SMUCKLER/TIM4 is a distinct member of TIM family expressed by stromal cells of secondary lymphoid tissues and associated with lymphotoxin signaling

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Lymphotoxin-α (LTα) was originally linked to delayed-type hypersensitivity and its production was later attributed to Th1, but not Th2 cells. Studies employing knockout mice demonstrated that LT signaling is essential for the development and functional compartmentalization of lymphoid tissues. Here, using gene expression profiling, we identified a novel gene termed SMUCKLER (spleen, mucin-containing, knockout of lymphotoxin), that is selectively down-regulated in spleens of LTα- or LTβ-deficient mice. The encoded transmembrane protein contains immunoglobulin V and mucin domains and is identical to TIM4, a predicted member of recently identified TIM family (T cell immunoglobulin- and mucin-domain-containing molecule). Unlike TIM1 and TIM3, which were implicated in T cell-mediated functions, SMUCKLER lacks tyrosine phosphorylation motif in its intracellular domain and is not expressed by bone marrow-derived cells. *In situ* hybridization of spleen sections demonstrated SMUCKLER expression by stromal cells predominantly in the marginal zone and to a lesser extent throughout the white pulp. Similarly to other TIM genes, SMUCKLER maps to a locus associated with predisposition to asthma both in mice and in humans (11.b1 and 5q33, respectively) and shows coding sequence variations between BALB/c and DBA mice. Therefore, SMUCKLER/TIM4 may be considered as a candidate disease-predisposition gene for asthma.

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1 Introduction

Lymphotoxin (LT) was initially discovered as a soluble cytotoxic factor implicated in delayed-type hypersensitivity [1, 2]. Molecular studies have later defined two distinct molecules, LTα (also known in literature as TNFβ) [3, 4] and LTβ [5]. LTβ is a membrane-bound protein, which can heterotrimerize with LTα and retain the latter on the membrane in the form of $LT\alpha_1LT\beta_2$ complex. The role of LT system in lymphoid organogenesis, as revealed initially in LTα knockout mice [6, 7], is largely linked to the function of this membrane-bound cytokine $LT\beta_2LT\alpha_1$ whose main signaling receptor is LTβR [8–10]. Signaling through LTβR, as well as through TNFR55 is essential for the development of peripheral lymphoid organs, such as lymph nodes and Peyer's patches, and for the development of follicular dendritic cell clusters and the establishment of correct compartmentalization of the spleen (for review see [11]). LTα is also one of the cytokines that shows distinct pattern of expression upon Th1/Th2 polarization of T helper cells, and serves as a Th1 marker [12]. Studies of TNF- and LT-deficient mice identified several key molecules mediating the effects of LT

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Abbreviation: LT: Lymphotoxin **TIM:** T cell immunoglobulin- and mucin-domain-containing molecule **SMUCKLER:** Spleen, mucin-containing, knockout of lymphotoxin **aa:** Amino acid **KO:** Knockout

LT signaling, in particular, chemokines specific for secondary lymphoid tissues, such as SLC, BLC and ELC [13, 14]. Independently, these and several other gene products were identified by expression profiling in TNF and LTα knockout mice [15, 16].

Proteins of the TIM (T cell immunoglobulin- and mucin-domain-containing molecule) family recently attracted much attention as potential surface markers of Th1 and Th2 cells (reviewed in [17]). In particular, TIM3 which is expressed only by differentiated Th1 cells [18] was recently shown to be critical for down-regulation of Th1-mediated immune responses [19, 20]. TIM family genes form a compact cluster on chromosome 5 in humans and on chromosome 11 in mice. Both in mice and in humans TIM locus belongs to a chromosomal interval linked to susceptibility to asthma, a disease characterized by disturbed Th1/Th2 balance [21]. There are three TIM genes in humans and eight in mice. Human TIM4 and mouse TIM4–8 were predicted based on protein homology [17] but have not been characterized yet.

In this study we describe a novel gene, which encodes a putative membrane-bound molecule, is predominantly expressed in lymphoid tissues, such as spleen, lymph nodes (LN) and Peyer's patches, and is strongly down-regulated in LT-deficient mice. In spleen this gene, SMUCKLER (spleen, mucin-containing, knockout of lymphotoxin), is expressed mostly by stromal cells in the marginal zone and by isolated stromal cells in T- and B-cell areas, but not by T or B lymphocytes. We show that SMUCKLER is identical to TIM4 and describe coding sequence polymorphisms between BALB/c and DBA mice, two mouse strains used in allergen-induced airway hypersensitivity model of asthma [21]. The recently discovered link between LT signaling, IgE balance, and airway inflammation [22] suggests that SMUCKLER may be a legitimate candidate for genetic predisposition to asthma.

