Human CD8α expression in NK cells but not cytotoxic T cells of transgenic mice

Lynda J. Kieffer¹, Jane A. Bennett³, Ann C. Cunningham³, Ron P. Gladue³, John McNeish³, Paula B. Kavathas¹,² and Jeffrey H. Hanke³

¹Section of Immunobiology and ²Department of Laboratory Medicine, Yale University, New Haven, CT 06520, USA
³Central Research Division, Pfizer Inc, Groton, CT 06340, USA

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Abstract

In our previous work, DNase hypersensitivity mapping was used to identify an enhancer within the human CD8α (hCD8α) gene which allowed T cell-specific expression of a reporter construct in transiently transfected cell lines. To study the role of this intronic enhancer in vivo, transgenic mice were made using human CD8 genomic constructs. We found that while a 14 kb wild-type human CD8α (WThCD8) genomic construct did not lead to expression in mature peripheral CD8⁺ T cells, this transgene was consistently expressed in small populations of T cells and B cells, and in a subset of mouse NK cells. While murine CD8 is not normally expressed on resting NK cells, expression of the human CD8 transgene on mouse NK cells is appropriate since CD8 is expressed on a subset of human NK cells. Deletion of the intronic enhancer resulted in a complete loss of transgene expression in most lines and a loss of expression only in NK cells in one line. Our results indicate, firstly, that cis-acting sequences within the 14 kb genomic fragment are sufficient for NK cell-specific expression. In addition, our results suggest that the enhancer may have dual roles in regulation of transgene expression. It may enhance general expression of the transgene and may also be required for NK cell-specific expression.

Introduction

The CD8 glycoprotein, expressed on the surface of cytotoxic/suppressor T lymphocytes, functions as a co-receptor with the TCR in recognition of antigens presented in the context of MHC class I molecules (1-5). CD8 function is mediated by direct binding of its extracellular region with both the α2 and α3 domains of MHC class I (6,7). The intracellular region of CD8 is capable of participating in signal transduction by interaction with the tyrosine kinase p56lk (8,9).

The developmental expression of CD8 is tightly controlled as thymocytes progress along the T-lineage differentiation pathway from immature T cells (CD4⁻CD8⁻) through an intermediate (CD4⁺CD8⁻) stage and finally to mature CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells (10-12). Although CD8 can be expressed on the cell surface either as an αα homo dimer or as an αβ heterodimer, it is predominantly found as the αβ heterodimeric form on TCRαβ⁺ peripheral T cells (13-18). Since the CD8α and CD8β genes are closely linked in both the mouse and human genomes (15,19,20), they may be coordinately regulated. However, CD8 is expressed only as an αα homodimer on certain cell types, such as TCRαβ⁺ gut intraepithelial lymphocytes and NK cells (18,21-24), suggesting that expression of CD8α and CD8β may also be independently regulated.

To gain insight into the mechanisms controlling transcription of the CD8α gene, we previously used DNase I hypersensitivity mapping in cell lines to identify regulatory regions in proximity to the human CD8 gene. Four hypersensitive sites were identified, three of which were T cell specific (25). In subsequent mapping studies, two of these hypersensitive sites (HS1 and HS2 in Fig. 1B) were also found in human peripheral T cells (data not shown). One of these sites (HS1), located in the last intron of the CD8α gene, was found to contain T cell-specific enhancer activity in transiently transfected cell lines (25). Deletion of the 5’ end of the enhancer indicated that it contained a negative regulatory element, which upon examination of the sequence, was composed of a half Alu and an inverted full-length Alu repeat. This led to the proposal that the upstream Alu element could form a cruciform or stem-loop structure by base pairing with the downstream Alu element. Using P1 nuclease mapping and site-directed mutagenesis, it was shown in transient transfection assays
Fig. 1. Constructs used for generation of transgenic mice. (A) The wild-type human CD8 (WThCD8), hCD8+CD2 LCR, hCD8 enhancer deletion (hCD8-ED) and hCD8 stem–loop deletion (hCD8-SLD) constructs are shown. The intronic enhancer is represented by the diagonally hatched box and the stem–loop region is represented by a ball on a stick. (B) Enlargement of the WThCD8 construct diagram. The positions of DNase I hypersensitivity are shown (HS1 and HS2).

