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LST1: A Gene with Extensive Alternative Splicing and Immunomodulatory Function[1]

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The gene of the leukocyte-specific transcript (LST1) is encoded within the TNF region of the human MHC. The *LST1* gene is constitutively expressed in leukocytes and dendritic cells, and it is characterized by extensive alternative splicing. We identified 7 different LST1 splice variants in PBMC; thus, 14 LST1 splice variants (LST1/A-LST1/N) have been detected in various cell types. These isoforms code for transmembrane as well as soluble LST1 proteins characterized by two alternative open reading frames at their 3′ end. We demonstrate the presence of the transmembrane variant LST1/C on the cell surface of the monocytic cell lines U937 and THP1. Recombinant expression of LST1/C permitted its profound inhibitory effect on lymphocyte proliferation to be observed. In contrast, the alternative transmembrane variant LST1/A, the extracellular domain of which shows no amino acid sequence homology to LST1/C exerted a weaker but similar inhibitory effect on PBMC. These data demonstrate the protein expression of LST1 on the cell surface of mononuclear cells, and they show an inhibitory effect on lymphocyte proliferation of two LST1 proteins although they have only a very short amino acid homology. *The Journal of Immunology,* **2000, 164: 3169–3176.**

The leukocyte-specific transcript (LST1)[3] gene is encoded on the short arm of chromosome 6 within the designated MHC class IV in the TNF complex (1–4). The MHC class IV region spans the telomeric part of the former MHC III complex, and it contains a high concentration of genes that may play a role in various aspects of stress, inflammation, or infection (4). The *LST1* gene is encoded ~9 kb centromeric of the TNF-α gene (*TNFA*), and it is flanked telomerically at a distance of 4 kb by *LTB*, which codes for lymphotoxin β and forms a heterotrimer with TNF-β (2, 5). Centromerically, the *LST1* gene is flanked by the *1C7* gene which is located immediately adjacent to LST1, encoded by the opposite DNA strand such that the 3′ ends of the two mRNA templates come within a few bases of overlapping (6). Several polymorphisms have been identified thus far within the *LST1* gene: intron 4 encompasses the polymorphic microsatellites TNFd and TNFe; and a polymorphic *Pvu*II site is located downstream of the LST1 polyadenylation signal that is linked to *1C7* (2, 7, 8). The d3 allele of TNFd has been associated with severe grade acute graft-vs-host disease in HLA-identical sibling transplants, whereas TNFd4 was negatively associated with rejection of renal transplants, implicating a role of LST1 in the immune response (9, 10).

Northern blot analysis has shown constitutive expression of the LST1 cDNA in T cells, macrophages, and U937 cells and strong induction of transcription by stimulation of monocytic cell lines with IFN-γ (2). Transcription was also detected in human tonsil, lung, and placenta, the liver cell lines Hep G2 and Hep 3B, and by means of expression-tagged sequences in fetal liver/spleen and adult brain. Because the hybridization signal of ~800 nucleotides is very broad in the Northern blot analysis, a variation in length of the LST1 mRNA has been suspected. In fact, previous studies have identified four protein-encoding exons and five alternative noncoding exons 1 leading to eight different transcripts expressed in various cell lines which encode five different proteins (2, 11).

To characterize the complex LST1 expression pattern, we analyzed LST1 transcription and protein expression in freshly isolated PBMC, T cells, and B cells and after incubation of these cells with various cytokines. We describe essential parts of the biological function of two LST1 protein isoforms representing the two groups of LST1 polypeptides.

Materials and Methods

Cell separation

PBMC were isolated from buffy coats of healthy volunteer donors by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). T cells, monocytes, and B cells were separated from PBMC by positive selection with the immunomagnetic bead system using the respective Dynabeads M-450 (CD4), M-450 (CD8), M-450 (CD14), and M-450 (CD19) (Dynal, Oslo, Norway) as described previously (12–14). After selection, the cells were treated with Detachabeads (Dynal) to remove the CD4, CD8, and CD19 Abs from the membrane receptors (15). The purities of the recovered cells were 98% for $CD4^+$ cells, 99% for $CD8^+$ cells, 99.6% for $CD19^+$ cells, and 80–90% for $CD14^+$ cells.

Culture of dendritic cells

Dendritic cells were generated from PBMC as described (16–18). Briefly, mononuclear cells were isolated from leukocyte-enriched buffy coats by

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[3] Abbreviations used in this paper: LST1, leukocyte-specific transcript 1; TPO, thrombopoietin; DHFR, dihydrofolate reductase; ORF, open reading frame.

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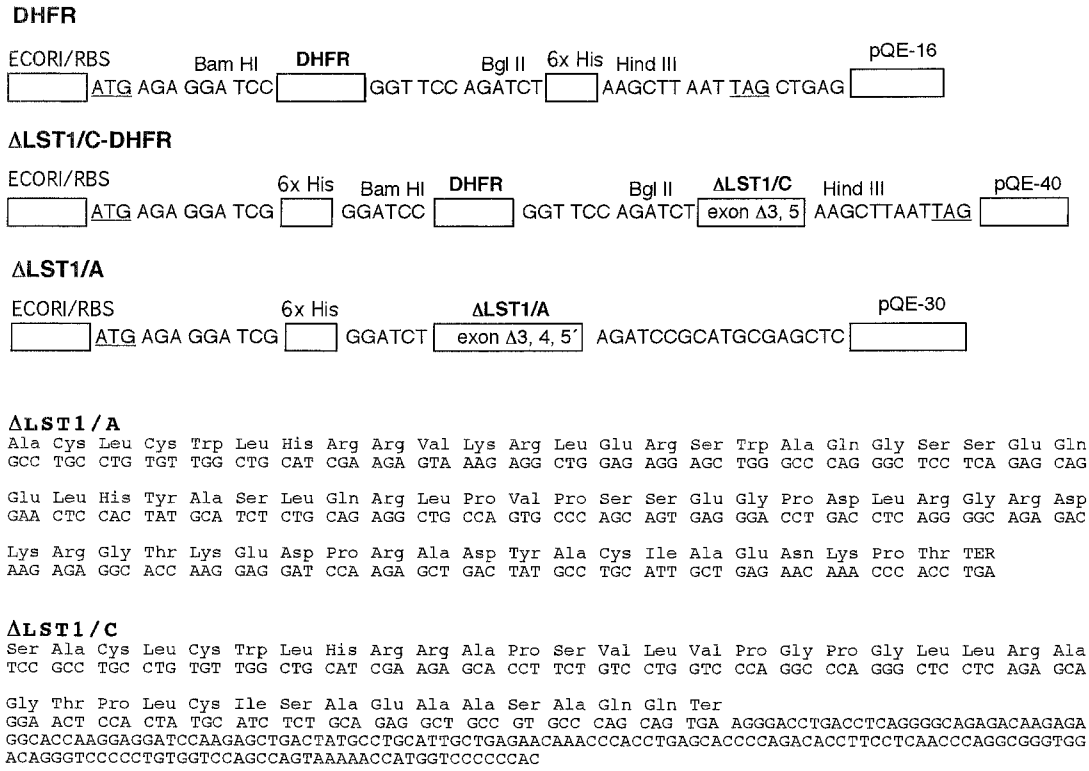

