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Trefoil Factor 2 (Tff2) Deficiency in Murine Digestive Tract Influences the Immune System

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Abstract
Background & Aims: The gastrointestinal trefoil factor family (TFF1, TFF2, TFF3) peptides are considered to play an important role in maintaining the integrity of the mucosa. The physiological role of TFF2 in the protection of the GI tract was investigated in TFF2 deficiency. Methods: *TFF2-/-* mice were generated and differential expression of various genes was assessed by using a mouse expression microarray, quantitative real time PCR, Northern blots or immunohistochemistry. Results: On an mRNA level we found 128 differentially expressed genes. We observed modulation of a number of crucial genes involved in innate and adaptive immunity in the *TFF2-/-* mice. Expression of proteasomal subunits genes (*LMP2*, *LMP7* and *PSMB5*) involved in the MHC class I presentation pathway were modulated indicating the formation of immunoproteasomes improving antigen presentation. Expression of one subunit of a transporter (*TAP1*) responsible for importing degraded antigens into ER was increased, similarly to the *BAG2* gene that modulates chaperone activity in ER helping proper loading on MHC class I molecules. Several mouse defensin (cryptdin) genes coding important intestinal microbicidal proteins were up-regulated as a consequence of TFF2 deficiency. Normally moderate expression of TFF3 was highly increased in stomach.

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Introduction

TFF peptides (trefoil factor family: TFF1, TFF2 and TFF3) belong to an evolutionary old [1] family of small (6-12 kDa) protease resistant peptides that share a conserved distinct motif of six cysteine residues forming characteristic disulfide bonds defining a so-called TFF domain [2]. TFF peptides are predominantly expressed by mucin-producing epithelial cells of the gastrointestinal (GI) tract but also in some other tissues [3]. In the GI tract TFF1 is expressed by gastric surface mucous cells [4] while TFF2 is present in antral and pyloric glands of

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Table 1. Primers used

Gene		Sequence 5	size	Conditions
Proteasome subunit 5 (PSMB5)	For	TTGGCTCGGCAGTGTCGAATCTAT	88bp	59°C
	Rev	ACACCATGTTAGCGAGCAGTTTGG		3.5mM $MgCl_2$
proteosome subunit, beta type 9 (LMP2)	For	ATGGGAGGGATGCTAATTCGACAG	139bp	59°C
	Rev	GAGTGATGGCATCTGTGGTGAAAC		2.5mM $MgCl_2$
20S proteasome subunit Lmp7 (PSMB8)	For	CTTGGCCGCAGAGCTATTGCTTAT	113bp	59°C
	Rev	ACATCGGAACTCTCCACTTTCACC		2.5mM $MgCl_2$
Mus musculus transporter (TAP1)	For	TCTCTACCAGCCTTCAGTTCACCCA	163bp	59°C
	Rev	AAGGCCTTTCATGTTTGAGGGTGC		2.5mM $MgCl_2$
Bcl2-associated athanogene 2 (BAG2)	For	GGTGTGCGTGGAAGCATGACAAAT	115bp	59°C
	Rev	AGCTCAAGGCAGCACATGAAGAGA		3.5mM $MgCl_2$
Cellular retinol binding protein 2 (CRBP2)	For	AAAGAAGTGATGGGCACGGGAAAG	133bp	59°C
	Rev	ACACTCCAGTTTAAACCCAGGCAG		3.5mM $MgCl_2$
Apolipoprotein A-IV (APO AIV)	For	AGAACATGCAGAAGTTGCAGGAGC	91bp	59°C
	Rev	TGGAGCTTCATTTCCTGGGTCTGT		3mM $MgCl_2$
Cathepsin C	For	CCCTACACAGCCAAAGATTCTCCA	103bp	67°C
	Rev	CATTGCAGCCACCATAGAAACCAC		3mM $MgCl_2$
Mouse cysteine-rich intestinal peptide (CRIP)	For	CGAGTGACGTCACTAGGCAA	82bp	57°C
	Rev	GACCCCCAGAGGTCAGTGTC		2.5mM $MgCl_2$
	Rev	CTGCAATTGATCCCACAGGCATTC		
Matrix Metalloproteinase7 (MMP7)	For	AACTTCCTGTTTGCTGCCACCCAT	148bp	56°C
	Rev	TCTGAATGCCTGCAATGTCGTCCT		4mM $MgCl_2$
Tissue inhibitor of metalloproteinase3 (TIMP3)	For	AGGGGCCCTTTGGCACTCTG	186bp	59°C
	Rev	TACACGCGCCCTGTCAGCAG		3.5mM $MgCl_2$
Mucin 3 (MUC3)	For	AGTGGTCATGTTACGATGGTAGGC	193bp	57°C
	Rev	TTGCCATGGAGATGGGAGGTAATC		3.5mM $MgCl_2$
Mouse Trefoil Factor 3 (mTFF3)	For	AACCGTGGCTGCTGCTTT	102bp	56°C
	Rev	CCTGGAGCCTGGACAGCTT		2.5mM $MgCl_2$
Mus musculus cryptdin genes (Cryptdins 1,2,3,5,6)	For	ACTCCCAGCCATGAAGACAC	857bp and 296bp	58°C
	Rev	CATGTTCAGCGACAGCAGAG		3mM $MgCl_2$
Mouse Trefoil Factor 1(mTFF1)	For	ATCCATCACTCGTGGTCTTC C	456bp Northern blot	56°C
	Rev	CAGGCCGAATTCGAGGACTA		3.5mM MgCl
Mouse Trefoil Factor 2 (mTFF2)	For	GCCCAGGTCCAGTGGAG	468 bp Northern blot	56°C
	Rev	GAACTTTCTTCTGGCTTGCAG		3.5mM $MgCl_2$
Maus Glyceraldehyd-3-Phosphat Dehydrogenase (mGAPDH)	For	AACGACCCCTTCATTGAC	191bp	59°C
	Rev	TCCACGACATACTCAGCAC		3,5mM $MgCl_2$
	Rev1	CATTGGGGGTAGGAACACGG	620bp	59°C 3,5mM $MgCl_2$
Mus musculus β-Glucuronidase (GUS)	For	TCAGCTCTGTGACCGATACG	168bp	59°C
	Rev	GCCACAGACCACATCACAAC		3,5mM $MgCl_2$
Mouse housekeeping protein (mHKG)	For	GGAGGGCCCATCAGTTTAAT	170bp	59°C
	Rev	AAACAATTGCATTGCATAGTGC		3,5mM $MgCl_2$