Fig. 2. Human CD8+ cells are predominantly in the lymphoid pool. Blood cells were stained with anti-hCD8-PE as described in Methods. (A) Ungated FACS analysis of blood from a WThCD8 H5 mouse. (B) Forward scatter/side scatter profile of hCD8+ cells from gate R2 in (A). (C) Ungated forward scatter/side scatter profile of blood cells.

Fig. 3. FACS analysis of blood of WThCD8 and hCD8+CD2 LCR mice with B and T cell markers. As described in Methods, blood cells were double stained with anti-hCD8-PE antibody and either anti-mouse CD3, anti-mouse CD4, anti-mouse CD8 or anti-mouse B220 antibodies conjugated to FITC. The data has been gated on lymphocytes (see Fig. 2).
that formation of a cruciform structure was necessary for repression of enhancer function (26).

To study the role of the enhancer in the regulation of CD8α gene transcription in vivo, transgenic mice were made using human CD8α genomic constructs, some of which contained deletions within the region of the enhancer. We found that a 14 kb wild-type human CD8α (WThCD8) genomic construct was expressed in a small population of lymphoid cells and did not lead to expression in the majority of mature peripheral CD8⁺ T cells, suggesting this fragment lacks control elements needed for high level CD8⁺ T cell-specific expression. Interestingly, however, the WThCD8 transgene was consistently expressed in a subset of mouse NK cells. Deletion of the enhancer resulted in a complete loss of transgene expression in all lines except for one. In this one enhancer deletion line, however, expression in NK cells was lost. These results suggest that NK cell-specific expression can be achieved with the 14 kb WThCD8 genomic construct, and that the intronic enhancer within this construct may be important for any expression of the transgene and may be indispensable for NK cell specific expression.

Methods

Construction of transgenes

The basic construct, WThCD8, was a 14 kb BamHI fragment of the human CD8α gene (Fig. 1B). The hCD8⁺CD2 locus control region (LCR) construct was prepared by first ligating a 5.5 kb BamHI-XbaI fragment containing the CD2 LCR into pBSSK⁺ (Stratagene, La Jolla, CA). The BamHI hCD8α 14 kb genomic fragment was subsequently ligated into the BamHI site at the 5' end of the CD2 LCR. For construction of the hCD8⁻SLD and hCD8⁻ED constructs, the Ndel–AatII fragment from the last intron of the hCD8α gene (see Fig. 1B) was first subcloned into pBSSK⁺. Deletions of a 211 bp Sphi–Thh111-I fragment (for hCD8⁻SLD) or of a 1064 bp Sphi–AflI fragment (for hCD8⁻ED) were made from the subcloned Ndel–AatII fragment. These Ndel–AatII fragments with the deletions were then used to replace wild-type Ndel–AatII sequence in an Ndel–BamHI fragment from the 3' end of the hCD8α gene (Fig. 1B) which had been subcloned into pBSSK⁺ via a linker. The Ndel–BamHI fragments containing the deletions were excised with Ndel and Kpnl, and used to replace the wild-type 3' Ndel–BamHI fragment of WThCD8 in pBSSK⁺.

Injection of transgene DNA and identification of positive mice

The WThCD8, hCD8⁻SLD and hCD8⁻ED constructs were excised from their vectors using BamHI to generate a linear transgene. The hCD8⁺CD2 LCR construct was excised from the vector using the unique sites, XbaI and Kpnl. Restriction plasmid DNA was run on an agarose gel in TAE buffer, the linearized transgene band excised from the gel, and the DNA electroeluted and ethanol precipitated. DNA was cleaned on an Elutip-D column (Schleicher & Schuel, Keene, NH) according to the basic protocol of the provided instructions, except that the Elutip prefilter was used after instead of before the column. DNA was ethanol precipitated and resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 7.5).

