Published in final edited form as:

Cytokine. 2013 July ; 63(1): 36–42. doi:10.1016/j.cyto.2013.04.004.

Multiple activating and repressive *cis*-promoter regions regulate *TNFSF15* expression in human primary mononuclear cells

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Abstract

TL1A/*TNFSF15* has been associated with IBD (inflammatory bowel disease) in GWAS (genome-wide association study) and plays a role mediating mucosal inflammation in IBD. Higher TL1A expression is associated with disease severity in both patients and mouse models. Although TL1A has been studied extensively for IBD-associated SNPs, the *cis/trans*-regulatory regions are poorly defined. Herein we identify response elements regulating *TNFSF15* in primary human myeloid cells. Peripheral mononuclear cells transfected with *TNFSF15* promoter constructs displayed 30-fold enhanced promoter activity in a minimal –74 bp region. Transactivation was mediated partly by AP-1, since mutation of the AP-1 site resulting in loss of promoter activity. Monocytes transfected with c-Jun siRNA or treated with TAT-TI-JIP (JNK Inhibitor VII TAT-TI-JIP) demonstrated reduced TL1A mRNA and protein levels. Surprisingly, constructs larger than –74 bp did not increase promoter expression (expression of –1275 bp construct was 25% of –74 bp activity), suggesting the presence of both activating and repressing TL1A promoter elements. In fact, mutation of the –210 bp NFκB site enhanced promoter activity (60-fold) suggesting a repressive role for this site. DNA–protein binding to the TL1A AP-1 and NFκB elements was inhibited by excess consensus or TL1A oligonucleotides and binding and confirmed by chromatin immuno-precipitation analysis. Yet, despite the fact that the –210 bp NFκB site acts as a suppressor element, overall mRNA and protein expression were inhibited in monocytes treated with MG132 (NFκB/proteasome inhibitor) or SN50 (NFκB-p50 blocking peptide), suggesting that NFκB acts as both an activator and silencer of TL1A expression. These data suggest that modulation of TL1A expression involves a complex interplay between positive and negative signals, binding to distinct regulatory regions.

Keywords

TL1A; *TNFSF15*; Gene regulation; Gene promoter; Inflammation

1. Introduction

TL1A (*TNFSF15*) is a tumor necrosis factor family member expressed by monocytes, macrophages, dendritic cells, T cells and endothelial cells [1–3]. Expression of TL1A is initiated via signaling of different molecular pathways depending on the cell type. TL1A was first reported to be expressed in human endothelial cells in response to TNFα or IL-1 [1]. In innate immune cells, such as monocytes, macrophages, and dendritic cells, TL1A

expression is induced following stimulation by immune complexes and through interaction with enteric microorganisms [2,3]. In T cells, expression of TL1A occurs as a consequence of inflammation or T cell receptor stimulation [4]. We have previously shown that TL1A augments inflammatory cytokine secretion in PB T cells through IL-12/IL-18-mediated production of IFN- γ and, augments both IFN- γ production and cytotoxicity in IL-12/IL-18-activated NK cells [5,6]. Recently, a role for TL1A was identified in regulation of Th17 responses [7–10].

Although TL1A has been implicated in the inflammatory process of several diseases, such as renal inflammation, rheumatoid arthritis, asthma and irritable bowel syndrome [10–14], its role in the context of inflammatory bowel disease is probably best established. Expression of TL1A is upregulated in macrophages and T cells of inflamed tissue from the colon and small bowel of CD (Crohn's Disease) patients compared to uninvolved areas, and in serum of patients with active UC (ulcerative colitis) [15–17]. TL1A is currently under development as a potential therapeutic target for treating IBD, since two different murine models of colitis have demonstrated the potential for anti-TL1A blocking antibodies to effectively reduce the severity of inflammation [10,18].

