \times DBA/2)F_1 \times C57BL/6$ embryos and backcrossed to C57BL/10 mice. The DR3 specificity was introduced into class II-negative mice by mating the $H2.Ab^0$ strain (24) with DR3.B10.M mice (23), as described for $HLA\text{-}DQ8.Ab^0$ mice (25). The $DR3.Ab^0$ mice used in this study were backcrossed for 10 generations with C57BL/10 mice and were eventually intercrossed. The resulting mice, therefore, are considered C57BL/10 congenic. During breeding, peripheral blood mononuclear cells were typed for expression and segregation of the transgene by flow cytometry and PCR (23). HLA-DQB1*0302/DQA*0301 (DQ8).Ab^0 mice were generated similarly (25). HLA-DR3/DQ8.Ab^0 mice were obtained by mating of $DR3.Ab^0$ and $DQ8.Ab^0$.

Peptides. Peptides were synthesized by simultaneous multiple peptide synthesis, as described (26). Homogeneity was confirmed by analytical HPLC and, for most peptides, by mass spectrometry. Fluorescence-labeled p3–13 was synthesized as described (27).

Ags. *M. bovis* BCG was purchased from the Statens Serum Institut (Copenhagen), recombinant hsp65 of *M. tuberculosis* was obtained from J. Van Embden (RIVM, Bilthoven, The Netherlands), *M. tuberculosis* from P. Klatser (Royal Tropical Institute, Amsterdam) and Dick van Soolingen (RIVM), and Ag85 was purified from an early culture filtrate of *M. tuberculosis* (28).

Immunizations. Emulsions comprising equal volumes of Ag in PBS and incomplete Freund's adjuvant (IFA; Difco) were prepared and administered as subcutaneous injections into tails and footpads (in total 100 μg of peptide, 10 μg of BCG or 10 μg of hsp65 per mouse). Seven days postinjection, inguinal, caudal, and lumbar lymph nodes were removed and cell suspensions were prepared for *in vitro* culture.

***In Vitro* Culture.** Cell suspensions (1×10^7 cells per ml) containing day 7 postinjection draining lymph node cells (LNCs) were resuspended in RPMI medium 1640 (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), 100 units/100 μg/ml penicillin/streptomycin solution (GIBCO), and 10% heat-inactivated fetal calf serum. The cell suspension (100 μl) was added to 96-well, flat-bottomed microtiter plates (Costar). Cells were challenged with 100 μl of medium (negative control), Con A (2 μg, positive control), peptide (10 μg), BCG (1 μg), *M. tuberculosis* (1 μg), or hsp65 (1 μg). For blocking experiments, antibody was added to the cells challenged *in vitro* with peptide, BCG, or hsp65. The following mAb were used: B8.11.2 (anti-HLA-DR), GK1.5 (anti-CD4), Lyt2 (anti-CD8), Y-17β (anti-I-E), and 14-4-4S (anti-I-Eα^k). After 24 h, 10 μl of a 180 μCi/ml solution of [^{3}H]thymidine was added to each well. After 18 h, cells were collected on glass fiber filter strips, and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. Results are the mean of triplicate cultures. SEM were <20%.

Cytokine Assays. Cytokine levels (IFN-γ, IL-2, and IL-4) were determined in the 24-h culture supernatants of LNCs that were treated as described for proliferation assays. Detection of cytokines was performed by ELISA (Genzyme), according to the manufacturer's instructions.

DR-Peptide-Binding Assay. As a source of DR3 molecules, the DRB1*0301- homozygous Epstein–Barr virus-transformed B lymphoblastoid cell line HAR was used. DR3 molecules were purified by affinity chromatography (29). Peptide binding to purified DR3 molecules (60–600 nM) was determined as described elsewhere (27). As a standard peptide, fluorescence-labeled hsp65 p3–13 was used. Peptide-binding affinity was defined as high (IC_{50} <1 μM), intermediate (1 μM to 10 μM), weak (10 μM to 100 μM) or null (>100 μM).

