The EMBO Journal vol.12 no.4 pp.1547-1553, 1993

Regulated expression of human CD4 rescues helper T cell development in mice lacking expression of endogenous CD4

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Communicated by D.Mathis

During T cell development, precursor thymocytes that co-express the CD4 and CD8 glycoproteins give rise to mature progeny expressing one of these molecules to the exclusion of the other. Continued expression of only CD4 is the hallmark of mature helper T cells, whereas cytotoxic T cells express CD8 and extinguish CD4. The differentiation program that generates the two T cell subsets is likely to be intimately tied to regulation of expression of these cell surface molecules. We now describe the use of a murine CD4 enhancer in the generation of transgenic mice expressing physiologic levels of human CD4. The transgene is appropriately regulated during T cell development and includes the necessary *cis*-acting sequences for extinguishing expression in the CD8 lineage. Furthermore, in mice whose endogenous CD4 gene is inactivated, the transgenic human CD4 mediates rescue of the CD4 lineage and restoration of normal helper cell functions. The generation of these mice exemplifies a general approach for developing reliable animal models for the human immune system.

Key words: CD4/CD8/T cell activation/T cell development

Introduction

Helper and cytotoxic subsets of T lymphocytes can be broadly distinguished by their surface expression of either the CD4 or CD8 glycoproteins (Fowlkes and Pardoll, 1989). Although of markedly different structure (Ryu *et al.*, 1990; Wang *et al.*, 1990; Leahy *et al.*, 1992), these molecules are thought to mediate related functions for the two types of T cells. On the outside of the cell, CD8 has been shown to bind a non-polymorphic region on the α-3 domain of MHC class I (Salter *et al.*, 1989, 1990; Connolly *et al.*, 1990). Similarly, the CD4 molecule engages an analogous region on the MHC class II β-chain (König *et al.*, 1992; Vignali *et al.*, 1992). Inside the cell, both CD4 and CD8 interact with p56^{lck} (Rudd *et al.*, 1988; Shaw *et al.*, 1989; Turner *et al.*, 1990; Glaichenhaus *et al.*, 1991), an *src*-related tyrosine kinase that has been shown to be essential for T cell signalling (Strauss and Weiss, 1992) and T cell development (Molina *et al.*, 1992). Thus, both of these molecules bind antigen-presenting structures with their ecto-domains and couple to a major T cell signalling component with their endo-domains.

The development and function of most T cells are critically dependent on the function of either CD4 or CD8. Antibodies against these molecules will block thymopoiesis and *in vitro* immune responses (Nakayama *et al.*, 1979; Webb *et al.*, 1979; Fan *et al.*, 1980; Sarmiento *et al.*, 1980; Dialynas *et al.*, 1983; Ramsdell and Fowlkes, 1989; Zúñiga-Pflücker *et al.*, 1990). More strikingly, CD4- or CD8-deficient mice show significantly impaired development of either helper or cytotoxic T cells, respectively (Fung-Leung *et al.*, 1991; Rahemtulla *et al.*, 1991). Furthermore, positive and negative selection of thymocytes is defective when the $\alpha\beta$ T cell antigen receptor (TCR) is restricted to a mutant class I molecule that does not bind CD8 (Aldrich *et al.*, 1991; Ingold *et al.*, 1991; Killeen *et al.*, 1992). These results support the notion that CD4 and CD8 function as co-receptors interacting simultaneously with the same MHC molecule recognized by the TCR (Janeway, 1989). From the crystallographic structure of MHC class I (Bjorkman *et al.*, 1987) and from models of MHC class II structure (Brown *et al.*, 1988), this seems feasible because the co-receptor binding sites are physically separated from the antigen-presenting grooves (Salter *et al.*, 1990; König *et al.*, 1992).

Developmental regulation of the genes encoding CD4 and CD8 follows an unusual pattern of coordinate expression on immature thymocytes ('double positive' cells) followed by mutually exclusive expression on their mature progeny ('single positive' cells). Silencing of either the CD4 or CD8 genes may impinge on a differentiative decision to acquire either a cytotoxic or helper phenotype. Indeed, it has been proposed that engagement of either CD8 or CD4 expressed on double positive thymocytes may be required for positive selection and leads directly to deactivation of the gene for the unused co-receptor (Borgulya *et al.*, 1991; Robey *et al.*, 1991a). Inappropriate silencing of the wrong co-receptor would presumably inacapacitate the thymocyte and arrest its development. Thus, the regulation of this transcriptional silencing is tied to pivotal decisions in the life of a T cell.

