SUPPLEMENTARY INFORMATION

Abnormal thymic stromal lymphopoietin expression in the duodenal mucosa of patients with coeliac disease

Running head: TSLP in coeliac disease

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Supplementary Methods

Generation of anti-TSLP antibody. For the preparation of the rabbit anti-TSLP antibody used for immunofluorescence, TSLP fragment aa 1-96 was cloned and expressed in BL21 competent *E.coli* cells with a DsbA tag. Rabbits were immunized with the tagged fragment in the presence of incomplete Freund's adjuvant and the resulting sera were depleted of anti-DsbA antibodies.

Short TSLP peptide synthesis. Recombinant short TSLP (aa 4-60, mw 7kDa, Uniprot: Q96AU7) was synthesized in Maria Rescigno's lab and resuspended in sterile water. Peptide was identified by MALDI-TOF mass spectrometry analysis. Endotoxin levels were below 0.1 ng/μg of peptide as determined by LAL test. The peptide was assembled by stepwise microwave-assisted Fmoc-solid-phase peptide synthesis (SPPS) on a Biotage ALSTRA Initiator^{+} peptide synthesizer, operating in a 0.12 mmol scale on a HMPB-ChemMatrix resin (0.45 mmol/g; Iris Biotech GmbH, Marktredwitz, Germany). Resin was swelled prior to use with a NMP/ dichloromethane (DCM; Sigma-Aldrich, Poole, UK) mixture. Activation and coupling of Fmoc-protected amino acids (Iris Biotech GmbH) was performed using ethyl cyanoglyoxylate-2-oxime (Oxyma; Novabiochem, Darmstadt, Germany) 0.5M / N,N'-diisopropylcarbodiimide (DIC; Sigma-Aldrich) 0.5M (1:1:1), with a 5 equivalent excess over the initial resin loading. Coupling steps were performed for 7 min at 75°C. Deprotection steps were performed by treatment with a 20% piperidine solution in N,N'-dimethylformamide (DMF; Carlo Erba, Rodano, Italy) at room temperature (1 x 3 min + 1 x 5 min). Following each coupling or deprotection step, peptidyl-resin was washed with DMF (4 x 5 ml). Following chain assembly, peptide was cleaved from the resin using a trifluoroacetic acid (TFA; Carlo Erba) 90%, water 5%, thioanisole 2.5%, TIS 2.5% mixture (3h, room temperature).

Following precipitation in cold diethyl ether, crude peptide was collected by centrifugation and washed with further cold diethyl ether to remove scavengers. Peptides was then dissolved in 50% aqueous acetonitrile (ACN) 0.07% TFA buffer and purified by preparative reversed phase high performance liquid chromatography (RP-HPLC). Analytical and semi-preparative RP-HPLC were carried out on a Tri Rotar-VI HPLC system equipped with a MD-910 multichannel detector for analytical purposes or with a Uvidec-100-VI variable UV detector for preparative purpose (all from JASCO, Tokyo, Japan). A Phenomenex Jupiter 5μ C18 90Å column (150 x 4.6 mm; Phenomenex, Torrance, CA) was used for analytical runs and a Phenomenex Jupiter 10μ C18 90Å (250 x 21.2 mm) for peptide purification. Data were recorded and processed with Borwin software. 2%/min linear gradient of 0-60% eluent B (eluent A = H_2O/3% CH_3CN/0.07% TFA, eluent B = 70% CH_3CN/30% H_2O/0.07% TFA) was employed at a flow rate of 1mL/min for analytic purposes. UV detection was recorded in the 220-320 nm range. Peptide purification was achieved by preparative RP-HPLC at a flow rate of 14 mL/min using a 100% A $\rightarrow$ 30% B gradient over 40 min. Pure RP-HPLC fractions (>95%) were combined and lyophilized. All solvents for solid-phase peptide synthesis (SPPS) were used without further purification. HPLC grade ACN and ultrapure 18.2Ω water (Millipore Milli-Q; Millipore, Bedford, MA) were used for the preparation of all buffers for HPLC.