2 Results

2.1 Identification of SMUCKLER through its link to lymphotoxin deficiency in knockout mice

To identify genes regulated by lymphotoxin signaling pathway, we compared splenic gene expression profiles of mice with single LTα, TNF and combined LT/TNF deficiencies with wild-type C57BL/6 controls. Using cDNA expression microarrays, we identified one of these differentially expressed genes, subsequently named SMUCKLER. SMUCKLER showed markedly reduced mRNA expression levels in mice with LT deficiency, a modest decrease in TNF knockout mice and a dramatic defect in mice with combined LT/TNF deficiency (Fig. 1A).

In addition to spleen, SMUCKLER is expressed in LN (Fig. 1B) and Peyer's patches (see below). Similar to spleen, SMUCKLER mRNA levels in mesenteric LN are reduced in TNF KO mice and further reduced in LTβ KO mice, as compared to wild-type controls (Fig. 1B).

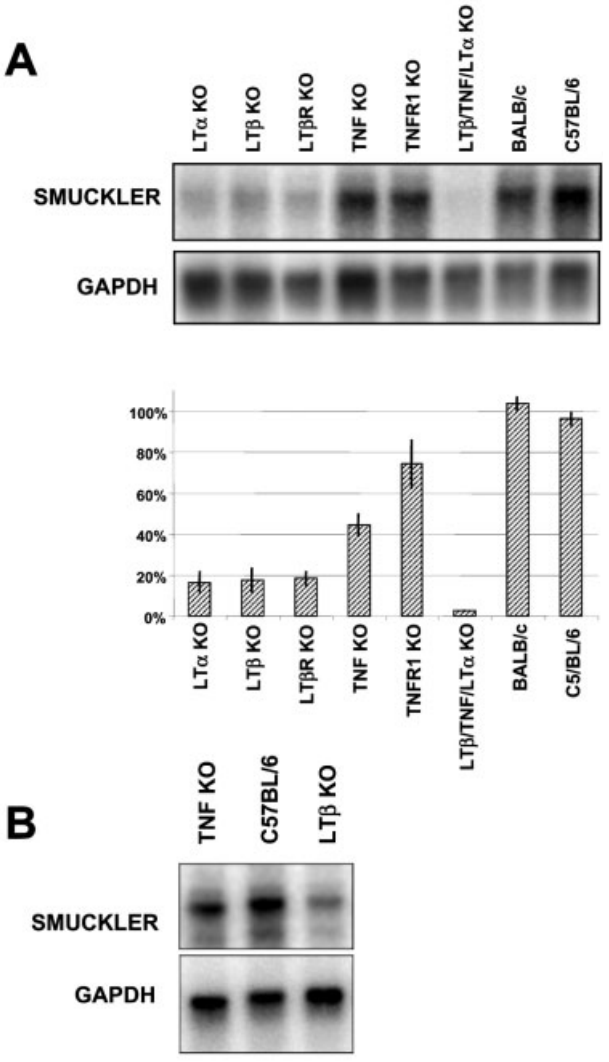

Fig. 1. Identification of SMUCKLER via link to LT deficiency. (A) Northern analysis of polyA$^+$ mRNA samples from spleen of wild type and several KO mice. Diagram shows data normalized for GAPDH expression. Mean SMUCKLER/GAPDH value between BALB/c and C57BL/6 mice was taken for 100%. Error bars indicate standard errors. (B) Northern analysis of polyA$^+$ mRNA samples from mesenteric lymph nodes.

2.2 SMUCKLER encodes a protein with putative transmembrane, immunoglobulin and mucin domains and belongs to TIM family

The 2.2-kb cDNA encoding SMUCKLER was amplified by reverse transcription (RT)-PCR from splenic mRNA of C57BL/6 mice and sequenced (Fig. 2) (GenBank AY376716*)*. Computer analysis revealed an open reading frame encoding a 343-amino acids (aa) protein with a 23-aa predicted signal peptide followed by an immunoglobulin V domain containing the RGD motif (aa 104–106), a mucin domain (aa 135–280), a putative transmembrane domain (aa 281–300), and a cytoplasmic region (Fig. 3A).

Database searches revealed significant homology of SMUCKLER to kidney injury molecule (KIM-1) [23] (also cloned as a receptor of hepatitis A virus [24]), and the recently identified family of TIM genes located on chromosome 11 in mice [21]. The gene encoding SMUCKLER maps to the same 11.b1 chromosome region. The murine TIM locus contains eight putative genes, only two of which have been previously characterized. Interestingly, the human locus apparently contains only three TIM genes: TIM1, TIM3, and TIM4. Alignment of mouse and human TIM families (Fig. 3A) and phylogenetic analysis (Fig. 3B) clearly indicated that SMUCKLER is a murine ortholog of human TIM4 and is identical to the predicted murine TIM4 [17].

