The trefoil peptides TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response

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Abstract Members of the trefoil factor (TFF) family are mucin-associated polypeptides that are expressed along the entire length of the gastrointestinal tract. TFFs have been proposed to play a role in mucosal defence through both protective and reparative mechanisms. The potential relationship between TFFs and mucins in non-gut glycoprotein-secreting epithelia has not been fully explored. In the present study we identified TFF2 and TFF3 mRNA and peptide in rat lymphoid tissues, demonstrated that TFF peptide expression in rat spleen increased 1.5- to 3-fold following experimental induction of the immune response, and showed that hTFF2 and hTFF3 (1–5 mg/ml) stimulated migration of human monocytes. Our data suggest that TFFs may in part be involved in the repair of injury through the modulation of the inflammatory response.

Key words: Trefoil factor; Lymphoid tissue; Inflammatory response

1. Introduction

Trefoil factors (TFFs) comprise a group of small polypeptides that are characterised by one or more three-looped structural motifs resembling a three leaf clover or trefoil [1]. There are three known mammalian members of the TFF family called TFF1, 2, and 3.

Collectively, the trefoil peptides are expressed along the entire length of the normal gastrointestinal tract, with TFF1 and TFF2 expressed primarily by the stomach [2–4], and TFF3 by the intestine and colon [5–7]. The cellular sites of trefoil expression are invariably associated with extensive glycoprotein biosynthesis and secretion, and trefoil expression has been localised to mucus-secreting cells of the gastrointestinal mucosa [2,4,5]. Both a structural [8] and functional [9–11] association between mucus glycoproteins and the TFFs have been proposed, and it is possible that this is of widespread physiological importance.

While the precise role of the trefoil peptides is still largely unknown, in vivo studies have shown that parenteral [12,13], as well as oral [14,15] administration of TFFs protect the rat gastric mucosa against NSAID-induced injury. This cytoprotective function is supported by the production of TFF transgenic animals in which loss of TFF1 or TFF3 leads to increased susceptibility to damage [16,17], and over-expression of TFF1 results in increased resistance to damage [18]. These observations are also underscored in in vitro systems where hTFF2 and rTFF3 were shown to act synergistically with mucin glycoproteins to enhance protection of intestinal epithelial cells against a variety of injurious agents [11]. A potential role for the TFFs in the reparative process through restitutive mechanisms is also inferred from observations demonstrating that TFFs stimulate the migration of epithelial cells in monolayer culture [10,12,13].

While it is likely that the TFFs major role is in the maintenance of gastrointestinal integrity, a potential relationship between TFFs and non-gastrointestinal glycoprotein-secreting tissues has not been fully explored. For instance, both endothelial cells and leukocytes express glycoproteins that may be defined as mucins by virtue of their O-glycosylation patterns and proline, serine and threonine rich domains [19]. These molecules, such as CD34, CD43, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and mucosal addressin cellular adhesion molecule-1 (Mad-CAM-1), act as ligands for the selectins and integrins and participate in the adhesion cascade by which peripheral leukocytes traffic to lymphoid organs and/or inflamed tissue [20]. Since TFF3 has been shown to regulate E-cadherin-catenin, and APC-catenin interactions [21], it is possible that the trefoil factors may also facilitate migration through an association with mucins in these extra-gastrointestinal sites.In this study we have unequivocally identified the expression of the trefoil peptides TFF2 and TFF3 in rat lymphoid tissues, shown that TFFs can be induced in splenic tissue by the bacterial cell wall endotoxin lipopolysaccharide, and demonstrated a potential function for these peptides as stimulators of monocyte migration.

2. Materials and methods

2.1. Animals

Six male and four female Long Evans rats (250–310 g) were used to investigate the distribution of trefoil factors in various tissues.

2.2. mRNA analysis

Total cellular RNA was prepared from 11 tissues by using the acid-phenol method developed by Chomczynski and Sacchi [22].

2.2.1. Identification of TFF2 mRNA by RNase protection assay (RPA). Antisense ^{32}P-UTP (Bresatec, SA, Australia) labelled TFF2 riboprobe (from Dr. G. Jeffrey) was incubated with 5–20 μg of total RNA and hybridised overnight at 45°C, essentially as described by Krieg and Melton [23], except that hybrids were digested with 20 μg/ml Rnase A and 1 μg/ml Rnase T1 (Promega, Madison, WI, USA) at 37°C for 30 min. Protected fragments were resolved on 5% acrylamide/8 M urea gels, vacuum dried and laid against autoradiographic film for 7–18 days.