the stomach and within the Brunner's glands of the duodenum [5]. TFF3 is expressed by goblet cells of the intestine and colon [6] and weakly in stomach [7, 8]. The GI epithelium is daily confronted with frequent insults and the maintenance of epithelial integrity is of crucial importance. Upon injury the epithelial cells spread and migrate across the basement membrane (known as restitution) within minutes to re-establish surface cell continuity. Regeneration occurs later on and involves proliferation and differentiation of epithelial cells and restoration of specialized elements. The TFF peptides are rapidly and strongly up-regulated after epithelial damage [9], in gastric ulceration [10] and inflammatory bowel disease [11, 12]. TFFs facilitate restitution [13] and re-epithelialization by stimulating cell migration [14, 15], inhibiting apoptosis [16, 17] and reducing antigen access to the healing epithelium by augmenting the barrier function of mucus through an increase of its viscosity [18]. In addition, they participate in the mucosal immune response by stimulating immunocyte migration [19] and are

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Fig. 1. Targeting strategy of the TFF2 gene and Genotyping of progeny from crosses. (A) The wild type TFF2 locus is shown in the upper line. To construct the targeting vector a proximal PstI/XhoI fragment and a distal 2.4-kb fragment, generated by PCR using the PK1 and PK2 primers, were inserted into appropriate restriction sites of the pL2-neo vector. The exon 2 and a part of exon 1 were deleted in this process (targeting vector pL2Δm2, middle). The targeted allele generated by homologous recombination is shown at the bottom. A, AatII; B, BamHI; C, ClaI; H, HindIII; K, KpnI; P, PstI; X, XbaI and XhoI. Horizontal arrows indicate primer (PK1 and PK2) orientations. (B) Southern blot analysis of the TFF2 locus from a BamHI digest for the null mutation. Genomic DNA was extracted from tail biopsies of wild-type (wt), heterozygous (het) and *TFF2-/-* mice. The fragment sizes are indicated. The 6-kb represents the targeted allele. (C) Northern Blot analysis of stomach RNA using TFF1 (top) and TFF2 (bottom) exon-probes. *TFF2-/-* mice show no expression of TFF2 mRNA

(bottom) compared with wild-type (wt) or heterozygous (het) animals. The identical blot probed with TFF1 cDNA displays expression of TFF1 mRNA (top) in all animals. Lane marked with an asterisk (*) is unloaded. (D) *TFF2-/-* mice express no TFF2 protein as shown by Western Blot in (A)-unboiled and (B) boiled tissue homogenates of stomach.
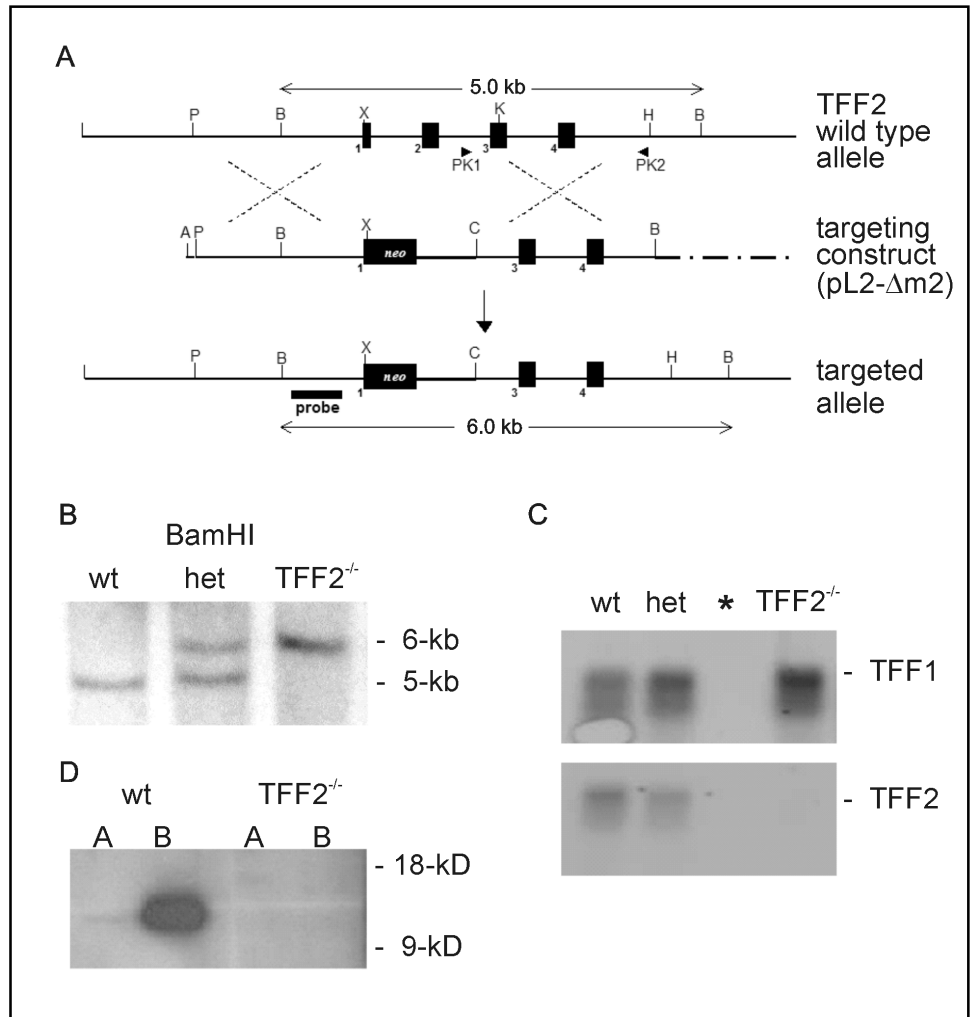
regulated by both pro-inflammatory [20, 21] and anti-inflammatory cytokine expression [22, 23] acting through diverse transcription factors [21, 24-26]. Treatment with exogenous TFF peptides demonstrated both protective and healing functions in stomach and colon [27-29].