TNFSF15 has been identified and confirmed in GWAS as an IBD-associated gene [19]. *TNFSF15* is one of the few genes that is associated with IBD in all ethnic populations studied. Although the functional significance of these polymorphisms remains unknown, expression of TL1A in monocytes from patients carrying disease-associated SNPs is enhanced and more rapid [20]. Furthermore, risk SNPs within the *TNFSF15* locus are associated with the development of severe, medically-refractive UC [21], as well as fibrostenotic CD [22,23]. In transgenic mice, constitutive TL1A expression in lymphoid or myeloid cells spontaneously leads to intestinal inflammation and fibrosis, and disease severity positively correlates with transgene expression levels [18,22,24]. Like-wise, protein expression is associated with IBD disease severity in both patients and mouse models, and is believed to play a key role in driving the inflammatory process.

Although *TNFSF15* has been studied extensively for IBD-associated SNPs, the *cis*- and *trans*-regulatory regions remain poorly defined. Two studies of TNF α mediated regulation of TL1A expression in murine and human endothelial cell lines identified NF κ B (–210 bp) and AP-1 binding (+12 bp) sites [25–27] within the *TNFSF15* promoter region. However, TNF α transactivation of the *TNFSF15* promoter resulted in a modest 1.5–5-fold increase of promoter activity. Likewise, activation of the human monocytic U937 cell line with 5 μ g LPS (lipopolysaccharide), resulted in less than 2-fold enhancement of promoter activity. Although the authors proposed a role for NF κ B in LPS-mediated activation of TL1A, the biological significance of this finding remains to be determined [27]. In the present study we identified regulatory response elements and pathways that mediate primary human myeloid expression of TL1A. In view of the importance of myeloid expression of TL1A as a modulator of inflammation in IBD, understanding the molecular mechanisms involved in transcriptional regulation of TL1A may help elucidate molecular pathways involved in the pathogenesis of disease.

2. Materials and methods

2.1. Study subjects

Human subjects were recruited through the IBD Center at Cedars- Sinai Medical Center. All controls subjects were healthy individuals, free of medication, and with no known personal or family history of autoimmune disease or IBD. Informed consent (approved by the Institutional Review Board at Cedars-Sinai Medical Center) was obtained from all participating subjects.

2.2. Isolation of monocytes

PBMC (peripheral blood mononuclear cells) were isolated from healthy volunteers by separation on Ficoll-Hypaque gradients. Monocytes were isolated using negative selection by depletion with magnetic beads (Stemcell Technologies, Vancouver, BC, Canada) and were at least 95% pure.

2.3. TL1A ELISA assay

TL1A was measured by an amplified ELISA. ELISA plates (Greiner Bio-One, Longwood, FL) were coated overnight with 100 μ L of 5 μ g/mL monoclonal anti-TL1A (Clone 04H08, Teva Pharmaceuticals, Irvine, CA). Samples and standards were added for 24 h followed by addition of 100 μ L of 2.5 μ g/mL biotinylated anti-TL1A (Clone 16H02, Teva) for 2 h. This was followed by addition of 100 μ L of 1/1000 diluted alkaline phosphatase-conjugated strepta-vidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h. Substrate, 0.2 mM NADP (Sigma–Aldrich, St. Louis, MO) was added for 30 min followed by addition of amplifier (3% 2-propanol, 1 mM iodonitrotetrazolium violet, 75 μ g/mL alcohol dehydrogenase, and 50 μ g/mL diaphorase; Sigma–Aldrich) for 30 min. Plates were read at 490 nm using an E max plate reader (Molecular Devices, Sunnyvale, CA).

2.4. Gel Mobility electrophoretic shift assay (EMSA)

Nuclear extract protein (3–6 μ g) or human recombinant c-Jun protein (Promega) was incubated at 25 °C with 0.25 mg/ml poly (dI-dC), in 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris pH 7.5 for 10 min. Oligonucleotides 5'-IRD700-labeled (Integrated DNA Technology, Coraville, Iowa) were then added (250 fmol) and the binding reactions incubated for an additional 30 min. Specificity was determined by the addition of 50–100-fold excess unlabeled oligonucleotide as competitor. The DNA–protein complexes were separated from unbound probe on a pre-run native 6% polyacrylamide gel in low ionic strength buffer (22.3 mM Tris pH 7.4, 22.3 mM Borate, 0.5 mM EDTA pH 8.0) and analyzed with Odyssey infrared imaging system (Li-Cor Biosciences). The elements used were:

- *TNFSF15* NF κ B 5'-TCCTTCAGGGACTTTCCTAACTTC-3',
- mut *TNFSF15* NF κ B 5'-TCCTTCaGACgcTCCTAACTTC-3',
- *TNFSF15* AP-1 5'-GGTGACTTTAATCACTCAGTCTCC-3',
- mut *TNFSF15* AP-1 5'-GGTtgcTTAATCACTCAGTCTCC-3'.

Consensus AP-1 and NF κ B oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, California).

2.5. Promoter cloning and reporter constructs

The *TNFSF15* promoter–reporter (Firefly luciferase) constructs were a generous gift from the laboratory of Steinberg et al. [26]. Constructs, originally numbered from first translated sequence, have been re-labeled to reflect their position relative to the transcriptional start site.

2.6. Transfection

Freshly isolated PBMC were transfected following overnight culture in RPMI 1640 medium containing 10% fetal calf serum. Cells were then washed and resuspended in 250 μ L fresh medium at 2×10^7 cells/ml and electroporated in the presence of 50 μ g of Firefly reporter construct (600 V, for 9 pulses of 500 μ s, with 100 μ s between pulses) using 4 mm (gap width) cuvettes in a BTX Electro Square Porator ECM 830 (Genetronics, Inc., San Diego,

CA). A control plasmid (2 µg) containing the β-actin promoter driving Renilla luciferase (provided by Dr. Christopher Wilson, University of Washington) was co-transfected as an internal standard and values were normalized to correct for transfection efficiency:

$$\text{Normalized Fold Increase} = \frac{\text{Test}_{\text{FF}} \times \text{pGL3}_{\text{Ren}}}{\text{Test}_{\text{Ren}} \times \text{pGL3}_{\text{FF}}} \quad (1)$$

After electroporation, the cells were diluted in fresh medium, allowed to rest for 1 h prior to plating, and then stimulated with 40 ng/mL PMA (phorbol 12-myristate 13-acetate, Sigma–Aldrich, St. Louis, MO) plus 1 µg/mL ionomycin (Sigma–Aldrich) for 4 h. Luminescence was measured using a Promega (Madison, WI) luciferase assay kit and counted on a 6-detector Perkin Elmer Life Sciences (Gaithersburg, MD) 1450 Microbeta liquid z deactivated.

2.7. siRNA

Monocytes were transfected with siRNAs for c-Jun, TL1A or non-target scramble control siRNA using two transfection modalities. Transductin (15 µM, Integrated DNA Technologies) was incubated with 0.5 µM siRNA (Integrated DNA Technologies) in PBS for 30 min at 4 °C. Complexed siRNA was incubated with 1×10^5 monocytes/well in 48-well cell culture plate for 4 h at 37°C in RPMI 1640 with 10% Q-depleted serum (Integrated DNA Technologies). Alternatively, monocytes were transfected using Lipofectamine RNAiMax (Invitrogen, Grand Island, NY) following the manufacturer's recommendations.

2.8. Flow cytometry

Membrane-bound TL1A was measured by flow cytometry following staining of monocytes with PE conjugated (EasyLink R-PE kit, Abcam, Cambridge, MA) anti-TL1A (clone 04H08, Teva) compared with PE-conjugated mouse IgG2b (Invitrogen). Cells were analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, CA).