In a previous study we characterized a T cell-specific transcriptional enhancer element located 13 kb upstream of the first exon of the mouse CD4 gene (Sawada and Littman, 1991). Although a positive regulator of the CD4 promoter, the enhancing activity of this element was not restricted to CD4$^+$ cells, indicating that a *cis*-acting element that silences the promoter in CD8$^+$ cells was absent. We now describe the use of this enhancer in transgenic constructs encoding human CD4 and show that in the appropriate context, the enhancer contributes to a regulated pattern of expression of the human CD4 gene. Furthermore, we show that expression of human CD4 corrects the defective helper cell development observed in mice lacking endogenous CD4. Mice in which helper cell development is dependent on human CD4 may be useful for studies of several human diseases in which the function of CD4$^+$ T cells is either impaired or enhanced.

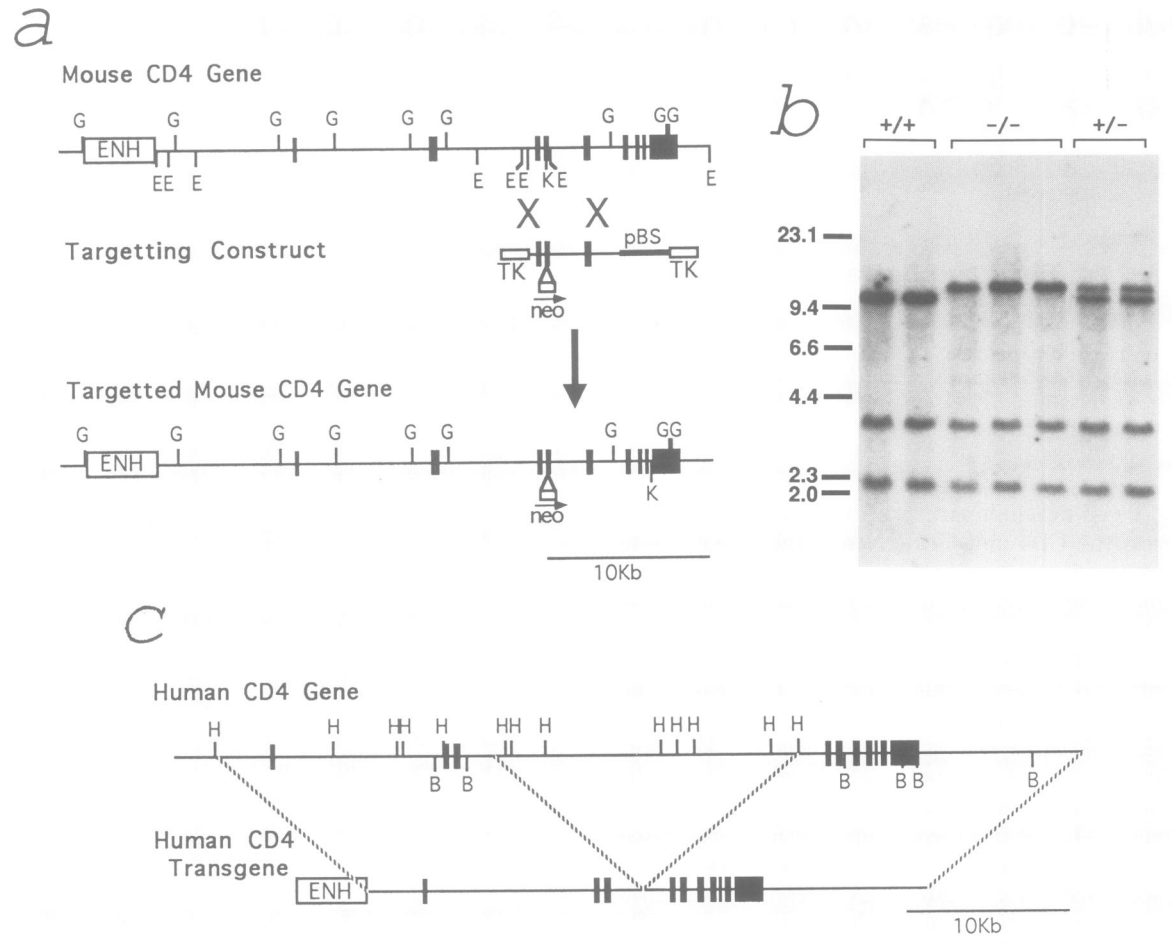

Fig. 1. Replacement of murine CD4 with the human homologue. **a**. Strategy for the disruption of the murine CD4 gene. The mouse CD4 gene was disrupted by insertion of the MC1neo cassette into a *Kpn*I site (K) of the fifth exon. *Bgl*II and *Eco*RI sites are denoted by G and E; only one *Kpn*I site is shown. **b**. Southern blot analysis of the mutant CD4 allele. *Bgl*II-digested mouse tail DNA was probed with a mouse CD4 cDNA probe spanning exons 2–10. The predicted structure of the targetted CD4 locus was also verified with several other digests and probes external to the region spanned by the targetting construct. **c**. Map of the human CD4 transgene. *Bam*HI and *Hin*dIII sites are denoted by B and H respectively.