Proliferation Assays of Human T Cell Lines. Proliferation was assayed by mixing 10^4 T cells, irradiated peripheral blood mononuclear cells (5×10^4/well), and Ag. After 66 h of culture, 0.5 μCi of [^{3}H]thymidine was added to each well, and 18 h later cells were collected on glass fiber filter strips and the radioactivity incorporated into the DNA was determined by liquid scintillation counting.

RESULTS

BCG Vaccination of HLA-$DR3.Ab^0$ Induces Specific T Cell Responses to *M. tuberculosis*, hsp65, and P1–20. To establish first whether routine BCG vaccination can induce efficient T cell responses to mycobacteria in $DR3.Ab^0$ mice, animals were immunized with *M. bovis* BCG and LNCs challenged *in vitro* with BCG, hsp65, or hsp65 p1–20. T cells from BCG-immunized $DR3.Ab^0$ mice strongly proliferated in response to BCG, hsp65, and p1–20, whereas control PBS/IFA-injected mice failed to do so (Fig. 1*A*). $DR3.Ab^0$ mice immunized with hsp65 encoding plasmid DNA similarly displayed significant T cell immunity to hsp65, *M. tuberculosis*, and p1–20, indicating that the same protein and peptide are presented to DR3-restricted T cells in $DR3.Ab^0$, irrespective of the mode of immunization (data not shown).

We next addressed whether hsp65 and p1–20 are efficient immunogens themselves and thus might represent potential candidate vaccines. A single immunization of $DR3.Ab^0$ mice with either hsp65 or p1–20, indeed, led to efficient T cell responses to *M. tuberculosis*, *M. bovis* BCG, hsp65, and p1–20 (Fig. 1*A*). Immunization of DR3.B10.M mice, in which both Ea and Eb genes are mutated, also induced strong T cell responses to p1–20 and hsp65, as well as to BCG, while no such responses were seen in Ab^0 mice that lack DR3. Collectively, these data demonstrate that the DR3$\alpha\beta$ tg molecule presents Ag to T cells in $DR3.Ab^0$ mice.

To characterize further the response in BCG-immunized $DR3.Ab^0$ mice, LNCs were restimulated *in vitro* with BCG, hsp65, or p1–20, in the absence or presence of mAbs specific for HLA-DR, H2-Eβ, H2-Eα, mouse CD4, or CD8. Only anti-DR and anti-CD4 mAbs could inhibit the response to BCG, hsp65, and p1–20 (Fig. 1*B*). Similar results were obtained for hsp65- or p1–20-immunized $DR3.Ab^0$ mice (data not shown). Furthermore, LNCs from BCG-, hsp65-, or p1–20-immunized $DR3.Ab^0$ mice were found to produce significant levels of IFN-γ after *in vitro* stimulation with these Ags (Fig. 1*C*), whereas no IL-4 could be detected (data not shown). Thus, T cell responses to BCG, hsp65, and p1–20 in $DR3.Ab^0$ mice are DR3-restricted, CD4-dependent, and accompanied by type 1 cytokine production.

HLA-DR3-Restricted T Cells in Immunized HLA-$DR3.Ab^0$ Mice Recognize the Same Epitopes as HLA-DR3-Restricted Human T Cells. To assess whether the specificity of DR3-restricted T cells in $DR3.Ab^0$ mice was indeed identical to that of human DR3-restricted T cells at the epitope level, we tested 54 peptides (20-mers, overlapping 10 residues) covering the entire *M. tuberculosis* hsp65 sequence. LNCs from BCG- or hsp65-immunized $DR3.Ab^0$ mice were tested for proliferation toward each individual peptide (Fig. 2). Also, peptide-binding affinity for DR3 was determined in a quantitative peptide-binding assay. p1–20 bound to DR3 with 5-fold higher affinity than the next best binding peptide (p41–60). Ten additional peptides bound well or intermediately to DR3, whereas the remaining peptides hardly bound to DR3 ($\geq$100 μM) (Table 1).

