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TECK: A Novel CC Chemokine Specifically Expressed by Thymic Dendritic Cells and Potentially Involved in T Cell Development

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Summary

A novel CC chemokine was identified in the thymus of mouse and human and was designated TECK (thymus-expressed chemokine). TECK has weak homology to other CC chemokines and maps to mouse chromosome 8. Besides the thymus, mRNA encoding TECK was detected at substantial levels in the small intestine and at low levels in the liver. The source of TECK in the thymus was determined to be thymic dendritic cells; in contrast, bone marrow–derived dendritic cells do not express TECK. The murine TECK recombinant protein showed chemotactic activity for activated macrophages, dendritic cells, and thymocytes. We conclude that TECK represents a novel thymic dendritic cell–specific CC chemokine that is possibly involved in T cell development.

Introduction

Chemokines belong to a family of small peptides (6–15 kDa) whose best described biological function is to control the migration of certain leukocyte populations to localized sites of inflammation. (Baggiolini et al., 1994; Schall and Bacon, 1994; Hedrick and Zlotnik, 1996). In the past few years many new members of the chemokine superfamily have been characterized. Initially, new chemokines were identified through their chemotactic effects on leukocytes (Baggiolini et al., 1994; Schall and Bacon, 1994) and were isolated mainly from blood leukocytes or cell lines. More recently, approaches based on the selective cloning of secreted molecules by signal sequence trap (Tashiro et al., 1993; Imai et al., 1996) or on the use of public and private databases of expressed sequence tags through bioinformatics (Hieshima et al., 1997; Patel et al., 1997; Rossi et al., 1997) have allowed the rapid identification of novel chemokines based on sequence and structural homologies.

These approaches take advantage of the fact that most of the chemokines are secreted factors whose protein sequences contain four conserved cysteines (Schall, 1994). In the CXC or α chemokine family, the two first N-terminal cysteines are separated by a non-conserved amino acid. In the CC or β chemokine family, these two cysteines are consecutive. A third type of chemokine, the C or γ family, is represented by lymphotactin, which conserves two cysteines (1 and 3) instead of the original four (Kelner et al., 1994). Finally, a recently identified chemokine with three amino acids separating the first two cysteines defines a fourth, CX_3C family (Bazan et al., 1997).

Some of the new chemokines discovered show a relatively restricted pattern of expression (Imai et al., 1996; Hieshima et al., 1997). It is tempting to suggest that these new approaches may lead to the discovery of tissue- or cell-specific chemokines. In addition, there is new biological evidence that chemokines have important roles in hemopoiesis (Cook, 1996; Nagasawa et al., 1996) and control of viral infections, including infection by the human immunodeficiency virus (Cocchi et al., 1995; Cook et al., 1995). Thus, the molecular cloning of novel chemokines through DNA-based strategies may uncover novel proteins belonging to the chemokine superfamily but whose physiological role extends beyond control of inflammation.

In an attempt to identify novel genes involved in T cell development, we analyzed a cDNA library from the thymus of recombinase activation gene-1 (RAG-1) deficient mice. We identified a novel CC chemokine, designated TECK (thymus expressed chemokine), based on sequence homology to other known chemokines. We subsequently isolated the human homolog of TECK. The pattern of expression of TECK mRNA is highly restricted to the thymus and small intestine in both human and mouse. Moreover, in the mouse thymus, TECK protein is produced by dendritic cells, whereas splenic dendritic cells do not express TECK mRNA. Recombinant TECK showed chemotactic activity on thymocytes, macrophages, THP-1 cells, and dendritic cells but was inactive on peripheral blood lymphocytes (PBLs) and neutrophils. The restricted pattern of expression of TECK together with its biological properties suggests a role for this novel dendritic cell–specific chemokine in T cell development.

Results

Cloning and Structural Analysis of Mouse TECK

A directional cDNA library was made from RAG-1–deficient mouse thymus and analyzed by random sequencing. One of the clones contained an open reading frame with significant homology to previously described CC chemokines. The full-length cDNA contains 1037 base pairs (bp), including an open reading frame of 426 bp encoding a protein of 142 amino acids, and is referred to in this report as mTECK (Figure 1). In the 3′ untranslated region, there is one unique polyadenylation signal consistent with the single mRNA species observed in Northern blots. The mTECK cDNA does not contain any ATTTA transcript destabilization motif (Shaw and Kamen, 1986). Comparison of the amino acid sequence of mTECK with previously described murine CC chemokines demonstrates conservation of the four cysteines present in all of these chemokines (Figure 2A). However,

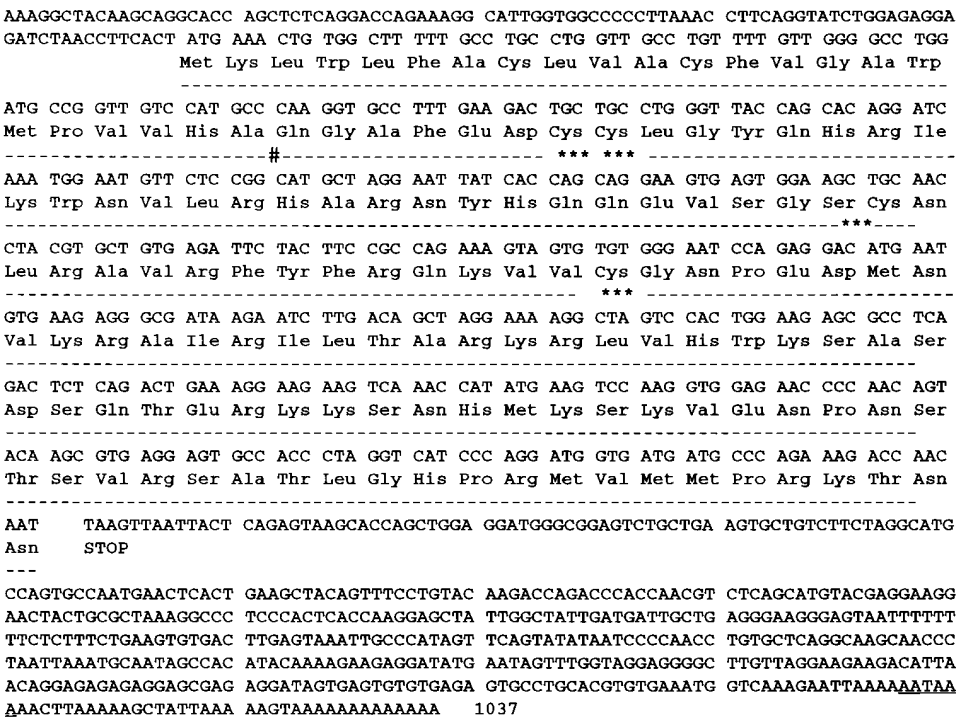

Figure 1. Nucleotide Sequence of mTECK cDNA

Sequence outlined in a three-nucleotide frame is the coding sequence. Underlining indicates the polyadenylation site. The putative cleavage site for the signal peptide (#) has been defined according to the SignalP World Wide Web server at http://www.cbs.dtu.dk/services/SignalP. Asterisks indicate the conserved cysteines. The mTECK sequence has been deposited in the GenBank database (accession number U86357).

mTECK shows few additional identities with these proteins (Figure 2B).