FIGURE 1. LST1 expression and the control vectors. The vector pQE-16 was used for expression of the control protein DHFR. The clone ΔLST1/C-DHFR was created by cloning ΔLST1/C cDNA into the *Bgl*II and *Hin*dIII restriction sites of the expression vector pQE-40, which contains the N-terminal part of the DHFR protein. The expression vector pQE-30 was used to generate the ΔLST1/A clone by cloning the *Bgl*II-restricted cDNA into the *Bam*HI site of pQE-30. All three expression vectors encode an N-terminal histidine tail (6× His). The DNA and amino acid sequences of ΔLST1/A and ΔLST1/C are indicated.

standard density gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and resuspended in complete medium (Biological Industries, Beth Haemek, Israel; RPMI 1640 supplemented with 10% heat-inactivated FCS (30 min, 56°C), 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml Fungizone, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM pyruvate, and 5×10^{-5} M 2-ME), and 5.0×10^7 PBMCs were allowed to adhere in 75-cm^2 cell culture flasks (2 h, 37°C). Nonadherent cells were removed, and the adherent fraction of the mononuclear cells was allowed to detach during overnight incubation in complete medium in the absence of exogenous cytokines. These cells were replated at high cell density (5×10^6 cells/ml) resulting in rapid readherence. Nonadherent cells were again removed, and an equal amount of medium containing 1000 U/ml of each GM-CSF and IL-4 was added. Dendritic cells were developed under essentially endotoxin-free conditions as indicated by the absence of spontaneous TNF-α production (<5 pg/ml TNF-α per 5×10^5 dendritic cells). On day 2, 5 ml of fresh medium containing 1000 U/ml of GM-CSF and IL-4 were added. After 5 days of culture, cells were harvested. These dendritic cells were immature and thus lacked CD83. However, they expressed all markers characteristic of dendritic cells (forward/side scatter properties, high levels of MHC class I and II, CD40, CD54, CD58 as well as absence of CD14) (16–18).

Cell stimulation

After cell isolation, a fraction of the cells was directly lysed in Trizol (Life Technologies, Paisley, U.K.) for further RT-PCR analysis, and the remaining PBMC, monocytic or lymphatic cells were incubated in McCoy's 5A (modified) medium (Life Technologies) with 50 μmol/ml 2-ME, 5×10^{-3} U/ml insulin, and 20% heat-inactivated FCS with or without addition of the indicated stimulating agents.

The following recombinant human cytokines were used in the concentrations indicated: IL-3 (Novartis, Basel, Switzerland), 25 ng/ml; IL-4 (Schering-Plough, Madison, NJ), 100 ng/ml; G-CSF (Roche, Basel, Switzerland), 100 U/ml; GM-CSF (Novartis), 100 ng/ml; thrombopoietin (TPO) (Genentech, South San Francisco, CA), 50 ng/ml; IFN-γ (Bender, Vienna, Austria), 200 U/ml. Further stimulating agents were: PHA (Difco Bacto, Detroit, MI), 0.02%; LPS (*Escherichia* serotype 0111:B4; Sigma, Darmstadt, Germany), 50 ng/ml.

RNA isolation and RT-PCR

With the use of Trizol, total RNA samples were prepared from cells at each time indicated, according to the manufacturer's instructions. To remove traces of contaminating DNA, 2 μg of total RNA were digested with 1 IU DNase (Promega, Madison, WI) for 1 h, phenolized, and precipitated as described previously (19). One reverse transcription reaction for every cell stimulation was performed with 2 μg RNA using avian myeloblastosis virus reverse transcriptase, oligo(dT)$_{15}$, and RNasin (all purchased from Promega) for 1 h at 37°C (19). The revealed cDNA was used for all PCR amplifications of the respective cell stimulations and control amplifications with G3PDH-specific oligonucleotides (Clontech, Palo Alto, CA) using the sense primer 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and antisense primer 5′-CATGTGGGCCATGAGGTCCACCAC-3′ of the cDNA and of DNase-treated not reverse transcribed RNA were performed. LST1 transcripts were amplified with the oligomers 5′-plst1 5-′ATGAGGAACT TGAGGCAAGTC-3′ encoded in exon 2 (nucleotides 1001–1021, Fig. 1) and 3′-plst1 5′-TCTCAGCAATGCAGGCATAGTC-3′ in exon 5 (nucleotides 2457–2446). For the PCR reaction mixtures, 80 ng reverse transcribed RNA were used in 25 μl PCR buffer containing 1 μM concentrations of each primer, 0.25 mM dNTP (Sigma, Vienna, Austria), and 1 IU *Taq* polymerase (Boehringer Mannheim, Vienna, Austria). After an initial step of denaturation (5 min at 94°C), 35 PCR cycles were performed (1 min at 94°C, 1 min at 56°C, 2 min at 72°C) followed by a final extension of 10 min at 72°C. PCR products were loaded on a 2% agarose gel together with a PCR m.w. marker (United States Biologicals, Cleveland, OH) and photographed.

Cloning of LST1 cDNAs

To analyze the splice transcripts expressed in freshly isolated monocytes, we subcloned 2.5 μl of the RT-PCR product that was revealed through amplification of reverse transcribed mRNA from freshly isolated monocytes with the oligonucleotides 5′-plst1 and 3′-plst1 into the TA cloning vector pCR II (Invitrogen, Leek, The Netherlands). The ligation product was transfected into INVa F′ bacteria (Invitrogen) following the manufacturer's recommendations.

