Human naive CD8 T cells down-regulate expression of the WNT
pathway transcription factors lymphoid enhancer binding factor
1 and transcription factor 7 (T cell factor-1) following antigen
encounter in vitro and in vivo

Citation for published version:
CD8 T cells down-regulate expression of the WNT pathway transcription factors lymphoid enhancer binding
factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo' Journal of
Immunology, vol 176, no. 3, pp. 1439-46.

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Journal of Immunology

Publisher Rights Statement:
© 2006 by The American Association of Immunologists, Inc.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s)
and / or other copyright owners and it is a condition of accessing these publications that users recognise and
abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer
content complies with UK legislation. If you believe that the public display of this file breaches copyright please
contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and
investigate your claim.
Human Naive CD8 T Cells Down-Regulate Expression of the WNT Pathway Transcription Factors Lymphoid Enhancer Binding Factor 1 and Transcription Factor 7 (T Cell Factor-1) following Antigen Encounter In Vitro and In Vivo

Tim Willinger, Tom Freeman, Mark Herbert, Hitoshi Hasegawa, Andrew J. McMichael and Margaret F. C. Callan

*J Immunol* 2006; 176:1439-1446; http://www.jimmunol.org/content/176/3/1439

References This article cites 32 articles, 13 of which you can access for free at: http://www.jimmunol.org/content/176/3/1439.full#ref-list-1

Subscriptions Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscriptions

Permissions Submit copyright permission requests at: http://www.aai.org/ji/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/cgi/alerts/etoc
The differentiation of T lymphocytes is mainly controlled by signals through their Ag-specific TCR. In the thymus, TCR signals regulate the development of a mature T cell repertoire via positive and negative selection. In the periphery, naïve T cells (T_N) further differentiate into effector and memory T cells when recognizing foreign Ags derived from infectious pathogens via their TCR (1). Apart from TCR signals and cytokines, other factors contributing to peripheral T cell differentiation are not well characterized at present.

In addition to TCR signals, major developmental pathways such as the WNT, Notch, and Hedgehog signaling pathways influence T cell development (2–4). Interestingly, there is recent evidence that these major developmental pathways also control the differentiation of peripheral T cells. Thus, Notch regulates the decision of CD4 T cells between the Th1 vs the Th2 fate (5), while the Hedgehog pathway can influence the proliferation and cytokine production of peripheral T cells (6, 7). In contrast, it is currently unknown whether the WNT pathway has any role in mature T cells.

The canonical WNT signaling pathway is a critical regulator of stem cell function, e.g., it controls the maintenance and self-renewal of hemopoietic stem cells (8–11). Furthermore, dysregulation of the WNT pathway commonly occurs in human cancers (11). In the absence of a WNT signal, cytoplasmic β-catenin is phosphorylated and targeted for degradation by the proteasome (3, 11). WNT signaling allows β-catenin to escape proteasomal degradation and to translocate to the nucleus. In the nucleus, β-catenin interacts with members of the lymphoid enhancer binding factor (LEF)/T cell factor (TCF) family of transcription factors (12). Importantly, these truncated isoforms can function in a regulatory full-length isoform, there are N-terminally truncated isoforms (12). In addition to the stimulatory full-length isoforms, there are N-terminally truncated isoforms with alternative C-termini (termed tails) known as N- or B-tailed isoforms. Currently, very little is known about the different functional properties of these multiple LEF1 and TCF7 isoforms with alternative C-termini (termed tails) known as N- or B-tailed isoforms. Currently, very little is known about the different functional properties of these multiple LEF1 and TCF7 isoforms.
Knockout and transgenic studies in mice have clearly shown a redundant and β-catenin-dependent role of LEF1 and TCF7 (TCF-1) in T cell development (3). TCF7 (TCF-1)−/− mice have impaired T cell development with a partial block at the intermediate single-positive to double-positive transition due to reduced thymocyte proliferation and survival (22–24). Although T cell development is normal in LEF1−/− mice, B cell development is impaired (25). Thymocytes from TCF7 (TCF-1)−/− LEF1−/− mice show a profound block at the intermediate single-positive stage with neither double-positive nor single-positive thymocytes present, and consequently no mature T cells in the periphery (26). Taken together, the WNT-β-catenin-LEF1/TCF7 (TCF-1) axis plays a pivotal role in T cell development. However, it is unknown whether LEF1, TCF7 (TCF-1), and the WNT pathway have a specific function in peripheral T cells.