2.9. qPCR

Total RNA was isolated from monocytes using the RNeasy kit (Qiagen, Inc., Valencia, CA, USA) and gene expression was measured by real-time quantitative RT-PCR. Five hundred nanograms of total RNA were used in each RT reaction, with oligo (dT) (Integrated DNA Technologies) as primer, using the Omniscript kit and protocol (Qiagen). Real-time PCR was performed using a Mastercycler® ep realplexPCR detection system (Eppendorf, Hauppauge, NY). Primer/probe sequences (Integrated DNA Technologies) spanned introns and were as follows: TL1A forward, 5'-CTTCCTTGCAAGGACTCACCAC-3', reverse 5'-GCTGATGTGAAGGTGCAAACCTC-3', probe 5'-FAM/ACCTGCTTGTCAGCCAGCTCCGG-3'BHQ_1. Levels of gene expression were normalized as a percentage of housekeeping gene *EF1a* expression (*EF1a* primer forward, 5'-GCAAAAATGACCCACCAATG-3', reverse 5'-GGCCTGGATGGTTCAGGATA-3', probe 5'-FAM/CACCTGAGCAGTGAAGCCAGCTGCT-3'BHQ_1). PCR assays were run in duplicate.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a ChIP assay kit (EMD Millipore, Billerica, MA). Briefly, monocytes untreated or activated with PMA and ionomycin were crosslinked with 1% paraformaldehyde. Sonicated chromatin from 5 to 8×10^7 cells was immunoprecipitated by incubation with anti-c-Jun, anti-p50 or anti-p65 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX) or with normal rabbit IgG. qPCR primer for c-Jun binding to the

TNFSF15 AP1 site were: forward primer 5'-TCTGTTGTAGGCGG TGCATTCTCT -3' and reverse primer 5'-CCCAAAGCTCAGTCCCAGATCCT -3', and for the -210 bp *TNFSF15* NFκB element: forward prime 5'- CCTCCAGTGCAGGGAACAG GTATATT-3' and reverse primer 5'-AGAGAATGCACCGCCTACAACAGA-3'. Data are expressed as a percentage of input DNA samples with binding to IgG subtracted.

2.11. Statistical analysis

Tests for statistical significance were determined by Student's T test using JMP Statistical Software.

3. Results

3.1. PMA/ionomycin signaling induces TL1A mRNA and protein expression in human monocytes

To investigate the regulation of myeloid expression of TL1A, human peripheral monocytes were stimulated with PMA/ionomycin as a broad activator. As seen in Fig. 1A, expression of TL1A mRNA was detectable within 8 h following activation by PMA/ionomycin and peaked at 36 h following stimulation. Likewise, activation by PMA/ionomycin resulted in both secreted and membrane expression of TL1A (Fig. 1B and C). The kinetics of activation following PMA/Ionomycin stimulation were similar to those previously reported for immune complex-induced expression of TL1A [2].

3.2. Functional mapping of *TNFSF15* AP-1 and NFκB cis-regulatory elements in human PBMC

In order to identify the response elements necessary for expression, a -1276 bp *TNFSF15* promoter-luciferase construct was transfected into human PBMC. Promoter expression was compared to constructs truncated to -1114 bp, -942 bp, -798 bp, -426 bp, -330 bp, 74 bp and 26 bp. Fig. 2A shows activation-dependent expression of the *TNFSF15* promoter. The -74 bp region possesses a critical minimal promoter element which produced a 30-fold enhancement over pGL3 basic vector following PMA/ionomycin activation. Surprisingly, the promoter constructs of longer length elicit less enhancement of expression. The longest -1276 bp construct exhibited only 20% promoter activity compared to the -74 bp construct. These results suggest the presence of both activating and repressing promoter elements within the *TNFSF15* promoter region.

AP-1 and NFκB sites previously identified in upregulating endothelial cell TL1A expression reside within the first 250 bp flanking the transcriptional start site (Fig. 2B). The NFκB site resides at -210 bp and the AP-1 site further downstream within the 5'UTR, adjacent to the first translated nucleotide. EMSA assays demonstrated PMA/ionomycin induced activation of nuclear protein binding the AP-1 (Fig. 3A) and NFκB (Fig. 3B) sites. As seen in Fig. 3A and B, PMA/ionomycin activation resulted in similar pattern of upregulation of *trans*-acting factors binding to both the AP-1 and NFκB elements, which was sustained up to 4 h. Upregulation of binding to the *TNFSF15* AP1 and NFκB sites was similar to that detected with consensus AP-1 or NFκB oligonucleotide (data not shown). The capacity of the *TNFSF15* 5'UTR site to function as an AP-site and bind c-Jun was confirmed utilizing recombinant c-Jun. Purified recombinant c-Jun protein effectively bound to the *TNFSF15* AP1 site and binding was selectively inhibited by excess cold consensus AP-1 but not mutant oligonucleotides. Likewise, binding was inhibited by excess *TNFSF15* AP-1 oligonucleotides (Fig. 3C).

