Human CD4–Major Histocompatibility Complex Class II (DQw6) Transgenic Mice in an Endogenous CD4/CD8-deficient Background: Reconstitution of Phenotype and Human-restricted Function

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Summary

To reconstitute the human immune system in mice, transgenic mice expressing human CD4 and human major histocompatibility complex (MHC) class II (DQw6) molecules in an endogenous CD4- and CD8-deficient background (mCD4/8$^{-/-}$), after homologous recombination, have been generated. We report that expression of human CD4 molecule in mCD4/8$^{-/-}$ mice rescues thymocyte development and completely restores the T cell compartment in peripheral lymphoid organs. Upon vesicular stomatitis virus (VSV) challenge, the reconstituted mature T cell population effectively provide T help to B cells in immunoglobulin class switching from IgM to specific IgG-neutralizing antibodies. Human CD4$^+$DQw6$^+$ double transgenic mice are tolerant to DQw6 and the DQw6 molecule functions in antigen presentation, effectively generating a human MHC class II–restricted T cell response to streptococcal M6C2 peptide. These data show that both the hCD4 and DQw6 molecules are functional in mCD4/8$^{-/-}$ mice, fully and stably reconstituting this limb of the human immune system in mice. This animal model provides a powerful in vivo tool to dissect the human CD4–human class II MHC interaction, especially its role in human autoimmune diseases, superantigen-mediated diseases, and acquired immunodeficiency syndrome (AIDS).

Surface expression of the CD4 or CD8 glycoprotein distinguishes the helper and cytotoxic subsets of T lymphocytes, respectively. CD4 is a member of the immunoglobulin gene superfamily, involved in recognition of MHC class II molecules on the surface of antigen presenting cells by T lymphocytes (1). The interaction between CD4 and MHC class II molecules is known to be an important event in both T cell ontogeny and peripheral activation of helper T cells (2–4). Extracellularly, CD4 interacts with MHC class II molecules via the MHC β chain (5) which contributes to the species barrier in the interaction between CD4 and class II MHC. Despite this species barrier, human CD4 molecules have been shown to function as coreceptors in mice, though less efficiently than murine CD4 (6, 7). Intracellularly, CD4 is associated with the src family protein tyrosine kinase p56^{lck} (8), which has been shown to be essential for antigen receptor–mediated signaling (9, 10) and T cell development (11). The cytoplasmic domain of CD4 is highly conserved across mammalian species with 79% homology between human and mouse sequences, but an overall homology of only 55% exists between human and mouse extracellular and transmembrane regions (12). The human CD4 molecule has been shown to be the principal cellular receptor for HIV (13). The external envelope glycoprotein of HIV, gp120, binds with high affinity to CD4, and is the prime determinant of HIV tropism, facilitating the binding and penetration of viral particles, which is central to the cytopathic process. Mice are not susceptible to HIV infection and the mouse CD4 molecule, L3T4, does not bind gp120 (14).

The family of MHC class II molecules is encoded on chromosome 17 in the mouse and chromosome 6 in humans. Four murine class II polypeptides Aα and Aβ, or Eα and Eβ pair to form I-A and I-E heterodimers on the cell surface, respectively. The human MHC class II locus contains the genes encoding the α and β chains for HLA-DR, DQ, and DP molecules, as well as, the DO and DZ genes, whose function

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is still unknown (15). The human equivalents of murine I-A and I-E molecules are DQ and DR, respectively. The DO and DZ mouse counterparts are Aβ2 and Eβ2 whose expression differs from that of classical I-A and I-E molecules (16, 17). Besides the role of HLA-DQ molecules in restricting T cell responses, many DQ alleles have generated interest because of their strong association with autoimmune diseases such as type I diabetes, coeliac disease, and multiple sclerosis (18). It is interesting to note that HLA-DQ–restricted cells may play a unique role in the immune response to some pathogens. Thus, in the general population there are genetically determined responders and nonresponders to streptococcal cell wall antigen, and nonresponsiveness can be abrogated by monoclonal antibodies to DQ, suggesting an immunoregulatory role provided by the DQ molecules (19, 20).

The purpose of the present study was to investigate the specific interaction between human CD4 and human MHC class II (DQw6), in an in vivo system. The endogenous CD4- and CD8-deficient mouse strain (mCD4/8$^{-/-}$)[1] was an ideal model system to use, as crossspecies molecular interactions are minimized. Reconstitution of the entire human CD4–MHC class II arm of the immune system in this knockout mouse, optimized the expression and function of the human CD4 molecule in this model. We provide evidence for full restoration of phenotype and function by both human transgenes and effective interaction between hCD4 and DQw6 in generating an immune response. This unique system provides a valuable in vivo model to evaluate the importance of human CD4–MHC class II interactions in the pathogenesis of human diseases including autoimmune diseases, superantigen-mediated diseases, and AIDS.

Materials and Methods

Mouse Strains. C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice homozygous for the murine CD4 and CD8 mutations have been described (21). Briefly, using the method of targeted gene disruption and homologous recombination in embryonic stem cells, mouse strains carrying mutations in the Lyt-2 (CD8α) gene (22) and L3T4 (CD4) gene (4) were bred together until homozygous for both mutations (mCD4/8$^{-/-}$ double knockout). The human CD4 transgenic mice (line 313, described in reference 23), with the CD4 transgene expressed under the control of the CD2 expression cassette (24), have lymphocyte-specific, copy number–dependent, integration site–independent expression of human CD4. These hCD4 transgenic mice were bred into the mCD4/8$^{-/-}$ double knockout mice. Concurrently, the human MHC class II DQw6 transgenic mice (19), with coinjected DQw6 α and β genomic DNA fragments under control of their endogenous promoters, were bred into the mCD4/8$^{-/-}$ background. These two strains of mutant mice were then bred together to obtain hCD4$^{+/-}$ DQw6$^{+/-}$mCD4/8$^{-/-}$ double transgenic, double knockout mice. Mice were typed by FACS® (Becton Dickinson & Co., Mountain View, CA) of PBL as described below. All mice used in the experiments had an H-2^b background unless otherwise stated. All animals were cared for in accordance with regulations under "The Animals for Research Act 1980," and the regulations of the Ontario Cancer Institute Animal Care Committee.