Immunofluorescence. Four 5 μm-cryostat sections were fixed in PFA 4% for 15 min and incubated overnight at 4°C with rabbit anti-TSLPR (1:400 dilution, LSBio, Seattle, WA), mouse anti-CD3 (1:10 dilution, Abcam, Cambridge, UK), mouse anti-CD11c (1:5 dilution, Abcam), or mouse anti-CD14 (1:100 dilution, Biolegend, San Diego, CA). Alexa555-conjugated donkey anti-rabbit (Life Technologies Ltd, Paisley, UK) and Alexa488-conjugated donkey anti-mouse (Life Technologies Ltd) were used as

secondary antibodies. Sections were counterstained by DAPI (1:1000 dilution; Life Technologies Ltd). For each fluorochrome label, isotype control antibodies were used on parallel sections. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and visualized under a Leica TCS SP5 laser confocal scanner (Leica, Wetzlar, Germany) mounted on a Leica DMI 6000B inverted microscope (Leica) equipped with motorised stage (20x or 40x magnification). Leica LAS AF was used for all acquisitions. ImageJ software package (ImageJ, Bethesda, MD) was used for image analysis.

Bidirectional mixed lymphocyte reaction. Peripheral blood mononuclear cells from heparinised peripheral venous blood of six donors were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich). Freshly isolated peripheral blood mononuclear cells from pairs of different donors were co-cultured (3×10^5 cells from each donor/well, in triplicate) with RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C, 5%CO_2 for 120h in 96-well plates (VWR International, Lutterworth, UK), in the absence or presence of 40 U/ml furin (Sigma-Aldrich), or increasing concentrations (0.0001-1 nM) of vitamin D3 (Sigma-Aldrich), or 25 ng/ml or 50 ng/ml short TSLP (manufactured and kindly provided by Maria Rescigno's lab; please see Supplementary Methods for details), or increasing concentrations (25-200 ng/ml) of long TSLP (R&D Systems, Abingdon, UK) pre-treated for 24h with furin (Sigma-Aldrich; TSLP and furin concentrations in the cleavage reaction 1 μg/ml and 200U/ml, respectively) or RPMI-1640 medium. IFN-γ was measured in culture supernatants by ELISA.