In agreement with our results in humans (21), only DR3-restricted murine T cells responded strongly to the N-terminal p1–20 (Fig. 2*A*). The high-affinity DR3 binder p41–60 (Table

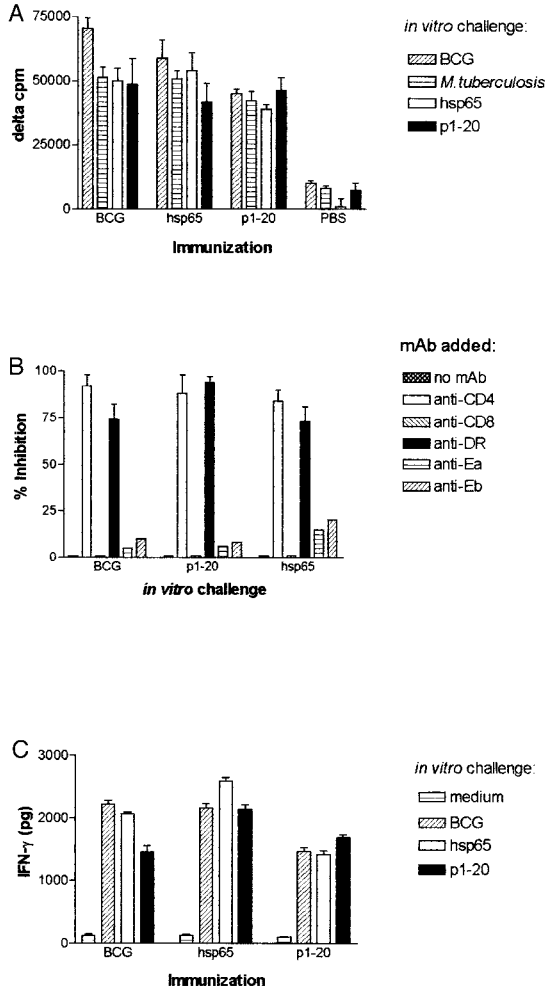

FIG. 1. (*A*) Proliferative T cell responses of LNCs from HLA-DR3.Ab^0 mice immunized with BCG (10 μg), hsp65 (10 μg), p1–20 (100 μg), or PBS, in IFA. Proliferation is indicated in delta cpm (mean cpm in experimental wells − mean cpm in control wells without Ag). (*B*) Percentage inhibition of proliferative T cell responses by mAb specific for HLA-DR, mouse CD4, and mouse CD8, H2-Eα, and H2-Eβ LNCs of BCG-immunized mice were challenged *in vitro* with BCG, hsp65, or p1–20 in the presence of indicated mAb. (*C*) IFN-γ production by DR3-restricted LNCs from DR3.Ab^0 mice immunized with BCG, hsp65, or p1–20. The *in vitro* challenge is indicated in the key.

1) clearly was immunogenic in the context of DR3, since it induced a T cell response to itself, yet it failed to immunize against BCG, thus identifying this peptide as a "latent" or "cryptic" epitope. Low levels of proliferation were observed to p171–190, but this peptide could not immunize against hsp65 or BCG (data not shown). Non-DR3-binding peptides such as the influenza peptide HAp307–319 completely failed to immunize DR3.Ab^0 mice (data not shown), further demonstrating the essential requirement for high-affinity DR3 binding in the induction of Ag-specific T cell responses in DR3.Ab^0 animals.

Identification of HLA-DQ8-Restricted T Cell Epitopes of Hsp65 in HLA-DQ8.Ab^0. T cells from BCG-immunized HLA-DQ8.Ab^0 mice also responded to BCG and to hsp65, but did not recognize p1–20 (Fig. 3*B*). Instead, when challenged *in vitro* with the 54 overlapping hsp65 peptides, six other peptides covering three different regions were recognized (amino acids 171–200, 311–340, and 411–440, Fig. 2*B*). Thus, three naturally processed antigenic determinants could be identified, that are seen in the context of DQ8.

Next, to explore the immunodominance of the DR3-restricted p1–20 and the DQ8-restricted epitopes in the presence of another HLA class II allele, double tg DR3/DQ8.Ab^0 mice were generated and immunized with hsp65. T cells from these animals proliferated in response to BCG, hsp65, and hsp65 p1–20, as well as to the hsp65-derived sequences 171–190, 311–340, and 421–440 (Fig. 3*C*). These results clearly show that no competition at the level of T cell epitope formation is observed in double HLA-tg animals, as both DR3- and DQ8-specific responses are maintained.