The direct interaction between AP-1 and NFκB binding to the TL1A promoter region was further confirmed by ChIP assays. Isolated chromatin from primary monocytes was

immunoprecipitated with anti-c-Jun or control IgG. Quantitative real-time qPCR analysis was carried out with primers targeted to the 5'UTR AP-1 binding site. As seen in Fig. 4, we detected a significant enrichment of c-Jun binding to the 5'UTR AP-1 binding site following activation. The NFκB transcription factor is composed of both homodimers and heterodimers of the Rel family of proteins therefore analysis of binding to the -210 bp NFκB site was carried out using chromatin immunoprecipitated with anti-p50 and p65 antibodies. As seen in Fig. 4, there is a significant increase in p50 protein binding relative to p65 to this region following activation. While most NFκB dimers are activators of transcription, the p50 protein dimer lacks a transcriptional activation domain and is believed to function as a repressor of transcription. The EMSA analysis in Fig. 3B is likewise supportive of a shift in balance of p50 and p65, with the intensity of the upper and lower bands binding to the NFκB -210 bp site displaying a decrease in the upper band (p50/p65) and increase in the lower band (p50/p50) following activation. Thus, a shift in the balance between p50 and p65 binding may partially explain the repressor activity of this site ultimately determining the level of gene expression.

To characterize the functional role of the AP-1 and NFκB sites in regulation of TL1A expression, cells were transfected with *TNFSF15* constructs in which the AP-1 or NFκB sites had been mutated. Only the AP-1 site was present in the -74 bp construct whereas the -330 bp construct contained both the AP-1 and NFκB sites. As seen in Fig. 5, mutation of the AP-1 site alone, within the context of the -74 bp construct, resulted in a loss of promoter activity, supporting a role for AP-1 as a positive regulator of TL1A expression. No decrease in promoter activity was seen when mutating the AP-1 site within the context of the -330 bp region. In contrast, mutation of the -210 bp NFκB site alone led to a marked enhancement of promoter activity, suggesting that the -210 bp NFκB site repressed TL1A expression. Mutation of both activating AP-1 and repressive NFκB sites resulted in expression similar to the wild type construct. These results suggest a complex regulatory process exists, which includes multiple activating and inhibitory response elements within the *TNFSF15* promoter.

3.3. Inhibition of AP-1 and NFκB pathways attenuates TL1A expression in human monocytes

The functional significance of the AP-1 transactivation in monocytes was confirmed using a selective JNK peptide inhibitor, TAT-TI-JIP, comprised of an inhibitory peptide corresponding to amino acids 143–153 of the c-Jun JNK-Interacting Protein 1 coupled to the cell-permeable TAT protein domain [28]. Monocytes were cultured with increasing concentrations of TAT-TI-JIP. As seen in Fig. 6A, TL1A mRNA expression and protein secretion were inhibited in a dose dependent manner. Expression of TNF-α mRNA was not inhibited by TAT-TI-JIP treatment (data not shown). The role of AP-1 in regulating TL1A expression was further confirmed using siRNA mediated silencing. A major challenge in working with primary monocytes is a difficulty in efficiently transfecting them. Traditional transfection techniques utilizing liposomal transfection reagents are largely inefficient and electroporated monocytes are resistant to activation. To bypass these difficulties, monocytes were transfected via a peptide-based transduction delivery method (see Section 2), in addition to a lipofection method. Human monocytes were transfected with siRNA targeting TL1A mRNA itself or siRNA targeting c-Jun. As seen in Fig. 6B, a 50% decrease in TL1A secretion was seen for the TL1A siRNA and a 35% reduction in TL1A secretion for c-Jun siRNA compared to control scrambled siRNA. The expression of c-Jun mRNA itself displayed a 37–50% inhibition by c-Jun siRNA (data not shown). To confirm the role of NFκB in regulating myeloid TL1A expression, we used the synthetic cell-permeable peptide SN50. SN50 contains the nuclear localization sequence of the p50 subunit of NFκB which interferes with NFκB's translocation through the nuclear pore [29]. Monocytes were treated in the presence or absence of SN50 or a control peptide (which was mutated in the nuclear