DNA was introduced into fertilized embryos from FVB mice (Taconic, Germantown, NY) by pronuclear injection. Embryos were implanted into pseudopregnant CD1 mice (Charles River, Wilmington, MA). Transgene incorporation was assessed by restriction of tail DNA with HindIII and BgIII and Southern blotting, according to the basic protocol in Current Protocols in Molecular Biology. Transgene integrity was assessed by Southern blots of DNA which had been digested with BgIII alone, yielding a band the size of the complete transgene, since all lines contained more than one copy of the transgene, in a tandem array. Transgene copy numbers were estimated by comparing hybridization signals from DNA isolated from tails with known standards of cloned plasmid DNA. The probe was a BssHII fragment corresponding to hCD8α exons I and II (Fig. 1B), which have the least homology to mouse CD8α, such that there was no cross-hybridization to mouse CD8α. Labeling of probe was performed using the Oligolabeling Kit (Pharmacia, Piscataway, NJ).

FACS analysis of tissues and blood

Transgenic mice were analyzed for hCD8α expression at 2–6 months of age. Spleens were ground through 100 mesh Collector tissue sieves (Bellco Glass, Vineland, NJ) in RPMI medium and the cell suspensions were passed through 45 μm Nitex cloth. Cells were pelleted and red blood cells were lysed by hypotonic lysis in water followed by adjustment of toxicity with 10×HBSS (Gibco/BRL, Grand Island, NY). Cells were again filtered through Nitex cloth. Lymph node (axillary, superficial inguinal and mesenteric) and thymus tissues were ground in RPMI medium between the frosted ends of glass slides. Bone marrow cells were obtained by flushing RPMI medium through the femur with a needle/syringe and filtration through Nitex cloth. Cells from all tissues were resuspended in FACS buffer (Ca²⁺/Mg²⁺-free PBS with 2% heat inactivated FCS and 0.01% sodium azide), and stained with phycoerythrin (PE)- or FITC-conjugated antibodies purchased from PharMingen (San Diego, CA), Amac (Westbrook, ME), Caltag (San Francisco, CA) or Becton Dickinson (San Jose, CA). Streptavidin–Red670 for three-color FACS analysis was purchased from Gibco/BRL. For analyzing peripheral blood, samples were washed in FACS buffer and stained with antibodies, followed by lysis of red blood cells with FACS Lysing Solution (Becton Dickinson). The resulting leukocytes were washed and resuspended in FACS buffer. FACS data was collected on a Becton Dickinson FACSort with Lysys II software or a Becton Dickinson FACSScan using Consort 30 software. All data was analyzed with Lysys II software. The percentage of cells in each quadrant is indicated, with the background from isotype control antibodies subtracted.

Preparation of lymphokine-activated killer (LAK) and NK cells

Spleen tissue was homogenized through sterile Collector tissue sieves and the resultant cell suspension was washed two times and resuspended in DMEM. Leukocytes (2×10⁷) were cultured in 10 ml of LAK cell medium (DMEM with non-essential amino acids, 10,000 U each of penicillin and streptomycin, 0.292 mg/ml l-glutamine, 10% FCS, 0.01 M HEPES, 0.05 M β-mercaptoethanol and 500 U/ml recom-
binit human IL-2 (R&D Systems, Minneapolis, MN) in 5% CO₂ at 37°C for 6 days. Expanded cells were collected and either stained with antibodies for FACS analysis or cytotoxicity was determined as described below. NK cells were prepared for functional analysis by culturing FACS-sorted hCD8⁺ or unsorted splenocytes in DMEM medium containing 50 U/ml recombinant human IL-2 for 18 h in 5% CO₂ at 37°C.

**Functional analysis for NK and LAK cell activity**

Cells plated in triplicate or quadruplicate starting at 50,000 cells/well were serially diluted 1:2. Then 5000 ⁵¹Cr-labeled target cells (YAC-1, NK and LAK sensitive or P815, NK insensitive and LAK sensitive) were added to each well and the amount of chromium released to the medium was determined after 4 h. Total possible ⁵¹Cr release was defined as the amount in the supernatant of target cells lysed with Triton X-100 detergent and background was the amount released in the absence of effector cells. The percent specific lysis was calculated according to the following formula: % specific lysis = (test release – background release)/total release.