Therefore, we undertook a detailed analysis of the expression of LEF1 and TCF7 (TCF-1) in human peripheral T cells. We found that both LEF1 and TCF7 (TCF-1) are expressed in mature CD8+ T cells and that their expression is down-regulated following TCR or IL-15R engagement in vitro and Ag encounter in vivo. Furthermore, T cell activation changed the balance of stimulatory vs inhibitory LEF1 and TCF7 (TCF-1) isoforms. Our results suggest that the WNT pathway, in addition to its well-known role in T cell development, is likely to be involved in regulating peripheral T cell differentiation.

Materials and Methods
Isolation of CD8 T cell subsets
For microarray and quantitative RT-PCR (qRT-PCR) experiments, CD8 T cell subsets were isolated from healthy donors in accordance with institutional ethics approval as previously described (27). CD8 T cells were sorted into either TN (CCR7+/CD45RA−), N-terminally derived memory RA T cells (TEM:CCR7−/CD45RA+), central memory T cells (TCM:CCR7−/CD45RA−), and effector memory RA T cells (TEMRA:CCR7−/CD45RA+) (microarray data set 1), or T cells (CCR7+/CD45RA+), central memory T cells (TCM:CCR7−/CD45RA−), TEm (CCR7−/CD45RA−), and TEMRA (CCR7−/CD45RA+) populations (microarray data set 2).

Microarray gene expression analysis
RNA extraction and labeling was performed as previously described (27, 28). For microarray data set 1, total RNA was isolated from several donors (replicate pool 1: n = 10; replicate pool 2: n = 6) and hybridized to Affymetrix HG-U95Av2 arrays. Two independent microarray experiments were performed with RNA from CD8+ T cells and TEMRA for data set 1. For microarray data set 2 (described in Ref. 27), total RNA from individual donors was used and hybridized to Affymetrix HG-U133 plus 2.0 arrays (Affymetrix). Four independent microarray experiments were performed with RNA from CD8+ T cells, TEM, and TEMRA for data set 2. We used GCOS software (Affymetrix) and the software package BRB-ArrayTools for data analysis and the identification of differentially expressed genes between CD8 T cell subsets (27).

qRT-PCR analysis
qRT-PCR was conducted on cDNA from the indicated CD8+ T cell populations with the 5′ nuclear TaqMan assay. Briefly, we prepared cDNA from 1 μg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies). We then performed quantitative PCR in a final volume of 25 μl with 300 nM of the forward and reverse primers and 100–250 nM of the fluorogenic TaqMan probes (Eurogentec) using 2× quantitative PCR Mastermix Plus (Eurogentec). Reactions were run on an ABI Prism 7700 Sequence Detection System machine (Applied Biosciences) in triplicate (initial steps: 50°C/2 min and 95°C/10 min, followed by 40 cycles: 95°C/15 s and 60°C/1 min). The following primers and probes were used: LEF1: forward, TGA CAGTGCCTTCATACCTGAAAC; reverse, GCTGCCTTTGCTTTCCTGAC; probe: FAM-TTTCACAGAAGCTCATTCCCA-TAMRA. TCF7 (TCF-1) isoforms 2, reverse, GCTGCCTTTGCTTTCCTGAC; probe: FAM-TTTCACAGAAGCTCATTCCCA-TAMRA. All probes span exon-intron junctions. We applied the comparative threshold cycle method for relative quantification of mRNA expression according to the manufacturer’s recommendations. Validation experiments demonstrated that the amplification efficiencies of LEF1 and TCF7 (TCF-1) were equal to that of the endogenous control hypoxanthine phosphoribosyltransferase.