Results

Disruption of the mouse CD4 gene by homologous recombination in ES cells

As a prelude to transgenic reconstitution experiments, the murine CD4 gene was inactivated by homologous recombination in embryonic stem cells (Capecchi, 1989), using the strategy depicted in Figure 1A. Mice bearing the desired mutant genotype were identified by Southern blot analysis (Figure 1B). The mutation, which was predicted to disrupt the protein coding region, eradicated cell surface expression of CD4 on all T cells and thymocytes of homozygous mutant mice. Similar mice have been independently generated by Rahemtulla *et al.* (1991) using a related strategy. CD4-deficient mice manifest a block in the development of the CD4 lineage (see Figure 3A and C) and consequently ~90% of peripheral $\alpha\beta$ T cells are $CD8^+$. A significant consequence of this mutation is the loss of helper T cell activity and of other class II-restricted T cell responses. For example, antibody titres are reduced at least 10-fold following immunization with foreign antigens and the T cells in these mice fail to proliferate when exposed to foreign MHC class II molecules, despite normal class I-dependent responses (Rahemtulla *et al.*, 1991; see also below).

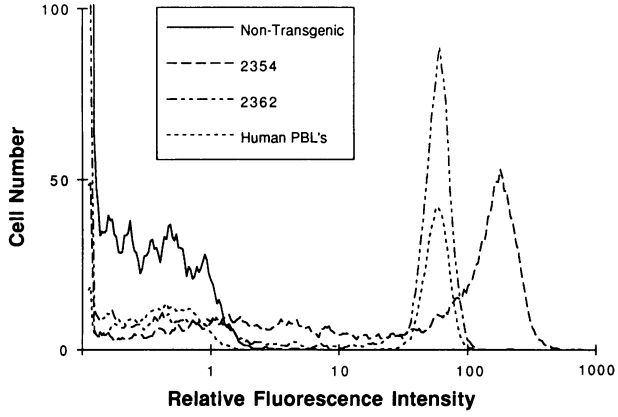

Fig. 2. Expression of human CD4 on splenic T cells from two lines of transgenic mice. The histogram shows expression of human CD4 on T cells from the two transgenic lines compared with transgene-negative T cells and human peripheral blood lymphocytes (PBLs). Mouse spleen cells and human PBLs were stained with FITC–anti-mouse Thy-1 and PE–anti-human CD4. Cells were analysed using the FACScan and histograms represent expression of human CD4 on mouse Thy-1 positive cells and total human PBLs isolated from a Ficoll gradient. Cells were gated for equivalent forward and side scatter and for exclusion of propidium iodide.