Most valuable data about TFF peptide functions were obtained from separate TFF knock-out animal models. TFF1-/- mice developed antropyloric adenomas and occasionally carcinomas, suggesting a tumour suppressor function of TFF1 [30]. TFF3 knock-out animals showed increased sensitivity to dextran sulphate sodium-provoked intestinal damage [31] with increased intestinal apoptosis [17]. TFF2 deficient mice appeared more susceptible to

ulceration upon indomethacine treatment [32]. These pleiotropic effects of TFFs are compatible with their high-resolution structures suggesting that each TFF peptide has a specific target or group of target molecules [33]. To study in detail the molecular events behind TFF2 deficiency, we investigated alterations of gene expression in response to lack of TFF2, by comparing 12,000 genes using mouse expression microarrays in tissues expressing TFF2 (pyloric antrum of the stomach, with Brunner's glands of the duodenum). Genes with significantly altered expression were further tested by quantitative real time PCR (qPCR) or by Northern blots. Protein expression was assessed immunohistochemically.

Materials and Methods

Generation of TFF2 -/- mice

All procedures involving living animals were approved by the local ethical committee (CRUK). Two fragments were subcloned into the AatII/XbaI and ClaI/BamHI sites of the pL2-neo vector (kindly provided by C. Dickson, CRUK). First, a 2.4-kb fragment containing exon 3 and 4 of TFF2 gene generated by PCR using PK1 (5-GGATCGATCCCCATCCCATACCC-3) and PK2 (5-CGGGATCCTATAGGGTCTGAGGTC-3) primers was subcloned into the ClaI/BamHI site of the targeting vector. Secondly, the PstI-XhoI fragment (3-kb), derived from 129/Sv genomic BAC-DNA harbouring genomic mouse DNA (Research Genetics, USA), containing the first four codons of exon 1, was first subcloned by shotgun cloning into the PstI-XhoI sites pBK-CMV (Stratagene, USA) vector, and after a AatII/XbaI restriction finally into the fitting sites of pL2-neo (Figure 1A middle). In the applied targeting vector (pL2- m2; Figure 1A middle) exon 2, which encodes one of the two functional TFF domains, and exon 1 were deleted. The deletion in exon 1 did not include the first 13 nucleotides up to the XhoI site. The targeting vector was linearized with AatII and electroporated into AB2.2 embryonic stem (ES) cells (Stratagen). After electroporation and positive selection (G418), ES cell clones were screened for incorporation of the targeting construct into the correct genomic locus. DNA was isolated from 552 ES cell clones and analyzed by both PCR (data not shown) and Southern blot (see below). One positive clone was identified. After microinjection into blastocysts from C57BL/6 donors, high percentage chimaeric animals were obtained which were mated to C57BL/6 mice, and germ line transmission of the disrupted TFF2 allele was confirmed by genomic Southern blot analysis of tail DNA from the progeny. Disruption by homologous recombination was confirmed by Southern blot analysis by using the diagnostic BamHI site. For blots, 15 µg of BamHI digested ES cell or tail genomic DNA was subjected to agarose (0.7%) gel electrophoresis and transferred to nylon membranes. A diagnostic probe was labelled by random priming using [32P] dCTP and hybridized to the blots. A 928-bp fragment (Figure 1A bottom; horizontal bar) of the TFF2 gene was used as a probe. The wild-type allele generates a 5-kb BamHI fragment and the heterozygous mutant contains an additional 6-kb BamHI fragment, representing the targeted allele (Figure 1B). Complete absence of TFF2 was confirmed by Northern (Figure 1C) and Western blot analyses (Figure 1D).

Northern and western blot analysis

Total RNA was isolated from frozen tissues of the stomach, particularly of pyloric antrum and Brunner's glands, from 3 month old mice (RNeasy; Qiagen) The quantity of RNA was determined spectrophotometrically (Nanodrop). TFF1, TFF2, and cryptdin cDNA were generated using 129/Sv mouse genomic DNA template (Table 1). cDNA probes were labelled by random priming using [32P] dCTP. Total RNA (15 µg per lane) was separated by electrophoresis and transferred to a nylon membrane and hybridized to specific probes. The membranes were hybridized overnight at 42 C and washed

before exposing and analyzing in the Phosphor-Imager (BioRad). To confirm absence of TFF2 protein mouse stomachs were homogenized in lysis buffer containing 300 mM mannitol, 12 mM HEPES/Tris (pH 7.4), 1 mM PMSF, 0.1 mM benzamidine, and 0.1µg/ml of antipain. Homogenates were cleared by centrifugation, resuspended in loading buffer, denatured by boiling and separated by SDS/PAGE before transferring to PVDF membrane. The membranes were blocked (5% non fat milk in PBS) and incubated over night with polyclonal anti-mouse TFF2 antibody (produced using peptide EVPWCFFP-QSVEDCHY for immunization). Signals were detected using the enhanced chemiluminescence method (Amersham).