**Results**

Transgenic mice were produced containing one of four different human CD8 genomic constructs (Fig. 1A). The wild-type 14 kb human CD8α genomic fragment (WT/hCD8, enlarged in Fig. 1B) contained the entire human CD8α gene as well as ~4 kb of the 5' and 3' flanking sequences. As a positive control, the hCD8+CD2 LCR construct was made. This construct consisted of the WT/hCD8 DNA with the LCR of the human CD2 gene added to the 3' end (Fig. 1A), providing a strong enhancer that should allow expression in all CD2⁺ T cells if the hCD8 genomic fragment was competent for expression *in vivo*. The *in vivo* regulatory effects of the intronic enhancer and stem–loop regions were addressed with mutant hCD8 genomic constructs (Fig. 1A). The hCD8–ED construct was identical to WT/hCD8 except that the entire enhancer including the stem–loop region was deleted. The hCD8–SLD construct contained a deletion of a portion of the Alu-containing stem–loop region which disrupted the ability to form a stem–loop. The presence of transgene DNA was detected in tail DNA preparations using Southern blots as described in Methods.

**hCD8 is predominantly expressed on lymphoid cells in all transgenic lines**

Mice which were positive for the transgene were initially screened for transgene expression using FACS analysis of blood. FACS profiles from blood of line H5, the WT/hCD8 line which expressed hCD8 at the highest levels, are shown in Fig. 2. As seen in Fig. 2(A), there was a small population of hCD8⁺ cells, and some highly autofluorescent cells, indicated by the diagonal pattern, which was also observed in unstimulated cells (data not shown). Gating on the hCD8⁺ cells (R2) and plotting them in a forward scatter/side scatter format (Fig. 2B) shows that the hCD8⁺ cells fall predominantly (>90%) in the lymphoid pool (R1 in Fig. 2B). By comparison, in an unengated sample (Fig. 2C), cells are additionally distributed in granulocytic, mononuclear and red blood cell ghost pools. A similar analysis of hCD8⁺ cells from blood, spleen, lymph nodes, thymus and bone marrow from all lines of all transgenic constructs also indicated that at least 90% were lymphoid (data not shown). FACS data from all of these tissues was therefore analyzed with a gate set on lymphocytes, removing highly autofluorescent cells from analysis so that double-positive populations could be more easily discerned.

**CD2 LCR provides T cell expression of the hCD8 transgene**

The CD2 LCR has been shown to yield T cell-specific, copy number-dependent, site of integration-independent expression of CD2, β-globin and CD8 cDNA (27–29). We therefore included the hCD8+CD2 LCR mice in our studies as a positive control. All of five founder lines containing the hCD8+CD2 LCR construct expressed hCD8 on mCD3⁺ cells in blood only two of these were carried to the F₁ stage. A representative hCD8/mCD3 profile for blood from one of these lines, line L3, is shown in Fig. 3(A). Consistent with expression patterns of other genes coupled to the CD2 LCR, line L3 expressed hCD8 on all of the CD3⁺ cells. This expression pattern is also observed in spleen, thymus and lymph nodes of these mice (data not shown). This indicates that (i) the promoter elements and CD8α exons in the 14 kb fragment are competent for expression *in vivo*, (ii) there is no dominant suppressor in the 14 kb BamHI fragment which can inhibit expression in the presence of the CD2 LCR, although the CD2 LCR may activate the CD8 gene by a mechanism which is different from that of the endogenous CD8 cis-acting sequences, and (iii) there is no selection against cells expressing the hCD8α gene product, as can also be inferred from the work of others who have used hCD8 cDNA to construct transgenes (29–31).