Cloning and Molecular Characterization of Human TECK

To investigate the possible existence of a gene homologous to mTECK in other mammalian species, a Southern blot with genomic DNA from various species was hybridized with the mTECK cDNA probe. Under high stringency conditions, hybridizing bands were detected in mouse, rat, hamster, and human genomic DNAs (Figure 3A). A single band was detected in human DNA, suggesting that a single gene encodes for TECK in this species. The multiple bands present in mouse, rat, and hamster DNA could be the result of a internal EcoRI site within the TECK gene. Alternatively, the TECK gene may have been duplicated in these species.

To clone the human homolog of mTECK, a blot of cDNAs from a panel of human cDNA libraries was hybridized with the mTECK cDNA probe. A signal was observed in a fetal small intestine cDNA library (data not shown). Screening of this library with the mTECK probe allowed the isolation of several identical clones of 1012 bp with an open reading frame of 453 bp (data not shown) encoding a protein of 151 amino acids. This protein had a much higher degree of homology at the nucleic acid level (71% nucleic acid identity for the open reading frame and 49.3% amino acid identity) to mTECK than to other known CC chemokines and was thus designated hTECK (Figures 2B and 3B).

Chromosomal Location of mTECK

It has been shown that within the genome the genes encoding for most chemokines are clustered. The genes encoding CC chemokines cluster on mouse chromosome 11 and on human chromosome 17q11–12 (Schall, 1994; Hedrick and Zlotnik, 1996). The chromosomal location of mTECK (designated *Teck*) was determined by interspecific backcross analysis between ([C57Bl/6J × Mus spretus]F1 × C57Bl/6J) mice (Jenkins et al., 1982). The mapping results indicated that the *Teck* locus is located on the proximal part of mouse chromosome 8 (data not shown). Although the chromosomal location of the human *TECK* locus could not be determined, this region of mouse chromosome 8 is syntenic to the human 19p13.3 and 13q34 regions (data not shown). However, the *Teck* locus is also very close to a region syntenic to human chromosome 4. The closest known gene, *Insr*, encodes the insulin receptor, and the genetic distance between *Teck* and *Insr* was estimated at 0.9 ± 0.9 cM (data not shown). We have compared our interspecific map of chromosome 8 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (Mouse Genome Database, The Jackson Laboratory, Bar Harbor, ME). *Teck* resides in a region of the composite map that lacks mouse mutations

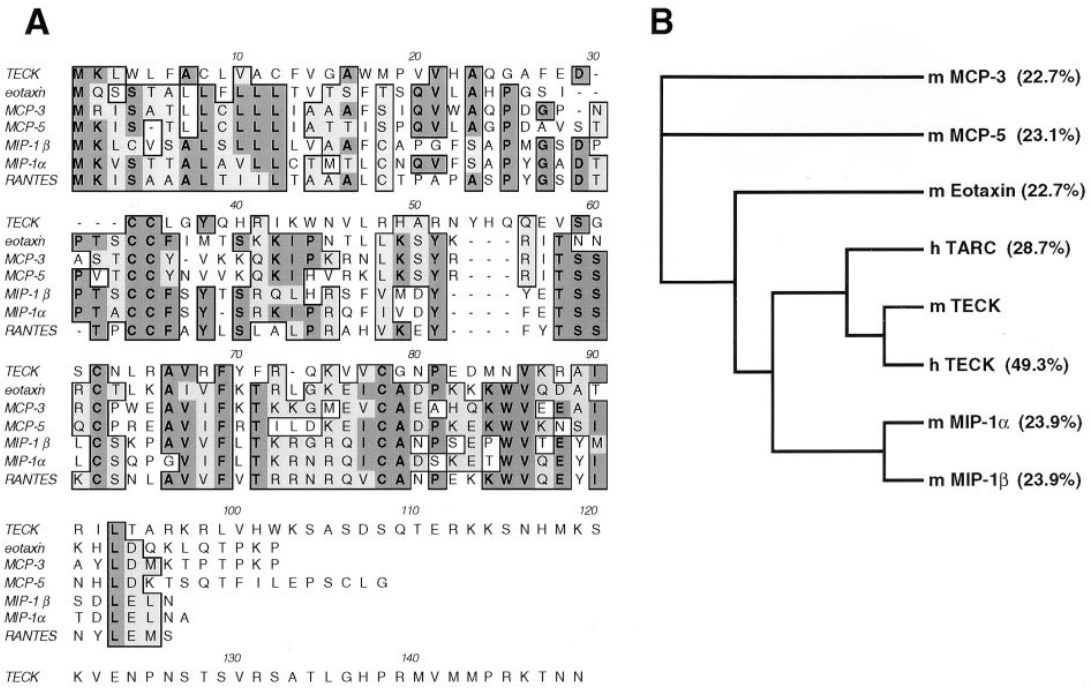

Figure 2. Comparison of the Amino Acid Sequence of TECK with Other CC Chemokines

(A) Multiple sequence alignment of mTECK with other murine CC chemokines. The protein sequences of eotaxin (Gonzalo et al., 1996), MCP-3 (Thirion et al., 1994), MCP-5 (Jia et al., 1996), MIP-1β (GenBank accession number X62502), MIP-1α (Grove et al., 1990), and RANTES (Schall et al., 1992) were compared using the Clustal W program (Thompson et al., 1994). Dark-shaded boxes indicate conserved anino acids between at least four sequences, including the four cysteine residues characteristic of the CC chemokine family; light-shaded boxes indicate conservative substitutions.

(B) Phylogenic tree of CC chemokines. According to their Clustal W alignment, evolutionary distances between mTECK and other chemokines were estimated. The location of the branch points is not to scale. Numbers in parentheses are the percentage amino acid identities between mTECK and the other chemokines. The percentage identities of human TARC (Imai et al., 1996) was calculated in comparison with hTECK.

with a phenotype that might be expected for an alteration in this locus (data not shown).

Analysis of mTECK and hTECK mRNA Distribution in Cells and Tissues

An analysis of the distribution of mTECK mRNA in tissues and cells by Northern blotting or by Southern blotting of mouse cDNA libraries revealed that mTECK was expressed at significant levels only in the thymus and to a lesser extent in small intestine (Table 1 and Figure 4A). Weak expression of mTECK mRNA was observed in brain, testis, and liver RAG-1$^{-/-}$ cDNA libraries. Interestingly, mTECK mRNA was detected in a cDNA library of activated pro-T cells. Pro-T cells represent an early stage of intrathymic T cell progenitors, not fully committed to the T cell lineage because they can give rise to natural killer and dendritic cells (Moore and Zlotnik, 1995; Wu et al., 1996). In contrast, mTECK mRNA was undetectable in resting or activated thymocytes, peripheral T or B cells, macrophages, PBLs, and splenic dendritic cells and in all other tissues tested, with the exception of spleens recovered from mice injected with lipopolysaccharide (LPS) (Table 1 and Figure 4A). mTECK mRNA was detected by polymerase chain reaction (PCR) in fetal thymi of day 14 of gestation (Figure 4B), indicating that mTECK is expressed in the thymus at the earliest stages of T cell development.