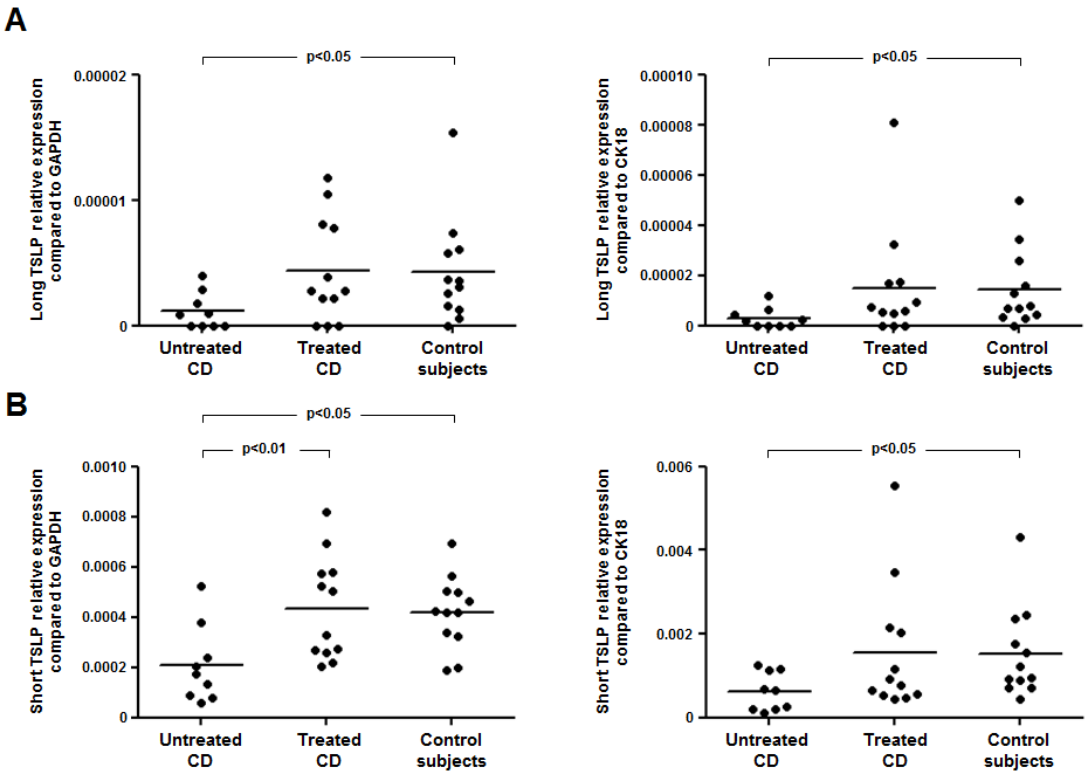
Supplementary Figure Legend

Supplementary Figure 1. *Long and short TSLP transcript expression in untreated CD, treated CD and control mucosa.* (**A**) Long and (**B**) short TSLP transcripts, quantified by qRT-PCR, in the duodenum of 9 untreated CD, 12 treated CD and 12 controls. Changes in transcript levels are normalised for either GAPDH or CK18. Horizontal bars are mean values.

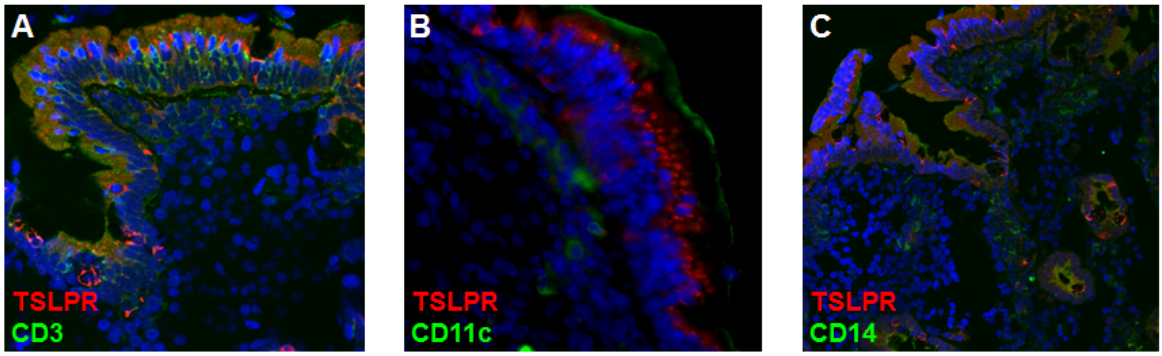
Supplementary Figure 2. *Immunofluorescence staining of the duodenal mucosa of an untreated CD patient with TSLPR versus CD3 (**A**), TSLPR versus CD11c (**B**), and TSLPR versus CD14 (**C**).* (**A**) TSLPR (red) is mostly expressed by enterocytes, while it is undetectable on the surface of CD3-positive (green) intraepithelial lymphocytes and lamina propria mononuclear cells. (**B**) Some LPMCs localised underneath the epithelium co-express CD11c (green) and TSLPR (red). (**C**) A few LPMCs co-express TSLPR (red) and CD14 (green). Nuclei were counterstained by DAPI (blue). These results are representative of the staining performed in six untreated CD patients. Original magnification 40x.

Supplementary Figure 3. *Ex vivo increased IL-8 production by untreated CD biopsies and down-regulatory effects of TSLP on IL-8 production by untreated CD myofibroblasts*. (**A**) IL-8 production by untreated CD biopsies cultured *ex vivo* for 24h, as evaluated on culture supernatants by ELISA. Values are mean ± SEM. (**B**) Effects of recombinant human long TSLP on the release of IL-8 by duodenal myofibroblasts isolated from 2 untreated CD patients, as evaluated on culture supernatants by ELISA. Six independent experiments were repeated using 2 different cell lines. Values are mean ± SEM.