Immunofluorescence. Blood samples (20 μl) were collected in heparinized capillary tubes, washed once in immunofluorescence staining buffer (Ca^{2+} and Mg^{2+} free PBS, 0.1% NaN_3, 5% FCS), and double stained with PE-conjugated anti–human CD4 (Leu3a; Becton Dickinson & Co.) and FITC-conjugated anti-HLA DQ (Leu10; Becton Dickinson & Co.), anti–human CD4-PE, and anti–mouse CD3-FITC (145-2C11, PharMingen, San Diego, CA), or anti–mouse L3T4-PE (Becton Dickinson & Co.) and anti–mouse Lyt-2-FITC (Becton Dickinson & Co.). Additionally, PBL from mCD4$^{-/-}$ single knockout mice were triple stained with anti–human CD4 (Leu-3a-PE), anti–mouse Thy 1.2-FITC (Becton Dickinson & Co.), and anti–mouse Lyt-2 Biotin (Becton Dickinson & Co.) which was visualized using Streptavidin Red 670 (GIBCO BRL, Gaithersburg, MD). Single cell suspensions of thymuses from 8–12-wk-old mice were resuspended in staining buffer and stained with anti–mouse TCR-α/β FITC (PharMingen). Similarly lymph node cells (mesenteric, inguinal, and paraaortic) from 8–12-wk-old mice were resuspended in staining buffer and incubated (4°C) with appropriate antibodies. Samples were analyzed using a FACScan® (Becton Dickinson & Co.).

Mixed Lymphocyte Reaction (MLR). Purified spleen cell responders (10^5 and 3×10^5) were cocultured with 10^6 irradiated (2,000 rad) splenic stimulator cells in 200 μl of IMDM supplemented with 10% heat inactivated FCS (Hyclone Laboratories Inc., Logan, UT), β mercaptoethanol (βME, 5×10^{-5} M), 0.01% penicillin, and streptomycin in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark). After 3, 5, 7, and 10 d of incubation at 37°C and 5% CO_2, cells were pulsed for 16 h with 1 μCi of [^{3}H]thymidine. For antibody blocking studies, purified anti-DQ mAb (Chemicon International Inc., Temecula, CA) or anti-I-A^b mAb (PharMingen) were dialyzed using microcollodion bags (Sartorius AG, Gottingen, Germany) as per protocol. Both mAb were added to irradiated splenic stimulator cells at a concentration of 0.1–10 μg/ml and incubated at 37°C for 1 h before addition of purified spleen cell responders. After 5 d of incubation, cells were pulsed for 16 h with 1 μCi of [^{3}H]thymidine.

T Cell Proliferation Assay. Experimental mice were immunized with 20 μg of soluble M6C2 peptide emulsified in 200 μl of complete Freund's adjuvant (CFA) at the base of the tail (100 μl per side). M6C2 is a 21-mer (AKKQVEKDLANLTAELDKVKE) derived from Streptococcal cell wall (SCW) extract from rheumatogenic strains of Streptococci. Inguinal and paraaortic lymph nodes were harvested after 9–12 d and single cell suspensions were prepared in 5 ml of RPMI 1640 media supplemented with 10% heat-inactivated FCS (Hyclone Laboratories Inc.), 100 U/ml penicillin, 100 μl/ml streptomycin, 2 mM L-glutamine, 50 μM βME, and 20 mM Hepes. T cell enrichment was accomplished via passage of single cell suspensions through T cell enrichment columns (R & D Systems, Inc., Minneapolis, MN) according to protocol. The enriched T cells (4×10^5) were cultured in 200 μl of complete medium (as described above), in triplicate in 96-well flat-bottomed tissue culture plates (Nunc), containing 8×10^5 irradiated (3,000 rad) syngeneic spleen cells as APC, with or without peptide, at

[1] *Abbreviations used in this paper:* BME, β mercaptoethanol; DQw6$^+$ mCD4/8$^{-/-}$ mice, mice expressing human DQw6 transgene in an endogenous murine CD4- and CD8-deficient background; hCD4$^+$DQw6$^+$ mCD4/8$^{-/-}$ mice, mice expressing human CD4 and human DQw6 transgenes in an endogenous murine CD4- and CD8-deficient background; hCD4$^+$mCD4/8$^{-/-}$ mice, mice expressing human CD4 in an endogenous mouse CD4- and CD8-deficient background; mCD4/8$^{-/-}$, mice with targeted gene disruption in both endogenous CD4 and CD8; MLR, mixed lymphocyte reaction; SCW, streptococcal cell wall; VSV, vesicular stomatitis virus.

37°C in a humidified atmosphere containing 5% CO_2 for 48 h and pulsed with 1 μCi [^{3}H]thymidine for an additional 12 h.

Detection of Neutralizing Serum Antibodies to Vesicular Stomatitis Virus (VSV). VSV Indiana (isolate P3) was obtained from Dr. Lud Prevec (McMaster University, Hamilton, Ontario, Canada). Seeds were grown on BHK 21 cells infected with low multiplicity of infection and plaqued on Vero cells. The determination of neutralizing anti-VSV serum antibody titers has been described previously (25). Briefly, sera were prediluted 80-fold in minimal essential medium, then heat inactivated for 30 min at 56°C. Twofold serial dilutions were mixed with equal volumes of virus containing 300 PFU/ml. The mixture was incubated for 90 min at 37°C. 100 μl of this serum virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl DME containing 1% methylcellulose. After incubation for 24 h at 37°C the overlay was flicked off and the monolayer was fixed and stained with crystal violet in formaldehyde. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer. To determine IgG titers undiluted sera were pretreated with an equal volume of 0.1 M βME in saline (26).

Cytotoxicity Assays. VSV-specific cytotoxicity was determined in a standard primary ^{51}Cr release assay (27, 28). Briefly, mice were immunized intravenously with 2 × 10^6 PFU of VSV. After 6 d spleen cells were coincubated for 5 h with ^{51}Cr-labeled target cells. VSV-specific cytotoxicity was measured on MC57G (H-2^b) fibroblasts infected with VSV. Noninfected MC57G cells were used as controls. Spontaneous release was <15% in all assays.

Histology. Thymuses of 8–12-wk-old mice were harvested and fixed in 10% formalin. All tissues were sectioned serially at different levels and conventionally stained with hematoxylin and eosin.