2.2.2. Identification of TFF2, TFF3 and GAPDH mRNA by RT-PCR. RT: Four μg of total RNA from rat stomach, intestine, thymus, spleen, heart and liver was reverse transcribed with MMLVre-

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Abbreviations: TFF, trefoil factor; RIA, radioimmunoassay; RNase, ribonuclease; RT-PCR, reverse transcription polymerase chain reaction; ir, immunoreactivity

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verse transcriptase (Gibco-BRL, Gaithersberg, MD, USA), after which the product was denatured at 95°C for 5 min and cooled on ice.

PCR: TFF2: A 519 bp fragment of the rat TFF2 sequence [3] was amplified from single stranded DNA by PCR using two oligonucleotide primers specific for the rat TFF2 cDNA sequence. Sense primer 5′-GTCCAGTGGAGCAGACAT (nucleotides 14–31) and antisense primer 5′-CACTGCTGAGGCTCAAGA (nucleotides 516–533; Gibco-BRL).

TFF3: A 422 bp fragment of the rat TFF3 sequence [7] was amplified from single stranded DNA by PCR using two oligonucleotide primers specific for the rat TFF3 cDNA sequence. Sense primer 5′-GTACCTCGAGATGGAGACCAGAGCCTTCTG (nucleotides 18–37) and antisense primer 5′-TCAGTCTAGAACAGCCTTGTGCTGACTGTA (nucleotides 401–420; Gibco-BRL). Bases underlined are added enzyme restriction sites. The sense primer contains an *Xho*I linker and the antisense primer contains an *Xba*I linker (underlined).

GAPDH: A 420 bp fragment of the rat GAPDH sequence [24] was amplified from single stranded DNA by PCR using two oligonucleotide primers specific for the rat GAPDH cDNA sequence. Sense primer 5′-TGTCAGCAATGCATCCTGCACCACC (nucleotides 503–527) and antisence primer 5′-GAAGTCACAGGAGACAACCTGGTCC (nucleotides 898–923; Bresatec).

Reagent conditions for PCR reactions were 5 μl or 10 μl of each RT reaction, 50 ng of each primer, 10 mM Tris pH 8.3, 50 mM KCl, 2.0 mM MgCl, 200 μM dNTPs and 1.25 U of *Taq* DNA Polymerase (Promega), in a total volume of 50 μl. Each amplification consisted of denaturing at 95°C for 40 s, annealing at 45°C (GAPDH) or 60°C (TFF2, TFF3) for 40 s, and polymerisation at 72°C for 1 min. Twenty five (GAPDH) and 30 (TFF2, TFF3) cycles of amplification were performed after kinetic analysis to ensure that DNA amplification was in the linear range for each template. PCR products (15 μl) were resolved on 2% agarose gels.

2.3. Peptide analysis

Antibodies were raised in rabbits against a synthetic peptide comprising the C-terminal 10 amino acid sequence encoded by either the rat TFF2 or TFF3 cDNA [25]. Rat tissues and the plasmacytoma cell line HPC108.1 (from Dr. A. Harris, Walter and Eliza Hall Institute, Australia) were extracted for TFF2 and TFF3 peptide analysis by RIA and WBA according to methods previously described [26].

Immunohistochemistry was performed on consecutive serial section of Bouin's fixed rat spleen so that a comparison of the cellular expression of TFF2 and ED-1 (an established marker of macrophages, monocytes and activated microglia) could be made. TFF2 immunoreactivity (ir) was assessed according to methods previously described [26]. ED-1-ir was assessed by pre-incubation with 5% normal goat serum for 30 min, followed by incubation with monoclonal anti-rat ED-1 antisera (neat; from Dr. P. Tipping, Monash Medical Centre, Australia) for 1 h. Sections were subsequently reacted with biotinylated goat anti-mouse IgG secondary antibody (diluted 1:50 in 1% BSA) for 30 min, followed by mouse peroxidase-anti-peroxidase (diluted 1:100 in 1% BSA) for 30 min, then DAB.