The distribution of hTECK mRNA was similarly analyzed. As with mouse, hTECK mRNA expression was highly restricted to the thymus and small intestine (Figure 5). Weak expression was also detected in inflamed tonsil and fetal spleen, but at much lower levels than that observed in the thymus since this particular blot was exposed for a long time. Of note, hTECK mRNA was absent from a series of cDNA libraries from dendritic cells derived in vitro from bone marrow CD34^{+} progenitor cells or peripheral blood monocytes (Figure 5A). In addition to the dendritic cell cDNA libraries displayed in Figure 5, hTECK mRNA was absent from libraries of monocyte-derived dendritic cells stimulated with LPS or a combination of tumor necrosis factor-α (TNFα), interleukin 1α (IL-1α), and monocyte supernatant for 4 and 16 hr (data not shown). Collectively, these data indicate that TECK mRNA is specifically expressed at high levels in thymus and small intestine in vivo.

Identification of mTECK-Producing Cells In Vivo

The abundance of mTECK mRNA expression in RAG-1–deficient thymus and its absence in thymic T cells suggested that mTECK was expressed by a thymic stromal component in normal mice. We performed in situ mRNA hybridization with sense or antisense mTECK probes generated by PCR. Thymic sections hybridized with the sense probe (negative control) demonstrated

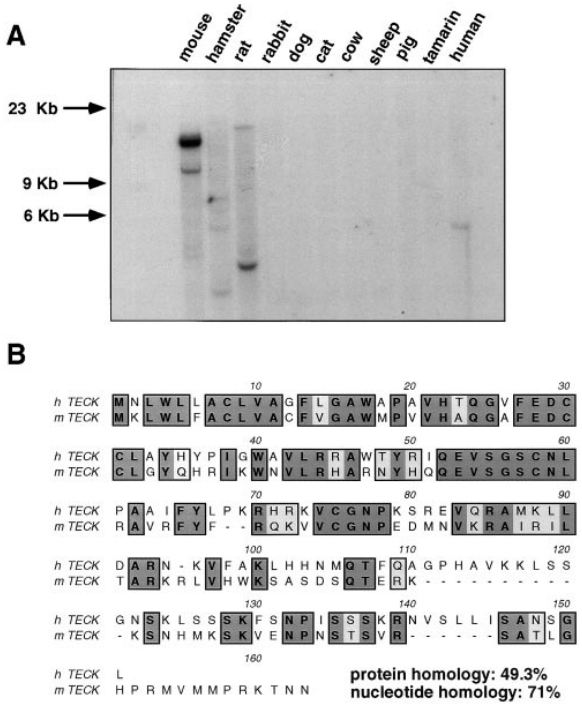

Figure 3. Conservation of TECK in Mammalians and Structure of Human TECK

(A) Zooblot of genomic DNA hybridized with a mTECK probe. A Southern blot of genomic DNA of different mammalian species digested with EcoRI was hybridized with the mTECK probe.

(B) Structure of hTECK and comparison of hTECK and mTECK genes. Protein sequences of mTECK and hTECK were compared using the Clustal W program (Thompson et al., 1994). Dark-shaded boxes indicate conserved amino acids; light-shaded boxes indicate conservative substitutions. The protein and amino acid homologies both were calculated for the coding sequence only. The hTECK sequence has been deposited in the GenBank data base (accession number U86358).

no specific staining (Figure 6A), while sections hybridized with the antisense probe at the same concentration showed specific staining in the thymic medulla (Figure 6B). At higher magnification (Figure 6C), positive cells appeared to have a nonlymphoid morphology with processes surrounding lymphoid cells. This experiment indicated that in vivo, mTECK mRNA is expressed by a nonlymphoid component of the medullary stroma, possibly dendritic cells.

The thymic stroma is composed mainly of epithelial cells, macrophages, dendritic cells, and fibroblasts, together with a network of vascular and nervous tissue (Boyd et al., 1993). Since we previously failed to detect mTECK mRNA expression in thymic epithelial or macrophage cell lines with or without activation with interferon-γ (IFNγ) (Table 1), we sorted thymic dendritic cells based on their high expression of major histocompatibility (MHC) class II and CD11c (N-418 antibody). Analysis of mTECK expression by reverse transcription PCR (RT-PCR) revealed that freshly isolated MHC class II$^+$ CD11c$^+$ thymic dendritic cells expressed mTECK mRNA (Figure 4C), whereas the MHC class II$^+$ CD11c$^-$ subset was negative. In contrast, mTECK mRNA was undetectable in a cDNA library made from freshly isolated splenic dendritic cells (Table 1).

We then performed immunostaining of thymic sections and purified thymic dendritic cells with a polyclonal antibody raised against a decapeptide corresponding to the C-terminus of mTECK. This polyclonal antibody reacted with recombinant mTECK in enzyme-linked immunosorbent assay and Western blot, while normal rabbit serum was negative (data not shown). In thymic sections, the polyclonal anti-peptide antibody reacted with a stromal component of the thymic medulla, consistent with the in situ hybridization data (Figures 6E and 6F), while staining with normal rabbit serum was negative (Figure 6D). The antibody also reacted weakly with some endothelial cells (Figure 6F), raising the possibility that mTECK can be produced by the thymic endothelium. Finally, the anti-mTECK polyclonal antibody stained sorted thymic dendritic cells, while the control serum was negative (Figures 6G and 6H). High magnification (Figure 6I) clearly showed intracellular staining of cells with characteristic dendritic morphology. Taken together, these results indicate that thymic dendritic cells and possibly thymic endothelial cells produce TECK in vivo.

Chemotactic Activities of mTECK Protein

To evaluate the biologic properties of mTECK, a recombinant protein with a N-terminal FLAG peptide was obtained in a bacterial expression system (see Experimental Procedures). In some experiments, a recombinant mTECK protein with a C-terminal FLAG was used and similar results were obtained (data not shown). mTECK induced the migration of mouse thymocytes (Figure 7A). The optimal response was obtained with a dose of 10 ng/ml TECK. Cell migration was determined to be chemotaxis and not chemokinesis through checkerboard analysis (data not shown). Furthermore, it is established that chemokines bind to specific receptors that are coupled through heterotrimeric G proteins to intracellular signal-transducing pathways (Murphy, 1994). To determine whether the chemotaxis of thymocytes involved a G protein–coupled receptor, cells were incubated prior to the assay with 10 ng/ml pertussis toxin, which ADP-ribosylates $G_{\alpha i}$ proteins (Katz et al., 1992). This pretreatment completely abrogated the chemotactic response of thymocytes to mTECK (Figure 7A).