Importantly, SMUCKLER/TIM4 is distinct from TIM3, as well as from TIM1, due to the absence of a putative tyrosine phosphorylation sequence in its intracellular domain which is implicated in TIM3 signaling [17].

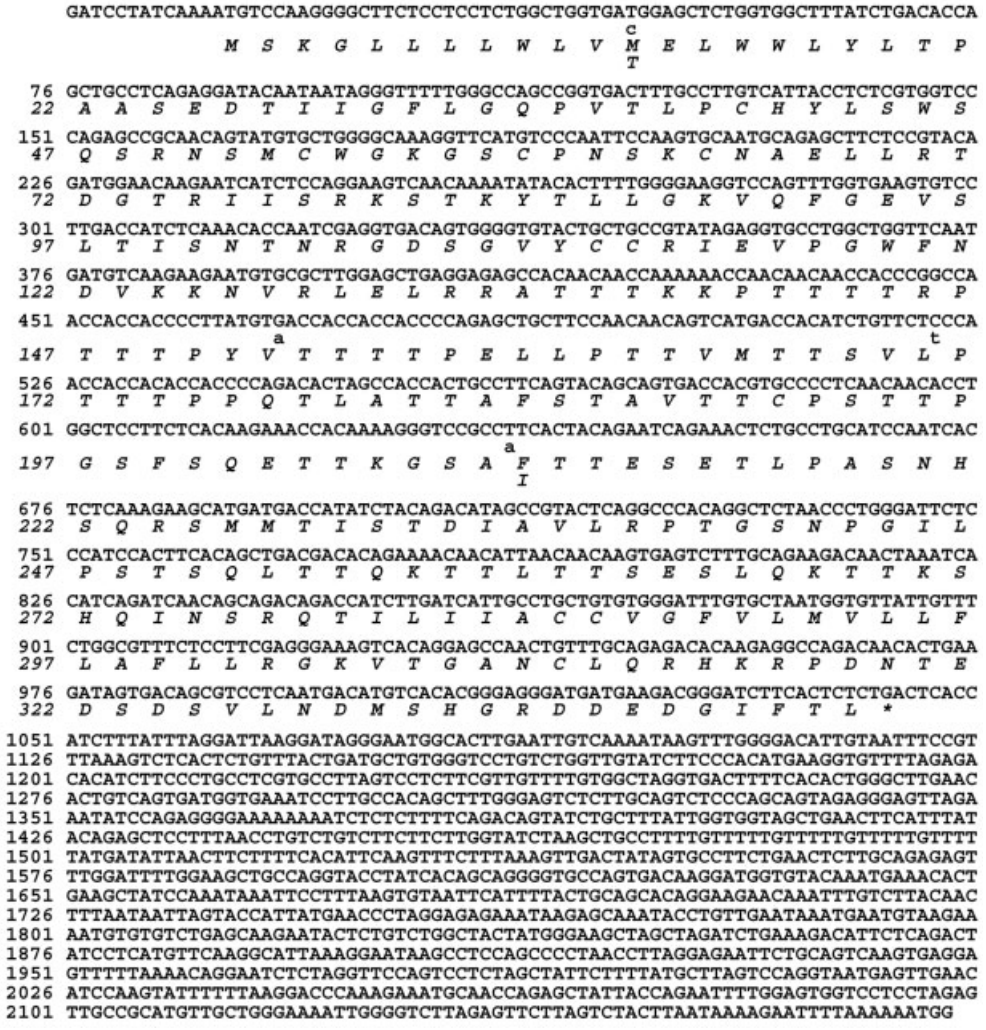

Fig. 2. Nucleotide sequence of cDNA encoding SMUCKLER from C57BL/6 strain. Nucleotide and amino acid variations with BALB/c strain are indicated.