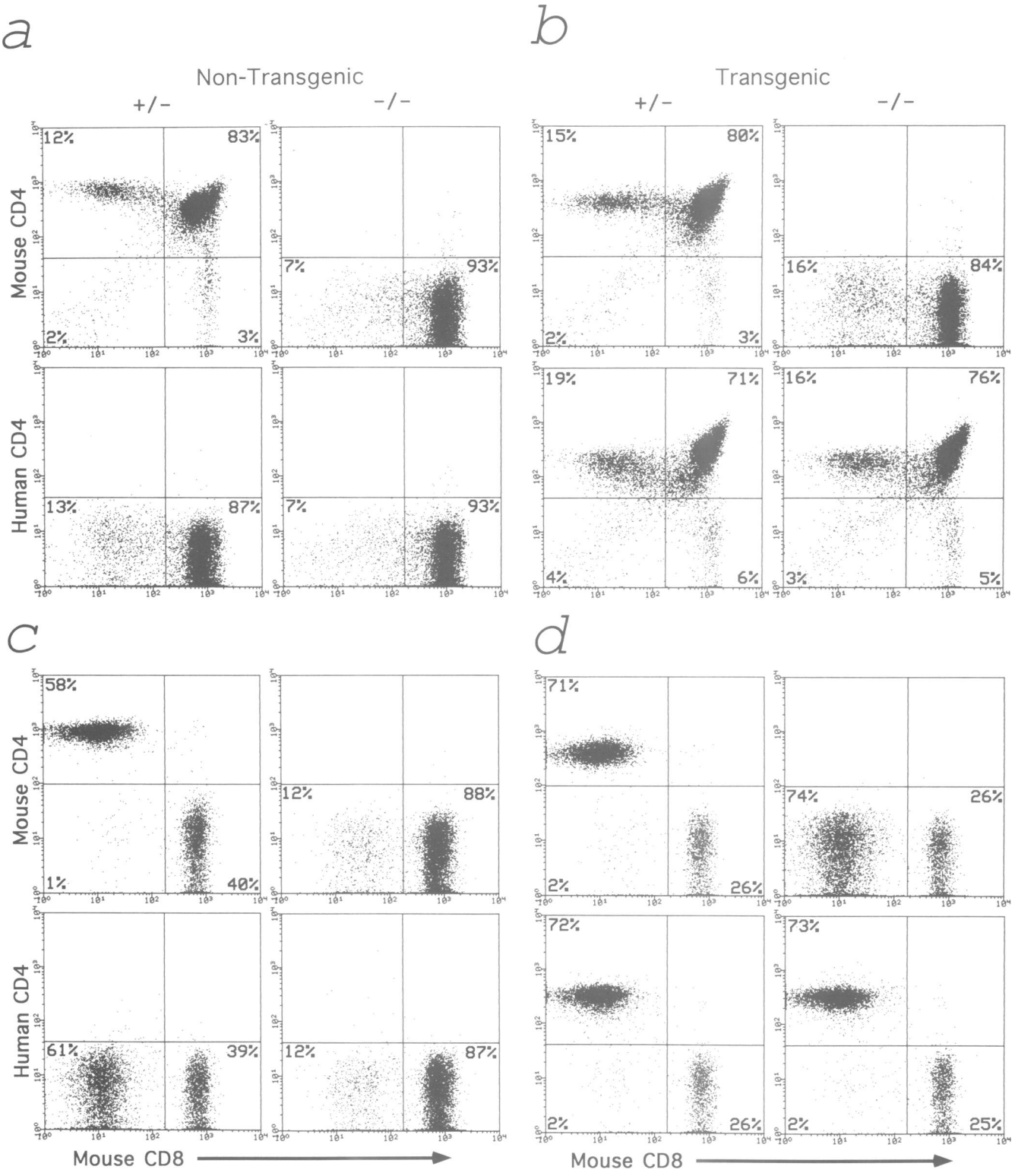

Fig. 3. Expression of human CD4 in CD4-deficient mice. The dot-plots show expression of endogenous mouse and transgenic human CD4 on thymocytes (**a** and **b**) and peripheral CD3$^+$ lymphocytes (**c** and **d**) from CD4+/− and −/− mice. Cells from progeny of founder #2362 were stained with FITC−anti-CD8, biotin−anti-CD3 and either PE−anti-mouse CD4 or PE−anti-human CD4 followed by streptavidin-PE−Texas Red tandem conjugate. Ten thousand cells were analysed using a Becton Dickinson FACScan and Lysys II software. Dot plots for peripheral lymphocytes were generated by gating for expression of CD3.

Regulated expression of human CD4 in transgenic mice

Previous attempts to achieve appropriate expression of CD4 transgenes in mice have not been successful. A genomic construct containing the human CD4 coding sequence with 3 kb of sequence upstream of the transcription initiation site and 8 kb downstream of the termination site was not expressed in T cells of transgenic mice (G.Wong, D.R.Littman and