2.4. Temporal expression of TFF2 and TFF3 in the rat spleen following LPS

In order to assess the effect of lipopolysaccharide (LPS) on trefoil expression, 32 male Long Evans rats were injected with 20 μg of LPS (*Escherichia coli* 127: B8 endotoxin; Difco, Detroit, MI, USA) i.p. 2 h–14 days before killing. Non-injected (0 h) rats were used as controls. Upon killing, rat spleens were excised and analysed for TFF2 and TFF3 peptide expression by RIA according to methods previously described [26].

2.5. Stimulation of human monocyte migration by recombinant TFF2 and TFF3

Ten ml of fresh human blood was heparinised, layered onto 8 ml of Ficoll-Paque and centrifuged at 1200 rpm for 30 min at 20°C. The mononuclear leukocyte rich layer was collected, washed three times in Hanks buffered salt solution (Trace Bioscientific, Castle Hill, Australia) and resuspended in Dulbecco's modified Eagle's medium (DMEM, Trace Bioscientific) at 1×10^6 cells/ml.

Monocyte migration was assayed in a 48-well micro-chemotaxis chamber (Neuro Probe Inc., Cabin John, MD, USA). The potential migratory stimuli (glycosylated human TFF2 and human TFF3 at concentrations of 0, 1, 2 and 5 mg/ml in DMEM), and a positive control (0.5 mg/ml Zymosan A), were added to the lower wells of the microchamber plate. A polycarbonate filter with 5 μm pores separated the lower wells from the upper wells. After the human mononuclear leukocyte solution was added to the upper wells (50 μl), the entire chamber was incubated for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Following incubation monocytes on the underside of the filter were fixed in methanol and stained with haematoxylin. The number of cells that had migrated toward the putative migratory factor were counted microscopically. Migratory activity was expressed as the relative percentage of Zymosan A activity.

3. Results

3.1. Identification of TFF2 and TFF3 mRNA in rat spleen

RPA was used to examine the distribution of rat TFF2

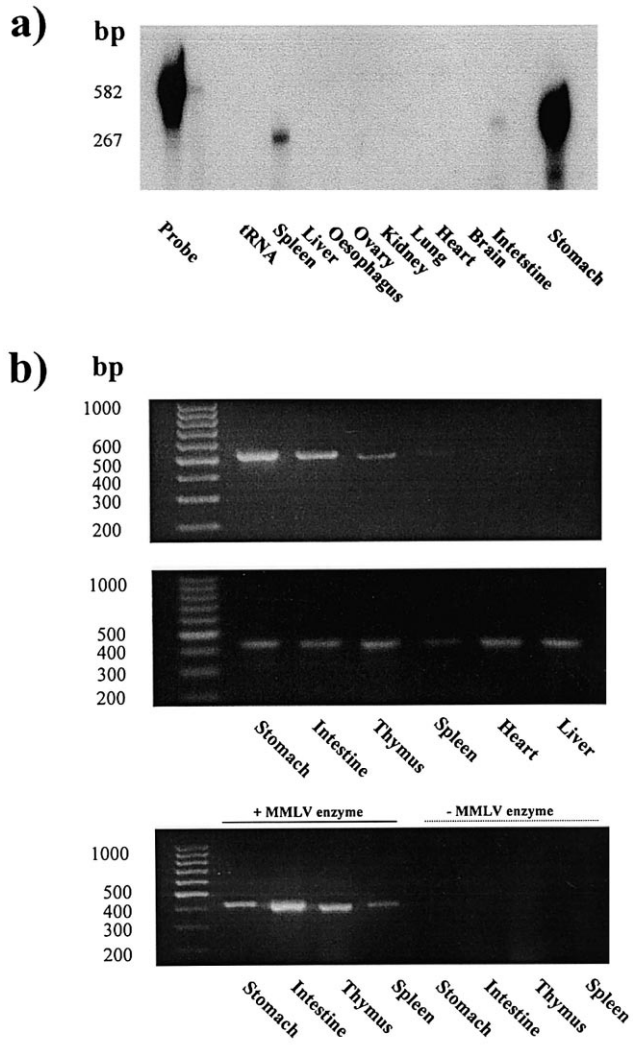

Fig. 1. a: RNase protection assay of total RNA from adult rat tissues (stomach 5 μg, other tissues 20 μg) hybridised with a rat TFF2 riboprobe (267 bp). The size of the free probe was 582 bp. Detectable levels of rat TFF2 mRNA were identified in spleen, intestine and stomach. Exposure, 18 days at −70°C. b: RTC-PCR analysis of TFF2 mRNA, GAPDH mRNA, and TFF3 mRNA in adult rat tissues. TFF2 mRNA was detected in the rat stomach, intestine, thymus and spleen but not the heart or liver. Rat GAPDH mRNA was detected in all tissues at similar levels. Rat TFF3 mRNA was detected in the rat stomach, intestine, thymus and spleen. Control reactions containing total RNA but without transcriptase demonstrate that the products were not amplified due to primer contamination.