The recombinant mTECK protein also induced migration of human monocytic THP-1 cells activated for 16 hr with IFNγ (Figure 7B), whereas it was not significantly active on resting THP-1 cells. This experiment showed that mTECK is active on human cells. In addition, mTECK induced activated mouse peritoneal macrophages to migrate as well as highly purified mouse splenic dendritic cells (Figure 7B). In all of these experiments, the optimal dose of mTECK was 10 ng/ml. In contrast, no chemotaxis was observed with bone marrow cells, purified neutrophils, splenic B cells, splenic T cells, or IL-2–activated RAG-1–deficient mouse splenocytes lacking mature T and B lymphocytes (Mombaerts et al., 1992) and therefore enriched in natural killer cells (data not

Table 1. mTECK mRNA Expression in Tissues and Cells

cDNA Libraries			Northern Blot		
Cell Type or Tissue	Negative	Positive	Cell Type or Tissue	Negative	Positive
Th2 $CD4^+$ T cells	X		Heart	X	
Th1 $CD4^+$ T cells	X		Brain	X	
Lung	X		Spleen	X	
L cells	X		Lung	X	
RAG-1 KO lung	X		Liver	X	
RAG-1 KO heart	X		Skeletal muscle	X	
RAG-1 KO brain		X (+)	Kidney	X	
RAG-1 KO spleen	X		Testis	X	
RAG-1 KO kidney	X		Thymus		X (+++)
RAG-1 KO testis		X (+)	Small intestine		X (++)
RAG-1 KO thymus		X (+++)	$CD4^+CD8^-$ thymocytes R/A	X	
RAG-1 KO liver		X (+)	$CD4^-CD8^+$ thymocytes R/A	X	
$CD4^-CD8^-$ thymocytes	X		$CD4^-CD8^-$ thymocytes R/A	X	
A20-J B cell lymphoma	X		$B220^+$ splenocytes R/A	X	
BW $CD4^-CD8^-CD3^-$ hybridoma	X		Thy-1^+ splenocytes R/A	X	
pro-T cells		X (+)	1G18LA macrophages R/A	X	
pre-T cells	X		Primary thymic stroma R/A	X	
30-R bone marrow stroma	X		3D.1 thymic epithelial R/A	X	
D10 T cell hybridoma	X		MTSC-C thymic epithelial	X	
CTLL T cell clone	X		30.R bone marrow stroma	X	
Peritoneal macrophages	X				
Splenic dendritic cells	X				

Analysis of mTECK mRNA was carried out as described in Experimental Procedures.
+ to +++, relative intensity of the signal; R/A, resting or activated.

shown). These results are consistent with the absence of in vivo accumulation of neutrophils, monocytes, or lymphocytes 2 and 5 hr following an intraperitoneal injection of 10 μg of mTECK (data not shown). Collectively, these data indicate that TECK is a chemotactic factor for thymocytes, macrophages, and dendritic cells.

Discussion

TECK, a Distant Member of the CC Chemokine Family

In this report, we describe the molecular isolation and characterization of TECK, a novel mouse and human CC chemokine. Analysis of its predicted amino acid sequence showed that TECK is distantly related to previously described CC chemokines. Conservation of particular amino acids among most CC chemokines may be related to their functional importance (Beall et al., 1992; Lusti-Narasimhan et al., 1995). In particular, a tyrosine residue between the second and third cysteines has been shown to be critical for monocyte chemotaxis (in position 50 of the multiple alignment, Figure 2A) (Beall et al., 1992). Although TECK does not have a tyrosine at this particular position, it has one in position 52 (Figure 2A) that may have the same function, since TECK is chemotactic for activated monocytes. In addition to these differences in the primary structure, the gene encoding TECK maps on chromosome 8 in the mouse, unlike most other CC chemokines, which are clustered on chromosome 11. This is not the first report of an unusual chromosomal location for a CC chemokine. We have cloned the human CC chemokine MIP-3β (macrophage inflammatory protein-3β) and showed that its gene was on chromosome 9 rather than chromosome 17 (Rossi et al., 1997), and the gene encoding the novel human CC chemokine MIP-3α/LARC (Rossi et al., 1997) has been mapped on chromosome 2 (Hieshima et al., 1997). It is likely that the CC chemokines on chromosome 11 in the mouse and chromosome 17 in the human have been generated through gene duplication of a primordial chemokine. Our results suggest that TECK may have been generated at an earlier stage during evolution. In this regard, the TECK gene may have evolved to ensure functions similar to other CC chemokines with a distant primary structure but through similar receptor(s) as dictated by its secondary and tertiary structures. Alternatively, the receptor(s) and physiological role of TECK may be unique among chemokines.

TECK Expression and Function Is Associated with T Cell Development

We observed that TECK was strongly expressed in the thymus, the primary lymphoid organ in which T cell development takes place. Recently, another CC chemokine highly expressed in the thymus, TARC (thymus- and activation-restricted chemokine), has been identified (Imai et al., 1996). However, TARC is also expressed in lung and colon as well as activated PBMCs (Imai et al., 1996), whereas TECK was absent from these tissues. Besides the thymus, numerous reports indicate that T cell development can occur in the small intestine (Poussier and Julius, 1994), where TECK is also expressed. The liver has also been suggested to support T cell development to some extent (Abo et al., 1994), and we observed a low TECK expression in a liver cDNA library. These data show that TECK expression correlates with organs that support T cell development.

While many molecular and cellular aspects of T cell

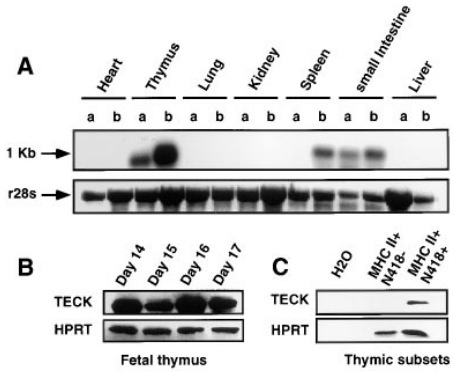

Figure 4. Expression of mTECK mRNA in Different Mouse Tissues and Cell Types

(A) Northern blot of RNA from different organs hybridized with the mTECK cDNA probe with or without in vivo LPS stimulation. RNAs from different organs were isolated 3 hr after intravenous injection of PBS (lanes a) or 50 μg LPS (lanes b). Each lane contained approximately 10 μg of total RNA. The hybridizing bands (top) corresponded to the predicted size of approximately 1040 bp for mTECK mRNA. Staining of the gel with ethidium bromide (bottom) showed a band corresponding to the ribosomal 28s RNA.

(B) mTECK mRNA expression in the mouse fetal thymus. RNAs from fetal thymic lobes were extracted on days 14–17 of gestation, and expression of mTECK mRNA was analyzed by RT-PCR. A unique band corresponding to the predicted size of the PCR product (~400 bp) was observed after ethidium bromide staining. The expression of HPRT mRNA in the same samples was analyzed by RT-PCR as a control.

(C) mTECK mRNA expression in thymic dendritic cells. A population enriched in thymic dendritic cells was prepared from 15 pooled adult thymi as described in Experimental Procedures. Dendritic cells (>99% pure) were then sorted by flow cytometry based on their MHC class II^+ $N\text{-}418^+$ phenotype. mTECK mRNA was then analyzed by RT-PCR, and an MHC class II^+ $N\text{-}418^-$ population sorted in the same experiment was used as a negative control. The expression of HPRT mRNA in the same samples was analyzed by RT-PCR as a control.