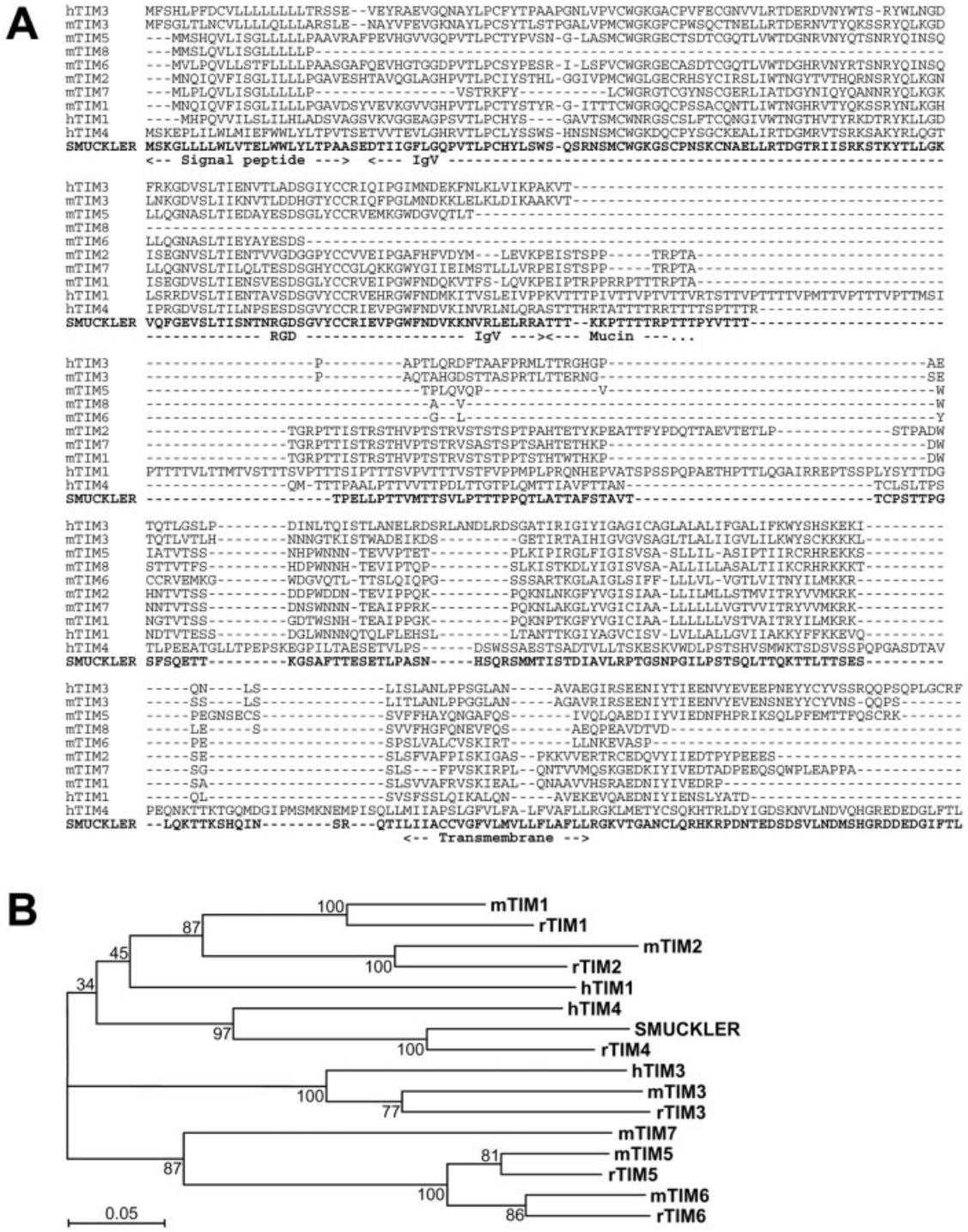

Fig. 3. The relation between SMUCKLER and TIM family members. (A) Alignment of SMUCKLER amino acid sequence with murine and human TIM. Signal peptide, IgV domain, mucin domain and transmembrane domain of SMUCKLER are indicated. (B) Phylogenetic tree of TIM proteins. A neighbor-joining tree is shown representing the relationship of the immunoglobulin V domain amino acid sequences of human, mouse and rat TIM proteins. A maximum parsimony analysis yields a tree with identical topology. Numbers at nodes indicate percent of bootstrap support based on 1,000 replicates. The scale bar indicates substitutions per site using the Poisson correction distance method. Putative TIM8 protein lacks IgV domain and therefore was not included in the analysis.

2.3 SMUCKLER expression in lymphoid organs

By Northern blot analysis we established that in wild-type C57BL/6 mice SMUCKLER is predominantly expressed in peripheral lymphoid tissues, such as spleen (Figs. 1A, 4), lymph nodes (Fig. 1B) and Peyer's patches (data not shown). We failed to detect any appreciable expression levels in T or B lymphocytes by Northern analysis (data not shown). Moreover, even 1 μg of polyA$^+$ mRNA prepared from total splenocyte suspension (obtained using a cell strainer, Sect. 4) failed to produce any detectable signal, in contrast to mRNA preparations from unfractionated homogenized spleen (Fig. 4 and 6A). Under these conditions the expression of many genes, known to be expressed by lymphocytes, could be easily detected (Fig. 6A and data not shown).