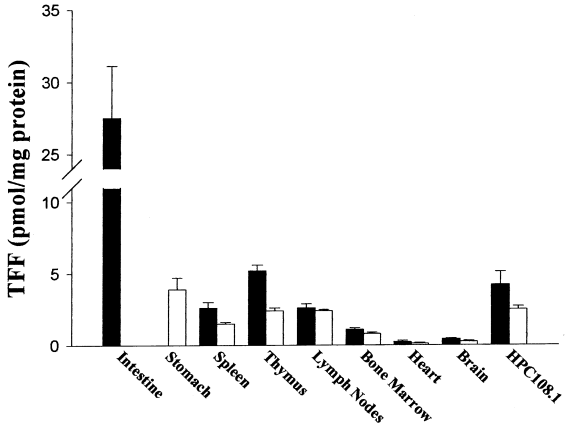

Fig. 2. TFF2 and TFF3 peptide expression in rat lymphoid tissues by RIA. TFF2 (□) and TFF3 (■) peptide was detected in stomach, spleen, thymus, lymph nodes and bone marrow and the plasmacytoma cell line HPC108.1, but not in brain or heart where levels fell below the sensitivity of the assay.

mRNA in adult rat tissues (Fig. 1). Protected fragments were detected in rat stomach, intestine and spleen but not liver, oesophagus, ovary, kidney, lung, heart or brain. Due to the low concentration of TFF2 message in the rat intestine and spleen, RT-PCR was used to produce enough cDNA for subsequent analyses. Using specific primers, we detected the predicted 519 bp TFF2 fragment in rat stomach, intestine, thymus and spleen, but not in heart or liver (Fig. 1b). The level of GAPDH was similar in all tissues (Fig. 1b), confirming the specificity of the PCR conditions for TFF2 detection.

To determine the identity of the 519 bp fragments detected in rat stomach, spleen and thymus, each amplified product was subcloned and sequenced (data not shown). Without exception, the sequences were the same in each tissue and identical to the nucleotide sequence reported for rat TFF2 [3].

Given the sensitivity of RT-PCR, we chose this technique to screen rat tissues for TFF3 expression. A 422 bp TFF3 amplified product was detected using specific primers in rat stomach, intestine, thymus and spleen (Fig. 1b).

3.2. Identification of TFF2 and TFF3 peptide in rat lymphoid tissues

TFF2 peptide was detected by RIA in rat lymphoid tissues at concentrations between 20% and 61% that of the fundic region of the rat stomach (Fig. 2). TFF3 peptide was detected by RIA in rat lymphoid tissues at concentrations between 4% and 19% that of the rat intestine (Fig. 2).

Expression of TFF2 peptide (at ~12 kDa) and the monomeric form of the TFF3 peptide (at ~7 kDa) was confirmed in the rat spleen by Western blot analysis (data not shown).Immunohistochemistry showed that TFF2-ir was present in large splenic cells, with eccentrically placed, oval, spherical or bilobed nuclei, characteristic of macrophage and/or B cells/plasma cells (Fig. 3a). Expression of the macrophage marker ED-1 was not co-localised with TFF2-ir in splenic white pulp (Fig. 3b).

3.3. Temporal expression of TFF2 and TFF3 in rat spleen following LPS (endotoxin) treatment

The time course of TFF2 and TFF3 peptide expression following exposure to LPS was quantified by RIA. There was a significant fall in TFF2-ir in rat spleen at 4 and 14 h after LPS treatment compared to controls ($55.3 \pm 8.3\%$ and $65.0 \pm 6.2\%$ of TFF2 basal levels, respectively; $P < 0.05$). TFF2-ir increased significantly in the rat spleen at 96 h, 7 days and 14 days after LPS ($132.5 \pm 8.1\%$, $147.8 \pm 10.7\%$ and $144.5 \pm 10.3\%$ of TFF2 basal levels, respectively; $P < 0.05$; Fig. 4a). TFF3-ir increased 2–3-fold in rat spleen between 14 h ($181.7 \pm 25.8\%$ of TFF3 basal levels, $P < 0.05$) and 14 days ($251.2 \pm 16.5\%$ of TFF3 basal levels, $P < 0.05$) post-LPS (Fig. 4a).