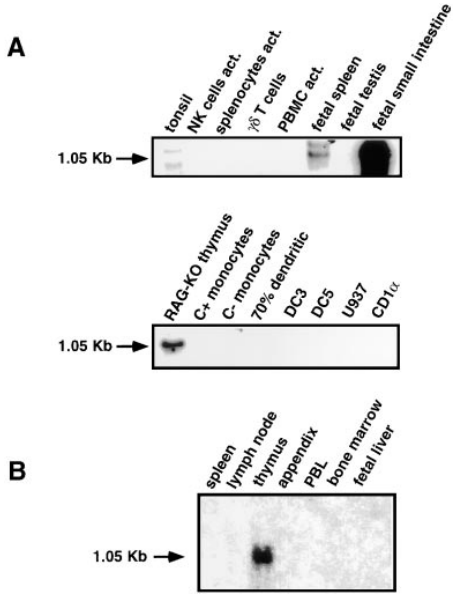

Figure 5. Expression of hTECK mRNA in Different Human Tissues and Cell Types

(A) Expression of mTECK mRNA in human cDNA libraries. Southern blots of human cDNA libraries digested with the appropriate restriction enzymes (10 μg/lane) were hybridized with the hTECK cDNA probe. A major hybridizing band corresponding to the predicted length of hTECK mRNA (~1040 bp) was observed, sometimes with some other bands that may represent incomplete cDNAs. RAG-KO thymus showed the cross-hybridization between hTECK cDNA and mouse RNA. NK cells correspond to pools of NK clones activated with PMA and ionomycin for 12 hr. Splenocytes were activated with anti-CD40 antibody and IL-4 for 6 and 12 hr. PBMC were activated with anti-CD3 and PMA for 6, 12, and 24 hr. C^+ monocytes consisted in elutriated monocytes cultured with IFNγ and IL-10; C^- monocytes consisted in elutriated monocytes cultured with IFNγ and anti-IL-10 antibody. The designation "70% dendritic" corresponds to a 70% $CD1\alpha^+$ dendritic cell population obtained by expansion of $CD34^+$ bone marrow cells with GM-CSF and TNF-α and resting; DC3 corresponds to a similar dendritic cell population stimulated with PMA and ionomycin for 1 and 6 hr. CD1α is similar to DC3 but the cells were 95% $CD1\alpha^+$; DC5 corresponds to dendritic cells obtained by culturing peripheral blood monocytes in the presence of IL-4 and GM-CSF. U937 is a premonocytic cell line.

(B) Expression of hTECK mRNA in human tissues. A northern blot made with poly(A)$^+$ RNA from different tissues (5 μg/lane) was hybridized with the hTECK cDNA probe. The hybridizing band corresponds to the predicted size of approximately 1040 bp for hTECK mRNA.

differentiation are well documented, the precise role of chemokines in T cell development is still unknown. Recently, it has been shown that the bone marrow stroma–derived CXC chemokine SDF-1 (stromal cell–derived factor-1) is important for B lymphopoiesis and myelopoiesis since SDF-$1^{-/-}$ mice are impaired for these functions (Nagasawa et al., 1996). Similarly, it is likely that chemokines act at different steps of T cell differentiation. Chemokines, together with the expression of appropriate adhesion molecules, may dictate the migration of uncommitted progenitors from the bone marrow to other anatomic locations. Indeed, SDF-1 is chemoattractant for human $CD34^+$ progenitor cells (Aiuti et al., 1997). The observation that TECK is chemoattractant for thymocytes but not for mature peripheral T cells suggests that TECK may attract T cell progenitors to the thymus. Such populations are very difficult to isolate in sufficient numbers to conduct in vitro chemotaxis experiments, but we are currently designing new strategies to address this question. In addition, we have not found significant chemotactic activity of TECK on bone marrow cells. SDF-1 was shown to be much less potent on $CD34^+$ progenitors from the peripheral blood than those from the bone marrow (Aiuti et al., 1997). It is possible that the sensitivity of progenitor cells to TECK would increase as these cells leave the bone marrow to colonize lymphoid organs. Of note, intrathymic maturation is also characterized by a directional migration from the subcapsular region, which contains the earliest progenitors to the cortex, and finally to the medulla, where thymocytes finish their maturation (Boyd et al., 1993). It is possible that the secretion of TECK by medullary dendritic cells may play a role in this directional migration. Yet another possibility is that TECK may play a role in the organization and development of the thymic stroma.

We also showed that TECK is chemotactic for activated macrophages and dendritic cells. These two cell types also play important roles in T cell development. Through a complex screening process involving positive