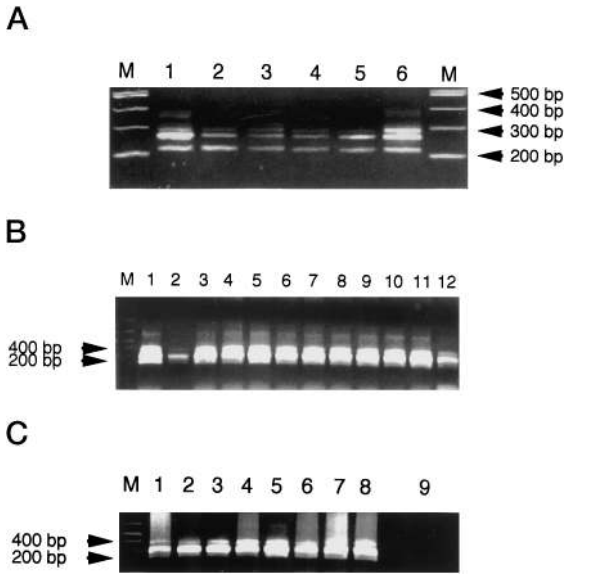

FIGURE 2. DNA gel electrophoresis of LST1-specific RT-PCR. Positively selected macrophages, B cells, and T cells as well as differentiated dendritic cells were used before or after incubation with various cytokines. No clear difference in alternative splicing of the LST1 mRNA can be detected between these cell types directly after separation or after cultivation for the times indicated. *A*, *Lane M,* DNA weight marker; *lane 1*, $CD4^+$ cells stimulated with PHA for 96 h; *lane 2*, dendritic cells; *lane 3,* $CD19^+$ cells incubated with LPS for 15 h; *lane 4,* $CD19^+$ cells incubated with LPS and IFN-γ for 48 h; *lane 5,* $CD8^+$ cells cultivated with thrombopoietin (TPO) for 48 h; *lane 6,* $CD14^+$ cells stimulated with GM-CSF for 24 h. *B*, RT-PCR of a freshly separated monocyte-enriched cell population (85% $CD14^+$ cells) (*lane 1*) or after incubation in medium alone for 17 h (*lane 2*), 24 h (*lane 7*), or after addition of TPO (*lane 3*), IFN-γ (*lane 4*), GM-CSF (*lane 5*), IL-4 (*lane 6*) for 17 h, and TPO (*lane 8*), IFN-γ (*lane 9*), GM-CSF (*lane 10*), IL-4 (*lane 11*), and LPS (*lane 12*) for 24 h. *C*, RT-PCR of positively selected $CD8^+$ cells directly after separation (*lane 1*), after incubation for 36 h in medium alone (*lane 2*), medium plus TPO (*lane 3*), PHA (*lane 4*), or IFN-γ (*lane 5*) and after incubation for 48 h in medium (*lane 6*), medium plus TPO (*lane 7*) or PHA (*lane 8*), negative control (*lane 9*).

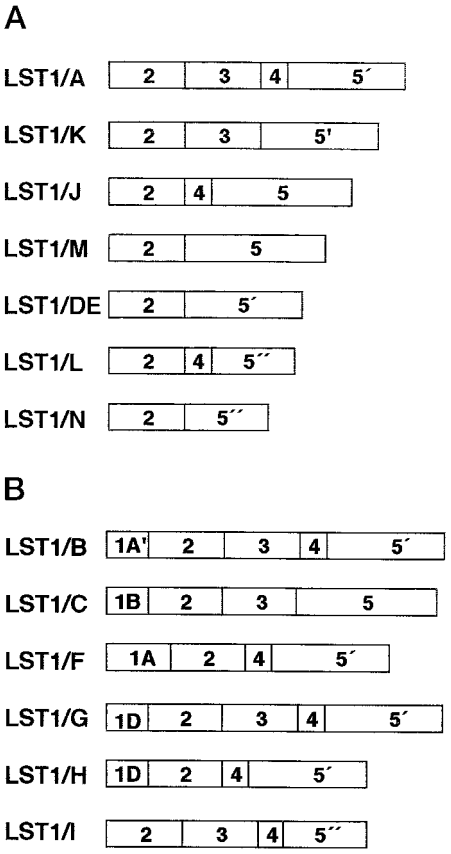

FIGURE 3. A. LST1 splice variants expressed in PBMC. Cloning and sequencing of the RT-PCR product from PBMC enabled identification of seven alternative LST1 splice variants. The schematic structure starts with exon 2, which encodes the start codon, whereas exon 3 contains a putative transmembrane region of 23 hydrophobic amino acids. Thus, LST1/J, LST1/M, LST1/DE, LST1/L, and LST1/N constitute putative soluble isoforms. Because exon 4 is 23 bp long, a different ORF is used in exon 5 in the variants LST1/A, LST1/J, and LST1/L, which span this exon in contrast to the remaining cDNAs. *B*, LST1 splice variants not expressed in PBMC. Five alternative noncoding exon 1 (1A, 1A′, 1B, 1C, and 1D) forms have been identified, and the splice variants LST1/B, LST1/C, and LST1/G constitute transmembrane variants.

DNA sequencing and data analysis

Plasmid DNA was prepared according to standard protocols, and DNA sequencing was conducted by the dideoxynucleotide chain termination method using either the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) with the Applied Biosystems DNA Sequencing System Model 373A or the Thermo Sequenase fluorescence-labeled primer cycle sequencing with 7-DEACA-DGTT (Amersham, Vienna, Austria) with the Li-Cor 4000L Sequencing System. Sequencing primers were the universal and reverse M13 primers. DNA sequences were analyzed with MacMolly software (Soft Gene, Bocholt, Germany)

Production of a polyclonal rabbit LST1 Ab

The 3′ end of the cDNA clone LST1/C (Fig. 3), which is expressed in U937 cells (2) and spans exon 2, 3, and 5, was amplified with the primer open reading frame 6 (ORF6) 5′-ACT AGATCTTCCGCCTGCCTGT GTT3′ containing a *Bgl*II restriction site (underlined) and the 3′-primer ORF4 5′-ACTAAGCTTGTGGGGGGACCATGG3′ introducing a *Hin*dIII site (underlined). The *Bgl*II- and *Hin*dIII-restricted amplification product that encompasses exon 5 and the region 3′-terminal of the transmembrane region of exon 3 were cloned into the respective cloning sites of the expression vector pQE-40, which contains a 6× His affinity tag at the N terminus as well as the N-terminal mouse dihydrofolate reductase (DHFR) carrier protein (Fig. 1) (Qiagen, Hilden, Germany). This construct fused the 4.1-kDa ΔLST1/C protein encoding the extracytoplasmic region of the 6.8-kDa LST1/C polypeptide (Fig. 4*A*) to the 24.5-kDa DHFR carrier protein, resulting in expression of a soluble ΔLST1/C-DHFR fusion protein with a histidine affinity tag. The expression vector pQE-30 was used to generate the ΔLST1/A clone (spans the 3′-end of exon 3, exon 4, and 5′) by cloning it into the *Bam*HI site of pQE-30, enabling the purification of the 8-kDa extracytoplasmic region of the LST1/A protein(10.8 kDa). The control vector pQE-16 (Qiagen) was used for expression of the 24.5-kDa DHFR control protein (Fig. 1). The K-12-derived *Escherichia coli* strain M15[pREP4] was used for high level expression of the proteins ΔLST1/C-DHFR, ΔLST1/A, and DHFR.