Intracellular FACS staining
We analyzed intracellular expression of LEF1 and TCF7 (TCF-1) in CD8 T cell subsets using the methanol permeabilization protocol as previously described (27). LEF1 and TCF7 (TCF-1) were detected by indirect staining using pretitrated mAbs REMB6 (OncoGene Research Products) and TH3 (Upstate Biotechnology), respectively. Briefly, 2 μg of primary mAb was added to 2 × 106 cells and incubated for 1 h at room temperature. After washing, this was followed by staining with PE-conjugated rabbit anti-mouse Ab (DakoCytomation) for 1 h at room temperature. Cells were subsequently washed in blocking buffer (PBS containing 2% mouse serum) before surface staining with directly conjugated mAbs specific for CD62 ligand (CD62L), CD45RA, and CD8 (all BD Biosciences). CD62L was used instead of CCR7 as a surface marker because CCR7 staining was compromised following methanol permeabilization (27).

Stimulation of cord blood (CB) CD8 T cells in vitro
We obtained CB samples from the John Radcliffe Hospital maternity unit, upon written consent and approval by the local Medical Ethics Committee. CB CD8 T cells were isolated by immunomagnetic selection as described above. Phenotyping was conducted using mAbs specific for CCR7 (R&D Systems), CD45RA (BD Biosciences), CD8 (BD Biosciences), CD3, CD25, and HLA class II (all DakoCytomation). We stimulated CB CD8 T cells (1 × 106) with either plate-bound anti-CD3 mAb OKT3 (1 μg/well), IL-15 (50 ng/ml), or TGFβ1 (3 ng/ml) in 24-well plates for the indicated time points. All cytokines were from obtained R&D Systems. Cells were cultured in complete medium (RPMI 1640 supplemented with 10% FCS, 1% sodium pyruvate, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in 5% CO2. Alternatively, CB T cells (1 × 106) were cocultured with irradiated allogeneic EBV-transformed B cells at a 1:1 ratio. From day 3 onward, CD8 cells represented >95% of live cells in these cocultures, i.e., the stimulator B cells had practically disappeared.

Cloning of LEF1 mRNA isoforms from primary CD8 T cells
Total RNA was extracted from primary CD8 T cells using TRI Reagent (Sigma-Aldrich) and cDNA was prepared as described above for the qRT-PCR experiments. RT-PCR was performed with the following primers: forward primers, CAGCGGAGCTCGATTACAGAG (full-length isoforms) and ACTCGAGCTCTTCCGGGTACATAATG (ΔCTNNB isoforms); reverse primers, CTTCGAAATTCCACATTGCTAGT (N-terminal isoforms) and GTGACATGTTCCCGTGAC (B-terminal isoforms). PCR products were cloned into the vector pRES2-EGFP (BD Clontech) followed by DNA sequencing.

Western blot analysis
We prepared protein lysates from 3 to 5 × 106 CD8+ T cells by washing the cells in PBS and resuspending them in an equal volume of 2× sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 8% 2-ME, 0.2% bromphenol blue, and 20% glycerol). After sonication for 2–5 s and boiling for 5–10 min, protein samples were separated by SDS-PAGE and gels blotted to nitrocellulose membranes. We performed immunodetection of LEF1 and TCF7 (TCF-1) with the REMB6 mAb (Exalpha) at 1/5000 dilution and with the TH3 mAb (Upstate Biotechnology) at 1/1000 dilution, respectively. This was followed by incubation with secondary HRP-conjugated anti-mouse Ig (DakoCytomation) and signal detection with ECL reagent (BD Amersham). Blots were stripped by incubating the membrane at 50°C for 30 min in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2.5% SDS, and 100 mM 2-ME) and reprobed with anti-β-actin mAb AB-15 at a 1/5000 dilution (Sigma-Aldrich).