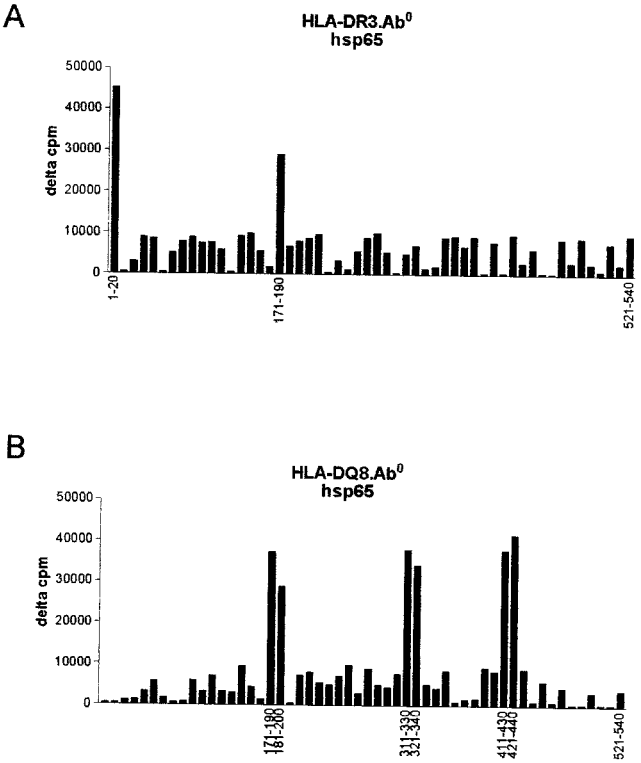

FIG. 2. (*A*) T cell reactivity of hsp65-immunized DR3.Ab^0 to a complete set of hsp65-derived 20-mer peptides containing 10 amino acids overlap. (*B*) T cell reactivity of hsp65-immunized DQ8.Ab^0 to a complete set of hsp65-derived, 20-mer peptides containing 10 amino acids overlap. Amino acid positions are indicated on the *x*-axis.

HLA-DR3-Restricted T Cell Responses in Ag85-Immunized HLA-DR3.Ab^0 Mice. Early culture filtrate Ags of mycobacteria, such as the Ag85 family, have been proposed as important candidate vaccine Ags. Like hsp65, Ag85 is frequently recognized by human T cells (28). Therefore, we first analyzed T cell responses to Ag85 in immunized DR3.Ab^0 mice. Routine BCG-vaccination led to a strong response against *M. tuberculosis* and Ag85. Moreover, Ag85 immunization of DR3.Ab^0 induced a strong response to BCG and *M. tuberculosis* (Fig. 4*B*).

Next, to assess whether determinants recognized by human T cells can be identified in the HLA-tg mice model, the epitope specificity of *M. tuberculosis* Ag85-reactive T cells in DR3.Ab^0 mice was mapped by using 28 peptides, covering the entire *M. tuberculosis* 85B sequence (Fig. 4*A*). T cell reactivity was observed to only one single peptide epitope, p51–70 (LQVP-SPSMGRDIKVQFQSGG) that was newly identified. Strikingly, similar to hsp65 p1–20, 85B p51–70 was the highest affinity DR3-binding peptide (400 nM) of all 85B sequences (Table 2). Also, immunization with this epitope induced T cell immunity to BCG, *M. tuberculosis,* and Ag85 in DR3.Ab^0 mice, although to a lesser extent than did Ag85 (Fig. 4*B*). Responses were again DR3-restricted, CD4-dependent, Th 1-like, and DR3-specific, since no such responses were detected in p51–70-immunized DQ8.Ab^0 mice (data not shown).