After protein purification according to standard protocols of the manufacturer (Qiagen), two rabbits were immunized with 2.2 mg of the ΔLST1/C-DHFR protein using Freund's adjuvant. The rabbits were injected s.c. on days 0, 28, 56, and 70 with a protein solution containing 880 μg/ml ΔLST1/C-DHFR. The polyclonal Abs received on day 85 were purified on a protein A column, and only the Ab LST1–25 was used for further analyses. This protein A purified Ab did not detect ΔLST1/A in Western blot analyses (Fig. 4*B*). To purify a highly specific LST1 Ab, we coupled the DHFR protein to Affi-Gel (Bio-Rad, Vienna, Austria) following the manufacturer's instructions, which allowed selective binding of the DHFR-specific Ab onto a column, whereas ΔLST1/C-specific Abs could pass the column. After a 3-fold passage of the Ab solution through the DHFR column, we recovered the ΔLST1/C-specific Ab LST1–25-Affi which did not detect the DHFR protein as well as ΔLST1/A in Western blot analysis.

The expression vector pQE-30 was used to express the α1 domain (exon 2) of HLA-G with a 6× His N-terminal affinity tag. The purified recombinant His-tagged α1 polypeptide was used to immunize a rabbit as described above. The antiserum raised did not recognize native HLA-G or class I molecules expressed on the cell surface but stained HLA class I

A

```
      ->exon 1A                                -> exon 1A´
CTGGGGGAGA AGTTAAAGCC AGAGGAGGGG CAGGAATGTC TGAGGTGGCA ACACTTCTCT TCAGCCAGAC AGCACTGGCC AGTTTGGAGT CTGTCCATCC   100

TGCAGGCCAC AAGCTCTGGG TAAGCTGGGA ATGGGCAGGG ACCTTGGTGG AAGGATGGTC ACACCCCAGA GTGGGGTGAA GCTAAGATGA GGGGAGGGAG   200
AGTATGGGTT TGAGTTTCCC TGGGCCGTCG AGGAATCCTC TGAGTCTCTG CTCCCCAAAG AAATTAAAGA CAATTCATTT CTGTGCCCAC GGCCCTTATG   300
GCTCCACCTG CACTTCTGCT CCCCACCCCC CAGAATTCCT CTTAAACCCA GAAGGTCCCA GTTTCCAGAC CCTAGTCAGT ATATCTGGCT CTGGGGTGAA   400
                                                                                    TATA like
GAGAACGGCC CCCTCTTCAC CCTCAAACAG GAACCAGTGG TTGGAGGGGA GGAAGTGCCT GAGGGGAAGT TATGGGGCCC CAGATACTCC TCCATGCCCC   500
-->  exon 1B
ACTTCAGCCC TAGCAGCATC TGCCTGTGGG AAGCAGCTCT CCACACCAGC CAAGGGGGCC CCCACACTCC CGCGCTGCTC TGCGGCTCAG GGAGCAGCCC   600
                                                -> exon 1C
ACCTGCTGGG TGTGCTGATA TCACCCTCCC TTCTTCCCCC CAGTGCCCAC ACCCACCCAG GCCCAGGCTC CTTCCCCTCC ATCATCCCCT TACCAGCACC   700
TAGAACCATC CAGGGCTGAA AAGTCCCCTC CAAACCACGT GGTCAGCCCA GGGCAGAGGA AAGGGCTGGG CTCTGGAGTT GGGCAGAGCT GGCCTTAAAC   800
                                      -> exon 1D
CCCAGCTCCA CCTTTCTGGG ATGGGTGACC TAGTAAAGTC CAGGCTTGAA TCTCGGGTCT TTACTTGGGC AACGGGCACC ATGATACCCT ATGTTCTGGG   900
GATTAGCAGT GAGGAATGGA AAGTGCCCAG CTCGGGTTGG CACATAAGGG AGGCTCCCCA GCCTGGGAAC GATTATAACA GAGGGCCCCT CACTTCACAG  1000
->  exon 2
-> 5´-plst1
ATGAGGAACT TGAGGCAAGT CACCAGCCCC TGATCATTTC GCCTAAAAGA GCAAGGACTA GAGTTCCTGA CCTCCAGGCC AGTCCCTGAT CCCTGACCTA  1100
ATGTTATCGC GGAATGATGG TAAGTAAAGT GTCTCTTGCA TCTGCATAGA GAGAGTCCTG GGAGCTTAGG AAGTGATGGG GAACAGTGAT GTATGCAGCT  1200

CATGACTAGG TGGACAGGCC TCTGGGGACA GCTGGTACAG GAGGGAAAGG GACCTCACGG GAGGCCCAGA AACCTGGTAA GAGGTGAGGT ATTAAGGTCT  1300
GGGATGGAGA AGCTCTGAGG GTATATTTTT CTGCCTCTAA AACTGTTGGA GAGGGAATCT GAGAAAGCTG CAACCAACCA GGAGGCTGGG GTACGCTGGA  1400
                                      -> exon 3
GAAGGAATGG GCTTCCTAAC CTTGAGCCCT CTTCCCTGAA GATATATGTA TCTACGGGGG CCTGGGGCTG GGCGGGCTCC TGCTTCTGGC AGTGGTCCTT  1500

CTGTCCGCCT GCCTGTGTTG GCTGCATCGA AGAGGTGAGC GCTGCACTCC CTCCCTCCCC CTGCAGCAGT GCCCCCTGTG CCCCCACCCC CACACGCTTT  1600

CCCACTGCTT TCCCAGAACA CTGCCTGGCC CTGGAGCCAC TGGGAAGCCA ACAGGGGAGT CCACGCCTGC TGGTGGGGGG AGCCCGGGAG GCGGGAGAAG  1700
          FcγR1
                                      -> exon 4
CACAAAGGGT GGGCTGTGTT GAGCTTCTTC TTTTCTTCCA GTAAAGAGGC TGGAGAGGAG CTGGGTGAGT CTGGGGACAG GGAAGGGGGA GGGCAAGAGA  1800
GATCCTGAGT GGGTGAGTGG GGAGAAGCAT GGCTGAGCGC TGAGAGGAGG GTTGGGGACG GGAGACAAGG AGAGAGAAAG TAGGAGCATG AGAGAGGCAG  1900
                                                  --> TNFe
AGAAAATCGA GGCAAAAGAG AAAGAGAAAA TGAGACAGAA ACCAAGAGAA AAAGTGAGAC AGAGGATAGG AGAGACAGGG AGAAAATGAG AGTGAGAGAG  2000
               ISGF-2
ACACAAAGAG AAGAGCAATG AAAGAGAGAG AGAGAGAGAG GCTCCAGAAC CAGGCACAGT GGCTCACGTC TGTCATTCCA CTATCGCAAG GCTGAGGCAG  2100
                                      --> TNFd
GAAGATAGCT TGAGCTCAGG GGTTGAAGAC AATCCTGGAC AACATAGTGG ACTCTGTCTC CAAAGAAAAA AGAGAGAGAG AGAGAGAGAG AGAGAGAGGG  2200
                                                                                          --> exon 5
AGAGAGAGAG AGAGAGAGGG AGAGAAGTAA GAAAGGCTGG AGGTGGGAGC AGAACTCACA GGGAAGGATC TGACGCATCG CCTCCCATCA GCACCTTCTG  2300
          -> exon 5´                                    -> exon 5´´
TCCTGGTCCC AGGCCCAGGG CTCCTCAGAG CAGGAACTCC ACTATGCATC TCTGCAGAGG CTGCCAGTGC CCAGCAGTGA GGGACCTGAC CTCAGGGGCA  2400
                                      3´-plst1 <-
GAGACAAGAG AGGCACCAAG GAGGATCCAA GAGCTGACTA TGCCTGCATT GCTGAGAACA AACCCACCTG AGCACCCCAG ACACCTTCCT CAACCCAGGC  2500
GGGTGGACAG GGTCCCCCTG TGGTCCAGCC AGTAAAAACC ATGGTCCCCC CACTTCTGTG TCTCAGTCCT CTCAGTCCAT CTCGAGCCTC CGTTCAAATT  2600
GATCATCATC AAAACTTATG TGGCTTTTTG ACCTTTGAAT AGGGAATTTT TTAAATTTTT TAAAAATTAA AATAAAAAAA ACACATGGCT CACCCTTCCA  2700
CCCACTCTGG GGTCAAATAG TAATTTGTTG GGTGAATGAC AGTGTTCAGG GACCCAAGCT CCCCTAACAG CCAGAAGAGG GTATGTGTGG GCCTGGCAGG  2800
AAAGGGCAGT TGCCAAGGAG GAGTCATATC TGATCCTTCC CATTTCTCAG GACAATCAGG CTCAGCCTCC TGGGACTGGG GGAAGCAGAT GTGCTGAGCT  2900
                                      Pvu II
CCCACATGGT GGTGGGAGGG GCGCTGGGAC CACAGCCGGC AGCTGCCTTC TTGGACCTTT CCAGGTCAGA CCTGGTGGAA GGGAAAGTTC AGAGTTGGGG  3000
```