The WT/hCD8 construct is not sufficient for expression in CD8⁺ T cells but can direct expression to NK cells

In order to determine whether the cis-acting elements within the 14 kb hCD8α genomic BamHI fragment were sufficient to direct tissue-specific expression, transgenic mice containing this construct were prepared. Of 10 F₁ WT/hCD8 lines which carried the transgene, six lines showed detectable levels of expression in blood by FACS analysis. There was no correlation between estimated copy number, which ranged from 2 to 50, and expression level of the transgene for any of the constructs (data not shown). In the blood of the highest expressing WT/hCD8 line (H5), hCD8 was not significantly expressed in mouse CD8⁺ (mCD8⁺) lymphocytes (Fig. 3B). As seen in Fig. 3(C), 46% [(1.9/(1.9 + 2.3))] of hCD8⁺ lymphocytes from blood of WThCD8 mouse H5 expressed mCD3, and these hCD8⁺mCD3⁺ cells could all be accounted for in the hCD8⁺mCD4⁺ population (Fig. 3D). Furthermore, all hCD8⁺mCD3⁺ cells were TCRαβ⁺ (not shown). Thus, 17% (0.93/(4.7 + 0.93)) of the hCD8⁺ blood lymphocytes from line H5 were positive for B220 (Fig. 3E), a mouse B cell marker. In the spleen of this mouse, 60% of the hCD8⁺ cells, representing ~6% of the total splenic lymphocytes, were B220⁺ (data not shown). Thus, the transgene appeared to be expressed on subpopulations of B cells and CD4⁺ T cells, as well as another lymphoid population not marked by CD3 or B220. We subsequently determined that this other population was NK cells. As shown in Fig. 4(A), 30% [(1.1/(2.5 + 1.1))] of the hCD8⁺
cells in blood of the WThCD8 H5 line co-stain for NK1.1 (a mouse NK cell marker), with the hCD8*NK1.1+ cells representing the majority of the blood NK1.1+ cells. Triple staining indicated that the hCD8*NK1.1+ population was distinct from the hCD8*mCD3+ population (data not shown). There was a similar co-staining pattern of hCD8 and NK1.1 in blood from all five of the other expressing WThCD8 lines, from all four of the expressing hCD8–SLD lines (data not shown) and from the two hCD8+CD2 LCR lines carried to the F1 level. Representative data for one of these hCD8+CD2 LCR lines is shown in Fig. 4(B). These data demonstrate that in addition to CD4+ T cells and B cells, hCD8 is also expressed on the majority of NK1.1+ cells in the blood.

Several tissues from all of the transgenic lines were analyzed for hCD8 expression. The expression level of hCD8 on peripheral blood lymphocytes, splenocytes and lymph node cells from all of the WThCD8 lines ranged from 0.5 to 16%. There was no expression of hCD8 on mouse CD8+ blood, lymph node or spleen cells in any of the WThCD8 lines, as was the case for WThCD8 line H5. Of all the WThCD8 mice, only the H5 line expressed hCD8 at high levels in the thymus (30% of total thymic lymphocytes), while others expressed at insignificant levels (data not shown). The mCD4/mCD8 profiles of total thymocytes and of the hCD8+ thymocytes from WThCD8 H5 were similar to that of a non-transgenic mouse (not shown) indicating that (i) presence of the transgene was not skewing the thymic populations and (ii) hCD8 was not selectively expressed on a particular thymic population. Together, the above data indicate that even in the highest expressing WThCD8 line, H5, the elements contained in the 14 kb BamHI fragment are not sufficient to direct expression to mature mCD8+ T cells. Although there are some hCD8+mCD8+ cells in the WThCD8 H5 thymus, most of these do not survive to populate extrathymic sites. However, we did observe expression in subpopulations of CD4+ T cells, B cells and NK cells.

Deletion of the stem–loop does not alter transgene expression

In transient transfection assays, deletion of the stem–loop region of the hCD8 enhancer indicated it had a negative regulatory effect (25). The potential influence of the stem–loop region on transcription in vivo was studied in transgenic mice containing a hCD8α construct with the stem–loop region deleted (hCD8–SLD). While four of seven hCD8–SLD founders expressed the transgene, one of these four expressing founders was sterile, producing no F1 offspring. Another of the expressing founders was mosaic, not transmitting the transgene to the F1 generation, leaving two expressing F1 lines for subsequent analysis. In comparing the expression levels and FACS profiles from blood and various tissues of F0 or F1 hCD8–SLD mice with those of WThCD8 mice, no differences were seen which could be attributed to the construct (data not shown). Although this data suggests that the in vivo transcription of this construct is not affected by the stem–loop region, it is possible that its effect on human CD8 expression could only be observed when appropriate expression on mouse CD8+ T cells is achieved.