Identification of HLA-DR3-Restricted 85B Epitopes in Human T Cell Lines. To determine whether p51–70 is also recognized by DR3-restricted, Ag85-reactive human T cells,

Table 1. HLA-DR3-restricted T cell responses and HLA-DR3 binding of hsp65-derived peptides in humans or HLA-DR3.Ab^0 mice

hsp65 amino acid sequence	Recognition by HLA-DR3-restricted T cells*		HLA-DR3 binding affinity, IC_{50}, μM†
	In humans	In tg mice	
1-20 MAKTIAYDEEARRGLERGLN	+++	+++	0.06
41-60 KKWGAPTITNDGVSIAKEIE	−	−	0.3
511-530 FLTTEAVVADKPEKEKASVP	−	−	0.7
201-220 YFVTDPERQEAVLEDPYILL	−	−	1.3
281-300 DRRKAMLQDMAILTGGQVIS	−	−	1.7
211-230 AVLEDPYILLVSSKVSTVKD	−	−	2
241-260 AGKPLLIIAEDVEGEALSTL	−	−	2
481-500 VYEDLLAAGVADPVKVTRSA	−	−	2.6
261-280 VVNKIRGTFKSVAVKAPGFG	−	−	5
81-100 DDVAGDGTTTATVLAQALVR	−	−	6.5
181-200 FGLQLELTEGMRFDKGYISG	−	−	9
321-340 VVVTKDETTIVEGAGDTDAI	−	−	14
191-210 MRFDKGYISGYFVTDPERQE	−	−	19
341-360 AGRVAQIRQEIENSDSDYDR	−	−	20
401-420 AKAAVEEGIVAGGGVTLLQA	−	−	20
441-460 KVALEAPLKQIAFNSGLEPG	−	−	22
491-510 ADPVKVTRSALQNAASIAGL	−	−	22
301-320 EEVGLTLENADLSLLGKARK	−	−	25
171-190 GVITVEESNTFGLQLELTEG	−	+	26
331-350 VEGAGDTDAIAGRVAQIRQE	−	−	28
31-50 GPKGRNVVLEKKWGAPTITN	−	−	30
All other peptides	−	−	>100

DR3.Ab^0 mice were immunized with BCG and hsp65 (10 μg per mouse). After 7 days, LNCs were challenged *in vitro* with hsp65− peptide (50 μg/ml).

*Results for hsp65-reactive human T cells are shown for reference purposes only (see ref. 19). +++ indicates SI (stimulation index) $\geq$ 4; + indicates 2 $\leq$ SI $\leq$ 3; − indicates SI < 2.

†Peptide binding affinity (IC_{50}) was defined as high-affinity (<1 μM), intermediate-affinity (1 μM to 10 μM), weak-affinity (10 μM to 100 μM), or nonbinding (>100 μM), according to ref. 27.

four Ag85-reactive T cell lines from three HLA-DR3+ and one HLA-DR3− individuals were generated by stimulation with *M. tuberculosis* and were tested for proliferation to all overlapping 85B peptides. In concordance with the above results in BCG/Ag85-immunized DR3.Ab^0 mice (Fig. 4), all HLA-DR3+ T cell lines efficiently recognized p51–70. This epitope was mapped as the core epitope in the p51–70 sequence by using *M. tuberculosis*/*Mycobacterium leprae* cross-reactive T cell clones (data not shown). Instead, the HLA-DR3-negative (DR1,DR7) T cell line proliferated in response to the DR1-restricted epitope p71–90 but not to p51–70 (Fig. 5). Thus, HLA-DR3-restricted human T cells from vaccinated or infected individuals recognize precisely the same epitope as HLA-DR3-restricted T cells from BCG- or hsp65-immunized DR3.Ab^0 mice, showing that HLA-tg mice can be used as an efficient model to define naturally processed epitopes for human T cells.

DISCUSSION

The expression of functional HLA class II transgenes in mice has been reported previously (22, 30, 31) but only very recently have HLA class II tg animals been generated that express HLA class II molecules in the absence of cell surface-expressed murine class II molecules (Ab^0) (23, 25). A unique advantage of this system is that all CD4 T cells in Ab^0 animals are restricted by the transgene-encoded HLA molecule. Thus, these animals offer a model to study Ag-specific T cell responses in the context of human HLA transgenes only. The expression of disease-associated HLA class II transgenes in Ab^0 mice has been shown to confer susceptibility to experimentally induced autoimmune disease, such as autoimmune thyroiditis (23), encephalomyelitis (32), and arthritis (25, 33). Thus, HLA polymorphism determines susceptibility to a variety of organ-specific autoimmune diseases. In this study, we have used the DR3.Ab^0 and DQ8.Ab^0 mice (*i*) to study vaccine-(live BCG, protein, peptide) induced T cell responses to mycobacteria in the context of HLA class II, (*ii*) to define novel DR3- and DQ8-restricted mycobacterial T cell epitopes, and (*iii*) to demonstrate recognition of such novel epitopes by human T cells.