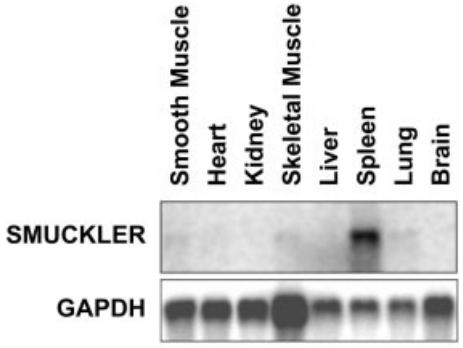

Fig. 4. Tissue specificity of SMUCKLER expression. Northern blot using polyA$^+$ mRNA samples from selected tissues of wild-type mouse.

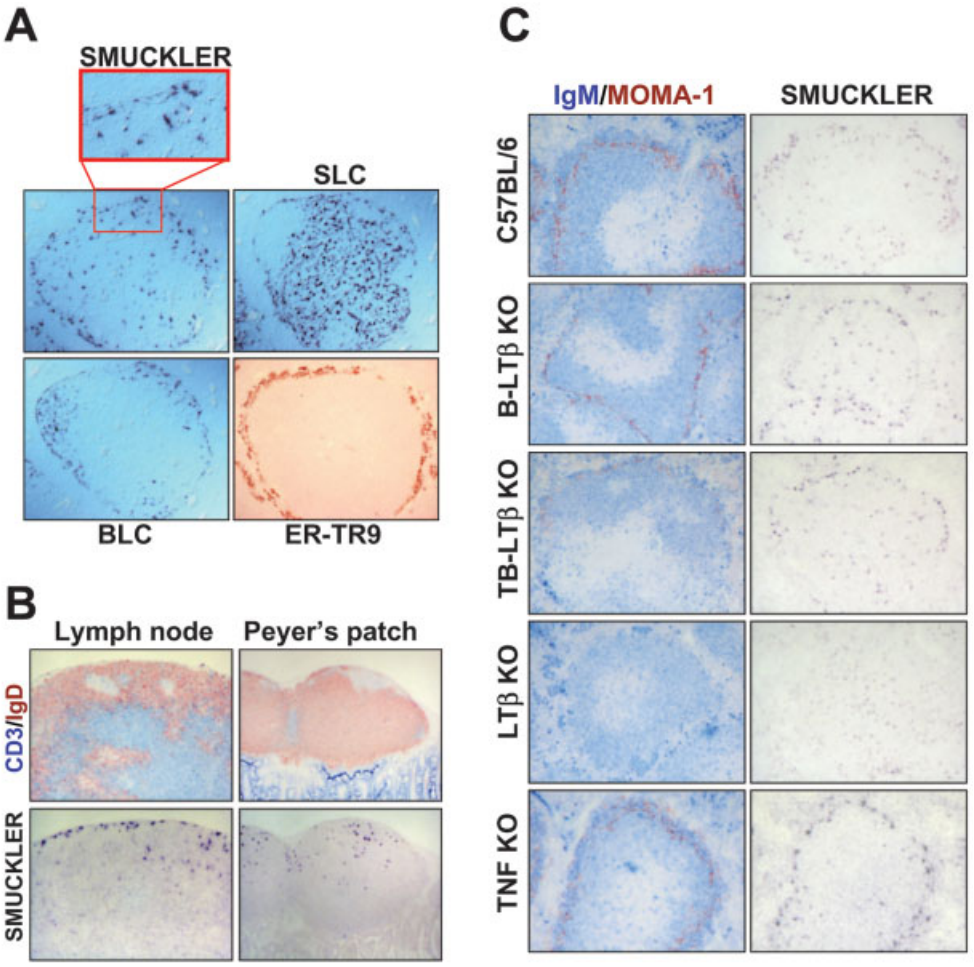

Fig. 5. In situ analysis of SMUCKLER expression. (A) Spleen. ER-TR9 antibody staining on serial sections demarcates marginal zone macrophages while probes for BLC and SLC chemokines stain B cell zones and T cell zones, respectively. Images were taken using differential interference contrast microscopy. (B) LN, PP. B cell follicles and T cell zones are labeled with two-color immunohistochemistry (anti-IgD, red; anti-CD3ε, blue). (C) *In situ* analysis of SMUCKLER expression in LT and TNF knockout mice (spleen). For marginal zone visualization on serial sections, MOMA-1 stains metallophilic macrophages, and IgM staining outside MOMA-1 indicates marginal zone B cells.