Statistical analysis
A two-sample, two-tailed t test assuming unequal variances was used to determine the significance of differences in mRNA expression between the two groups (α = 0.05). For multigroup comparisons, we applied one-way ANOVA with post hoc testing using Tukey’s significant difference test (α = 0.05).
Results

CD8+ T_N down-regulate expression of LEF1 and TCF7 (TCF-1) upon Ag encounter in vivo

We used microarray technology to screen for genes that are differentially expressed between human T_N and Ag-primed CD8 T cells. Our first, exploratory, microarray data set compared gene expression in purified CD8+ T_N (CCR7+/CD45RA+) and CD8+ T_EM/EMRA (CCR7-CD45RA-) populations using RNA pooled from several donors. We also generated a more detailed second data set that analyzed the gene expression profiles of CD8+ T_N (CCR7+/CD45RA+) in relation to that of CD8+ T_EM (CCR7-CD45RA-) and T_EMRA (CCR7-CD45RA-) subsets. Technical advances allowed us to use RNA from individual donors instead of pooled RNA and to perform a greater number of replicate experiments (four instead of two) for the second data set. Furthermore, gene chips (HG-U133 Plus 2.0 instead of HG-U95Av2) with a greater number of probes and greater coverage of the human genome were available for this data set. Using this data set, we have previously investigated the molecular relationships between TN and memory (Ag-primed) CD8 T cell subsets and the molecular basis for their different functional properties (27). We now aimed to examine selected differentially expressed genes in more detail to gain further insight into the molecular basis of CD8 T cell behavior.

We identified LEF1 as a gene highly expressed in T_N, compared with Ag-primed CD8 T cell subsets. In both microarray data sets, LEF1 was found to be in the top three most differentially expressed genes when comparing the different mature CD8 T cell populations (Table I). Indeed, within data set 2, probe identifications for LEF1 accounted for two of the top three differentially expressed genes. LEF1 mRNA levels were 5- to 10-fold higher in T_N, compared with the three Ag-experienced subsets, with T_EM expressing LEF1 more strongly than T_EMRA and T_EMRA (Fig. 1A). Interestingly, we found that TCF7 (TCF-1) was also expressed at higher levels in T_N, compared with T_EMRA and T_EMRA (Fig. 1B).

qRT-PCR experiments confirmed the differential expression of LEF1 and TCF7 (TCF-1) in T_N and Ag-primed CD8 T cell subsets (Fig. 1). We also examined LEF1 and TCF7 (TCF-1) protein expression in peripheral CD8 T cell subsets by intracellular flow cytometry. Importantly, as shown in Fig. 2, T_N (and T_EMRA) expressed significantly higher levels of both LEF1 and TCF7 (TCF-1) protein than T_EM and T_EMRA (p < 0.05, one-way ANOVA/Tukey's post hoc test). In summary, we found that the WNT pathway effectors LEF1 and TCF7 (TCF-1) are expressed in peripheral T cell subsets and that LEF1 is the most differentially expressed transcription factor between T_N and Ag-primed CD8 T cell subsets.

Expression of LEF1 and TCF7 (TCF-1) in CD8+ T_N is down-regulated by TCR signals and IL-15 in vitro

We studied the regulation of LEF1 mRNA expression in peripheral CD8 T cells in vitro by qRT-PCR. We observed down-regulation of LEF1 mRNA expression in CD8 T cells in response to TCR triggering (Fig. 3A). This down-regulation was rapid (within 12 h) and persisted for >48 h. Stimulation with homeostatic cytokines, such as IL-15, also led to a persistent decrease in LEF1 mRNA levels (Fig. 3B), with IL-2 having a similar effect (data not shown). In contrast, stimulation with TGFβ1 increased LEF1 expression in CD8 T cells, compared with the medium control (Fig. 3C).