localization sequence residues), prior to activation with PMA/ionomycin. As seen in Fig. 7A, treatment of cells with SN50 markedly inhibited the expression of TL1A mRNA and protein secretion. To further confirm the contribution of functional activation of NF κ B on regulation of TL1A expression, we utilized an additional NF κ B inhibitor, MG132. The level of TL1A mRNA and protein secretion inhibition following treatment with MG132 is shown in Fig. 7B. Likewise, membrane TL1A expression was inhibited in monocytes treated with MG132 (Fig. 7C).

4. Discussion

In UC and CD, TL1A expression by activated monocytes and T cells is up-regulated during chronic intestinal inflammation [19]. In this study we examined the transcriptional regulatory regions mediating expression of TL1A in human PBMC. Following PMA/ionomycin activation, an increase in TL1A mRNA levels was detected by 6–8 h while secreted TL1A and membrane protein was expressed by 12 h. The kinetics of expression is similar to that observed following activation with immune complexes. Significant promoter transactivation (a 30-fold increase) was mediated through the –74 bp minimal promoter region (located immediately upstream of the transcription start site). No further increase in promoter activity was detected in constructs up to –1 kb in length. However, a significant decrease in promoter activity was observed for promoters of longer length, with a decline down to 20-fold enhancement for the –1114 bp, and less than 10-fold for the –1276 bp promoter construct. These results suggest the presence of both activating as well as repressive *cis*-acting elements within the first –1276 bp of the TL1A promoter region.

Previous studies have proposed the presence of putative conserved AP-1 and NF κ B *cis*-regulatory binding sites within the first –250 bp of the TL1A promoter, utilizing either endothelial or monocytic cell lines [25–27]. The difference in promoter activity reported when comparing control to activated cells was minimal (1.5- to 3-fold) raising the question as to the functional significance of these results. In this study, we used human primary mononuclear cells and demonstrated a 30-fold enhancement of promoter activity, suggesting that regulation of TL1A gene expression in primary cells differs from that observed in cell lines. The AP-1 site previously believed to reside within the TL1A promoter region actually lies within the 5'UTR of the TL1A mRNA sequence. This AP-1 site functions as a regulator of TL1A expression, particularly in the context of the –74 bp region. Electrophoretic mobility shift assays, using nuclear extracts from activated PBMC or recombinant c-Jun protein, demonstrated binding to this AP-1 site. Likewise, electromobility shift assays demonstrated that binding was competed by excess TL1A AP-1, but not mutant oligonucleotide. The functional significance of this site in modulating TL1A expression was complex. Promoter transactivation was reduced virtually down to baseline levels when PBMC were transfected with –74 bp promoter constructs mutated at the AP-1 site. This finding supported an activating role for AP-1 in enhancing TL1A expression. Indeed, siRNA- or peptide-mediated inhibition of AP-1 resulted in reduced TL1A mRNA and protein. However, within the context of the longer –330 bp construct, mutation of the AP-1 site alone did not significantly alter promoter expression levels. Rather, mutation of the NF κ B site resulted in a marked enhancement of promoter activity. This finding suggested an inhibitory role for –210 bp NF κ B site in regulating TL1A expression. In fact, mutation of both the activating AP-1 and inhibitory NF κ B sites restored promoter levels to that observed in the wild type –330 bp constructs. The EMSA and ChIP data suggest that following activation there is a shift in the composition of the NF κ B complex binding to this region to one comprised of a more inhibitory p50/p50 composition. However, the functional consequence of global inhibition of NF κ B following treatment with multiple inhibitors of the NF κ B pathway resulted in reduced expression of TL1A mRNA and protein. These results demonstrated that while the –210 bp NF κ B site serves as a suppressor element,