First, our results show that immunization of HLA-DR3.Ab^0 mice with either hsp65 protein or Ag85 induces efficient DR-restricted, CD4-dependent T cell responses to *M. tuberculosis*, comparable to the level induced by BCG immunization. A second important observation is that the Ag and peptide epitope specificity of DR3-restricted T cells in tg mice appears to be indistinguishable from that of DR3-restricted T cells in humans: the same immunodominant epitopes (hsp65 p1–20 and the newly identified 85B epitope p51–70) are processed and presented in HLA-DR3.Ab^0 mice as in HLA-DR3$^+$ humans. Thus, immunization of DR3.Ab^0 mice was used successfully to define an epitope for human T cells. Third, our results reveal a lack of competition at the level of T cell epitope formation in hsp65-immunized DR3/DQ8.Ab^0 mice, as both DR3- and DQ8-specific responses are maintained. These results demonstrate the lack of allelic competition at the level of T cell epitope formation in double HLA-tg mice. Since both DR and DQ alleles are present in humans, these double HLA-tg mice represent a good model to determine *in vivo* T cell responses against mycobacterial Ags in the context of HLA polymorphism. Furthermore, the additive effect of DR3- and DQ8-restricted T cell responses in DR3/DQ8.Ab^0 mice underlines the potential of hsp65 in human vaccines against *M. tuberculosis*, as it induces T cell responses against several HLA-restricted epitopes.

The above results imply that the major MHC class II Ag-processing pathways (endosomal/lysosomal proteases such as cathepsin S, B, and D), and peptide/class II loading systems (Ii chain, H-2M/HLA-DM) in mice can cooperate efficiently across a species barrier with human HLA molecules. These results parallel previous observations in HLA-A*0201 tg mice whose cytotoxic T lymphocyte recognized the same naturally processed epitopes as human CD8$^+$ cytotoxic T lymphocytes (34). Our

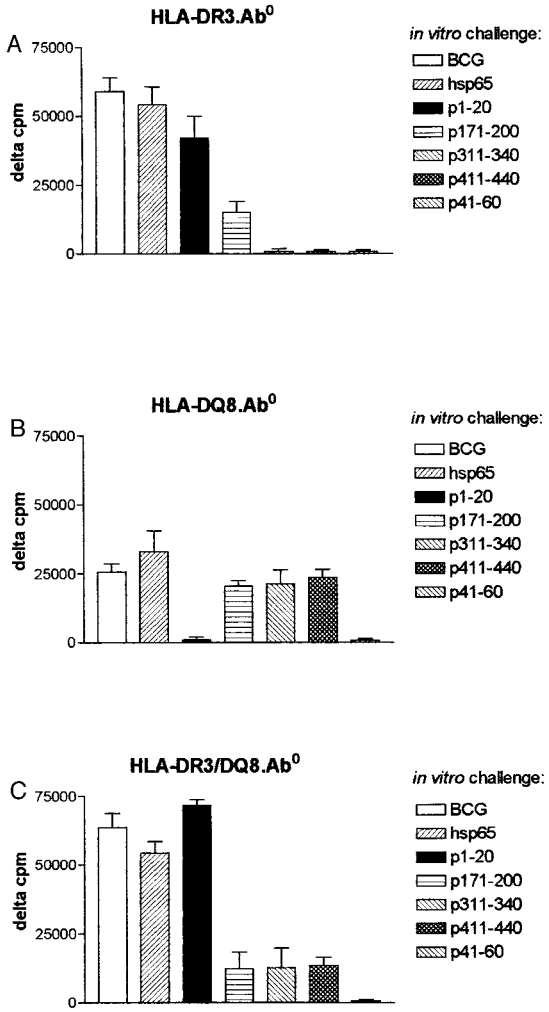

FIG. 3. T cell recognition of specific peptide epitopes by DR3.Ab^0 (*A*), DQ8.Ab^0 (*B*), and DR3/DQ8.Ab^0 (*C*) mice, after immunization with hsp65 (10 μg) in IFA. The *in vitro* challenge is given in the keys.