Microarray analysis

Total RNA from pyloric antrum with the Brunner's glands (TFF2 expressing tissues) was isolated (RNeasy kit, Qiagen) from 3 wild-type (wt) and 3 TFF2-/- mice. RNA was processed and independently hybridized on a mouse expression array (MGU74A) containing 12,000 genes (Affymetrix). Scanned images were processed using Microarray Suite 5.0. The overall fluorescence intensity across each chip was scaled to target intensity 150 and pair-wise comparison of mRNA levels was performed with the wild type samples as a baseline. We additionally analyzed our microarray data using Significance Analysis of Microarrays (SAM) [34] with an acceptable false discovery rate (rough equivalent to P-value) of 0.004%. Significant alterations were compared with results of Affymetrix software and only genes displaying signals > 100 in either wt or TFF2 -/- and changed by factor > 1.6 were accepted.

Quantitative real time PCR

To confirm data of microarray analysis we tested expression of a number of selected genes using SYBR Green-based qPCR in 12 wild type and 12 TFF2 deficient animals. Total RNA was isolated from tissue portions containing (1) pyloric antrum of stomach with Brunner's glands (pyloric antrum/Brunner's glands) (as used for Microarray analysis);

Fig. 2. Data set of signal log ratios of different comparisons between wild-type and *TFF2-/-* mice. The presented genes have a signal intensity >100 and are significantly changed according to one class analysis of the Significance Analysis of Microarrays (SAM) program, with a false discovery rate of 0.004%. Changes in expression ≥1.6 were considered. The expression pattern of the genes is represented in the horizontal strips where the tones of red and green represent respectively the degrees of up- and down-regulation. More intense colour indicates a higher degree of change: red for up- and green for down-regulation. The difference in expression of some genes (see K3xWT1; lane 7) is due to individual variability.

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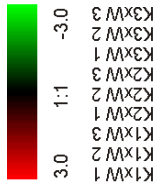
Immune response

U43084: INF-induced protein with tetratrieopeptide repeats 1
"J00475: IgH chain gene, DJC region-sebment D-FL16.1"
U12560: Cryptdin 5 (Defcr5)
U03003: Cryptdin 6 (Defcr6)
U02997: Cryptdin-2 (Defcr2)
M62541: CD20 antigen
M14167: Adenosine deaminase
V00817: Ig heavy chain 6 of IgM
AJ235940: Ig kappa chain variable B
UD2999: Cryptdin-3 (Defcr3)
M16118: T-cell receptor insulin (A-chain) reactive alpha chain
U-48716: Ig heavy chain 4 (serum IgG1)
AF059706: Immunoglobulin heavy chain VDJ region gene
U73037: INF regulatory factor 7
X00652: Ig-kappa light chain V-J kappa 5 joining region
X67210: Rearranged Ig gamma 2b heavy chain
U02995: Defensin related cryptdin peptide
V00821: Ig heavy chain 6 (heavy chain of IgM)
X67210: Rearranged Ig gamma 2b heavy chain
X00651: Ig kappa chain variable 8 (VB)
X82691: Ig Vkappa-PCG-4gene
U53219: Interferon gamma induced GTPase
AB016496: Intelectin
U16985: Lymphotexin B
"M90388: Protein tyrosine phosphatase, non-receptor type 8 (PEP)"
X88903: mRNA for variable light chain
"NM_009034: Retinol binding protein 2,cellular"
AF099973: Schlafen 2
U23095: Ig heavy chain 4 (serum IgG1)
"NM_013683: Transporter 1, ATP-binding cassette, sub-familly B"
M13018: Cysteine-rich protein 1
"U21906: Histocompatibility 2, D region locus 4"
AB023418: Chemokine (C-C motif) ligand 8
"M27134: Histocompatibility 2, Q region locus 10"
AI838195: Opioid growth factor receptor
U22033: 20 S proteosome subunit LMP7 (Psmb8)
U15635: IFN-gamma induced (Mg11) mRNA
"NM_013585: Proteosome subunit, beta type 9 (LMP2)
M58156: MHC (A.CAJ/(H-2K-f) class I antigen
M55561: CD52 antigen; phosphatidylinositol-linked antigen (pB7)
U26437: Tissue inhibitor of metalloproteinase 3
NM_145392: Bcl2-associated athanogene 2
AB003305: Proteasome beta type subunit 5
AI842667: Cathepsin C (Lysosomal dipeptidyl transferase)
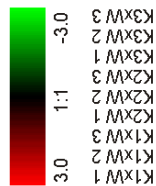
## Detoxification

"M27168: Cytochrome P450, cyp2d9"
AI848952: Glyoxalase 1
"AF103875: ATP-binding cassette, sub-familly G, member 2"
AA790008: Reduced expression:Rex3
U20611: Peroxiredoxin 2

Cell signaling and transcription factors

AW123408: KDEL(Lys-Asp-Glu-Leu) ER protein retention receptor 2
"U29055: Guanine nucleotide binding protein, beta 1
"AF085219: Diacylglycerol kinase, alpha"
NM_011546: Zinc finger homeobox 1a
AF038995: DEAD (Asp-Glu-Ala-Asp) box polypetide 6
X17320: Purkinje cell protein 4
Y08027: ADP-ribosyltransferase 3
"AL078630: Gamma-aminobutyric acid (GABA-B) receptor, 1"
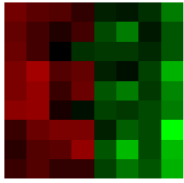
Digestion and nutrient transport