Results

Human CD4 Rescues Thymocyte Development. Mice deficient in both CD4 and CD8 expression (21) have normal total number of thymocytes, but a marked reduction of mature TCR-α/β^{high} thymocytes. In accordance with a block in thymocyte maturation, the size of the thymic medulla is largely diminished on histologic sections. To investigate whether the introduction of the human CD4 molecule into mCD4/$8^{-/-}$ mice could restore thymocyte maturation, thymocyte populations of 8–12-wk-old mice were analyzed by flow cytometry with the use of various monoclonal antibodies as described in Materials and Methods. Introduction of the human CD4 transgene, alone and in combination with HLA-DQw6, rescued T cell development and restored the thymic population corresponding to TCR-α/β^{high} HSA$^-$

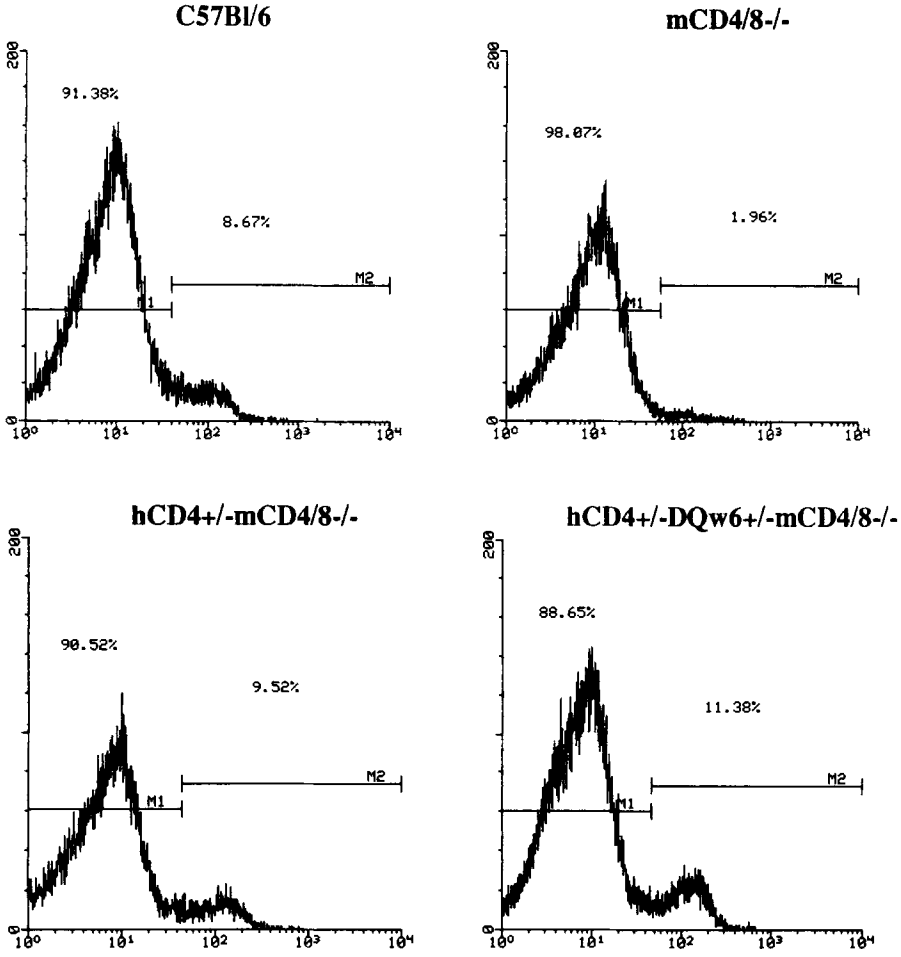


Figure 1. TCR-α/β expression on thymocytes. Single cell suspensions of thymuses from 8–12-wk-old mice were prepared and stained with anti-TCR-α/β FITC as described in Materials and Methods.