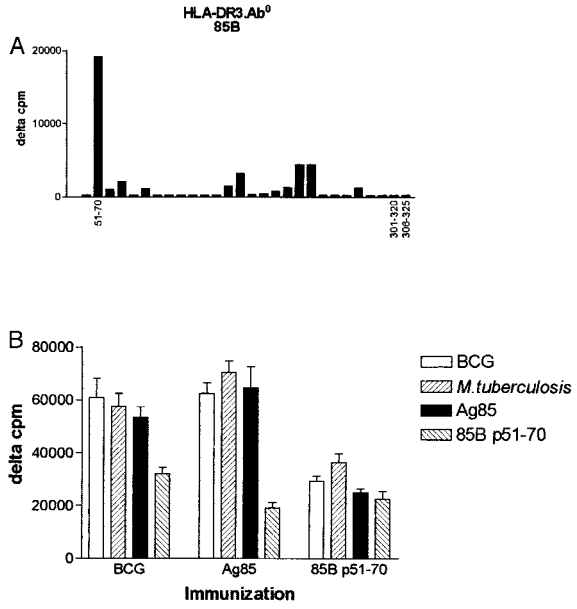

FIG. 4. Reactivity of Ag85-immunized DR3.Ab^0 mice to 85B-derived, 20-mer peptides containing 10 amino acids overlap (*A*). *In vitro* proliferation of LNCs from DR3.Ab^0 mice immunized with BCG (10 μg), Ag85 (10 μg), or p51–70 (100 μg) in IFA (*B*).

results indicate the potential of HLA class II tg, class II $-/-$ mice as a preclinical model to define the immunogenicity and protective efficacy of candidate vaccine Ags in the context of HLA polymorphism.

What controls the immunodominance of the epitopes p1–20 and p51–70 in the context of DR3? Since p1–20 and p51–70 are the highest-affinity HLA-DR3 binders among all hsp65 and 85B peptides, respectively, MHC-binding affinity must be an important factor, in agreement with findings on other class II- and class I-restricted epitopes (35, 36). Furthermore, peptide binding may also explain the slightly stronger response to BCG induction by p1–20 immunization, compared with p51–70 immunization, since p1–20 binds 10-fold better to DR3 than does p51–70. Several other DR3-binding hsp65 peptides, however, were not recognized by T cells and also failed to induce efficient T cell responses to hsp65, defining these peptides as cryptic or latent epitopes. One reason why such peptides could remain cryptic is that they are destroyed during Ag processing, as reported recently for viral MHC class I binding peptides (37). Indeed, several of the DR3-binding hsp65 peptides, but not p1–20, contain cathepsin D sites (38). A second factor that might contribute to the immunodominance of p1–20 is related to its position based on the three-dimensional structure of GroEL: the *Escherichia coli* GroEL (hsp65) crystal consists of a highly ordered dimer of heptamers, which forms a cylindrical structure (39). The N-terminal hsp65 residues, however, are disordered in the crystal and project into the central channel where they are exposed to the solvent. Thus, p1–20 may be well accessible to processing proteases and may be processed rapidly compared with other hsp65 peptides. A functional cathepsin D site is indeed located between residues 25 and 26 of the mycobacterial hsp65 molecule. Strikingly, hsp65 pcDNA-immunized DR3.Ab^0 also responded to

Table 2. HLA-DR3-restricted T cell responses and HLA-DR3 binding of 85B-derived peptides in humans or HLA-DR3.Ab^0 mice