To investigate the expression of LEF1 and TCF7 (TCF-1) in CD8+ T_N, we used CD8 T cells isolated from CB. CB CD8 T cells have a predominant CCR7+CD45RA- phenotype similar to adult T_N (Fig. 4A) with some CCR7+CD45RA- cells also present. Importantly, it has been shown that CB T cells (including the CCR7+CD45RA- subset) are functionally naive (29). CB CD8 T cells showed strong up-regulation of activation markers such as CD25 (IL-2Rα; Fig. 4A) and HLA class II (data not shown) in response to TCR (allogeneic) stimulation. At later time points, poststimulation CD8 T cells converted back to a resting state as shown by the absence of activation marker expression (Fig. 4A). Similar to bulk peripheral CD8 T cells, we observed down-regulation of LEF1 mRNA expression in CD8+ T_N (from CB) following TCR stimulation as measured by qRT-PCR (Fig. 4B). Importantly, at the time points examined (more than day 3) allogeneic stimulator B cells had practically disappeared from the T cell-B cell cocultures. Interestingly, after the initial down-regulation, there was a progressive increase in LEF1 mRNA expression at

---

Table I. LEF1 is among the 10 most differentially expressed genes between T_N and Ag-primed CD8 T cell subsets

<table>
<thead>
<tr>
<th>No.</th>
<th>GenBank Identification</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>T_N</th>
<th>T_EMRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microarray data set 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AB018295</td>
<td>NY-REN-7</td>
<td>NY-REN-7 Ag</td>
<td>93.0</td>
<td>6.6</td>
</tr>
<tr>
<td>2</td>
<td>M95178</td>
<td>ACTN1</td>
<td>Actin α</td>
<td>376.7</td>
<td>29.4</td>
</tr>
<tr>
<td>3</td>
<td>AL049409</td>
<td>LEF1</td>
<td>Lymphoid enhancer-binding factor-1</td>
<td>853.2</td>
<td>65.3</td>
</tr>
<tr>
<td>4</td>
<td>D90144</td>
<td>CCL3</td>
<td>MIP-1 α</td>
<td>66.4</td>
<td>491.0</td>
</tr>
<tr>
<td>5</td>
<td>U11276</td>
<td>KLRB1</td>
<td>CD161</td>
<td>232.5</td>
<td>1155.1</td>
</tr>
<tr>
<td>6</td>
<td>L31584</td>
<td>CCR7</td>
<td>CCR7</td>
<td>1385.7</td>
<td>238.6</td>
</tr>
<tr>
<td>7</td>
<td>X76220</td>
<td>MAL</td>
<td>T lymphocyte maturation-associated protein</td>
<td>1242.9</td>
<td>106.9</td>
</tr>
<tr>
<td>8</td>
<td>AJ223603</td>
<td>C4A4</td>
<td>GPI-anchored metastasis-associated protein homolog</td>
<td>367.0</td>
<td>41.2</td>
</tr>
<tr>
<td>9</td>
<td>AA478904</td>
<td>KLF7</td>
<td>Kruppel-like factor-7</td>
<td>189.2</td>
<td>38.0</td>
</tr>
<tr>
<td>10</td>
<td>X02910</td>
<td>TNF</td>
<td>TNF-α</td>
<td>93.9</td>
<td>704.7</td>
</tr>
<tr>
<td>Microarray data set 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AA992805</td>
<td>LEF1</td>
<td>Lymphoid enhancer-binding factor-1</td>
<td>421.5</td>
<td>52.4</td>
</tr>
<tr>
<td>2</td>
<td>X76220</td>
<td>ACTN1</td>
<td>Actin, α</td>
<td>928.9</td>
<td>47.4</td>
</tr>
<tr>
<td>3</td>
<td>AF294627</td>
<td>LEF1</td>
<td>Lymphoid enhancer-binding factor-1</td>
<td>1666.5</td>
<td>208.2</td>
</tr>
<tr>
<td>4</td>
<td>NM_014795</td>
<td>ZFHX1B</td>
<td>Zinc finger homeobox 1b</td>
<td>20.8</td>
<td>127.0</td>
</tr>
<tr>
<td>5</td>
<td>NM_006144</td>
<td>GZMA</td>
<td>Granelyme A</td>
<td>98.3</td>
<td>503.9</td>
</tr>
<tr>
<td>6</td>
<td>AI636759</td>
<td>SLC15A4</td>
<td>Solute carrier family 15, member 4</td>
<td>108.3</td>
<td>191.6</td>
</tr>
<tr>
<td>7</td>
<td>AI356412</td>
<td>LYN</td>
<td>v-yes-1 Yamaguchi sarcoma viral-related oncogene homolog</td>
<td>22.0</td>
<td>23.6</td>
</tr>
<tr>
<td>8</td>
<td>BC004344</td>
<td>CTSC</td>
<td>Cathepsin C</td>
<td>1181.2</td>
<td>240.1</td>
</tr>
<tr>
<td>9</td>
<td>AI246687</td>
<td>CTSC</td>
<td>Cathepsin C</td>
<td>286.7</td>
<td>529.8</td>
</tr>
<tr>
<td>10</td>
<td>NM_002835</td>
<td>PTPN12</td>
<td>Protein tyrosine phosphatase, non-receptor type 12</td>
<td>65.3</td>
<td>87.5</td>
</tr>
</tbody>
</table>