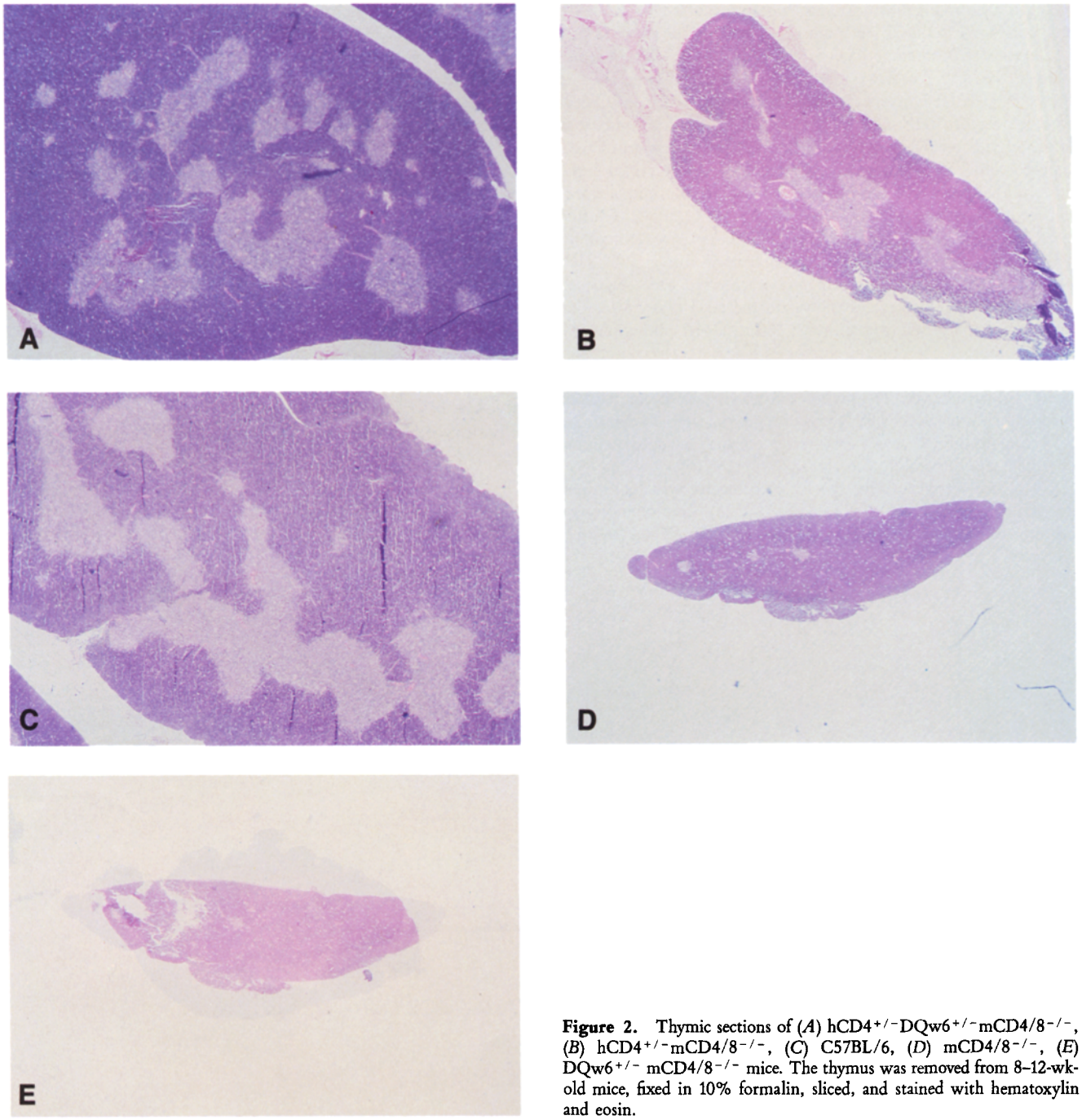


Figure 2. Thymic sections of (*A*) hCD4$^{+/-}$DQw6$^{+/-}$mCD4/8$^{-/-}$, (*B*) hCD4$^{+/-}$mCD4/8$^{-/-}$, (*C*) C57BL/6, (*D*) mCD4/8$^{-/-}$, (*E*) DQw6$^{+/-}$ mCD4/8$^{-/-}$ mice. The thymus was removed from 8–12-wk-old mice, fixed in 10% formalin, sliced, and stained with hematoxylin and eosin.

(J11d^{-}) mature thymocytes (Fig. 1). Moreover, these mice showed a dramatic increase in medullary size and the appearance of numerous Hassell's corpuscles on serial histologic sections (Fig. 2).

In peripheral lymphatic organs the T cell population was also rescued with the lymph node T cell population increasing from 10 to 20% in double knockout mice to 70% (wild-type levels) in mice expressing the hCD4 transgene (Fig. 3). A similar increase in the peripheral T cell population was seen in the spleen (data not shown). The decreased level of TCR/CD3 expression on peripheral T cells from mCD4/8$^{-/-}$ mice previously observed (21) was also restored to normal levels in the presence of hCD4 (data not shown). It should be noted that the presence of the DQw6 molecule alone in the CD4- and CD8-deficient background had no effect in restoring the mature TCR-α/β^{high} T cell population within the thymus (Fig. 2) or in restoring peripheral T cell populations.

Increased hCD4 Expression on T Cells in the Absence of mCD4 and mCD8. The expression of hCD4 on T cells varied de-

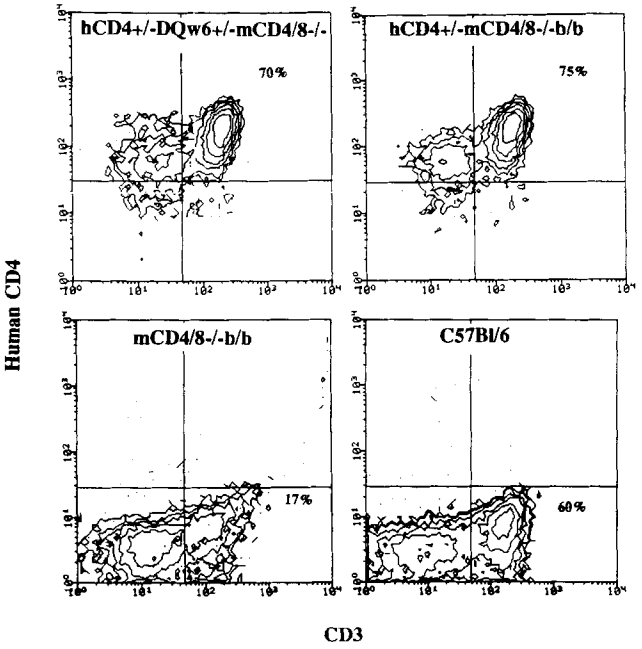


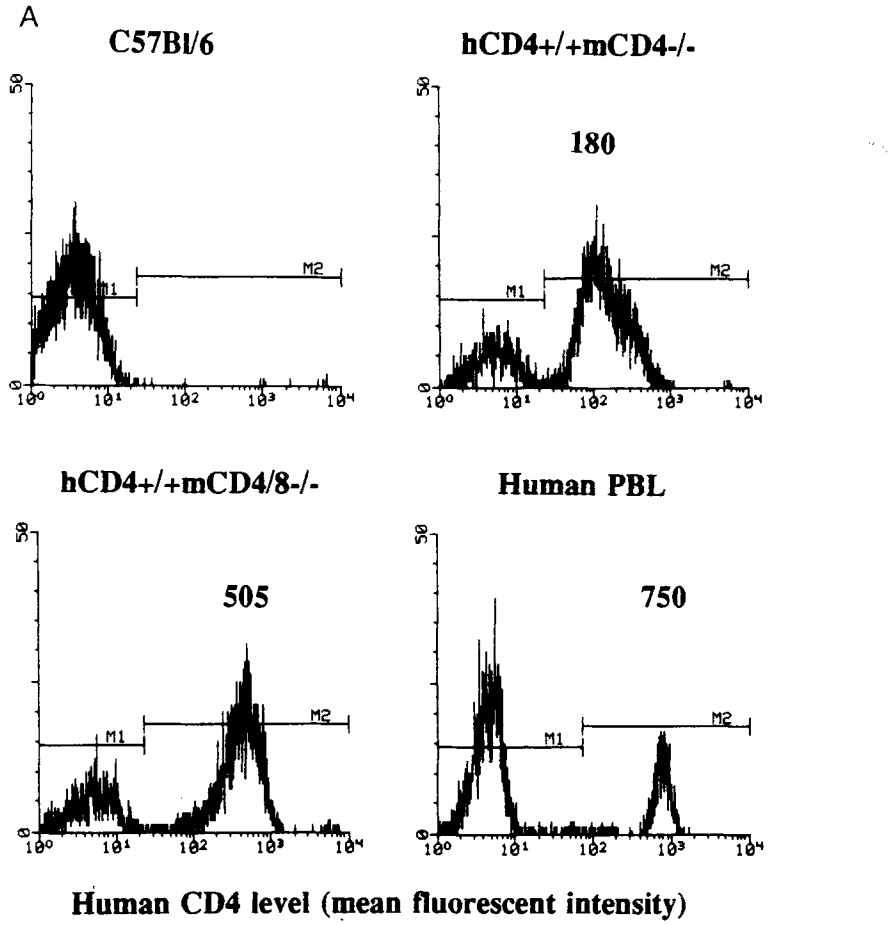
Figure 3. Rescue of the mature peripheral T lymphocyte population by the human CD4 transgene. Single cell suspensions were made from mesenteric, paraaortic, and inguinal lymph nodes and stained with anti-CD3 FITC and anti-human CD4-PE, as described in Materials and Methods. Please note in *upper panels*, human CD4 expression on B lymphocytes, as CD2 expression cassette drives expression of human CD4 at low levels in B cells.