"U95182: Guanylate cyclase activator 2b, Uroguanilyn"
"X15684: Facilitated glucose transporter, member 2"
Z22216: Apolipoprotein C-II
NM_026918: Zymogen granule membrane protein 16
M64248: Apolipoprotein A-IV
"AF031467: Branched chain aminotransferase 2, mitochondrial"
D13139: Dipeptidase 1
U30509: Gamma-glutamyltransferase 1
AB013095: Heme binding protein 1
"U63146: Retinol binding protein 4, plasma"
NM_009381: Thyroid hormone responsive SPOT14 homolog
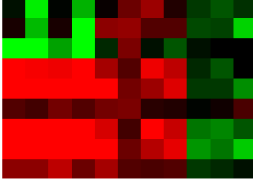
Diverse

M16360: Major urinary protein 5
M16358: Major urinary protein 4
M65237: Mouse murinoglobulin (MUG3)
AV355798: Major urinary protein 2
M16359: Major urinary protein 3
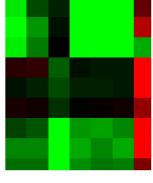M16357: Major urinary protein 1
D38410: Trefoil factor 3

Fig. 3. Cryptdin level in GI-tract showed by Northern blot. Compared to β-actin as control gene, cryptdins are up-regulated in the duodenum and especially in the distal small intestine of TFF2 deficient mice.
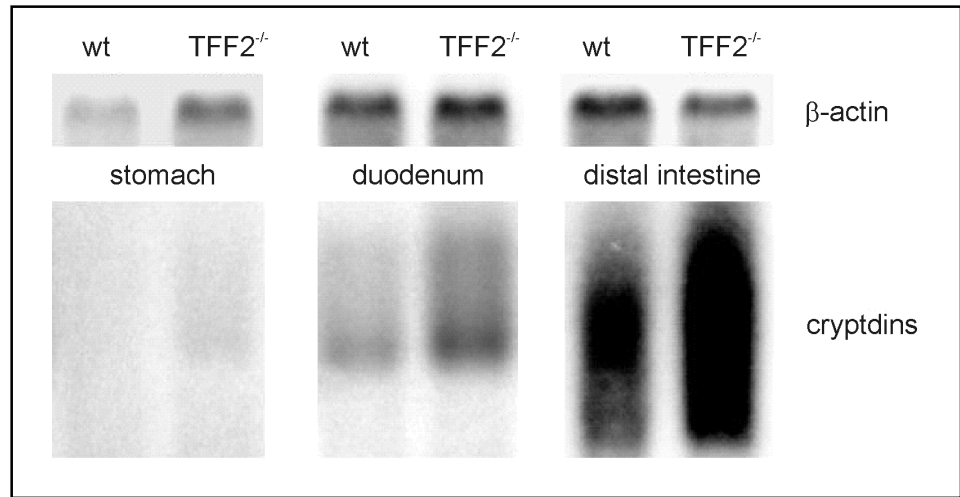

Fig. 4. Immunoreactivity of anti-cryptdin1 prosegment in intestine and TFF3 in stomach of normal and *TFF2-/-* mice. Immunoreactivity of the cryptdin prosegment localized in intestinal Paneth cells (arrows) is more pronounced in TFF2 deficient mice (B) than in wild type animals (A). TFF3 in wt animals is not (or very slightly) expressed in the pyloric junction (C), but is highly present in the pyloric junction of TFF2 deficient mice. Brunner's glands in (A) and (D) are marked by arrow.
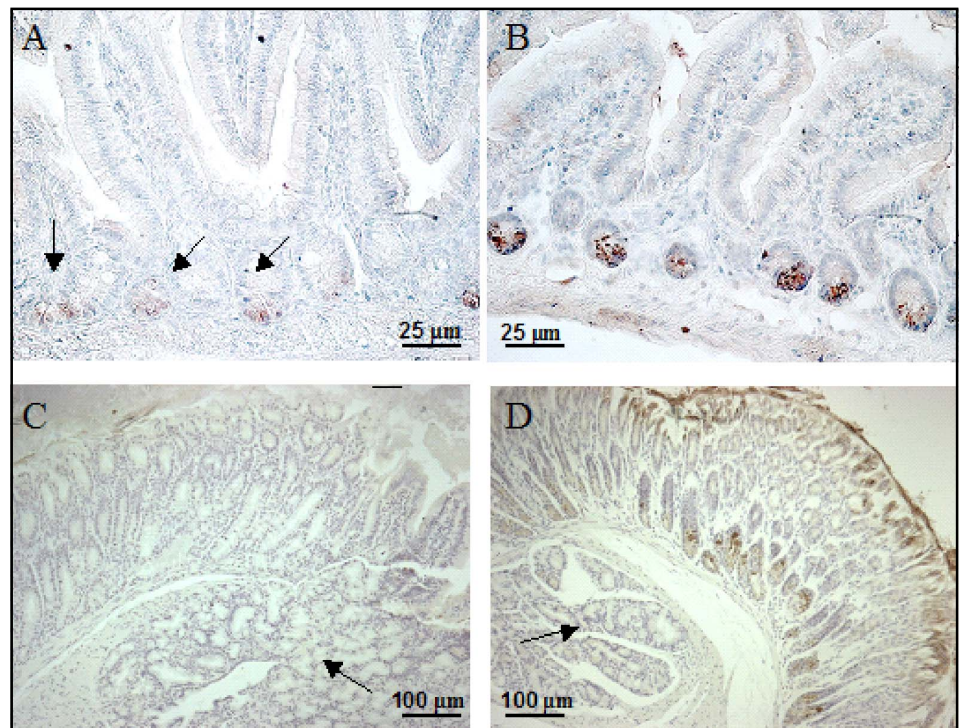
(2) mid-portion of duodenum and (3) distal small intestine (jejunum/ileum). RNA was DNase treated prior to cDNA synthesis using the iScript TM cDNA synthesis kit (BioRad). Primer pairs were designed to give only one band (confirmed by gel electrophoresis and melting curve analysis) and conditions of reactions were optimized so that efficiency of PCR reaction was 95-100% (Table 1). Relative quantification values were obtained from the threshold cycle number of 12 different animals measured in triplicate and normalized with 2 most stable out of 3 tested control genes using the geNorm program [35]. As control genes we used mouse house keeping gene (mHKG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-glucuronidase (GUS). Significance was assessed by Student's t-test.