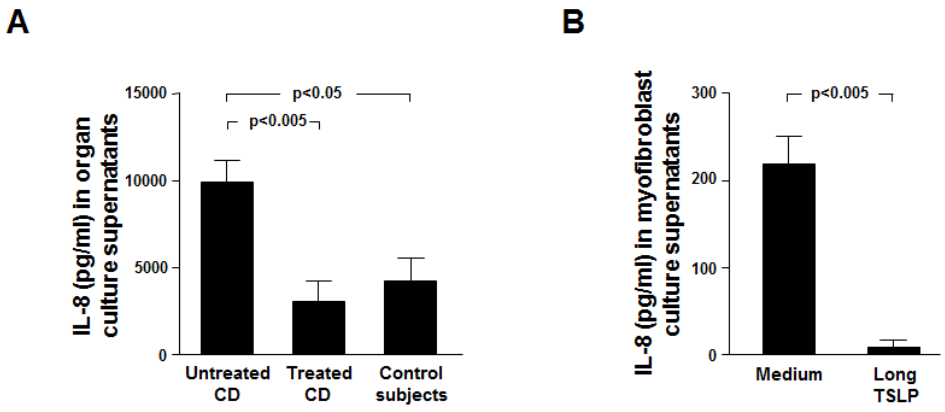
Supplementary Figure 4. *Effect of furin-exposed long TSLP on IFN-γ production in a bidirectional mixed lymphocyte reaction.* Freshly isolated peripheral blood mononuclear cells from pairs of different donors were co-cultured in a bidirectional mixed lymphocyte reaction in the absence or presence of 40 U/ml furin, or increasing concentrations (0.0001-1 nM) of vitamin D3, or 25 ng/ml or 50 ng/ml short TSLP, or increasing concentrations (25-200 ng/ml) of long TSLP, pre-treated for 24h with furin or RPMI medium. IFN-γ was measured in culture supernatants by ELISA. Culture with furin-exposed long TSLP led to a significant ($p<0.05$) reduction in IFN-γ release compared to culture with RPMI medium-exposed long TSLP at all concentrations tested. Results of three independent experiments are shown. Values are mean ± SEM. *$p<0.05$ *versus* medium alone; $^{\#}p<0.05$ *versus* RPMI medium-exposed long TSLP.



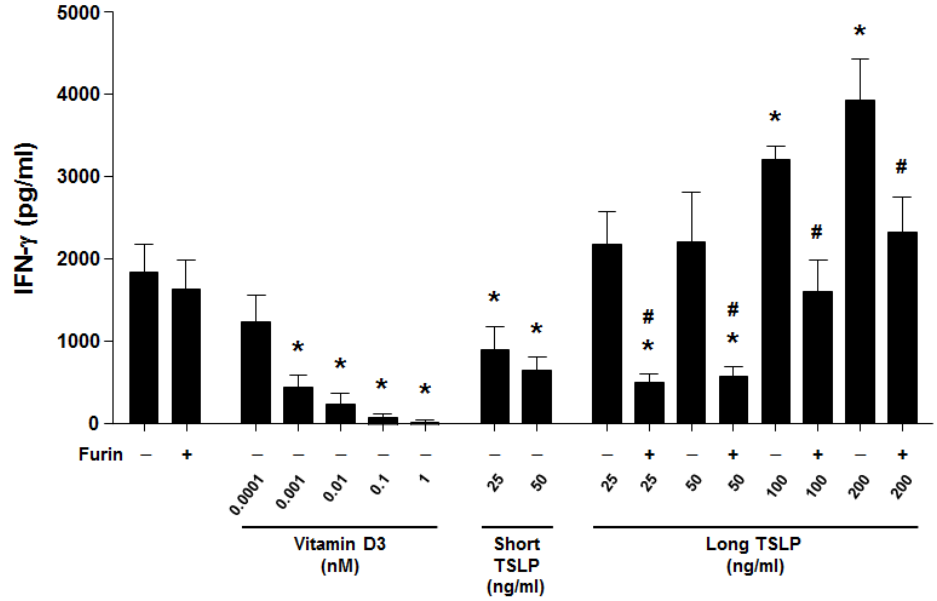
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4