* Listed are the 10 most differentially expressed genes between CD8 T cell subsets. Mean expression values (data set 1: n = 2; data set 2: n = 4) are shown.
later time points poststimulation, although it varied between donors. A further set of experiments corroborated these results: in addition to TCR triggering, homeostatic signals such as IL-15 also decreased LEF1 and TCF7 (TCF-1) mRNA expression in CD8^+ T_N from CB (Fig. 4C). The level of LEF1 down-regulation (5- to 10-fold) was similar to that observed when comparing T_N and CD8 T cells primed with Ag in vivo (Fig. 1A). Thus, the signals that control mature CD8 T cell differentiation, i.e., TCR triggering and homeostatic cytokines, also regulate the expression of LEF1 and TCF7 (TCF-1) in CD8^+ T_N.

TCR and IL-15 stimulation of peripheral CD8 T cells lead to a relative decrease in inhibitory LEF1 and TCF7 (TCF-1) isoform expression

Several LEF1 and TCF7 (TCF-1) isoforms with different functional properties have been described (13–21), but which of these different isoforms are expressed in CD8 T cells is unknown. Therefore, we analyzed LEF1 and TCF7 (TCF-1) isoform expression in mature T cells. First, cloning of LEF1 mRNA isoforms by RT-PCR and DNA sequencing showed that peripheral CD8 T cells express both stimulatory full-length and inhibitory LEF1 mRNA isoforms (Fig. 5A). Furthermore, we found that CD8 T cells preferentially express N-tail LEF1 mRNA isoforms, although isoforms carrying a B-tail could also be detected (Fig. 5B). These mRNA isoforms were distinguished by the presence (N-tail isoforms) or absence (B-tail isoforms) of exon 11 (16). The predominance of N-tail isoforms applied to both full-length and CTNNB LEF1 mRNA isoforms (Fig. 5B).

Second, we investigated LEF1 and TCF7 (TCF-1) protein isoform expression by Western blot. Jurkat cells, a transformed immature T cell line with a high proliferative capacity, predominantly expressed stimulatory full-length LEF1 isoforms of 48–55 kDa (Fig. 6A) as previously reported (17). In contrast, primary resting CD8 T cells showed predominant expression of a 38-kDa LEF1 protein isoform, although isoforms carrying a B-tail could also be detected (Fig. 5B). These mRNA isoforms were distinguished by the presence (N-tail isoforms) or absence (B-tail isoforms) of exon 11 (16). The predominance of N-tail isoforms applied to both full-length and CTNNB LEF1 mRNA isoforms (Fig. 5B).