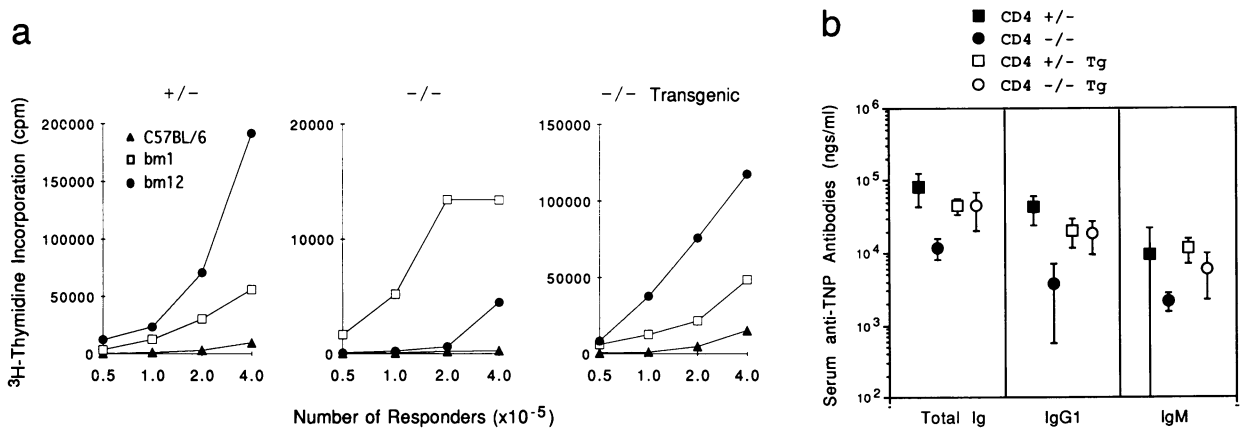

Fig. 4. The human CD4 transgene restores helper cell function in CD4-deficient mice. **a.** Restoration of bm12-reactivity in mixed lymphocyte cultures. Lymph node cells from mice of the indicated CD4 genotypes were stimulated *in vitro* with irradiated anti-Thy-1-depleted spleen cells from C57BL/6 mice or from the two MHC congenic strains B6.C-H-2^{bm1}/ByJ and B6.C-H-2^{bm12}/KhEg. [^{3}H]thymidine was added after 4 days and the assay was harvested 18 h later. **b.** Restoration of antibody response. Mice were immunized intraperitoneally with 10 μg TNP-KLH in alum and sera were drawn on days 0, 10 and 14. Plots show mean TNP-specific antibody titres at day 10 measured by indirect ELISA for groups of four mice (except for the CD4+/− controls where only three mice were analysed). Means for pre-bleed titres were: total Ig, 2099 ng/ml; IgG1, <94 ng/ml; IgM, 1185 ng/ml.

E.Lacy, unpublished results). Use of heterologous T cell-specific enhancers and promoters has resulted in expression of CD4 in thymocytes and T cells that do not normally express this protein, such as mature $CD8^+$ cells, as well as in inappropriate levels of CD4 expression (Robey *et al.*, 1991a,b; Teh *et al.*, 1991; Barzaga-Gilbert *et al.*, 1992).

By focusing on linked DNase hypersensitivity sites, we previously identified a T cell-specific enhancer element located ~13 kb upstream of the transcription initiation site in the murine CD4 gene (Sawada and Littman, 1991). On the assumption that this enhancer might facilitate expression of CD4 transgenes, a 4.5 kb *Bgl*II−*Eco*RI fragment encompassing it was ligated to a 30 kb human CD4 minigene that includes the promoter and all of the protein coding region (Figure 1C). Vector sequences were deleted from this construct and transgenic mice were created by standard pronuclear injection (Hogan *et al.*, 1986).

Of three transgenic founders, two expressed significant levels of the human CD4 molecule on the surface of a subset of their peripheral T lymphocytes as determined by flow cytometry. One of these (#2362) carried approximately six copies of the transgene and expressed a level of CD4 equivalent to that seen on human peripheral $CD4^+$ T cells (Figure 2). The other founder mouse (#2354) carried ~30 copies of the transgene and expressed ~3-fold more human CD4 per cell. Importantly, both transgenic mice and their offspring lacked human CD4 on their peripheral $CD8^+$ T cells (Figure 3), consistent with appropriate *cis*-acting transcriptional silencing of the human CD4 transgene.

Two other constructs, employing the same human CD4 minigene, were used to generate transgenic mice. One construct lacked any added enhancer and was not expressed in T cells (not shown). The other construct included a 0.3 kb CD4 enhancer fragment from within the 4.5 kb *Bgl*II−*Eco*RI fragment shown in Figure 1C. Transgenic mice bearing this last construct expressed human CD4 on mouse $CD4^+$ T cells, but not on $CD8^+$ T cells (not shown). Thus, in the context of the human sequences shown in Figure 1C, the 0.3 kb mouse CD4 enhancer fragment is sufficient for subset-specific expression of human CD4.

Human CD4 rescues helper cell development in CD4-deficient mice

With the goal of effectively replacing mouse CD4 with its human homologue, the male transgenic founder #2362 was back-crossed onto the murine CD4-deficient background. This was achieved in a single generation, because one of the founder's parents had been a CD4−/− male. Crosses between male #2362 and CD4−/− females yielded equal numbers of mice displaying the four distinct phenotypes shown in the flow cytometric analysis of Figure 3. Mice homozygous for the CD4 gene disruption have few $CD8^-$ thymocytes (Figure 3A) or $CD8^-$ peripheral T cells (Figure 3C) compared with CD4+/− controls. In the transgenic mice, human CD4 is expressed on these $CD8^-$ cells and restores their numbers to approximately the levels seen in the presence of mouse CD4 (Figure 3B and D). Similar to thymocytes from normal mice, those from the transgenic mice can be subdivided into mature and immature sub-populations based on expression of CD4 (human) and CD8 (mouse). Similarly, peripheral T cells express either human CD4 or mouse CD8, but not both, reflecting appropriate regulation of this transgene.