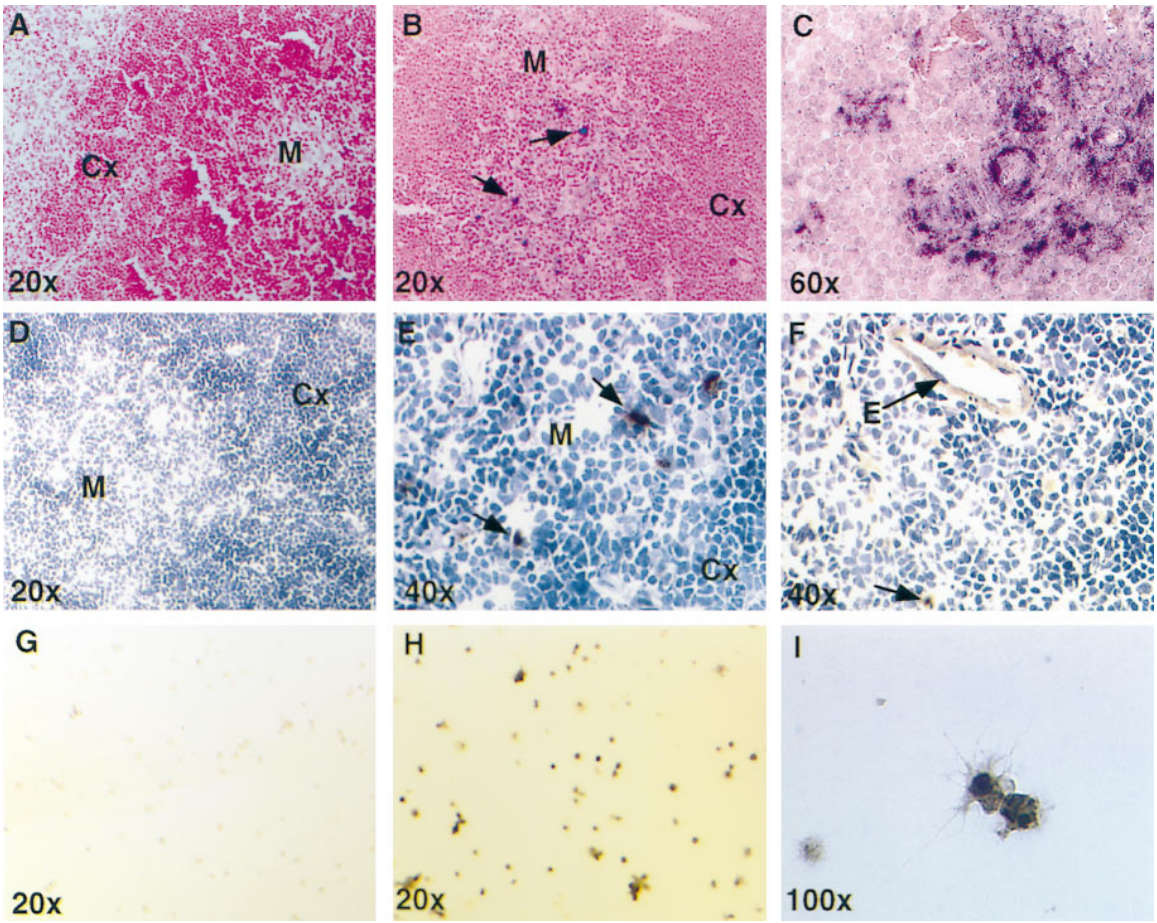

Figure 6. Expression of mTECK mRNA and Protein In Vivo

(A–C) Expression of mTECK mRNA by in situ hybridization. In situ hybridization was performed as described in Experimental Procedures. (A) Negative control sense probe. Cx, thymic cortex; M, medulla. (B) Antisense probe and nonlymphoid positive cells in the thymic medulla (arrows). (C) Antisense probe at a higher magnification.

(D–F) Expression of mTECK protein in the thymus. Intracellular staining for mTECK expression with a specific polyclonal antibody was performed as described in Experimental Procedures. (D) Staining with a control rabbit serum. (E) Staining with anti-mTECK polyclonal antibody showing positive nonlymphoid cells in the thymic medulla (arrows). (F) Staining with anti-mTECK polyclonal antibody at a higher magnification, showing expression by endothelial cells (arrow E).

(G–I) Expression of mTECK protein in thymic dendritic cells. Highly purified (>99%) dendritic cells were sorted by flow cytometry and stained with normal rabbit serum (G) or anti-TECK polyclonal antibody (H). A higher magnification (I) allows dendrites characteristic of dendritic cells and intracellular staining for mTECK to be distinguished.

and negative selection events, most of the antigenic specificities randomly generated in the thymus are eliminated by programmed cell death (Janeway, 1994). The efficient scavenging of dead thymocytes is probably mediated at least in part by thymic macrophages, and thus TECK may play an important role through its action on activated macrophages. Further along, T cells that have a high affinity for self-antigens and thus are potentially harmful are eliminated through negative selection (Janeway, 1994). It is believed that thymic dendritic cells are primarily responsible for the negative selection of thymocytes and therefore play a major role in the establishment of tolerance (Inaba et al., 1991). An efficient mechanism of central tolerance should eliminate T cells potentially reactive against autoantigens that are not expressed in the thymus, such as organ-specific autoantigens. Several known chemokines induce the migration of dendritic cells and may therefore contribute to their recruitment during peripheral immune responses (Sozzani et al., 1995; Xu et al., 1996). Similarly, dendritic cells presenting organ-specific or other antigens may be recruited to the thymus or the small intestine and induce negative selection of T cells specific for these antigens. It is possible that thymus- and small intestine–specific chemokines active on dendritic cells such as TECK play an important role in the establishment of tolerance. Thus, TECK could potentially interact at several important steps of T cell development. Future experiments will aim to define the precise role of TECK in T cell development and other physiological processes through the use of genetically modified mice.

TECK Is Specifically Expressed by Thymic Dendritic Cells

Dendritic cells represent an heterogeneous cell population derived from bone marrow progenitors. They are

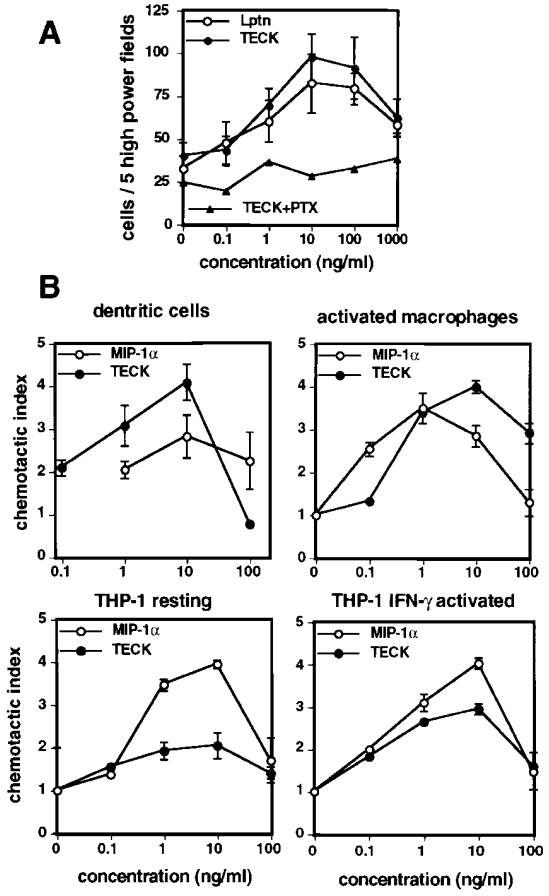

Figure 7. Chemotactic Properties of mTECK Recombinant Protein

(A) Migration of mouse thymocytes to recombinant mTECK and effect of pertussis toxin.

Chemotaxis assays were performed as described in Experimental Procedures. Recombinant mouse lymphotactin (Lptn) was used as a positive control. Data are expressed as the mean (± SEM) of cell counts obtained from three separate experiments in duplicate. In one experiment, cells were preincubated 1 hr with 10 ng/ml pertussis toxin (PTX) prior to the assay.

(B) Migration of other leukocyte subsets to recombinant mTECK

Mouse splenic dendritic cells and mouse activated macrophages were obtained as described in Experimental Procedures. THP-1 human monocytic cells were used without or with a 16 hr activation with IFNγ. Results are obtained as the mean (± SD) of the chemotactic index from three separate experiments per cell type in duplicate. The number of cells migrating to medium alone was greater than 40 cells per five high-power fields in each experiment. Recombinant MIP-1α was used as a positive control.