Deletion of the enhancer region leads to loss of hCD8 expression in NK1.1+ cells

Transgenic mice which contained the WThCD8 14 kb genomic construct with the enhancer deleted (hCD8–ED) were made to assess in vivo the role of this enhancer in regulation of gene expression. We obtained six F1 hCD8–ED lines which carried the transgene, but only one of these lines (G2) showed detectable levels of expression in blood by FACS analysis. In line G2, 4.2% of peripheral blood, 9.5% of splenocyte and 4.4% of the lymph node lymphocytes expressed hCD8 levels comparable to the highest expressing WThCD8 H5 line. Transgene expression levels in the thymus of this line, however, were low (1%), as seen for all of the WThCD8 lines except for line H5 (data not shown). Interestingly, line CD8–ED G2, the only hCD8–ED line that expressed hCD8, had no hCD8+ cells which specifically stained for NK1.1 (Fig. 4C). We also observed that NK1.1+ cells from bone marrow lymphocytes of line CD8–ED G2 lacked expression of the transgene, while bone marrow NK1.1+ cells from all enhancer-containing lines expressed it (data not shown). Since the spleen contains a high level of NK cells compared to other tissues, we next analyzed splenocytes for hCD8/NK1.1 expression. Figure 5 shows FACS profiles of splenocytes from a negative control mouse, WThCD8 H5 and hCD8–ED G2. As observed in blood, WThCD8 line H5 expressed hCD8 on a significant portion of its splenic NK cells (Fig. 5B). In contrast, the hCD8–ED line G2 was missing this splenic population of hCD8*NK1.1+ cells, even though the hCD8–ED mouse had a total hCD8+ population almost as large as the highest expressing WThCD8 line H5 (Fig. 5B and C).

To further study hCD8α expression on NK1.1+ cells, we expanded the NK1.1+ population with IL-2. It has been established that IL-2-induced murine LAK cells are enriched in NK1.1+ cells compared to the precursor splenocytes.
from which they are derived (32–36). To obtain LAK cells, splenocytes were cultured for 6 days in 500 U/ml recombinant human IL2. The hCD8/NK1.1 FACS profiles from LAK cells of control animals, WThCD8 H5 and hCD8-ED lines are shown in Fig. 5(D–F). As expected, the NK1.1+ population was greatly expanded in LAK cells compared to the splenocytes from which they were derived (Fig. 5A–C). Figure 5 also shows that while LAK cells from WThCD8 line H5 contain 9.2% hCD8+NK1.1+ cells, LAK cells from the CD8–ED mouse contained almost no hCD8+NK1.1+ cells. It is also noteworthy that the LAK cell population from hCD8-ED contained very few single-positive hCD8+ cells (Fig. 5F), despite a significant hCD8+ population in the splenocytes of this mouse (Fig. 5C). Therefore, expression of the hCD8 transgene in this CD8–ED mouse was missing not only in NK1.1+ LAK cells, but also in a population of LAK cells which is not marked by NK1.1.

Figure 6 summarizes the expression of hCD8 on spleen (left) and LAK (right) NK1.1+ cells for all lines analyzed. All of the WThCD8, hCD8–SLD and hCD8–CD2 LCR lines had a population of hCD8+NK1.1+ cells. Only the hCD8–ED line lacked the hCD8+NK1.1+ population. These findings were reproducible in a separate LAK cell experiment with different mice from these lines (data not shown). We know that LAK cells were indeed generated in the CD8–ED mouse, because (i) the NK1.1+ population was greatly expanded, from 1.9 to 22%, in going from splenocytes to LAK cells as seen in Fig. 5(C and F), and (ii) cytotoxic activity against P815 or YAC-1 target cells was the same among IL-2 expanded cells generated from WThCD8, hCD8+CD2 LCR, hCD8–SLD and hCD8–ED mice (data not shown).

Together, our data indicate that all enhancer-containing lines express human CD8 on NK cells and that deletion of the hCD8 intronic enhancer extinguishes CD8 expression in NK1.1+ cells, whether from circulating blood, bone marrow, spleen or cytokine-induced (LAK) cells.