pending on the presence or absence of mCD4 alone or in combination with mCD8 (Fig. 4, *A* and *B*). The level of hCD4 expression in normal C57BL/6 mice was much lower compared with that in human peripheral blood lymphocytes. T lymphocytes with deficient mCD4 had an increased level of expression of hCD4. This increase was even more dramatic in the mCD4/8$^{-/-}$ double knockout mice (Fig. 4 *A*). It is interesting to note that the expression of the hCD4 transgene in mCD4$^{-/-}$ mice varied depending on whether the T cells expressed endogenous CD8 or not. In the absence of mCD8, hCD4 expression level was much higher than in T cells coexpressing the endogenous mCD8 (Fig. 4 *B*). The high level of hCD4 expression on the double knockout T cells were comparable (70% of the mean fluorescent intensity) to that on human PBL (Fig. 4 *A*). The hCD4 expression remained stable in these transgenic knockout mice studied up to the age of 18 mo (data not shown).

Human CD4 Restores T Help for B Cells. We previously reported that T help for B cells is markedly reduced but not completely absent in mice without CD4 alone (29), or in mice without both CD4 and CD8 molecules (21). This T help is provided by CD4/CD8 double negative TCR-α/β^+ T cells, which are restricted to class II MHC molecules (29). To test whether the human CD4 molecule could restore T helper cell functions to normal levels, hCD4$^+$mCD4/8$^{-/-}$ mice were compared with mCD4/8$^{-/-}$ mice and hCD4$^+$mCD4$^{-/-}$ mice compared with mCD4$^{-/-}$ for their efficiency in immunoglobulin class switching after VSV challenge. The neutralizing IgM response which peaks at day 4 after immunization is independent of T help (30, 31), and comparable titers were reached in all groups of mice tested (Fig. 5), confirming that all mice were adequately immunized. The immunoglobulin class switch from IgM to neutralizing IgG is strictly dependent on T help (30, 31). As reported previously, IgG class switching in mCD4/8$^{-/-}$ mice was more efficient than in mCD4$^{-/-}$ mice most likely due to the higher numbers of double negative T cells in the double knock out mice (21). However, in both groups of mice the human CD4 transgene fully restored T help for B cells back to normal levels when compared with mCD4$^{+/+}$ control mice (Fig. 5).

Human CD4 Restores T Help for CTL. The generation of VSV-specific CTL responses depends on T help (32). We therefore investigated the ability of the human CD4 molecule to restore T help for CTL in hCD4$^{+/-}$mCD4$^{-/-}$ mice compared with mCD4$^{-/-}$ single knockout mice. Mice were immunized intravenously with VSV on day 0 and primary VSV-specific cytotoxicity was assessed on day 6. Fig. 6 shows that the presence of the human CD4 transgene increased cytotoxic T cell activity by a factor of 3 and cytotoxicity in hCD4$^{+/-}$mCD4$^{-/-}$ mice was comparable to mCD4$^{+/+}$ controls.

Acquisition of Immunologic Tolerance to DQw6. During thymic ontogeny, developing T lymphocytes undergo positive selection for self-MHC restriction, and clonal deletion to eliminate thymocytes expressing self-reactive T cell receptors. To study whether T cells developing in DQw6 transgenic mice are tolerant against the human MHC class II molecules, peripheral T lymphocytes were isolated and stimulated with irradiated splenic stimulator cells expressing DQw6 molecules. Elimination of self-reactive T cells was evidenced by the lack of stimulation by DQw6$^+$ splenic stimulators when cocultured with T responders from hCD4$^+$DQw6$^+$mCD4/8$^{-/-}$b/b mice. In contrast, T cells from hCD4$^+$mCD4/8$^{-/-}$b/b littermate controls responded readily to the same DQw6 stimuli. Both groups were tolerant to cells from the H-2^b background and to the human CD4 molecule (Fig. 7 *A*), but responded vigorously to allogeneic H-2^k stimulators (data not shown). It should be noted that T cells from mCD4$^+$mCD8$^-$b/b and C57BL/6 mice also responded to DQw6 positive APCs (data not shown). T cells from DQw6$^+$mCD4/8$^{-/-}$b/b mice did not respond to DQw6 positive stimulators, and their littermate controls, mCD4/8$^{-/-}$b/b mice demonstrated only a weak response to DQw6 stimulation (data not shown). T cells from mice lacking both CD4 and CD8 molecules have previously been reported to have only very weak alloresponsiveness (21). Anti-DQ monoclonal antibodies specifically blocked the hCD4$^+$mCD4/8$^{-/-}$b/b T cell proliferative response to DQw6$^+$ stimulators in a dose-dependent fashion, but anti-I-A^b monoclonal antibodies were not inhibitory (Fig. 7 *B*). A time course on the MLR using all the mouse strains described above showed maximal response to DQw6 at day 7 and no difference in kinetics of response between all of the mouse strains tested