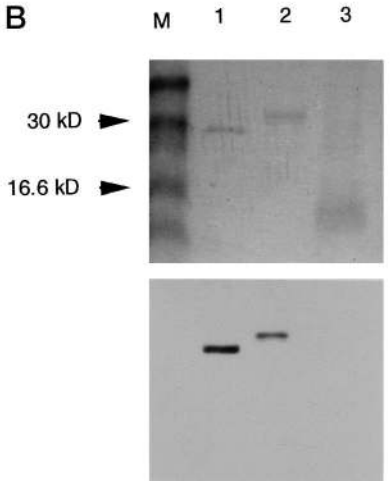

FIGURE 4. A. The LST1 genomic sequence. Protein-encoding exon sequences of LST1 are boldface; the putative noncoding alternative exons 1A-1D and the noncoding part of exon 2 are underlined. Exon 1A′, exon 5′, and 5″ mark alternative shorter variants of the respective exon sequences. The start and stop codons of the LST1 cDNAs and its short upstream ORF are outlined. The ORF encoding a nonapeptide is located at the 5′-end of exon 2, whereas the putative start codon of all LST1 cDNAs identified thus far can be found at its 3′-end (position 1101). The stop codons of the two different ORFs are outlined in exon 5 (LST1/A at position 2469 and for LST1/C at 2378) which are used according to the presence or absence of exon 4 in the various cDNAs. The 3′-end of exon 5 encodes the polyadenylation signal AATAAA. The polymorphic *Pvu*II restriction site and the polymorphic TNF microsatellites TNFd

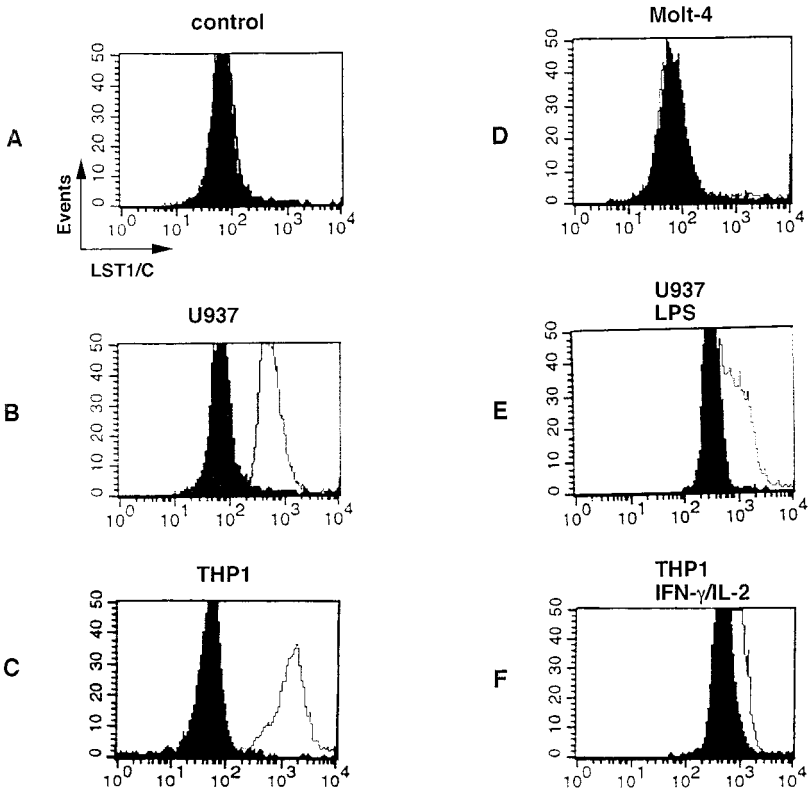

FIGURE 5. LST1 surface expression. FACS analyses were assessed with the primary Ab LST1–25-Affi and a PE-conjugated Fab fragment of a goat anti-rabbit Ig as secondary Ab. Black areas, results obtained by sole labeling with the secondary Ab. The control (*A*) compares single labeling of the cells with double-labeling of U937 cells with the HLA-G rabbit Ig as primary Ab. No difference between these controls can be seen, proving the specificity of the LST1–25 Ab. LST1 surface expression of the cell lines U937 (*B*), THP1 (*C*), and Molt-4 (*D*) and after cultivation of U937 cells with LPS (*E*) and THP1 with a combination of IL-2 and IFN-γ (*F*) is shown.

heavy chains only in Western blot analyses. Because HLA-G is not recognized on the cell surface of U937, Molt-4, and THP-1 cells with this rabbit antiserum, it was used as control Ab in our experiments (Fig. 5).