Based on antibody staining patterns, it appeared that the WThCD8 lines expressed the hCD8 transgene in NK cells and that the hCD8–ED lines were missing this population of hCD8+NK1.1+ cells. To further study the NK cell expression of the hCD8 transgenes, we determined whether hCD8+ cells had NK functional activity. To ascertain the NK functional activity of the hCD8+ populations in WThCD8 and hCD8–ED mice, 51Cr-release assays were performed using hCD8+ enriched splenocytes as effector cells. A representative

![Fig. 5. FACS analysis of hCD8a expression on spleen and LAK NK1.1+ cells. LAK cells were generated and cells were stained with anti-hCD8-FITC and anti-NK1.1-PE antibodies as described in Methods. Spleen data has been gated on lymphocytes (see Fig. 2). For the LAK cell data, since hCD8+ cells were not confined to the lymphoid pool, highly autofluorescent cells were removed from analysis by gating out cells which had no FL1 (hCD8-FITC) or FL2 (NK1.1-PE) signals, but which had a FL3 signal (for which there was no antibody–marker conjugate).](https://academic.oup.com/intimm/article-abstract/8/10/1617/681384/1622)

![Fig. 6. Summary of hCD8 expression on NK1.1+ splenocytes and LAK cells. Procedures were as described for Fig. 5 Note different x-axis scales for spleen and LAK graphs](https://academic.oup.com/intimm/article-abstract/8/10/1617/681384/1622)
Fig. 7. NK functional activity of hCD8-enriched splenocytes from WThCD8 versus hCD8-ED lines. Splenocytes from WThCD8 H5 or hCD8-ED mice were stained with anti-hCD8-PE antibody, then enriched by FACS for cells expressing hCD8 (A and C). The ability of these hCD8-enriched cells from WThCD8 H5 (B) or hCD8-ED (D) mice to lyse 51Cr-labeled YAC-1 target cells was compared to that of unsorted splenocytes from the same mouse or from a negative control mouse. Open squares, unsorted splenocytes from a negative control mouse; open triangles, unsorted splenocytes from a WThCD8 mouse (B) or from a CD8-ED mouse (D); closed circles, hCD8-enriched splenocytes from a WThCD8 mouse (B) or from a CD8-ED mouse (D).

Discussion

The aims of the present study were to determine if a 14 kb genomic human CD8α construct could direct tissue-specific human CD8α expression in transgenic mice and to address in vivo the potential contribution of the human CD8α intronic enhancer and its 5′ flanking stem-loop region, previously identified by DNase I hypersensitivity mapping and a reporter-based expression assay in transiently transfected cell lines (25). To this end, constructs with the stem-loop (hCD8-SLD) or the stem-loop plus the enhancer deleted (hCD8-ED) were used to make transgenic mice. The expression patterns of these constructs were analyzed by FACS and compared to those from the 14 kb wild-type construct (WThCD8) and from a positive control construct in which the CD2 LCR was added to the wild-type construct (hCD8+CD2 LCR) to provide a strong tissue-specific enhancer. While the hCD8+CD2 LCR construct was expressed in all T cells, the WThCD8 construct was not expressed in mouse CD8+ peripheral T cells. However, it was expressed in small lymphoid populations, which included subsets of the CD3+ CD4+ T cell (mainly CD4+), B cell and NK cell populations. The hCD8-SLD mice had similar expression patterns to those of WThCD8 mice. In contrast, five of the six hCD8-ED mice had no detectable expression of the transgene. Interestingly, in the one hCD8-ED line (G2) which did express hCD8, there was a distinct lack of expression in NK cells, as determined by FACS analysis of blood, bone marrow, spleen and LAK cells. NK functional studies confirmed that the hCD8+ splenocytes identified by FACS in the WThCD8 mice contained NK cells. In contrast, hCD8+ splenocytes isolated from the hCD8-ED line G2 lacked enhanced NK functional activity, further supporting the absence of hCD8 expression on NK cells in this line.