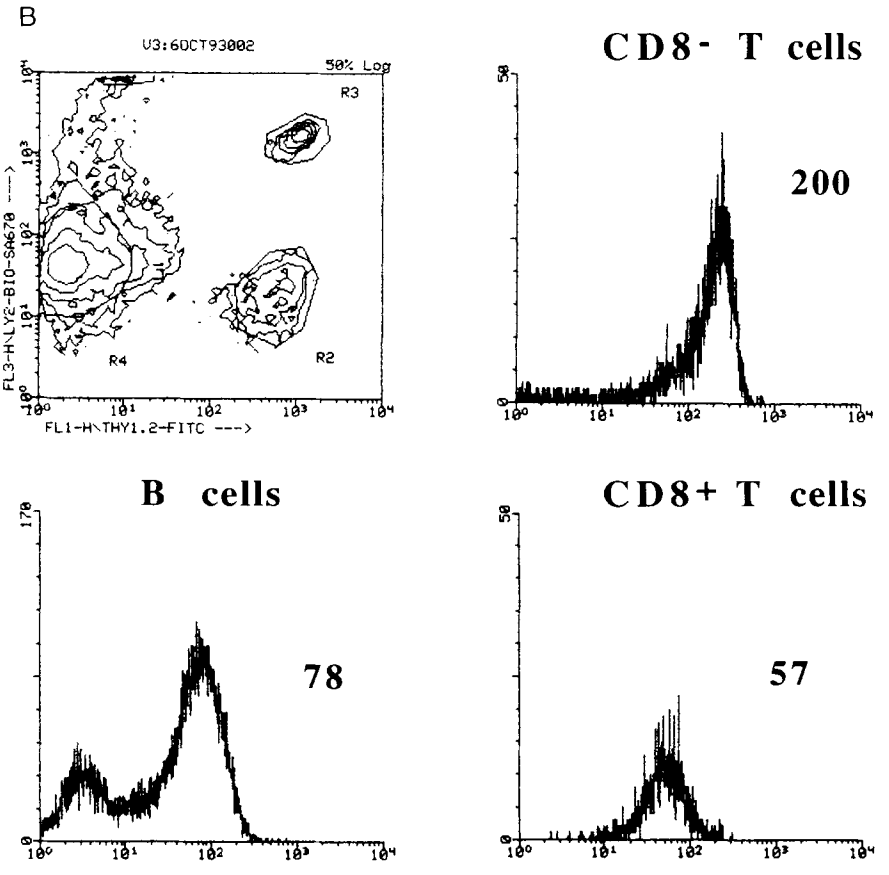


Figure 4. Human CD4 expression on T cells from PBL. Peripheral blood samples were obtained from tail vein venipuncture in mice, and from capillary samples from healthy human volunteers. Histograms depict fluorescence intensity staining with human CD4-PE and numbers represent mean fluorescence intensity. (*A*) PBL were stained with anti-human CD4-PE as described in Materials and Methods. Representative histograms from individual mice are shown as fluorescence intensity of human CD4-PE staining. (*B*) PBL from hCD4$^+$mCD4$^{-/-}$ mice were triple stained with anti-Thy 1.2 FITC, anti-Lyt2 Biotin plus streptavidin RED 670, and anti-human CD4-PE as described in Materials and Methods. Histograms showing human CD4-PE fluorescence intensity for the following lymphocyte subsets were obtained by gating on the appropriate cell populations on the contour graph in the *upper left panel*: CD8$^+$ T cells, gate R3 (Thy 1.2$^+$ Lyt2$^+$), CD8$^-$ T cells, gate R2 (Thy 1.2$^+$ Lyt2$^-$), B lymphocytes, gate R4 (Thy 1.2$^-$ Lyt2$^-$).

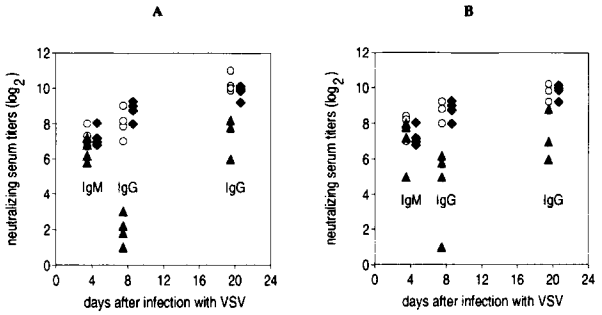


Figure 5. Neutralizing antibody response to VSV. Mice were immunized intravenously with VSV (2×10^6 pfu) on day 0. After 4 d, neutralizing IgM titers were determined. Neutralizing IgG titers were measured after 8 and 20 d. The values shown represent the number of twofold dilution steps starting at a predilution of 1/80. (*A*) $hCD4^{+/-}mCD4^{-/-}$ (*open circle*); $mCD4^{-/-}$ (*solid triangle*); $mCD4^{+/+}$ ($mCD8^{+/+}$) (*solid diamond*). (*B*) $hCD4^{+/-}$ $mCD4/8^{-/-}$ (*open circle*); $mCD4/8^{-/-}$ (*solid triangle*); $mCD4^{+/+}$ ($mCD8^{+/+}$) (*solid diamond*).

(data not shown). Response to H-2^k stimulation showed similar kinetics between all mouse strains but peaked at day 5.

DQw6-restricted Antigen Presentation. To study whether DQw6-restricted T cell responses can be generated in this double transgenic knockout system, the T cell proliferative response to antigen presentation by the human MHC class II molecule was studied. Previously it has been shown that the mouse I-A^b molecule does not effectively present M6C2, a 21-mer peptide found in SCW extract from rheumatogenic strains of Streptococcus (33). By contrast, the same peptide is presented by the human DQw6 molecule but not by other human DQ molecules such as DQw4 (33). T cells from $hCD4^+DQw6^+mCD4/8^{-/-}$b/b mice, demonstrated a marked proliferative response to M6C2 peptide when presented by autologous splenocytes, throughout varying concentrations (Fig. 8). Conversely, in the absence of $DQw6^+$ antigen presenting cells, T cells from the $hCD4^+mCD4/8^{-/-}$ H-2^b single transgenic littermates did not respond to M6C2 peptide (Fig. 8). This response is specific to DQw6 as the DQw4 molecule is unable to elicit the same T cell response in the presence of M6C2 peptide (33). It should be noted that T cells from $DQw6^+mCD4/8^{-/-}$ single transgenic

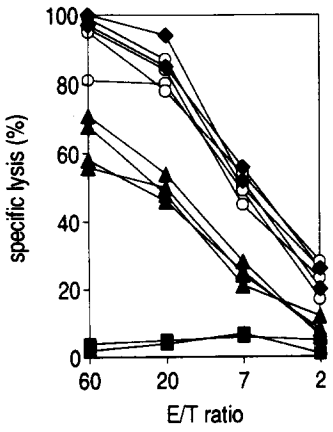


Figure 6. Primary CTL responses against VSV. Mice were infected intravenously with 2×10^6 pfu of VSV. After 6 d, primary cytotoxicity was assessed in a standard ^{51}Cr release assay. Syngeneic MC57G (H-2^b) fibroblasts infected with VSV were used as target cells. $hCD4^{+/-}$ $mCD4^{-/-}$ (*open circle*); $mCD4^{-/-}$ (*solid triangle*); $mCD4^{+/+}$ (*solid diamond*); unprimed (*solid square*). Specific lysis of noninfected control MC57G was below 5% for all effectors shown.