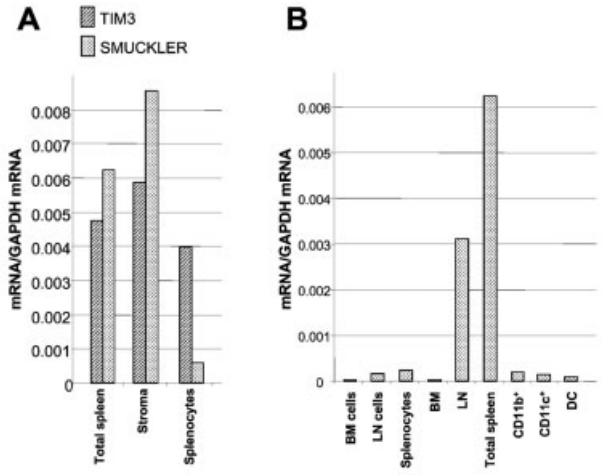

Fig. 6. SMUCKLER is expressed by stromal cells. (A) Differential expression of SMUCKLER/TIM4 and TIM3 in spleen. Spleen fractions were separated by a cell strainer (see Sect. 4). (B) SMUCKLER expression in lymphoid organs. "BM cells", "LN cells" and "splenocytes" cell suspensions were obtained by passing the corresponding organs through cell strainer. "$CD11b^+$" is and "$CD11c^+$" are cell fractions obtained by MACS from the total splenocyte populations. "DC" is a population of bone marrow-derived dendritic cells from BALB/c mice. Results of representative experiments are shown.

To precisely define the expression pattern of SMUCKLER in lymphoid tissues we performed *in situ* hybridization. This method allowed detection of SMUCKLER expression in splenic white pulp, inside the area demarcated by the labels specific for the splenic marginal zone, such as MAdCAM-1 and MOMA-1 (Fig. 5A). In addition, a few bright SMUCKLER-expressing cells were observed in T and B cell areas. In LN, SMUCKLER expression was clearly detected in the subcapsular sinus and in the paracortex area. In Peyer's patches, SMUCKLER expression was observed in isolated cells in lymphoid follicles (Fig. 5B). SMUCKLER-expressing cells appeared to have a stromal morphology (Fig. 5 A, B)

Importantly, the patterns of TIM3 and SMUCKLER expression are strikingly different. Both genes are expressed at similar levels in total mRNA preparations from unfractionated spleen (Fig. 6A). However, after separation of splenocytes and stroma using a cell strainer most of TIM3 expression was recovered in the pass-through fraction which contained lymphocytes, dendritic cells and macrophages, while the expression of SMUCKLER was lost. Similarly, we failed to detect any significant levels of SMUCKLER mRNA in lymph node cells passed through a cell strainer or in bone marrow (Fig. 6B). These observations were consistent with the reported expression of TIM3 on T lymphocytes, but clearly indicated that SMUCKLER is expressed by stroma of lymphoid tissues, or by cells tightly associated with stroma.

2.4 SMUCKLER expression in spleen correlates with the presence of the marginal zone

In order to determine the cellular source of LT required for SMUCKLER expression, we used tissue-specific knockout mice [25, 26]. Genetic ablation of LTβ expression either in B cells or in B and T cells together had little effect on SMUCKLER expression, as detected by *in situ* hybridization (Fig. 5C). B-LTβ KO and T,B-LTβ KO mice gradually lose organized B cell follicles and germinal centers in spleen, but retain distinct staining for marginal zone markers (although of reduced intensity). In contrast, LTβ KO mice completely lack marginal zone, and only few SMUCKLER-positive cells scattered throughout white pulp can be observed in the LTβ KO spleen (Fig. 5C). At the same time, SMUCKLER expression is preserved in the marginal zone of TNF KO mice which also lack organized B cell follicles and germinal centers (Fig. 5C). Thus, SMUCKLER expression in spleen appears to require the presence of marginal zone but does not correlate with intact B-cell follicles and germinal centers. Surface lymphotoxin expressed by T and B lymphocytes is not required for SMUCKLER expression in spleen, suggesting that the corresponding signals are coming from yet unidentified cell types.

2.5 TIM locus in BALB/c mice shows sequence variations in the coding regions of SMUCKLER gene, as compared to DBA or C57BL/6 mice

To address the possible role of SMUCKLER in genetic predisposition to asthma in a mouse allergen-induced airway hyperreactivity model [21], we sequenced all nine exons of this gene in three mouse strains, C57BL/6, BALB/c and DBA which strongly differ in their susceptibility to experimental asthma.