3.4. Recombinant hTFF2 and hTFF3 stimulate human monocyte migration

Glycosylated recombinant human TFF2 and recombinant human TFF3, at concentrations of 1, 2 and 5 mg/ml, were evaluated for their ability to recruit human monocytes. Cell migration in DMEM alone was $2.0 \pm 0.2\%$ that of the activity of Zymosan A and this dose dependently increased to $4.7 \pm 0.9\%$ (1 mg/ml), $16.0 \pm 6.1\%$ (2 mg/ml) and $27.5 \pm 7.1\%$ (5 mg/ml) after treatment with TFF2 (Fig. 4b). TFF3 also

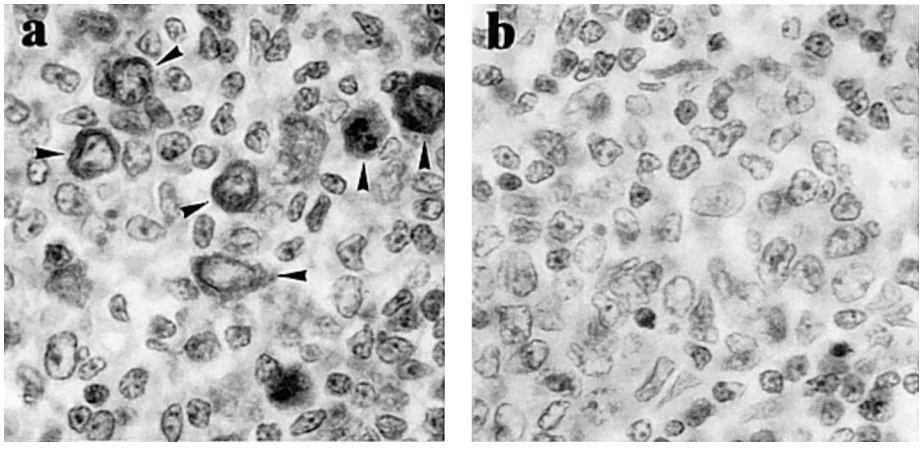

Fig. 3. Immunohistochemical localisation of a: TFF2 and b: ED-1 in consecutive sections of the rat spleen. TFF2 was localised to large cells with eccentric nuclei located mainly in the periphery of the white pulp (arrows). These cells lacked ED-1 immunoreactivity. Counterstained with haematoxylin (×1000).

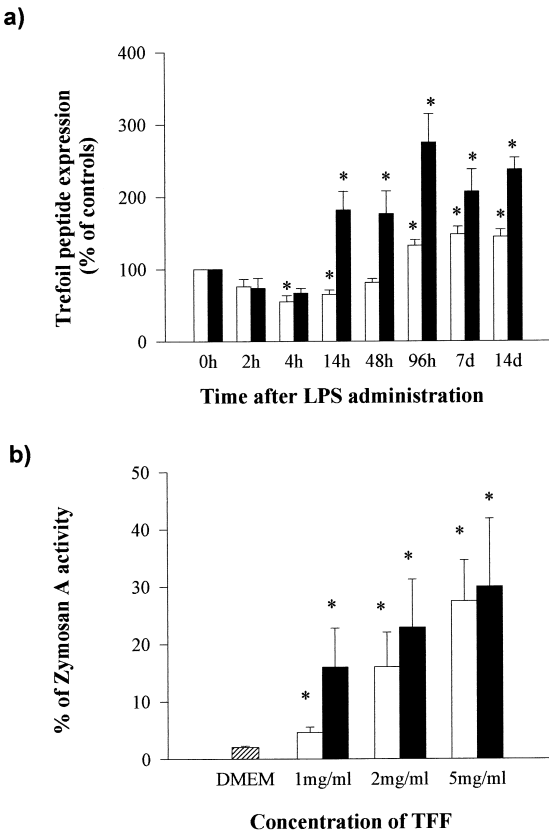

Fig. 4. a: Time course of expression of TFF2 (□) and TFF3 (■) in rat spleen following LPS administration. Each bar represents the mean ± S.E.M. of four rats. (*$P < 0.05$, *t*-test). b: Stimulation of migration of human monocytes (% Zymosan A) by media alone (striped bar), glycosylated human TFF2 (□) and human TFF3 (■). Each treatment was run in triplicate. Bars denote mean ± S.E.M. for five separate experiments. (*$P < 0.05$, *t*-test).

increased monocyte migration to 16.0 ± 6.8% (1 mg/ml), 22.9 ± 8.4% (2 mg/ml) and 30.0 ± 11.8% (5 mg/ml) that of Zymosan A-induced activity. All doses of TFF2 and TFF3 produced significantly greater activity than media alone ($P < 0.05$).