Single-step purification and solubilization of the LST1 proteins

All functional assays with the LST1 proteins described in this work were conducted with solubilized proteins. For solubilization of the LST1 proteins ΔLST1/C-DHFR, DHFR, and ΔLST1/A, a single-step purification/refolding procedure based on modification of existing solid phase solubilization procedures was performed (20). Briefly, the pellet from 1 liter bacterial expression culture was lysed in 50 ml lysis buffer (6 M guanidine, 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 4 mM octylglucoside), sonicated, and centrifuged at 20,000 × *g* for 30 min. The Ni-NTA agarose column was equilibrated with the lysis buffer and the supernatant was applied to the column. After a washing with 50 ml buffer 2 (6 M urea, 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 20 mM imidazole), the protein was eluted with buffer 2 containing 50 mM EDTA.

Flow cytometric measurement of surface Ag expression

To determine surface LST1 expression, cells (5–10 × 10^5) were suspended in FACS buffer (PBS containing 1% BSA), labeled with the protein A-purified primary Ab LST1–25 (see above) for 30 min at 4°C followed by a second labeling step with PE-conjugated Fab fragments of a goat anti-rabbit Ig (Southern Biotechnology, Birmingham, AL). In parallel, a single labeling without the primary Ab was performed as control as well as a two-step labeling with a rabbit Ig control as primary Ab. It was raised against the recombinant α1 sequence of HLA-G expressed in the expression vector pQE-30 containing a 6× His N-terminal affinity tag. Because HLA-G is not recognized on the cell surface of U937, Molt-4, and THP-1 cells with this rabbit antiserum, this primary Ab was used as control Ab in our experiments. Labeling of the cells with this Ab was followed by staining with the secondary anti-rabbit Ab used in all of our experiments.

All positive results were controlled with the LST1–25-Affi Ab as primary Ab. The following FITC-conjugated murine mAbs (Becton Dickinson, Mountain View, CA) were utilized in a third labeling step: Leu2/CD8, Leu-3/CD4, Leu-12/CD19, and Leu-M3/CD14. After each incubation, cells were washed in FACS buffer and then analyzed after three final washings. To determine cell purities, the same respective FITC-conjugated mAbs were used.

Lymphocyte proliferation assay after stimulation with PHA and candidin

PBMC were isolated on Lymphoprep as described. Cells were resuspended in RPMI 1640 medium containing 1% L-glutamine (ICN, Asserelegem, Belgium), 1% penicillin/streptomycin (Sigma, Bornem, Belgium), and 10% autologous (assays 1 and 2) or pooled human AB serum (ICN) in assays 3–5. Triplicate cultures were set up in a 200-μl volume in 96-well round-bottom plates (Nunc, Mereldeke, Belgium) at a concentration of 5 × 10^5 cells/ml. Cultures were incubated either in medium alone or with optimally stimulating doses of PHA (1/100 dilution of reconstituted Bacto-hemagglutinin P (Difco Bacto)) or 50 μg/ml candidin (Stallergènes, Fresnes, France) as a control. To evaluate an immunomodulatory activity of the LST1 variants, 5–50 ng of the renaturated proteins ΔLST1/C-DHFR, ΔLST1/A, DHFR, or the buffer used for elution of these proteins from the Ni-NTA column as negative control were added either to medium alone, or together with PHA or candidin at the onset of triplicate cultures. After incubation for 6 days in a humidified 5% CO_2, 95% air atmosphere, DNA synthesis was measured by adding 0.5 μCi [^{3}H]thymidine (40–60 Ci/mmol; Amersham, Arlington Heights, IL) during the final 12 h of culture. Cells were harvested with a semiautomatic cell harvester, and thymidine incorporation was assayed in a liquid scintillation counter (Beckmann LS 1801, Galway, Ireland). Proliferation is given as the mean of triplicates ± SE.

and TNFe are underlined, as are the IFN-γ-activated site (FcγR1) and the IFN-stimulated gene factor-2-responsive element (ISGF-2). *B*, SDS-PAGE and Western blotting of the LST1 proteins. The SDS-PAGE in the *top panel* shows 280 ng of the 24.5-kDa protein DHFR (*lane 1*), 400 ng of the 28.6-kDa fusion protein ΔLST1/C-DHFR (*lane 2*), 3 mg of the 8-kDa protein ΔLST1/A (lane 3), and the standard polypeptide-weight marker (M). The m.w. of these proteins was estimated according to their known DNA sequence showing a small discrepancy with the sizes of the protein marker. *Bottom panel*, Western blot analyses of the gel after hybridization with the LST1–25 Ab. It did not detect the protein ΔLST1/A, proving the specificity of the LST-25 Ab for the LST1/C protein group.

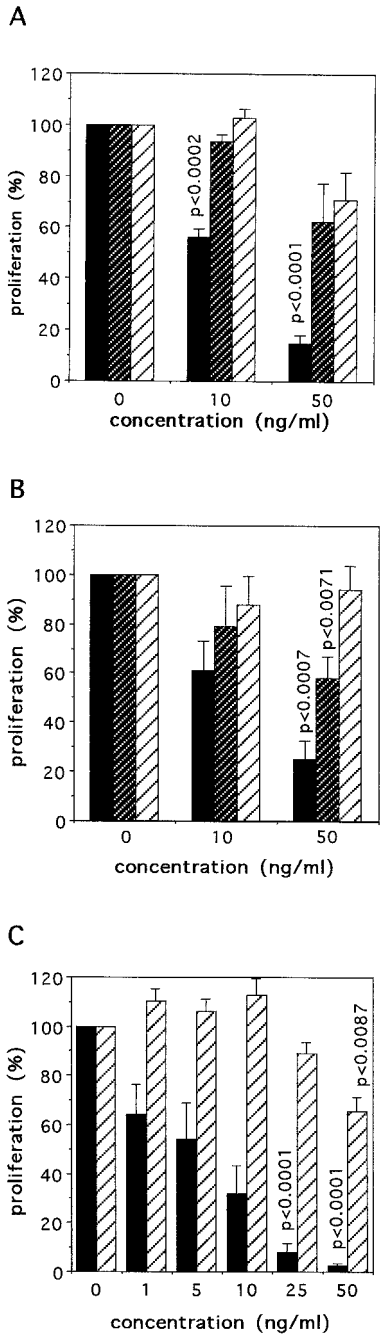

FIGURE 6. Proliferative response of PHA-, candidin-, and alloantigen-stimulated lymphocytes. Mean values and SE of lymphocyte proliferation in response to PHA (*A*) and candidin (*B*), as well as in MLR (*C*) are presented with or without addition of various concentrations of DHFR (*right columns*), ΔLST1/C-DHFR (*left columns*), and ΔLST1/A (*middle columns*). The controls were performed with the buffer in which the proteins were dissolved.