Immunohistochemistry

Stomach and intestine sections (4 μm) were stained with haematoxylin-eosin and assessed for pathomorphological examination. Tissues were stained with antibodies against TFF3 and anti-cryptdin1 prosegment, so called procryptdin (kindly provided by AJ Ouellette) specifically reacting with all mouse defensin family precursors [36]. Antigen unmasking for

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Table 2. Relative changes of mRNA expression between wild type and *TFF2-/-* animals obtained by micro-array analysis (n=3 each) and quantitative real time PCR (qPCR) (n=12 each) in different gastrointestinal regions. Data are expressed as mean fold changes ±SD. Significantly changed expression compared to wt mice is marked with (*)(p<0.05), (n.a. not available). Abbreviations: Proteasome subunit 5 (PSMB5); Proteosome subunit, beta type 9 (LMP2); Proteasome subunit Lmp7 (PSMB8); Mus musculus transporter 1(TAP1); Bcl2-associated athanogene 2 (BAG2); Cellular retinol binding protein 2 (CRBP2); Apolipoprotein A-IV (APO AIV); Cysteine-rich intestinal peptide

GENE	Pyloric antrum with Brunner's glands		qPCR	
	Microarray	qPCR	Duodenum	Jejunum/ Ileum
PSMB5	-4.0	-2.2±0.05*	-2.1±0.92*	-1.9±0.85*
LMP2	2.0	1.5±0.16*	1.5±0.31*	1.3±0.28*
LMP7	2.0	1.0±0.46	1.2±0.34	1.5±0.26*
TAP1	2.4	2.0±0.25*	1.1±0.67	1.1±0.75
BAG2	-2.0	-1.6±0.67*	-1.5±0.55*	-1.8±1.20*
CRBP2	2.6	3.3±0.22*	2.0±0.37*	1.7±0.22*
APO AIV	3.7	3.0±0.34*	1.7±0.17*	1.5±0.30*
CATHEPSINC	-4.8	-1.2±1.41	2.0±0.21*	1.5±0.19*
CRIP	2.3	2.2±0.27*	2.3±0.10 *	1.0±0.47
MMP7	n.a.	-1.0±1.2	-1.1±0.65	-1.4±0.59
TIMP3	-1.8	1.5±0.45	1.3±0.46	1.5±0.27
TFF3	5.6	41.0±0.04*	1.9±0.26*	-1.4±0.68
MUC3	4.9	1.2±1.08	2.1±0.83*	2.0±0.26*

(CRIP); Matrix Metalloproteinase7 (MMP7); Tissue inhibitor of metalloproteinase3 (TIMP3); Mucin 3 (MUC3); Trefoil Factors (TFF); Glyceraldehyd-3-Phosphat Dehydrogenase (mGAPDH); β-Glucuronidase (GUS); Mouse housekeeping protein (mHKG).

immunohistochemistry was performed by microwaving for 10 min (TFF3) or 3 times for 5min (procryptdin) in 10 mM sodium citrate, pH 6. The slides were incubated overnight at 4°C in a humid chamber with anti-TFF3 rabbit serum or with sheep anti-procryptdin serum. Specific antibody binding was visualized by biotin-conjugated sheep anti-rabbit IgG (DAKO) or rabbit anti-sheep IgG (Vector), followed by a streptavidin-biotin-horseradish peroxidase complex (DAKO) and diaminobenzidine.

Results

Generation of TFF2 -/- mice

Northern blot and Western blot analysis showed successful disruption of TFF2 gene and complete absence of TFF2 protein (Figure 1). Pathological analysis of the animals did not reveal specific gross or microscopic abnormalities.

Microarray data and confirmation of relevant genes

Analysis of mRNA levels in TFF2 expressing tissue of wt and TFF2 -/- animals showed 128 genes with significantly changed expression according to Affymetrix and SAM data analysis. We found 78 genes to be up-regulated and 50 genes down-regulated by factor >1.6. Further analysis involved grouping genes according to their function: a) immune response, b) detoxification, c) cell signalling and transcription factors, d) digestion and

nutrient transport. Signal log ratios of all 9 comparisons are visually represented (Figure 2) using Genesis software [37]. When matching wt/TFF2-/- crosswise fashion, expression levels for some of the genes were not uniform possibly reflecting biological variability. The majority of the genes concerned are involved in immune response. Among the most prominently up-regulated genes are several members of the cryptdin family (cryptdin 2,3,5,6 and defensin related cryptdin peptide); mouse orthologues of human defensins playing a crucial role in innate immunity. Genes coding for different Ig chains were also up-regulated.

Microarray analysis revealed significant change in several genes involved in MHC class I antigen presentation such as: different proteosomal subunits (PSMB5, LMP2 and LMP7), TAP1 (subunit 1 of transporter associated with antigen processing) and BAG2 (BLC2-associated athanogene 2), a member of the BAG protein family regulating chaperone activities. In addition to the expected complete absence of TFF2 mRNA, members of the less known family of murine urinary proteins (MUPs) were down-regulated as well. To substantiate the microarray analysis we analyzed some of the interesting genes by qPCR in 12 different animals (Table 2).