We identified several DNA sequence polymorphisms. The coding portions of C57BL/6 and DBA genes are identical (GenBank AY376716), and differed from the BALB/c gene (GenBank AY376717) by two aa variations: in the leader peptide (aa 12, methione to threonine), and in the mucin domain (aa 209, isoleucine to phenylalanine). Additionally, there were sequence variations in the third codon position at aa 152 and 170 which did not result in an aa substitution (Fig. 2).

3 Discussion

Identification of genes associated with LT deficiency in lymphoid organs may help to unravel molecular signaling pathways essential for functional compartmentalization of lymphoid tissues, germinal center formation, FDC differentiation and immune responses. The signals ablated in LT and/or TNF knockout mice may relate to functional interactions between cells of hematopoietic origin, such as T cells and B cells, or between T cells and antigen-presenting cells, such as macrophages and dendritic cells. Alternatively, LT-mediated interactions may involve lymphocytes and stromal components of spleen and other lymphoid tissues, known to express LTβR and TNFRp55.

By searching for genes whose expression in spleen is inversely associated with a splenic phenotype in LTα– and LTβ-deficient mice, we identified a cDNA encoding a novel mucin-containing transmembrane protein, named SMUCKLER, which is a member of the TIM family of genes [18, 21] and is identical to TIM4 gene previously predicted based on phylogenetic analysis [17]. The murine TIM cluster is located at the distal part of murine chromosome 11, and the human counterpart, on chromosome 5. Interestingly, the human genomic locus appears to lack the TIM2 gene, whose partner is a member of the semaphorin family expressed on antigen-presenting cells [27]. This interaction was implicated in the costimulation of T cells. Identification of a mouse ortholog of TIM2 in humans awaits further study, but SMUCKLER clearly is not a candidate due to the lack of a phosphorylation-signaling motif and due to its distinct pattern of expression.

When compared across the entire panel of TNF and LT KO mice and their corresponding receptor KO mice, it appears that the level of SMUCKLER expression in spleen correlated with the degree of disruption of splenic microarchitecture, which is lowest in TNF and TNFR KO, and highest in mice with triple deficiency in TNF/LTα/LTβ (Fig. 1A). Systematic comparison of expression levels in LN and PP across the panel was not possible, since many of these knockout models lack all or at least some of these peripheral lymphoid tissues.

Interestingly, the presence of marginal zone is required for SMUCKLER expression. It is tempting to speculate that SMUCKLER may be one of the molecules mediating yet poorly understood role of LT in marginal zone development. Further studies, including SMUCKLER biochemical and genetic inactivation, will be required to test this hypothesis.

Thus, SMUCKLER/TIM4 is a novel member of the TIM family. However, the lack of expression on T cells (the reason for T in the TIM abbreviation) and the lack of identifiable tyrosine phosphorylation motif suggest that SMUCKLER/TIM4 may be functionally distinct from the two previously characterized TIM genes.

The observations that SMUCKLER is linked to LT deficiency and thus may be involved into regulation of the Th1/Th2 balance is intriguing. The presence of tyrosine-phosphorylation motif in intracellular domain of TIM3 suggested that it may be a receptor for an unknown ligand [18]. Indeed, TIM3 signaling in mice can be effectively blocked by systemic administration of TIM3-Ig soluble protein [19, 20]. Future studies will show whether TIM4 which lacks any identifiable signaling motif in its cytoplasmic portion acts as a ligand, a decoy receptor or whether it recruits additional adapter molecules via yet unknown protein-protein interactions.

Recently Fu and co-workers have linked LTα signaling to IgE production [22] and showed that LTα deficient mice show unexpectedly decreased IgE levels and develop an airway inflammation related to asthma. Since low expression of SMUCKLER is associated with LT deficiency, it appears that its normal expression correlates with protection. Polymorphisms in the coding sequence of SMUCKLER between mouse strains differing in predisposition to disease in experimental asthma model make this gene a legitimate candidate for future genetic studies.

4 Materials and methods

4.1 Mice

LTα and other KO mice were described previously [6, 8, 28–32]. All mouse strains were maintained under specific pathogen-free conditions.

4.2 Preparation of primary cells

Spleen cells were obtained using 70-μM nylon Falcon cell strainer (BD Biosciences, San Jose, CA). Cell suspension and the stromal fraction retained on the strainer were collected separately and washed once with PBS before use. Dendritic cells were obtained from bone marrow by cultivation in the presence of IL-4 and GM-CSF. Separation of cells based on the surface markers was performed by MACS following the manufacturer's protocol (Miltenyi Biotec Inc., Auburn, CA).