The FACS data shown in Figure 3 indicate that human CD4 can rescue a lineage of cells whose development is otherwise prematurely arrested due to the absence of endogenous CD4. To determine whether these cells have properties of helper T cells that are absent in the CD4−/− mice, their functions were analysed in two helper cell-dependent experimental systems. To test for MHC class II-specific allogeneic responses, CD4−/− transgenic and control lymphocytes were challenged *in vitro* with irradiated cells from either the B6.H-2^{bm1} or B6.H-2^{bm12} mouse strains. These mice are congenic with C57BL/6 and bear mutations in either the class I K^b or class II I-A^b genes, respectively. As shown in Figure 4A (left panel), T cells from control CD4+/− H-2^b mice were tolerant to cells from MHC-syngeneic C57BL/6 mice, but proliferated in response to stimulation with either the bm1 or bm12 mutant haplotype cells. Whereas CD4−/− cells also responded to the class I alloantigen K^{bm1}, they did not proliferate when challenged with the class II alloantigen,

I-A^{bm12} (Figure 4A middle panel). There was restoration of a strong bm12 response in CD4−/− mice transgenic for human CD4, providing evidence for rescue of class II-restricted responses by the product of the human transgene and indicating that human and murine CD4 are functionally equivalent in their interactions with murine MHC class II (Figure 4A, right panel).

Due to the absence of $CD4^+$ helper T cells, CD4−/− mice are deficient in generating T cell-dependent antibody responses (Rahemtulla *et al.*, 1991). To determine whether this defect is corrected by expression of the human CD4 transgene, mice were immunized with the hapten TNP coupled to keyhole limpet hemocyanin (TNP-KLH) and sera were collected at days 0, 10 and 14 and assayed for the presence of TNP-specific antibodies using an isotype-specific indirect ELISA. Figure 4B shows that the reduction in antibody titre observed in CD4−/− mice was largely corrected by expression of human CD4. Levels of specific antibodies in the CD4−/− transgenic mice were similar to those obtained in CD4+/− control mice.

Discussion

Progenitor thymocytes expressing rearranged $\alpha\beta$ T cell receptor genes are bipotential cells that can differentiate along either helper or cytotoxic lineages. Initially, these cells co-express the CD4 and CD8 surface glycoproteins, but as they differentiate they extinguish expression of one or the other molecule. Retention of CD8 correlates with the adoption of a cytotoxic phenotype, whereas a helper phenotype is usually associated with CD4 expression. Gain-of-function analysis with mice expressing transgenes encoding class I or class II-restricted T cell receptors shows that there is strong selection for class I-restricted cells to express CD8 and for class II-restricted cells to express CD4 (Sha *et al.*, 1988; Teh *et al.*, 1988; Berg *et al.*, 1989; Kaye *et al.*, 1989). Loss-of-function mutants that do not express CD4 or CD8 manifest abortive development of either the CD4 or CD8 lineages respectively (Fung-Leung *et al.*, 1991; Rahemtulla *et al.*, 1991). Thus determination of helper or cytotoxic T cell development appears to involve co-receptor function. How CD4 and CD8 influence the differentiation program has yet to be resolved. It has been proposed that these co-receptors participate in reciprocal signalling events that lead to their mutually exclusive expression on mature T cells, by either directing their own continued expression or by silencing the inappropriate co-receptor (Borgulya *et al.*, 1991; Robey *et al.*, 1991a; Seong *et al.*, 1992). Alternatively, silencing of co-receptor genes may occur by a random mechanism that is followed by selection for appropriate pairing of co-receptor and TCR (Chan *et al.*, 1993; Davis *et al.*, 1993).

In this paper, we describe a gene rescue experiment involving reconstitution of a CD4-deficient mouse with a correctly regulated human CD4 transgene. The appropriate expression pattern of this transgene in double positive thymocytes and helper T cells appears to require the use of a T cell-specific enhancer, normally located 13 kb upstream of the mouse CD4 transcriptional initiation site. This follows from the observation that the same human CD4 minigene without the 4.5 kb enhancer fragment is not expressed in T cells (not shown). Significantly, the combination of enhancer, promoter and perhaps other *cis*-acting elements within the CD4 gene, results in extinction of expression in the $CD8^+$ cytotoxic lineage of T cells. Transient transfection studies in $CD8^+CD4^-$ T cell lines failed to demonstrate transcriptional silencing activity by the 0.3 kb minimal enhancer element contained within the 4.5 kb piece of DNA used here (Sawada and Littman, 1991), so it is reasonable to assume that a second element acts to suppress transcription in cells which express only CD8. The exact location of this putative second element is at present a matter for conjecture, but should be discernible using transgenic constructs related to the one described in this paper. For example, a related construct employing the 0.3 kb minimal enhancer element in place of the above 4.5 kb fragment is also subset-specific in its expression pattern, indicating that the putative *cis*-acting element involved in extinction of CD4 transcription maps to a different part of the gene than that immediately surrounding the enhancer (not shown). Other derivative enhancer-containing constructs including only the CD4 promoter and first intron have also shown deactivation in the CD8 lineage (not shown). Thus it seems that the activity of the enhancer may be subject to negative regulation by an element either immediately upstream or downstream of the transcription start site. That the CD4 enhancer/promoter is not inherently silent in $CD8^+$ T cells is supported by an analysis of still other transgenic constructs that contain both elements, but are expressed in both $CD4^+$ and $CD8^+$ T cells (not shown). The eventual characterization of the putative CD4 silencer element will contribute to a greater understanding of how this gene is regulated and will provide a useful tool for genetic manipulation of the immune system.