The finding that hCD8 is expressed on mouse NK cells in the WThCD8 and hCD8-SLD lines indicates that the element(s) necessary for NK cell-specific expression is localized to the 14 kb hCD8 genomic fragment. While the transgene expression pattern we observed on B cells and CD4+ T cells is not normally seen, CD8 is normally expressed as an αα homodimer on human NK cells. In the WThCD8 lines, six of 10 expressed the transgene in freshly isolated and IL-2 expanded NK cells. Although up to 40% of human NK cells express CD8 (37,38), freshly isolated mouse NK cells do not normally express this gene product (32-36,39-42). Our data suggests that this differential expression of CD8α on mouse and human NK cells may be explained by species divergence of cis-acting regulatory sequences with conservation of cell-type-specific trans-acting factors. Such a phenomenon has been observed for the Thy-1 gene, which is expressed in different tissues in mice versus humans. Human Thy-1 transgenes have the human tissue distribution pattern in
transgenic mice (43). The human gene contains elements which direct expression to the kidney or spleen while the murine gene lacks these elements but instead contains a thymocyte specific enhancer (44).

Our data implicates involvement of the human CD8 intronic enhancer in NK cell-specific expression of CD8. There was no NK cell expression of the transgene in any of the six hCD8-ED lines, including the one hCD8-ED line, G2, which, as observed in the WThCD8 lines, expressed on small populations of T and B cells. Interestingly, a sequence comparison between the last introns of murine and human CD8α, shows that the murine CD8 gene lacks a region homologous to the human enhancer (25). We also searched the murine intron for protein binding motifs that are present in the human intron, but none were found, indicating that the enhancer is completely absent in the last intron of the murine CD8α gene. Since the human CD8 intronic enhancer appears to be required for expression in NK cells, our data suggests that the evolutionary inheritance of the intronic enhancer may endow human NK cells with the ability to express the CD8α gene.

Another potential function of the enhancer may be to increase the likelihood of any expression of the transgene. Since our hCD8-ED lines had a low percentage of expression compared to the WThCD8 lines (one out of six versus six out of 10 lines expressed, respectively) and the hCD8-SLD lines (four out of seven lines expressed), it appears that the hCD8 intronic enhancer influences the general expressability of the transgene.

The lack of expression of the WThCD8 construct in the mCD8+ T cell population is most likely not attributable to a non-functional promoter nor non-functional coding exons, since the addition of CD2 LCR to the wild-type construct afforded expression in all CD3+ cells. Likewise, the hCD8 expression pattern in CD8+CD2 LCR mice rules out the possibility that expression of hCD8 gene product interferes with mouse T cell development. It is possible that mouse trans-acting factors do not cross react with the necessary human cis-acting elements to elicit CD8+ T cell specific expression. However, transgenic mice have been used with success to elucidate cis-acting elements responsible for developmental and tissue-specific expression of the T cell specific human genes, CD2 (27,45,46) and CD4 (47-49), as well as a number of other human genes, including those for globins (50,51), P53 (52), myelin (for review see 53), loop explains the lack of expression of the WThCD8 transgene (four out of seven lines expressed), it appears that the hCD8 intronic enhancer influences the general expressability of the transgene.

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Barring the possibility that a species-specific regulatory loop explains the lack of expression of the WThCD8 transgene in mouse T cells, it is most likely that the WThCD8 construct is simply missing enhancer(s) necessary for expression in CD8+ T cells. The low level expression in CD4+ T cells and B cells also suggests important regulatory elements are absent in the WThCD8 construct. Given the positive effect of the hCD8 intronic enhancer on transcription in transiently transfected T cells (25), it is still possible that this enhancer may act in concert with other as yet unidentified elements to elicit expression on CD8+ T cells in vivo. Although we have identified an enhancer that may be required for NK cell expression of hCD8α, it remains possible that other elements in the 14 kb genomic fragment in addition to the intronic enhancer are also required for NK cell-specific expression. Answers to these questions must await identification of the other cis-acting elements involved in regulation of CD8 gene expression.

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Abbreviations

hCD8-ED human CD8 with enhancer deleted
hCD8-SLD human CD8 with stem-loop deleted
HS1 hypersensitive site 1
HS2 hypersensitive site 2
LAK lymphokine-activated killer
LCR locus control region
PE phycoerythrin
WThCD8 wild-type human CD8

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