4.3 RNA preparation

Total cellular RNA was extracted with Trizol™ reagent (Gibco BRL, Gaithersburg, MD) and used for microarray hybridizations and Northern analysis. Poly A^+ RNA for cDNA synthesis was prepared by using the mRNA Separator from CLONTECH Laboratories, Inc. (Palo Alto, CA), following the manufacturer's protocol.

4.4 Northern blots

Ten micrograms of total or 1 μg of poly A^+ mRNA was separated on 1.5% denaturing agarose gels and transferred to Supported Nitrocellulose-1 (Gibco BRL) membrane. Hybridization with ^{32}P-labeled probes was performed in ExpessHyb™ solution (CLONTECH) and washed following the recommended protocol. Radioactivity was quantified using Molecular Dynamics screens and ImageQuant software. One microgram of poly A^+ RNA from the spleens of different KO or wild-type mice was separated by gel electrophoresis, transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and probed using randomly primed ^{32}P-labeled mouse cDNA probes in ExpessHyb™ solution from CLONTECH Laboratories, Inc. (Palo Alto, CA). To control for loading, the blots were rehybridized using mouse GAPDH cDNA as a probe. GAPDH blots were exposed for shorter times as compared to SMUCKLER. To quantitate the bands, Northern blots were developed using a PhosphorImager ™ SF, and the data were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The levels of mRNA were normalized by dividing the signal for each sample by the GAPDH signal for that sample.

4.5 Gene profiling analysis

InCyte (Genome System, Inc.) gene microchips (Mouse GEM1 Microarray) were used that contained 10,000 murine genes and expressed sequences tags (EST). The microarrays were probed with cDNA derived from splenic polyA$^+$ mRNA (0.6 μg) from both wild-type (Cy3) and triple KO (Cy5) mice as a custom service by the company. After hybridization, the data were posted at the company web site and the results were viewed using the provided software.

4.6 *In situ* hybridization analysis and immunohistochemistry

In situ hybridization analysis and immunohistochemistry were performed as described [15, 25]. All antibodies were from BD Biosciences PharMingen (San Diego, CA) except anti-MOMA-1 which was from Serotec Inc. (Raleigh, NC), and ER-TR9 which was from Bachem/Peninsula Laboratories Inc. (San Carlos, CA).

4.7 5' Nuclease Taqman real-time PCR assay

Real-time PCR assay was performed according to manufacturer's instructions (Applied Biosystems, Foster City, CA) with the following oligonucleotides: TIM3, 5'-ggcgatctcaacaaaggaga, 5'-ggagggtcaccagtgtctgt, FAM-5'-tatcctgcagcagtaggtcccatg-3'-TAMRA; TIM4, 5'-acaccaccccagacactagc, 5'-gtcgtcagctgtgaagtgga, FAM-5'-ttgagagtgattggatgcaggcaga-3'-TAMRA.

4.8 Sequencing

Platinum Taq DNA Polymerase High Fidelity (Gibco BRL) was used for amplification of genomic DNA. Primers for PCR and sequencing: MT4F1 gttgagcgtcccaccctg; MT4R1 gaaggaggcaggctttag; MT4F2 gacactccctcatctcctg; MT4R2 gcccagtcttgtcaccag; MT4F3 ggatgggaagggaaagatc; MT4R3 ggttgcccactgtagaaga; MT4F4 tgactctcaccacccactg; MT4R4 ctgagctcatctctggatc; MT4F5 gcatgtagcatttctggtatg; MT4R5 gcggactggtctcatgg; MT4F6 tctggctgaacaggtggc; MT4R6 tctcccgagcgcatcagtg; MT4F7 ttagaaggatgtgtcccaag; MT4R7 actacgcccctcccatga; MT4F8 ctgatgctagtctactttctg; MT4R8 caagtcactaaaccactgag; MT4F9 tgctcagcctgctttagtg; MT4R9 aacggaaattacaatgtccc. PCR fragments were purified and the same primers were used for sequencing with a DNA Sequencing Kit (Applied Biosystems), sequencing reactions were resolved on an ABI 373A automated sequencer.

4.9 Signal peptide prediction

The SignalP server was used with hidden Markov model and neural networks (http://www.cbs.dtu.dk/services/SignalP-2.0/) [33].

4.10 Phylogenetic analysis

Immunoglobulin V domains of TIM proteins were aligned using ClustalX program [34]. The resulting multiple alignment was analyzed using Mega2 program (http://www.megasoftware.net/) [35].

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