4. Discussion

The cellular sites of TFF expression in the gut are mainly associated with extensive glycoprotein biosynthesis and secretion, and TFFs have been localised by both immunohistochemistry and in situ hybridisation to mucus-secreting cells [2,4,5]. While exploring the expression of trefoils in other non-neoplastic mucin glycoprotein-secreting tissues, such as the lung, ovary and lymphoid organs, TFF2 and TFF3 mRNAs were detected in the rat spleen and thymus. While a previous study by Hirota et al. reported TFF1 mRNA in mouse spleen [27], this is the first study to unequivocally demonstrate TFF2 and 3 gene expression by rodent lymphoid tissues. Subsequent experiments revealed that both these peptides were also expressed by other lymphoid tissues including lymph nodes and bone marrow.

Trefoil peptide immunoreactivity has been localised to a small number of unidentified cells in rat spleen and thymus. These cells were negative for the macrophage marker ED-1, but displayed cellular morphology characteristic of plasma cells. Both TFF2 and TFF3 have been detected by region-specific RIA in the plasmacytoma HPC108.1, however the lack of a specific immunohistochemical marker for rat plasma cells has so far precluded the unequivocal localisation of the trefoil peptides to this cell type.

What is the normal function of the trefoil peptides in the spleen and other lymphoid organs? One possible implication from our data showing trefoil peptide expression by tissue plasma cells is that these peptides act in concert with secreted immunoglobulins. This is plausible since most immunoglobulins, like mucins, are variably glycosylated. However, in unpublished studies using the sensitive technique of plasmon resonance spectroscopy to detect weak interactions, we have been unable to show a direct binding interaction between recombinant TFF2 or TFF3 and IgG or IgA.

An alternative explanation may be that trefoil peptides aid in the immune response by regulating leukocyte recruitment at sites of severe epithelial damage and bacterial invasion. Evidence that trefoil peptides stimulate cell migration [10,12,13] and modulate epithelial cell adhesion and migration through regulation of the APC-catenin and E-cadherin-catenin complexes [21], together with the studies in this paper demonstrating that trefoils stimulate monocyte migration at concentrations similar to those found in gastrointestinal secretions [3], suggests that these peptides are capable of playing a role in regulating leucocyte migration in vivo. This might be accomplished directly, of by an interaction with one or more of the cell surface mucins which participate in the regulation of leukocyte adhesion to the endothelium.

Migration of various leukocyte subpopulations from the bloodstream into inflamed tissues or recirculation into lymphoid tissues is controlled by adhesion receptors and chemoattractants which direct them to the site of injury. The selectins and integrins are among the first adhesion molecules acting in this process. They aid in the docking of leukocytes to endothelial cell surfaces and participate in leukocyte rolling. GlyCAM-1, CD34 and MAdCAM-1 are sialomucins which contain large clusters of O-linked carbohydrate side chains, and which are expressed on the apical membrane of endothelial cells where they act as ligands for L-selectin (GlyCAM-1 and CD34) and the integrin alpha 4 beta 7 (MadCAM-1), and initiate weak tethering of leukocytes to the endothelium [28]. Whether or not the trefoils interact with these mucin glycoproteins to facilitate leukocyte migration and adhesion remains to be elucidated, and is the focus of ongoing investigation. However, the fact that trefoil peptides are actively secreted after synthesis [29] and their expression is upregulated several-fold by bacterial wall LPS, is consistent with such a role in the inflammatory response. Whatever their physiological role, we have shown that the trefoil peptides TFF2 and 3 are expressed by the main organs involved in immune regulation, that they can be induced by bacterial endotoxin, and are able to stimulate monocyte migration. Together, these observations suggest a potential role for the trefoil peptides in the immunological response to tissue injury.

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