Several LEF1 and TCF7 (TCF-1) isoforms with different functional properties have been described (13–21), but which of these different isoforms are expressed in CD8 T cells is unknown. Therefore, we analyzed LEF1 and TCF7 (TCF-1) isoform expression in mature T cells. First, cloning of LEF1 mRNA isoforms by RT-PCR and DNA sequencing showed that peripheral CD8 T cells express both stimulatory full-length and inhibitory LEF1 mRNA isoforms (Fig. 5A). Furthermore, we found that CD8 T cells preferentially express N-tail LEF1 mRNA isoforms, although isoforms carrying a B-tail could also be detected (Fig. 5B). These mRNA isoforms were distinguished by the presence (N-tail isoforms) or absence (B-tail isoforms) of exon 11 (16). The predominance of N-tail isoforms applied to both full-length and CTNNB LEF1 mRNA isoforms (Fig. 5B).

Second, we investigated LEF1 and TCF7 (TCF-1) protein isoform expression by Western blot. Jurkat cells, a transformed immature T cell line with a high proliferative capacity, predominantly expressed stimulatory full-length LEF1 isoforms of 48–55 kDa (Fig. 6A) as previously reported (17). In contrast, primary resting CD8 T cells showed predominant expression of a 38-kDa LEF1 protein isoform (Fig. 6A). It has previously been demonstrated that this 38-kDa LEF1 band corresponds to the CTNNB LEF1 isoform that has a dominant-negative function in the WNT signaling pathway (17, 18). The predominance of inhibitory LEF1 protein isoforms also applied to resting CD8^+ T_N from CB (Fig. 6B, lane 1). In line with our mRNA expression results (Fig. 4), there was an overall down-regulation of LEF1 protein expression following TCR triggering or stimulation with IL-15 in vitro (Fig. 6B, lanes 2 and 3).
Similar to LEF1, we observed differences in the stimulatory vs inhibitory TCF7 (TCF-1) isoform balance between primary T cells and transformed Jurkat T cells. Jurkat cells showed predominant expression of stimulatory full-length TCF7 (TCF-1) isoforms of 42–48 kDa (Fig. 7A). In contrast, the ratio of stimulatory full-length (42–48 kDa)/inhibitory CTNNB (26–32 kDa) TCF7 (TCF-1) isoforms (14, 15) was about equal in resting (Fig. 7A) and CD8+ T cells (from CB; Fig. 7B, lane 1). Clevers and colleagues (15) have previously demonstrated that the 26- to 32-kDa bands correspond to CTNNB TCF7 (TCF-1) isoforms that have a dominant-negative function (19, 20). Interestingly, TCR and IL-15 stimulation of CD8+ T cells (from CB) lead to preferential down-regulation of the inhibitory TCF7 (TCF-1) isoforms following activation. In conclusion, our data suggest that the negative effects of inhibitory LEF1 and TCF7 (TCF-1) isoforms prevail in resting CD8 T cells. In activated cells this negative effect seems to be relieved by down-regulation of overall LEF1 protein expression and by specific down-regulation of inhibitory TCF7 (TCF-1) isoform expression.

**Discussion**

Ag-experienced T cells are better able than TNa to respond to Ag. This reflects both an increase in their frequency and a change in their state of differentiation. The molecular mechanisms that underpin the changes in cellular state and hence the development of T cell memory remain poorly understood. The capacity of transcription factors to modulate many different aspects of T cell function makes them an attractive candidate for study in this respect. In two separate microarray studies, we observed that the Wnt pathway effector, LEF1, was expressed at higher levels in TNa, compared with Ag-experienced CD8 T cells. Indeed, in both studies, it proved to be one of the most differentially expressed genes and the most differentially expressed transcription factor between these two populations of cells. A second effector of the same pathway, TCF7 (TCF-1) was also differentially expressed.