present in nonlymphoid organs as immature dendritic cells (such as Langerhans cells in the skin), where they display a high ability for antigen capture (Cella et al., 1997). Subsequent to antigen challenge, they migrate to secondary lymphoid organs and acquire a high capacity to present processed antigens to naive T cells to initiate a specific immune response (Cella et al., 1997). It has been shown that dendritic cells can derive from $CD34^+$ progenitors cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNFα (Caux et al., 1992; Caux et al., 1996) or from monocytes in the presence of GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). There is also evidence for a lymphoid dendritic cell precursor in thymus and bone marrow that is able to derive both lymphocytes and dendritic cells in the absence of GM-CSF (Ardavin et al., 1993; Galy et al., 1995; Marquez et al., 1995; Wu et al., 1996). These lymphoid-derived dendritic cells may have different functional properties such as a negative regulation of T cell responses, since they express FasL in the mouse (Suss and Shortman, 1996). We found that TECK was expressed at high levels in mouse thymic dendritic cells but was absent in cDNA libraries from mouse splenic dendritic cells or from human dendritic cells generated in vitro from $CD34^+$ precursors or monocytes. mTECK mRNA was present at a low level in a population of early thymocyte progenitors still able to derive dendritic cells (Wu et al., 1996). Thus, it would be tempting to suggest that TECK could be a specific marker of lymphoid-derived dendritic cells. However, we observed that TECK was absent from splenic dendritic cells that likely contain lymphoid-derived dendritic cells. The expression of TECK mRNA in the spleen of mice injected with LPS would suggest that peripheral dendritic cells may express TECK upon activation, but we found that TECK was not expressed in cDNA libraries of bone marrow–derived dendritic cells activated with LPS, phorbol myristate acetate (PMA), and ionomycin or IL-1α and TNFα. It is possible that the normal expression of TECK is specific for lymphoid-derived dendritic cells or, alternatively, that it is up-regulated by very specific stimuli present in the thymic and intestinal microenvironment under physiological conditions. Consistent with the latter hypothesis is our observation of specific staining of thymic endothelial cells with anti-TECK antibody, since we have not been able to find TECK expression in human HUVEC endothelial cells by Northern blot analysis, without activation or following a 16 hr activation with various combinations of IL-1, TNFα, IL-4, IL-7, and oncostatin (data not shown), while some of these stimuli induce the expression of other CC chemokines in endothelial cells (Rollins and Pober, 1991; Marfaing-Koka et al., 1995; Garcia-Zepeda et al., 1996a, 1996b). Taken together, our data strongly suggest that TECK is a novel chemokine specifically expressed by activated lymphoid-derived dendritic cells.

Through their function of antigen presentation, dendritic cells play major roles in the establishment of tolerance and in the initiation of an antigen-specific immune response. The use of purified dendritic cells has been recently proposed in different therapeutic protocols (Cella et al., 1997). The discovery of factors with a regulated expression in dendritic cells such as the novel CC chemokine TECK will improve our knowledge of the biology of dendritic cells and lead to the design of relevant in vivo applications.

Experimental Procedures

Mice and In Vivo Experimental Procedures

Four- to eight-week-old and time-pregnant BALB/c mice were purchased from Simonsen Laboratories (Gilroy, CA). RAG-1–deficient mice (Mombaerts et al., 1992) were purchased from The Jackson Laboratory. To analyze TECK expression after in vivo activation, various organs were recovered from pools of two mice 3 hr after intravenous LPS injection (50 μg of LPS in 200 μl of phosphate-buffered saline [PBS] or 200 μl of PBS for controls).

Cell Purification, Culture, and Stimulation

THP-1 cells (TIB-202 from the American Type Culture Collection, Rockville, MD) were cultured in complete medium that consisted in RPMI 1640 medium (JRH BioSciences, Lenexa, KS) supplemented with 10% fetal calf serum, 200 mM L-glutamine, 5×10^{-5} M mercaptoethanol, minimal essential medium amino acids and vitamins, sodium bicarbonate, penicillin, streptomycin (all from Sigma, St. Louis, MO), and gentamycin (Boehringer, Indianapolis, IN). To obtain activated mouse macrophages, 10 ml of cold PBS was injected into the peritoneum and the collected cells allowed to adhere to plastic for 24 hr in complete medium. The adherent fraction, mostly macrophages, was then collected. To obtain splenic dendritic cells, a splenocyte cell suspension was prepared in RPMI 1640 Dutch modified medium (Life Technologies, Paisley, Scotland) as described previously (Macatonia et al., 1987). Splenocytes were incubated at 37°C for 16 hr, and the cell suspension was collected and laid over Metrizamide (Nycomed Pharma, Oslo, Norway). After centrifugation for 10 min at 1700 × g, the low interface was collected and stained with anti-Mac-1 (Pharmingen, San Diego, CA) and the anti-CD11c N-418 antibodies (Macatonia et al., 1993). Splenic dendritic cells were sorted by flow cytometry on a FACStar plus cell sorter (Becton Dickinson, Mountain View, CA) to a purity greater than 98% upon reanalysis in all the experiments included in this report. To obtain thymic dendritic cells, thymi were cut in small fragments and resuspended in 10 ml of RPMI-1640 + 10% fetal calf serum containing 1 mg/ml collagenase and 0.02 mg/ml DNase I (both from Sigma) and digested with continuous agitation at room temperature for 30 min (Shortman et al., 1995). One milliliter of 0.1 M EDTA (pH 7.2) was added for an additional 5 min. Cells were then washed in complete medium, resuspended in complete medium, and overlaid onto Metrizamide. The thymic dendritic cell–enriched preparation was then stained with anti-IA^d and N-418 antibodies and the dendritic cells sorted by flow cytometry.

Molecular Cloning of Mouse and Human TECK

The cDNA encoding mouse TECK was obtained by random sequencing of a RAG-1 knockout mouse thymic directional cDNA library. In brief, mRNA was extracted using RNAzol B (Tel-Test, Friendswood, TX) and then oligotex-dT mRNA kit (Quiagen, Chatsworth, CA) following the manufacturer's instruction. A directional cDNA library was prepared using the Superscript Plasmid System (Gibco–BRL, Grand Island, NY) and cloned into the pME18s plasmid vector. Sequencing was done using the TaQ DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). To determine whether TECK was present in other mammals, including human, a Southern blot containing EcoRI-digested genomic DNA from different species (Bios Laboratories, New Haven, CT) was hybridized with the full-length mouse TECK cDNA.

The cDNA encoding human TECK was found by screening of a small intestine cDNA library using the full-length mouse TECK cDNA as a probe, following standard procedures.

Northern Blot Analysis of RNA and Southern Blot Analysis of cDNA Libraries

All RNAs were isolated from tissues or cells using RNAzol B (Tel-Test) and analyzed after electrophoresis in a 1% formaldehyde-agarose gel (10 μg/lane). RNAs were then blotted onto a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL). Some Northern blots of mRNA were bought from Clontech (Palo Alto, CA). To analyze the expression of TECK in cDNA libraries (obtained from T. MacClanahan, DNAX), 10 μg of DNA was digested with the appropriate restriction enzymes to release their inserts and analyzed by Southern blotting onto nylon membranes. Northern blots and blots of cDNA libraries were hybridized for 16 hr at 65°C with a ^{32}P-labeled probe consisting in the full-length cDNA encoding for mouse or human TECK and then washed and exposed, according to standard protocols.

Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57Bl/6J × M. spretus)F1 females and C57Bl/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Teck* locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization with the full-length mTECK cDNA probe were performed as described (Jenkins et al., 1982). Fragments of 7.5, 6.9, and 2.5 kb were detected in HincII-digested C57Bl/6J DNA, and fragments of 8.8 and 5.4 kb were detected in HincII digested M. spretus DNA. The presence or absence of the 8.8 and 5.4 kb HincII M. spretus–specific fragments, which cosegregated, was followed in backcross mice. A description of the probes and restriction fragment length polymorphisms for two of the loci linked to *Teck*, including *Insr*, has been provided previously (Ceci et al., 1990). Recombination distances were calculated as described (Green, 1981) using the computer program Spretus Madness.