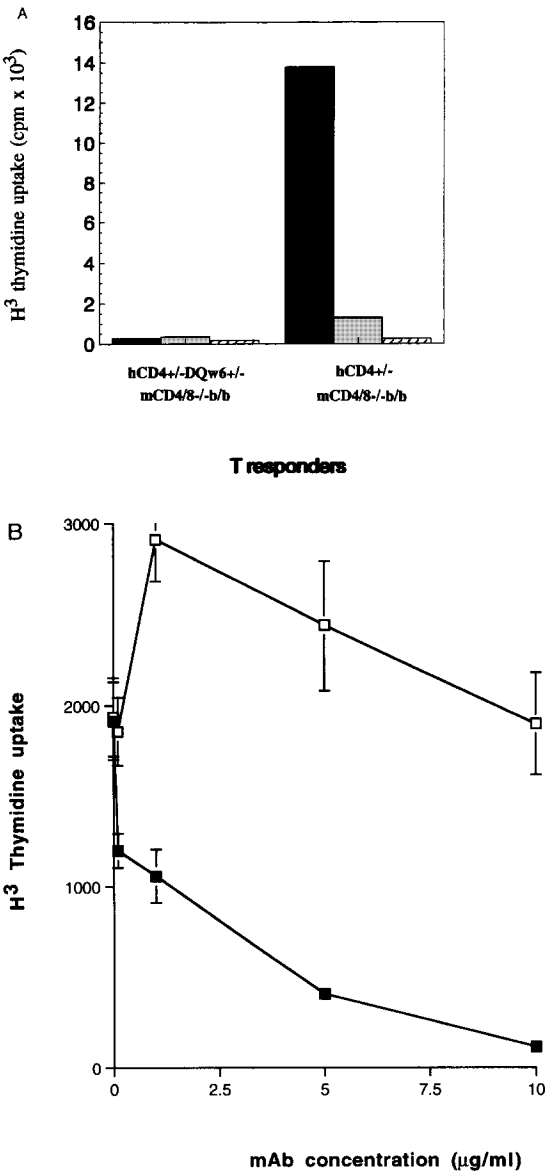


Figure 7. (*A*) Primary MLR. Spleen cell responders were cocultured with irradiated splenic stimulators as described in Materials and Methods. Results depicted represent data from 7-d MLR cultures. Lymphocytes from $hCD4^{+/-}DQw6^{+/-}mCD4/8^{-/-}$b/b mice were tolerant to DQw6 molecules as compared with their $hCD4^{+/-}$ $mCD4/8^{-/-}$b/b littermates which responded vigorously to DQw6. Both strains of mice were tolerant to hCD4 and to H-2^b syngeneic stimulator cells. Irradiated splenic stimulators: $hCD4^{+/-}$ $DQw6^{+/-}$ $mCD4/8^{-/-}$b/b (*solid black bar*); $hCD4^{+/-}$ $mCD4/8^{-/-}$ b/b (*shaded bar*); $mCD4/8^{-/-}$b/b (*striped bar*). (*B*) Antibody blocking. Spleen cell responders from $hCD4^+mCD4/8^{-/-}$b/b mice were cocultured with irradiated splenic stimulators from $DQw6^+mCD4/8^{-/-}$b/b mice in the presence of anti-DQ (*solid squares*) or anti-I-A^b (*open squares*) control antibodies as described in Materials and Methods. Anti-DQ monoclonal antibodies were able to block the T cell response in a dose dependent fashion, whereas, anti-I-A^b monoclonal antibodies were not. Plotted values represent the mean counts per minute of triplicate cultures in 96-well tissue culture plates with background counts substracted. *Error bars* represent standard deviation. Statistical analysis using paired Student's *t* test indicated that the differences shown were statistically significant ($p < 0.05$).

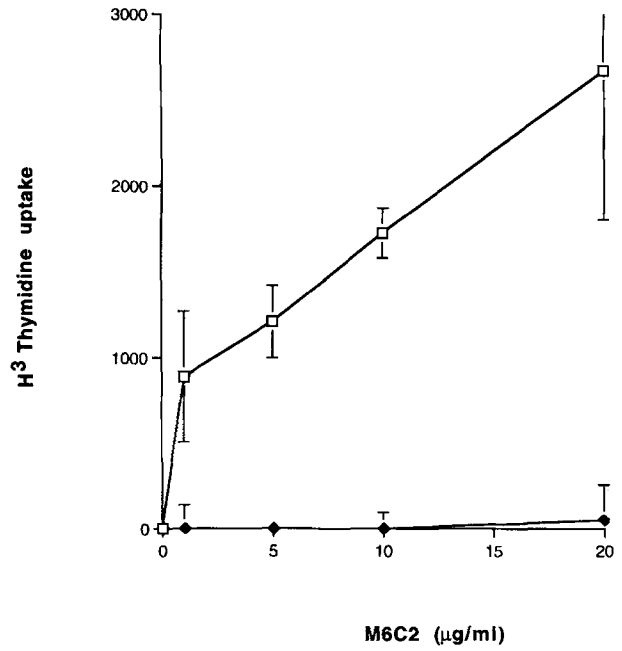


Figure 8. T cell proliferative responses to M6C2 peptide antigen in hCD4+DQw6+mCD4/68$^{-/-}$ b/b (*open square*), and hCD4+mCD4/8$^{-/-}$b/b (*solid diamond*) mice. Mice were immunized with M6C2 peptide as described in Materials and Methods. 9–12 d later inguinal and paraaortic lymph nodes were harvested. Lymphocytes were enriched for T cells, as described, and 4 × 10^5 T cells put into culture with 8 × 10^5 autologous T cell depleted, irradiated splenocytes with or without M6C2 peptide. T cell proliferation was measured by [^{3}H]thymidine uptake. Plotted values represent the mean of triplicate cultures in 96-well tissue culture plates with background counts substracted. The experiment shown is representative of seven experiments involving immunization of three to four mice per group in each experiment. *Error bars* represent standard deviation. Statistical analysis using paired Student's *t* test showed that the differences between experimental groups were statistically significant ($p < 0.05$).

mice did not show significant proliferation above baseline in the presence of the M6C2 peptide (data not shown).