We tested expression in tissues of pyloric antrum/ Brunner's glands, duodenum and jejunum/ileum. To get the most reliable data, qPCR measurements were

normalized against the 2 most stable out of 3 control genes using the geNorm program. Pyloric antrum/Brunner's glands were normalized against GAPDH/GUS; duodenum against GUS/mHKG and jejunum/ileum against GAPDH/mHKG normalization factors. Possible differences in sampling the tissues of pyloric antrum/Brunner's glands was excluded by monitoring the expression of Mucin 3 exclusively present in intestine [38]. QPCR data showed that there was no significant difference in Mucin 3 expression (excluding variable presence of intestinal tissues) between wt and TFF2-/-tissue samples.

Real time qPCR data confirmed changes in mRNA levels of proteosomal subunits. Constitutive PSMB5 subunit was significantly down-regulated (~2-fold) in all tissues analyzed. Substituting subunit LMP2 was up-regulated (~1.5-fold) in pyloric antrum/Brunner's glands and duodenum and slightly, but significantly, up-regulated (1.3-fold) in the distal portion of the small intestine. In contrast, subunit LMP7 was significantly up-regulated (~1.5-fold) but exclusively in the jejunum/ileum.

TAP1 gene showed significant (~2-fold) up-regulation only in tissues from pyloric antrum/Brunner's glands. BAG2 protein that regulates chaperone activities showed significant down-regulation between ~1.5-fold (duodenum) and ~1.8-fold in the jejunum/ileum. Cellular retinol binding protein, type 2 (CRBP2) that complexes vitamin A [39] was significantly up-regulated (~1.7-3.3 fold) as was apolipoprotein AIV (APO AIV) involved in fat metabolism (~ 1.5-3.0 fold).

Cathepsin C or dipeptidyl peptidase I (DPPI), a lysosomal cysteine protease involved in protein turn over in cells [40], showed significant up-regulation in the duodenum and the distal small intestine (~1.5-2.0 fold).

Some of the genes expressed in Paneth cells, providing host defense against microbes, were significantly changed in TFF2 -/- animals. Cysteine rich intestinal protein (CRIP) was up-regulated about 2-fold in pyloric antrum/Brunner's glands and the duodenum.

Several cryptdins, microbe killing molecules produced in Paneth cells, showed strong up-regulation (~4.0-9.0 fold). A Northern blot probe hybridizing with the most relevant cryptdins corroborated the same up-regulation of cryptdins in ileum/jejunum of TFF2 -/- animals (Figure 3). In addition, immunostaining with procryptdin1 antibody indicated an increased amount of defensin precursors (Figure 4A&B). The bactericidal activity of some of the cryptdins requires post-translational proteolytic activation of precursors by matrix metalloproteinase-7 (MMP-7) [41] which can be specifically inhibited by tissue inhibitor of metalloproteinases 3 (TIMP3). However, qPCR showed no significant change in level of MMP-7 and TIMP3 mRNA between wt and TFF2 -/- animals (Table 1).

TFF3 expression was more than ~40 fold up-regulated at the junction of the pylorus and duodenum, and ~2 fold up-regulated in the duodenum. While hardly present in the pyloric antrum of wt animals, TFF3 was strongly expressed in the same region of TFF2 -/- mice (Figure 4C&D). Mucin 3 (MUC3) is prominent in the intestine as a component of the protective mucus layer [42, 43]. It showed a ~2 fold up-regulation in the duodenum and in the end portion of the small intestine (Table 2).

Discussion

Anti-apoptotic and motogenic TFF peptides play an important role in the maintenance of epithelial integrity, protection and wound healing [3, 13]. Present data suggest that TFFs are multifunctional peptides. Many of these facts have been collected thanks to TFF knock-out animal models. While TFF1 deficient mice [30] display gross morphological changes (including gastric tumour development), TFF3-deficient animals show mild phenotypes unless chemically challenged [16]. TFF2-deficient animals, compared to wild type, showed greater mucosal inflammation in response to DSS treatment or Helicobacter felis infection, while LPS treatment resulted in increased circulating levels of proinflammatory cytokines (T. Wang, personal communication, 2004).

Monitoring altered mRNA levels in gastric mucosa using microarray technology revealed some interesting consequences of TFF2 deficiency pointing to its role in immune defence. An important part of immune surveillance is the presentation of endogenous and exogenous antigenic peptides to immune cells. Almost all nucleated cells express on their surface MHC class I molecules, glycopeptides presenting different endogenous peptide antigens produced within the cell to cytotoxic lymphocytes. These endogenous peptides are degraded via the normal intra-cellular protein degradation pathway and presented at the cell surface by MHC class I molecules. Proteins targeted for proteolysis are mainly ubiquitinated and degraded by large multifunctional protease complexes termed proteasomes [44]. The immune system appears to modify the proteasome by displacing the housekeeping subunits PSMB5 and PSMB 6 with LMP2 and LMP7

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subunits whose expression can be induced by increased levels of IFN-α [45] or by TNF-α [46]. These immunoproteasomes facilitate antigen presentation presumably by altering the cleavage specificities of proteasomes [45, 47]. Degraded short peptides are moved from the cytosol into the lumen of endoplasmatic reticulum (ER) by the TAP transporter composed of TAP1 and TAP 2 subunits and finally transported through the Golgi complex to the plasma membrane. Proper functioning of this system is crucial for combating infections and neoplastic changes.