The major function of the CD4 glycoprotein is to collaborate with the TCR−CD3 complex in the transduction of signals reflecting specific interaction with MHC class II molecules. The effect of this collaboration may be to increase the magnitude of the overall signal above a critical threshold, resulting in either activation of peripheral helper T cells or initiation of a cell differentiation program in thymocytes. In the absence of CD4 the development of the helper T cell lineage is severely impaired (Rahemtulla *et al.*, 1991), supporting the contention that CD4 plays a crucial role in delivering an appropriate differentiative signal. In this paper we have shown that regulated expression of human CD4 can overcome this defect and rescue helper cell development. This demonstration of human CD4 function in a developing murine immune system is consistent with previous *in vitro* experiments showing that human CD4 can interact with mouse MHC class II molecules and can signal through mouse $p56^{lck}$ (Glaichenhaus *et al.*, 1991; von Hoegen *et al.*, 1989; König *et al.*, 1992; Vignali *et al.*, 1992). Restoration of class II-specific allo-recognition and antigen-specific antibody responses indicates that human CD4 is functional in mice and mediates selection of a diverse and capable repertoire of helper T cells.

Mice whose helper T cells depend on the human CD4 protein for their development represent a useful experimental system for studying human diseases that involve $CD4^+$ T cells, such as AIDS and autoimmunity, and for developing vaccines specific for human MHC class II molecules. Whereas human CD4 can functionally replace murine CD4, the reverse is not the case, since murine CD4 cannot interact productively with human MHC class II molecules (Vignali *et al.*, 1992). For this reason, mice expressing human class II transgenes

have not yet proved useful for studying normal or autoimmune responses restricted to human MHC. Provision of appropriately regulated human CD4 in such animals is expected to circumvent this barrier and allow the development of mouse models for human class II-restricted diseases.

The hallmark of HIV disease is the specific depletion of $CD4^+$ helper T cells and the consequent increased susceptibility to opportunistic infections. Although helper cells are a primary target of HIV due to the interaction between CD4 and the viral envelope glycoprotein, gp120, the mechanism of cell death is not understood. There is evidence that the gp120-CD4 interaction is critical, not only for viral entry, but also for the destruction of $CD4^+$ helper T cells (Siliciano *et al.*, 1988; Lanzavecchia *et al.*, 1988; Koga *et al.*, 1990; Groux *et al.*, 1992). The mice described in this paper may provide a valuable tool for studying the mechanism of HIV envelope glycoprotein-mediated pathogenesis and for testing therapies designed to prevent death of helper T cells.

Materials and methods

Inactivation of the mouse CD4 gene

The CD4 targetting vector was constructed using a CD4 genomic clone isolated from a Balb/c library in pWE14 provided by Dr G.Evans (The Salk Institute, La Jolla) and plasmids pMC1neopolyA (Stratagene) and pIC19R/MC1-TK (from Dr K.Thomas, University of Utah). D3 ES cells (Doetschmann *et al.*, 1985) were kindly provided by Dr T.Doetschmann at the University of Cinncinnati. These were grown according to the protocols described by Robertson (1987) except that the culture medium was 15% fetal calf serum with no additional newborn calf serum. 2×10^7 cells were transfected by electroporation of 20–25 μg *Cla*I-linearised CD4-targetting vector in 0.8 ml of PBS at 250 V/500 μF. 5×10^6 cells were seeded on 10 cm plates containing 4×10^6 mitomycin C-inactivated Neo^r STO feeder cells (gift of Dr E.Robertson, Columbia University, New York). Selection with 150 μg/ml of G418 was imposed after 36 h. Gancyclovir was not used. Colonies were transferred into 96 well plates containing feeder cells after 12–14 days, at which time selection was removed. Two days later, each colony was split in two parts; half of each clone was returned to culture while the rest was used in pools of 10–30 clones for PCR analysis (Kim and Smithies, 1988). DNA was extracted from these pools according to a modification of the protocol described by Bowtell (1987). Briefly, the cells were pelleted and resuspended in a small vol of PBS. 7–10 vol of 6 M guanidine hydrochloride–0.1 M CH_3COONa were added and the mixture was rotated for 1 h before precipitating with ethanol and resuspending in TE at 55°C (1–2 hours) and 95°C (10 minutes). PCR was performed for 35 cycles with denaturation for 1 min at 94°C, annealing at 55°C for 1 min and extension at 72°C for 3 min. PCR-positive pools were expanded and reanalysed in smaller pools until the individual clones contributing the PCR signal were identified. DNA was extracted for Southern blot analysis and the cells were frozen at the earliest possible passage. Typically, 1 in 30 G418-resistant colonies had undergone homologous recombination at the CD4 locus.