Discussion

The results presented here demonstrate the appropriate reconstitution of phenotype and function by the hCD4 and hDQw6 transgenes in mice which lack expression of endogenous mCD4 and mCD8 molecules after targeted gene disruption and homologous recombination in embryonic stem cells. T cell development in the thymus is rescued, as evidenced by the dramatic increase in size of the thymic medulla on serial histologic sections and reconstitution of the mature TCR-α/β^{high} thymocyte population on flow cytometric analysis. The mature peripheral T cell population was also restored with the reemergence of normal numbers of TCR-α/β^+ T lymphocytes in lymph nodes and spleen. In mCD4$^{-/-}$ single knockout mice, the peripheral T cell pool consists of a majority of CD8$^+$ T cells and few (10%) of CD4$^-$8$^-$ α/β T cells. The presence of the hCD4 molecule normalized the ratio of CD4/CD8 T cells by decreasing the number of CD8$^+$ T cells back to that in normal mice. The mCD8$^+$ cells, however, expressed low levels of hCD4 due to the CD2 expression cassette (Fig. 4 *B*). These data clearly show the human CD4 molecule functioned as a positive selecting element, interacting with endogenous mouse I-A^b molecule, rescuing T cell maturation in the mCD4/8-deficient mice.

The human MHC class II molecule DQw6 functioned effectively in T lymphocyte development resulting in mice that were unresponsive to DQw6 in MLR assay, indicating that the hCD4 and DQw6 molecules interact during T cell development to induce T cell tolerance to self DQw6. Human CD4 recognition of DQw6 molecules was specific, as anti-DQ monoclonal antibodies were able to abolish reactivity to DQw6 in a dose-dependent fashion, whereas, anti-I-A^b monoclonal antibodies were not inhibitory. Additionally, hCD4 interacted with mouse I-A^b molecules resulting in T cell tolerance to murine self-MHC class II. It should be noted that mouse CD4 was also able to interact with the human DQw6 molecule, generating an effective T cell response to DQw6 in an MLR assay and induction of tolerance to DQw6 during thymic development (19). Interestingly, the kinetics of mCD4 and hCD4 interaction with DQw6 in MLR are similar and peak at day 7, as opposed to day 5 for MHC discordant MLRs. It is well known that DQ antigens are weak alloantigens (18), which may account for this difference in response times. As previously published, mCD4/8$^{-/-}$ mice have a weak but demonstrable alloreactivity (21). Consistent with this observation was the low T cell response of mCD4/8$^{-/-}$ mice upon DQw6 stimulation. Appropriate tissue-specific expression of the DQw6 alleles comparable in distribution to that of mouse I-A^b molecules has previously been shown (19). DQw6 expression was found on the cortex and medulla of the thymus, in lymph nodes, spleen, bone marrow, skin, and lungs, and in APCs such as B cells, and macrophages, but was absent from liver, kidney, muscle, and uterus (19).

Most importantly, the DQw6 molecule functioned in antigen presentation, as measured by specific T cell proliferative responses to M6C2, a 21-mer peptide derived from the M protein of rheumatogenic strains of Streptococcus. Thus, in this double transgenic knockout system, the DQw6 molecule was functional in antigen presentation, generating a human class II MHC–restricted T cell response, suggesting that human CD4 functionally interacted with human MHC class II molecules. This response is specific to the human DQw6 molecule as other DQ molecules, such as, DQw4 are unable to elicit the same response from immunized DQw6$^+$ mice (33). Although murine CD4 can interact with human MHC class II molecules as detected by both MLR and T cell responses to peptide presentation, further analysis of the human CD4–human MHC class II interaction vs. the interspecies mouse–human interactions in the context of other antigens and superantigens will enable us to dissect the nature of species-specific molecular interactions.

As previously reported (21), T help for B cells is markedly reduced but not completely absent in mice without CD4 alone

or in mice deficient in both CD4 and CD8 molecules. This residual T help is provided by CD4 and CD8 double negative TCR-α/β^+ T cells, which are restricted to class II MHC molecules (29). The level of T help as measured in an in vivo assay was higher in the mCD4/8$^{-/-}$ mice compared with the mCD4$^{-/-}$ single knockout mice presumably due to the higher numbers of double negative cells in the periphery of the double knockout mice (29, 21). The hCD4 transgene restored T helper function for B cells, as demonstrated in immunoglobulin class switching from IgM to neutralizing IgG immunoglobulins after VSV infection. The hCD4 transgene also restored T help for CTL in the mCD4-deficient mice. After immunization with VSV, the generation of VSV-specific CTL responses, which depend on T help (32), returned to normal levels.

Interestingly, the expression level of human CD4 varied inversely with the presence of mouse CD4 and CD8. Dramatic increases in human CD4 surface expression occurred in the absence of both mouse CD4 and CD8 coreceptors, while a milder increase occurred in the absence of mouse CD4 alone. In the hCD4$^+$mCD4$^{-/-}$ single knockout mice, two T cell compartments exist, one with murine endogenous CD8 and one without. The hCD4 transgene is expressed on both of these T cell subsets, and the level of hCD4 expression was dramatically higher in the CD8$^-$ T cell compartment compared with the CD8$^+$ cells (Fig. 4 *B*). The cytoplasmic domain of CD4 is known to associate with the protein tyrosine kinase p56^{lck} (8, 34), and 79% homology exists between cytoplasmic portions of human and murine CD4. The absence of murine CD4 and/or CD8 coreceptors may facilitate the association of p56^{lck} with human CD4. P56^{lck}-mediated signaling has recently been shown to regulate the intensity of surface expression of the CD4 molecule in thymocytes (35). Thus we propose that quantitative or qualitatively altered hCD4/p56^{lck} interactions may stabilize surface expression of hCD4 accounting for the increased expression in knockout mice. Additionally, the high expression of hCD4 on T cells in the double knockout mice more closely models CD4 intensities on human T lymphocytes.

Our results indicate that human CD4 and human MHC class II molecules are able to interact and effectively function in mice, reconstituting phenotype and function to the CD4$^+$ T helper compartment in an endogenous mouse CD4 and CD8 deficient background. Both the human CD4 and DQw6 molecules participated in thymic development and T cell function in peripheral lymphoid organs. The stable, long-term expression of both hCD4 and human MHC class II molecules in the absence of mCD4 and mCD8 provide a novel animal model to study the human CD4–MHC class II limb of the immune system during physiological responses and human disease. Various autoimmune diseases and superantigen-mediated diseases have human MHC class II associations. The fact that human CD4 and MHC class II molecules can fully function in mice may also provide an animal model to study the pathogenic mechanisms of AIDS, because of the key role played by the hCD4 molecule as the receptor for gp120 (14), and the speculations that AIDS may be a superantigen-mediated disease (36). This new system modeling the human CD4–MHC class II limb of the immune system in a knockout mouse background is a step towards reconstituting a stably expressed human immune system in mice and may prove to be a powerful tool in dissecting human disease mechanisms.

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