Mixed leukocyte reaction

Proliferative responses of PBMC to allogeneic stimulation were determined in one-way MLR, as described (21). Briefly, 5×10^4 responder PBMC were cocultured with 5×10^4 mitomycin C-treated (Sigma, St. Louis, MO) allogeneic PBMC for 6 days at 37°C in a humidified 5% CO_2, 95% air atmosphere at a volume of 150 μl culture medium in U-bottom 96-well microtiter plates (Costar, Cambridge, MA). The medium was composed of RPMI 1640 supplemented with 2 mM L-glutamine (Biochrom, Berlin, Germany), 100 U/ml penicillin (Biochemie, Vienna, Austria), 100 μg/ml streptomycin (Grünenthal, Stolberg, Austria), and 10% heat-inactivated human pooled AB serum. Various final concentrations of the renaturated proteins ΔLST1/C-DHFR and DHFR were added at the onset of triplicate cultures, whereas the buffer used for elution of these proteins from the Ni-NTA column was used as negative control. During the last 12–16 h, each well was exposed to 2 μCi [^{3}H]thymidine and harvested, as described above. The proliferation was displayed as mean of triplicates ± SE.

Results

Detection of various LST1 transcripts in PMNC and dendritic cells

RT-PCR analysis of a freshly isolated monocyte-enriched cell population (85% $CD14^+$ cells) was used to detect four different LST1 splice variants 220, 265, 286, and 381 bp long (Fig. 2). Constitutive LST1 transcription was observed in positively selected $CD4^+$, $CD8^+$, and $CD19^+$ cells, as well as in dendritic cells (Fig. 2).

No difference in size of the LST1 transcripts after stimulation

After stimulation of the PBMC, B cell, and T cell populations, no significant change in length of the LST1 mRNAs was detected by RT-PCR in comparison with freshly isolated cells (Fig. 2). After stimulation with various cytokines and agents (IL-3, IL-4, G-CSF, GM-CSF, TPO, IFN-γ, PHA, or LPS) for up to 96 h, the same transcripts 221, 265, 286, and 381 bp long were amplified in the monocyte-enriched cell population tested.

Sequencing of different protein-encoding transcripts in PBMC

To characterize the transcripts amplified by RT-PCR, the amplification products of an unstimulated monocyte-enriched cell population (80% $CD14^+$ monocytic cells) were subcloned and sequenced. By analyzing 30 different cDNA clones, we identified 7 different transcripts (Fig. 3*A*), of which 243-, 309-, and 358-bp mRNAs had not been detected by gel electrophoresis. Only two of these seven different transcripts had been previously described (LST1/A and LST1/DE (Fig. 3)). According to the proposed nomenclature of de Baey et al. (11), we named the newly detected variants LST1/J (248 nt), LST1/K (297 nt), LST1/L (182 nt), LST1/M (225 nt), and LST1/N (202 nt); (Fig. 3*A*). Because we amplified only the protein-coding sequences of the LST1 transcripts (exons 2–5), we could not distinguish between the variants LST1/D (exons 1A, 2, 5′) and LST1/E (exons 1C, 2, 5′) differing only in the noncoding exon 1. Therefore, the transcript spanning exon 2 and 5″ was called LST1/DE. We confirm the presence of a third alternative splicing acceptor site of exon 5 in our cDNA clones that had only been presumed previously (Fig. 4*A*) (11). However, expression of the splice variant LST1/C (2, 11) was not identified in PBMC. The sequences of the isoforms LST1/J–LST1/N have been submitted to the EMBL database under the respective submission numbers Y18486–Y18490.

LST1 transcripts encode different proteins

Only two of the transcripts expressed in PBMC contain exon 3 (LST1/A, LST1/K), which encodes a stretch of 23 hydrophobic amino acids spanning the transmembrane region (2). Thus, all the remaining transcripts probably constitute soluble cytoplasmic isoforms, because they do not contain a signal sequence necessary for secretion. Only the cDNA clones LST1/A, LST1/DE, and LST1/J encompass exon 4. As exon 4 spans 23 nt, its presence in LST1 transcripts leads to a frame shift. Thus, an alternative open reading frame (ORF) is present in the C terminus of the clones spanning exon 4 in comparison with the remaining transcripts, LST1/K–LST1/N. The two different ORFs encode putative soluble as well as transmembrane isoforms, which further increase the LST1 protein spectrum.

LST 1/C is expressed on the cell surface of U937 and THP1 cells

To analyze whether cells express the LST1 protein at the cell surface, we used LST1-specific antisera raised against recombinant LST1 polypeptides in FACS analysis. The Ab LST1–25 showed specific binding of the proteins ΔLST1/C and DHFR and did not detect the ΔLST1/A protein in Western blot analysis (Fig. 4*B*). FACS analysis revealed no surface expression of the LST1/C protein in PBMC from healthy individuals, and it was not induced after in vitro stimulation of the cells with the following substances or combinations for up to 72 h: PHA, TNF-α, IL-2 plus IFN-γ, IL-4, IL-4 plus LPS (data not shown). We also observed no constitutive or inducible (PHA, IL-2, IFN-γ) LST1/C cell surface expression in the T-lymphocytic cell line Molt 4. The histiomonocytic cell line U937, however, showed constitutive expression of the LST1/C protein (Fig. 5), which was not clearly up-regulated by incubation of these cells with LPS. All positive results were confirmed by performing the flow cytometric measurements with the ΔLST1/C-specific LST1–25-Affi Ab. Constitutive expression of the LST1/C protein was also observed in the monocytic cell line THP1 (Fig. 5).

Inhibition of lymphocyte proliferation exerted by LST1

To identify immunomodulatory activities of the variants ΔLST1/A and ΔLST1/C-DHFR, the proliferative response of PBMC stimulated with PHA, candidin, or allogeneic stimulator cells in the MLR was tested with regard to these proteins.