Blastocysts were harvested from C57BL/6 mice ~3.5 days post coitus. These were injected with targetted ES cells as described by Bradley (1987) and Hogan *et al.* (1986). Injected blastocysts were reimplanted into the uterii of pseudopregnant C57BL/6xDBA/2 F1 females. Chimeric progeny were identified by coat colour and the males were mated to C57BL/6 or C57BL/6xDBA/2 F1 females. Germline transmission of the agouti marker and the neo^r gene identified mice carrying the CD4 disruption and these were intercrossed to produce homozygous null mice. Screening of mice for the CD4 mutation was achieved by PCR, Southern blot or routinely by FACS analysis of peripheral blood, which can identify all genotypes (CD4+/− mice express ~2-fold less cell surface CD4 than CD4+/+ mice).

Generation of transgenic mice

A transgene was constructed by ligating a 4.5 kb *Eco*RI–*Bgl*II fragment containing the murine CD4 enhancer element (Sawada and Littman, 1991), to a human CD4 minigene that includes all of the coding region exons and ~3 kb of upstream sequence. B6/SJL F2 eggs or B6/SJL F1×CD4−/− eggs were microinjected with the human CD4 transgene according to standard procedures (Hogan *et al.*, 1986). Founders were identified by Southern blotting using a human CD4 cDNA probe and by FACS analysis of peripheral blood using anti-human CD4 monoclonal antibodies.

Antibodies and flow cytometry

$1-2\times10^6$ cells were stained with saturating levels of antibodies and 5000–10 000 gated events were acquired and analysed using a Becton-Dickinson FACScan flow cytometer and Lysys II software. FITC-conjugated anti-CD8 (53–6.7), PE-conjugated anti-mouse CD4 (GK1.5) and PE-conjugated anti-human CD4 (leu3a) were from Becton Dickinson. Biotin–anti-CD3 (145–2C11) was from Pharmingen and PE–anti-human CD4 (BF-5) was from BioSource International. Biotin groups were detected with streptavidin conjugated to PE coupled to Texas Red (Southern Biotechnology).

Mixed lymphocyte cultures

Responder cells were isolated from cervical, brachial, axillary and mesenteric lymph nodes. Stimulator splenocytes were treated for 30 min with anti-Thy-1 monoclonal antibody and guinea pig complement (Gibco or Cedarlane) followed by irradiation (~2000 rad) from a ^{137}Cs source. Culture medium was RPMI supplemented with 10% fetal calf serum, 100 μM non-essential amino acids, 110 μg/ml pyruvate, 5×10^{-5}M β-mercaptoethanol, penicillin and streptomycin. Proliferation assays were set up in a volume of 200 μl in round-bottom wells of microtiter plates and incubated for 4 days before pulsing for ~18 h with 1 μCi of [^{3}H]thymidine per well. Cellular radioactivity was harvested using a Pharmacia microtiter plate cell harvester.

Immunizations and ELISAs

Reagents for immunizations and ELISAs were kindly provided by Dr R.Coffman of DNAX (TNP-KLH, TNP-FγG, IgM and IgG1 anti-TNP control antibodies) or purchased from PharMingen (biotin–Rabbit anti-mouse Igs, biotin–anti-IgG1 and biotin–anti-IgM), Jackson ImmunoResearch (streptavidin-conjugated horse-radish peroxidase), Southern Biotechnology (biotin–goat anti-mouse IgG1) and Sigma [ABTS, 2′,2′-Azino-*bis*(3-ethylbenzthiazoline sulfonic acid)]. Mice were immunized intraperitoneally with TNP-conjugated KLH in potassium alum and bleeds were taken at 0, 10 and 14 days. Sera were analysed for TNP-specific antibodies by ELISA (Coffman and Carty, 1986) using plates coated with TNP-FγG, biotinylated secondary antibodies, streptavidin peroxidase and ABTS. Optical densities were read using a Molecular Devices ELISA reader and SOFTmax software. The assay was calibrated using known concentrations of U7.6 (IgG1) and U13.6 (IgM) anti-TNP monoclonal antibodies (gift of Dr R.Coffman, DNAX).

Acknowledgements

We thank Drs Thomas Doetschman, Elizabeth Robertson, Jonathan Sprent, Debbie Law and Susan Stuart for generous provision of advice and reagents; Dr Robert Coffman and the members of his laboratory for much help with ELISAs and Drs Tony DeFranco and Arthur Weiss for comments on the manuscript. This work was supported by the Howard Hughes Medical Institute and an EMBO postdoctoral fellowship to N.K.

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Received on December 3, 1992