TFF2 -/- animals showed significantly changed expression of important genes involved in MHC class I molecule presentation. Changes in mRNA levels suggest possible differences in proteasome composition. A constitutive PSMB 5 subunit is substituted by LMP2 (pyloric antrum and duodenum) and LMP7 (distal small intestine) subunits forming immunoproteasomes. Additionally, the TAP1 subunit, often down-regulated in several tumours and infections [48] is up-regulated in pyloric antrum and Brunner's glands. Forming MHC class I-protein complexes depends on the proper folding of proteins promoted by chaperones. Microarray analysis revealed no changes in the expression of calnexin and tapasin chaperones, both important for MHC class I presentation. Interestingly, we discovered a significant reduction (~1.5-1.8 fold) of chaperone cofactor BAG-2 as confirmed by qPCR in all tissues examined. The BAG family of proteins regulates chaperone activities through their interactions with Hsc70/Hsp70 molecules that are involved in diverse cellular processes [49, 50]. It is known that BAG-family proteins are over-expressed in several cancers and it has been demonstrated in vitro that they enhance cell survival and proliferation [50]. Recently BAG2 was recognized as a member of possible novel signalling pathway for response to cellular stresses [51]. Remarkably, molecular analysis of antropyloric tumours of TFF1 deficient mice showed an accumulation of misfolded proteins in the ER, which connected TFF1 deficiency to the unfolded protein response [52].

TFF2 deficient animals displayed increased CRBPII and APO AIV mRNA, genes which were found to be up-regulated in intestine as a part of intestinal adaptation [53, 54]. CRBP II, abundantly expressed in intestinal epithelial cells, plays a pivotal role in absorption and metabolism of retinol and beta-carotene which are involved in maintaining immunity and epithelial turn over [55]. Animal models showed that vitamin A deficiency inhibits intestinal adaptation following partial small intestine resection by reducing crypt cell proliferation, enhancing early crypt apoptosis and reducing enterocyte migration [56]. Recently it was recognized that retinoids act as multistep modulators of the MHC class I presentation pathway up-regulating proteosomal LMP2 and LMP7 subunits, increasing the half-life of MHC class I complexes and enhancing the sensitivity of neuroblastoma cells to lysis by cytotoxic lymphocytes [57]. Up-regulation of CRBPII in intestine of TFF2 deficient animals probably reflects an increased need for retinoids that could influence expression of genes involved in MHC class I antigen presentation like LMP2, LMP7 and TAP1.

First steps in the defence against invading microorganisms belong to innate immunity that involves products of Paneth cells located deep in the intestinal crypts. Their major products secreted into the lumen of intestinal crypts are the α-defensins, in the mouse called cryptdins. Defensins are vital contributors to innate host antimicrobial defense (direct microbicidal effect) and recent evidence points to their role as enhancers of adaptive immunity [58]. Some of the cryptdins (cryptdin 2&3) show paracrine actions [59]. They are able to form pores in epithelial cells and stimulate secretion of some interleukins [60]. It seems that they are able to coordinate an inflammatory response with the antimicrobial secretions of Paneth cells. TFF2- deficient animals show highly up-regulated levels of cryptdin pre-protein, especially in the distal small intestine. The bactericidal activity of some of the mouse cryptdins is dependent on processing of cryptdin precursors by matrix metalloproteinase-7 (MMP-7) [36, 41]. MMP-7 and some other matrix metalloproteinases can be irreversibly inactivated by tissue inhibitor of metalloproteinases-3 (TIMP3). We did not observe any significant change in expression of MMP-7 and TIMP 3 by qPCR, although microarray analysis showed an almost ~2 fold down-regulation for TIMP3. This points to a more complex regulation of cryptdin activity than one based solely on changes of gene expression.

Cysteine-rich intestinal peptide (CRIP), another highly expressed product of Paneth cells, was also up-regulated in the duodenum and distal small intestine of TFF2 deficient mice. CRIP plays a significant role in the regulation of cytokine balance and the immune response. Transgenic animals over-expressing CRIP (~3-7 fold) have changed numbers of different white cell types [61] and altered cytokine patterns and immune responses [62]. Dipeptidyl peptidase I (DPPI or Cathepsin C), important for MHC class II antigen presentation [63], was up-

regulated in intestine of TFF2-/- mice. DPPI, as a sole activator of several serine proteases plays an important role in processing lysosomal serine-proteases contained in immune/inflammatory cells [64].

TFFs expressed in the murine stomach seem to depend on each other. TFF3 deficient mice showed reduced synthesis of both gastric TFF1 and TFF2 [65] while ~70 % of TFF1-deficient mice failed to express gastric, but not pancreatic, TFF2 [30]. Our TFF2-deficient mice showed no change in TFF1 level and increased TFF3 expression in the pyloric antrum. This substitution of TFF2 loss with additional TFF3 at the pyloric antrum and duodenum could explain the failure of developing a drastic phenotype similar to TFF1-/- deficiency. Modulation of cryptdins in Paneth cells is not surprising when considering that injected 125I-TFF1 and TFF3 bind to Paneth cells [66]. The de-novo expression of TFF3 strongly induced in the pyloric junction and duodenum suggests the functional cross-regulation between the members of the TFF family.

Altered expression of different immune response relevant genes in the gastrointestinal mucosa of TFF2 deficient mice are in line with recent data of T. Wang and colleagues. They found that macrophages from TFF2-/- mice had a gene expression pattern consistent with constitutive activation of IL-1 receptor signalling, and enhanced phosphorylation of IκB-alpha (T. Wang, personal communication, 2004).

In summary, molecular alterations of immune response relevant genes as a consequence of TFF2-deficiency strongly indicate the involvement of TFF2 in the regulation of intestinal immunity.

In conclusion, TFF2 deficiency caused specific modulations of immune system relevant genes demonstrating an indirect interplay of TFF2 with the immune response.

Acknowledgements

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