The differential expression of these molecules in the different CD8 T cell subsets proved to be consistent and robust; it was confirmed at the level of mRNA using qRT-PCR and at the level of protein using intracellular staining in many different donors.
Furthermore, in a longitudinal study in an in vitro system, we were able to show that stimulation of Tsc, by alloantigen, led to a down-regulation/partial recovery of LEF1 expression upon activation. Interestingly, it has been shown that IL-15 and TCR stimulation induce very similar changes in gene expression in human CD8 T cells (30). This would suggest that IL-15 and TCR stimulation probably activate common signaling pathways, and this could also apply to the regulation of LEF1/TCF7 (TCF-1) expression in CD8 T cells. Furthermore, one recent study reported that another IL-2 family member, IL-7, can also inhibit LEF1 and TCF7 (TCF-1) expression (31). In contrast, we found that TGFβ1, which is known to inhibit T cell differentiation and maintain T cell quiescence (32), increased LEF1 expression. Interestingly, we noted a partial recovery of LEF1 expression in CD8 T cells in vitro when the cells converted back to a resting state following Ag stimulation. Similarly, one murine microarray study demonstrated initial down-regulation/partial recovery of LEF1 expression upon naive → effector → memory CD8 T cell differentiation in vivo (33).

The observed correlations between expression of LEF1 and TCF7 (TCF-1) and T cell naivety and quiescence were not consistent with published data showing that LEF1 and TCF7 (TCF-1) are able to drive cellular proliferation. However, this paradox was resolved by additional experiments that analyzed the expression of the different LEF1 and TCF7 (TCF-1) isoforms. Our work shows that, compared with Jurkat cells, resting CD8 T cells express relatively more of the inhibitory LEF1 and TCF7 (TCF-1) protein isoforms. T cell stimulation results in down-regulation of this inhibitory isoform. Importantly, it has previously been shown that although stimulatory full-length LEF1 and TCF7 (TCF-1) isoforms drive the proliferation of Jurkat T cells, dominant-negative ΔCTNNB isoforms inhibit proliferation (20). Furthermore, colon cancer cells predominantly express full-length LEF1 isoforms while down-regulating the expression of ΔCTNNB isoforms (17). Finally, inhibitory ΔCTNNB isoforms are the most abundant TCF7 (TCF-1) isoforms in the intestine and TCF7 (TCF-1)−/− mice develop intestinal and mammary adenomas (19). Thus, it is been suggested that the balance between stimulatory and inhibitory
LEF1 and TCF7 (TCF-1) is a checkpoint for cellular proliferation in the context of malignancy.

In a conceptually similar way, the experiments we describe lead us to formulate the hypothesis that the balance between stimulatory and inhibitory LEF1 and TCF7 (TCF-1) isoforms represent a checkpoint for the quiescence of peripheral T cells. Direct evidence for this will require future studies, in which expression of individual LEF1 and TCF7 (TCF-1) isoforms or combinations of individual isoforms is manipulated in primary mature T cells. The functional redundancy between LEF1 and TCF7 (TCF-1) and the presence of numerous isoforms will make such experiments difficult to design and perform. Consistent with this, in preliminary experiments, we did not find a clear phenotype when knocking down total LEF1 (i.e., all isoforms) by RNA interference in human peripheral T cells. Studies in knockout and transgenic mice are probably more suited to address the role of the WNT pathway in peripheral T cells. Although the conditional knockout of individual LEF1 and TCF7 (TCF-1) isoforms will be challenging.

In conclusion, our study identifies LEF1 as the most differentially expressed transcription factor between T\(\text{N}\) and Ag-experienced CD8 T cells. It shows that, compared with a Jurkat cell line, CD8\(^+\) T\(\text{N}\) express more of the inhibitory isoform of this and another TCF7 (TCF-1) member of the WNT signaling pathway. We provide evidence that down-regulation of these inhibitory isoforms is associated with T cell stimulation. Our results suggest that the WNT pathway may have a specific function not only in immature, but also in mature T cells and provide a strong rationale for further molecular studies aimed at directly investigating the functional importance of individual isoforms of members of the WNT-LEF1/TCF7 (TCF-1) signaling pathway in peripheral T cell differentiation.

Acknowledgments
We thank Reg Boone, Manuela Herber, and Ann Atzberger for expert cell sorting, as well as Laurie Scott and Hayley Wolfenden of the Leukaemia Research Fund Microarray Facility for assistance in performing the GeneChip analysis.

Disclosures
The authors have no financial conflict of interest.

References