85B amino acid sequence	Recognition by HLA-DR3-restricted T cells*		HLA-DR3 binding affinity, IC_{50} $\mu M^{\dagger}$
	In humans	In tg mice	
34-53 LQVPSPSMGRDIKVQFQSGG	+++	++	0.4
154-173 SAMILAAYHPQQFIYAGSLS	–	–	3
164-183 QQFIYAGSLSALLDPSQGMG	–	–	8
134-153 ANRAVKPTGSAAIGLSMAGS	–	–	9
144-163 AAIGLSMAGSSAMILAAYHP	–	–	15
284-303 THSWEYWGAQLNAMKGDLQS	–	–	25
All other peptides	–	–	>100

DR3.Ab^0 mice were immunized with BCG and Ag85 (10 μg per mouse). After 7 days, LNCs were challenged *in vitro* with 85B– peptide (50 μg/ml).

*+++ indicates SI $\geq$ 4; ++ indicates 3 $\leq$ SI $\leq$ 4; – indicates SI $<$ 2.

†Peptide binding affinity (IC_{50}) was defined as high-affinity ($<$1 μM), intermediate-affinity (1 μM to 10 μM), weak-affinity (10 μM to 100 μM), or nonbinding ($>$100 μM), according to ref. 27.

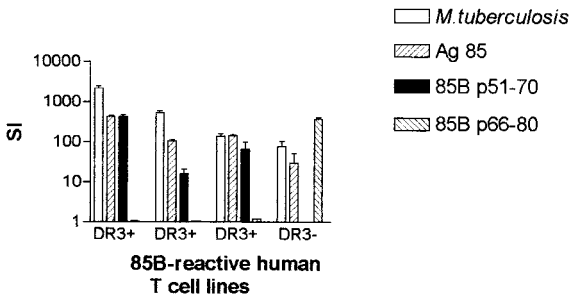

FIG. 5. Reactivity of *M. tuberculosis*-induced, 85B-reactive human T cell lines, derived from three DR3+ and one DR3 − (DR1/DR7) individuals. T cell proliferation induced by *M. tuberculosis*, Ag85, or peptide epitopes restricted by DR3 (p51–70) or DR1 (p71–90) are shown. The highly crossreactive *M. leprae* peptide was used, which differs only at two residues from the *M. tuberculosis* peptide but is crossrecognized. T cell reactivity is indicated as stimulation index (SI). Background values were <300 cpm.

p1–20 (data not shown), demonstrating that the same T cell epitope is recognized in the context of the DR3 molecule, irrespective of the mode of immunization. Thus, MHC binding affinity, site accessibility, and processing kinetics may all contribute to epitope dominance.

It is of considerable interest that immunization of DR3.Ab^0 animals with the immunodominant hsp65 p1–20 and with 85B p51–70 induced efficient CD4$^+$ Th 1 responses to whole *M. tuberculosis*. Well defined immunodominant T cell epitopes thus may be efficient immunogens and may provide useful templates for subunit vaccine design. Such epitopes could, for instance, be incorporated in synthetic or genetic (DNA, recombinant viral) vaccines. Challenge experiments in DR3.Ab^0 mice are planned for the near future to determine and compare the protective efficacy of peptide, protein, and DNA vaccination to infection with *M. tuberculosis*. Future studies will have to extend these results to other major HLA-DR (DR1, DR2, DR4) alleles to reach large vaccine coverage for the world population. Besides CD4$^+$ T cells, CD8$^+$ T cells also are involved in protection against mycobacterial infections, as is evident from experiments in β_2-microbulin −/− mice (40) and as is suggested by *in vitro* analyses of human T cells (41, 42). Effective tuberculosis vaccines thus need to induce CD4$^+$ as well as CD8$^+$ T cell immunity.

In conclusion, our results show that HLA-DR-restricted Th 1 cells from DR3.Ab^0 or DR3/DQ8.Ab^0 mice recognize exactly the same immunodominant *M. tuberculosis* Ags and epitopes as do human T cells, and that HLA-tg mice can be used to define novel naturally processed peptides that are recognized by human T cells. Furthermore, we demonstrate the lack of allelic competition at the level of epitope formation in double HLA-tg mice. Thus, HLA class II tg Ab^0 mice constitute highly useful tools for examining the immunogenicity of candidate vaccine Ags in the context of HLA polymorphism.

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