Measurement of TECK mRNA Expression by RT-PCR

RNAs from sorted thymic dendritic cells or fetal thymi were prepared with the RNeasy total RNA kit (Quiagen, Chatsworth, CA), following the manufacturer's instructions. First-strand cDNAs were generated by reverse transcription with a random hexamer in a 10 μl reaction, and 1 μl of this reaction was used as a template for PCR. TECK expression was compared to the expression of hypoxanthine-guanine phosphoribosyl transferase (HPRT). Primer sequences were as follows: TECK: 5′ primer, 5′CCTTCAGGTATCTGGAGAGGAGATC3′ and 3′ primer, 5′CACGCTTGTACTGTTGGGGTTC3′; HPRT: 5′ primer, 5′GTAATGATCAGTCAACGGGGGAC3′ and 3′ primer, 5′CCAGCAAGCTTGCAACCTTAACCA3′. Samples were submitted to 25 cycles of amplification, each composed of 94°C for 1 min, 57°C for 30 s, and 72°C for 2 min. PCR products were then separated by electrophoresis in 2% agarose gels and stained with ethidium bromide.

In Situ Hybridization

Biotin-14-CTP–labeled sense and antisense riboprobes were generated using a nonradioactive RNA labeling system (Gibco, Gaithersburg, MD) and the plasmid PCRII (InVitrogen, Carlsbad, CA) containing a 400 bp TECK cDNA fragment inserted by PCR and cloned into the PCRII vector using the TA cloning kit (InVitrogen). Paraffin-embedded tissues were cut in 3–5 μm sections, mounted on slides, baked at 60°C for 1 hr, deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA), and immersed in 100% ethanol. Sections were then incubated for 10 min at 37°C in proteinase K solution (40 mg/ml) (Gibco) in PBS and rinsed for 2 min in PBS at room temperature before being refixed in 10% formalin (Fisher Scientific) in PBS for 1 min. Next, the sections were dehydrated through graded solutions of ethanol and air dried. Hybridization was carried out using the Gibco in situ hybridization and detection system kit. Vanadyl ribonucleoside complex (Gibco) was added to the hybridization solution (39 mM final). A 0.1 μg/ml concentration of each probe was used during an 18 hr hybridization at 42°C. Posthybridization washes used room temperature 0.2× sodium chloride/sodium citrate. Following detection and substrate visualization, the slides were counterstained with 1% nuclear red stain (Sigma, St. Louis, MO).

Immunohistochemistry

A polyclonal antibody specific of a synthetic decapeptide identical to the C-terminus part of murine TECK (Figure 1) was prepared in rabbits by Research Genetics (Huntsville, AL). Normal rabbit serum from a pool of 50 different animals (Research Genetics) was used as a negative control. To study TECK protein expression in the mouse thymus, 6 μm thick cryostat sections were thaw mounted on organosilicone subbed slides (American Histology Reagent, Stockton, CA) and fixed in 3% formalin (Fisher Scientific, Springfield, NJ) in Hank's balanced salt solution (HBSS) with 0.01 M HEPES (HBSS–HEPES) (pH 7.4), for 15 min at room temperature. The sections were sequentially blocked for endogenous biotin binding using the Vector blocking kit (Vector Laboratories, Burlingame, CA) and for endogenous peroxidase activity with a 1% hydrogen peroxide, 0.2 M sodium azide solution, in HBSS-HEPES with 0.1% saponin (staining buffer). Nonspecific antibody binding sites were then blocked with 10% normal goat serum (Sigma) in staining buffer. Sections prepared as above were first incubated for 18 hr at 25°C with 1:500 dilution of polyclonal antibody or control rabbit serum in staining buffer. In the second step, the sections were incubated for 1 hr at room temperature with biotin-labeled goat anti-rabbit IgG (2 μg/ml) (Vector Laboratories) in staining buffer and then for 30 min

at room temperature with the Vectastain Elite ABC Kit (Vector Laboratories) in staining buffer. The sections were then rinsed in HBSS-HEPES without saponin. Immunoenzyme tissue staining was revealed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (0.5 mg/ml) (Sigma) in 0.05 M Tris (pH 7.4) containing 0.0075% hydrogen peroxide. The substrate reaction was stopped by rinsing the sections in distilled water. The sections were then counterstained with Harris' hematoxylin (Shandon Lipshaw, Pittsburg, PA).

Production of Recombinant Mouse TECK in Escherichia coli and Other Chemokines

Mouse recombinant TECK was produced in E. coli as a N-terminal FLAG (DYKDDDDKL) fusion protein. In brief, the fusion construct containing FLAG followed by the mTECK sequence minus the leader peptide (Figure 1) was obtained by PCR amplification of the TECK cDNA to flank the coding sequence with HindIII and EcoRI sites and subsequent ligation in the pFLAG.1 vector, which contains the FLAG sequence and an OmpA signal sequence. Electrocompetent UT 4400 E. coli were transformed with the pFLAG.1-mTECK plasmid. The cells were grown in 2× Luria broth plus 50 μg/ml ampicillin, induced at an OD of 2.3 with 400 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested. The cell pellet was resuspended in cold lysis buffer (20 mM Tris [pH 8], 2 mM EDTA, 20% sucrose, 0.1 mg/ml lysozyme, 100 μl benzonase), homogenized, and allowed to sit for 30 min. Then the same amount of a 1:4 dilution of cold lysis buffer without lysozyme was added for an additional 10 min. The solution was spun, and the supernatant was filtered through a 0.2 μm membrane and then diluted 1:1 in 50 mM Tris (pH 7.5). The diluted osmotic extract was submitted to chromatography on a Q-sepharose column equilibrated with 50 mM Tris (pH 7.5) and eluted with a linear salt gradient. The fractions containing the recombinant protein were pooled. The fractions were then loaded onto a S-sepharose column equilibrated with 20 mM acetate (pH 4.0). The column was eluted with a linear salt gradient and then with a 1.5 M NaCl wash that contained the protein. Finally, the eluate was loaded onto a reverse phase column. The column was eluted with a linear gradient of 20%–80% acetonitrile + 0.1% trifluoroacetic acid. The concentration of the mTECK protein was estimated by Comassie blue staining and densitometric scanning of a 10% Nu–polyacrylamide gel electrophoresis gel with lysozyme as a standard. The purity was estimated at 100% by sequencing of the N-terminus of the recombinant protein. Recombinant murine MIP-1α (R and D Systems, Minneapolis, MN) and lymphotactin (Hedrick et al., 1997) were used as controls.

Assay for Chemotaxis

The in vitro migration of cells isolated as described above in response to TECK or other factors was assessed in a modified Boyden micro chamber (Neuroprobe, Cabin John, MD) as described previously (Kelner et al., 1994). In brief, factor dilutions in Dulbecco's modified Eagle's medium (DMEM) (Gibco) were loaded in the lower compartment in duplicate, and 10^5 cells in a 50 μl volume of DMEM were loaded in the upper compartment. The two compartments were separated by a 5 or 8 μm pore polycarbonate filter (Nucleopore, Pleasanton, CA). After incubation at 37°C for 80 min (or 120 min for lymphocytes), the filters were fixed in methanol and stained with fields A and B. Cell migrated on the other side of the membrane were counted per five high-power fields (100×) under a microscope. The chemotactic index was calculated from the number of cells counted with the test sample divided by the number of cells counted with medium alone.

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