The PHA-induced proliferation rate of PBMC (Fig. 6*A*) was significantly reduced by the addition of 10 or 50 ng/ml ΔLST1/C-DHFR at the beginning of the culture period from 100% to 56 and 15%, respectively. In contrast, DHFR and ΔLST1/A showed no significant inhibitory effect on PHA-induced stimulation. Thus, the LST1/C portion is responsible for the observed inhibition whereas the recombinant proteins ΔLST1/A and DHFR that were isolated by the same procedure had no effect.

The candidin-induced proliferation of lymphocytes was significantly inhibited by ΔLST1/C-DHFR and to a lesser extent by ΔLST1/A, whereas no significant change was effected by DHFR (Fig. 6*B*).

A highly significant immunosuppressive response was also detected when ΔLST1/C-DHFR was added to MLCs, revealing a dose-dependent inhibition of the alloantigen-induced proliferation of lymphocytes. A concentration of 1 ng/ml reduced proliferation by 38%. In the presence of 50 or 100 ng/ml ΔLST1/C-DHFR, the proliferative response was totally blocked (Fig. 6*C*). Addition of DHFR to the MLR did not show a significant inhibition. Only at the highest concentrations (50 and 100 ng/ml) was proliferation reduced.

Discussion

Our studies show constitutive transcription of the *LST1* gene in peripheral mononuclear cells, T cells, B cells, and dendritic cells. RT-PCR analysis and subsequent cloning and sequencing of the cDNA from a monocyte-enriched PBMC cell population led to the identification of seven different splice variants (LST1/A, LST1/DE, LST1/J–N). Only two of them (LST1/A and LST1/DE) have been previously detected (11). The LST1/A mRNA is also present in several human tissues and cell lines, such as placenta, thymus, liver, lung, kidney, tonsil, Hep G2 and Hep 3B, U937, and Jurkat, whereas the LST1/D isoform has been detected only in brain tissue and mononuclear cells (11). The transcripts identified in our studies increase the number of LST1 splice variants to 14, which cover all but 2 of the possible polypeptides based on the known exon/intron organization (Fig. 3). However, we cannot exclude the presence of further splice variants in PBMC. The same is true of $CD4^+$, $CD8^+$, $CD19^+$, and dendritic cells in which transcription of at least four different splice variants were detected by RT-PCR and agarose gel electrophoresis. These data confirm the complex mRNA expression pattern of this gene and show the constitutive expression of several LST1 splice variants in PBMC and dendritic cells.

Although splicing of the LST1 RNA seems to be complex, no difference can be found in freshly isolated PBMC, B and T lymphocytes or after stimulation of these cells in vitro for up to 96 h (Fig. 2). We found no change in expression of the different splice variants in $CD8^+$ cells after stimulation with IFN-γ or PHA for up to 48 h. These results contrast the results of de Baey et al. (11), who observed a loss of the putative membrane-anchored isoforms containing exon 3 in the monocytic cell lines U937 and MonoMac 6 and exclusively detected the LST1/A cDNA after activation of a $CD8^+$ T cell clone with IFN-γ. These differences can be explained by the different primers used. The primer pair of de Baey et al. coded in the most 5′ of the alternative noncoding exon 1 sequences (exon 1A) and in exon 5′, which allowed detection only of LST1 transcripts controlled by the first promoter of the *LST1* gene causing the alternative exons 1A′, 1B, 1C, and 1D not to be amplified. In contrast, the oligonucleotides of our RT-PCR studies were located in exon 2 and exon 5″. All splice variants analyzed thus far span these exons, because exon 2 contains the start codon and exon 5″ contains the stop codons of both alternative ORFs.

Also, preferential expression of various isoforms in certain cells does exist. No surface expression of the LST1/C variant was seen in peripheral monocytes, T cells, and B cells in our FACS studies, whereas the monocytic cell lines THP1 and U937 show constitutive expression of this protein. These data are in good correlation with the fact that LST1/C mRNA was detected in U937 cells but not in PBMC. No clear influence on the cell surface expression has been observed by treating THP1 and U937 cells with IFN-γ, IL-2, or GM-CSF.

Because the *LST1* gene is located within the TNF complex in close vicinity to the genes for TNF-α, TNF-β, and lymphotoxin-β and its transcription is induced by IFN-γ, an immunomodulatory function has been suspected (2). Also, a positive as well as a negative association with bone marrow and renal transplant rejection was found with two different alleles of TNFd microsatellites which are located in intron 4 (9, 10). To examine an immunomodulatory effect of LST1, we expressed the two LST1 variants ΔLST1/A and ΔLST1/C as soluble molecules with an N-terminal His epitope in *E. coli* (Fig. 1). These two isoforms only share nine amino acids at the N terminus but differ completely in the remaining molecule. They represent the two groups of LST1 proteins with regard to the two alternative ORFs used in the last exon caused by presence or absence of exon 4. The DHFR sequence does not exert an immunomodulatory effect enabling its use for elongation of the 4.1 kDa protein ΔLST1/C, which was indispensable for its expression. Therefore, the 8-kDa ΔLST1/A and the 28.6 kDa ΔLST1/C-DHFR proteins were used for studying their effect on lymphocyte function.

We found that the two groups of LST1 polypeptides differ in function. The ΔLST1/C-DHFR molecule inhibited the PHA- and candidin- as well as alloantigen-stimulated lymphocyte proliferation highly significantly. This inhibition was dose dependent and exerted at low concentrations (1–10 ng/ml), whereas a significant effect was exerted by ΔLST1/A only with regard to the stimulation of PBMC with candidin but not with PHA.

These results show that PBMC do not present the LST1/C protein on their cell surface, whereas they respond to the presence of

this protein in soluble form. Interestingly, only two monocytic cell lines expressed LST1/C on their cell membrane, but it was not detected on peripheral monocytes of 24 individuals or after in vitro stimulation of these cells with various cytokines. This is in good correlation with the failure to amplify the LST1/C transcript in these cells. The LST1/M variant (Fig. 3), however, which constitutes the soluble homologue of LST1/C, is transcribed in PBMC. This demonstrates the complex splicing of the LST1 transcripts and its distinct regulation in different cell types.

Although ΔLST1/A and ΔLST1/C show only a short amino acid homology of nine amino acids, both isoforms inhibit the proliferation at a varying extent. Therefore, alternative splicing seems to result in LST1 isoforms of a probably varying immunosuppressive function. However, further studies of the various LST1 variants must be done to analyze the regulation of alternative splicing and the difference in function of all 10 LST1 polypeptides identified.

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