overall expression of TL1A involves activation of the NF κ B pathway. Although the TL1A AP-1 site participated in induction of expression of TL1A, it alone is not capable of activating the promoter. Thus, a complex interaction of both AP-1 and NF κ B as well as additional, as of yet unidentified, regulatory sites likely determine the level of TL1A expression.

UC and CD are multifaceted diseases triggered by interactions between genetic, environmental and immune components. TL1A was initially identified as a CD-associated gene [17,30]. Subsequently, additional GWAS studies extended this finding to association with UC, as well, and established TL1A as an IBD severity gene [21,22,31]. Although much progress has been made to define disease-associated TL1A risk haplotypes, the functional significance of these SNPs is complex and remains largely unknown. A study of CD patients carrying the TL1A risk haplotype demonstrated a more robust and rapid expression of TL1A compared to non-risk haplotype patients [20]. Expression of TL1A was likewise increased when comparing Jewish to non-Jewish CD patients, particularly in Jewish patients exhibiting antibody responses to an *E. coli* microbial antigen. Thus, expression of TL1A is multifaceted and dependent upon haplotype, ethnicity, as well as antibody response to microbial antigens. In a study examining the possibility that the risk SNPs themselves may function as *cis*-regulatory sites, only a small difference was detected when comparing TL1A expression from activated T cells in healthy individual possessing the risk versus the non-risk haplotype [32]. Likewise, transfection of promoter-reporter constructs carrying the different risk allele haplotype combinations demonstrated no differences for the 1 kb promoter and only minor differences for the 500 bp region. Thus, the interrelationship between the GWAS-identified polymorphisms and regulatory element remains unknown. Our data suggested that a complex interplay exists between positive and negative signals, binding to distinct *cis*-regulatory regions, which ultimately modulate TL1A expression. Coordinate fine mapping of the TL1A locus for both IBD-associated SNPs and *cis*-regulatory regions is underway to identify critical regions mediating the levels of TL1A expression in IBD.

Acknowledgments

This work supported by the F. Widjaja Inflammatory Bowel and Immunobiology Research Institute, USPHS Grants R37DK43211, PO1DK046763, and National Center for Research Resources, Grant UL1TR000124. We wish to thank Carrie Derkowski for assisting in tissue procurement and Patricia Lin for help with flow cytometry. We also thank Surinder Safaya for generating *TNFSF15* promoter-reporter constructs.

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Fig. 1.

PMA/ionomycin activation induced myeloid expression of TL1A. Monocytes were stimulated with PMA/ionomycin for indicated periods of time. (A) TL1A mRNA measured by real time RT-PCR; (B) secreted TL1A protein measured by ELISA; (C) TL1A expression on monocytes measured at 24 h by flow cytometry. Data is a representative experiment of four time-course experiments.

Fig. 3.

EMSA analysis of *TNFSF15* regulatory binding proteins. PBMC were stimulated up to 4 h with PMA/ionomycin and nuclear protein extracts were obtained. EMSAs were performed for binding activity to the (A) *TNFSF15* 5'UTR AP-1 and NFκB sites. (B) The functional capacity of the *TNFSF15* 5'UTR site to bind AP-1 was confirmed utilizing recombinant c-Jun protein. EMSA binding activity of recombinant c-Jun protein to the *TNFSF15* 5'UTR AP-1 site was assayed for specificity by competition with excess unlabeled consensus AP-1 or mutant AP-1 oligonucleotide or increasing amount of excess *TNFSF15* AP-1 oligonucleotide. Data are representative of three experiments with similar results.

Fig. 4. Chromatin immunoprecipitation (ChIP) analysis. Monocytes were activated for 8 h with PMA/ionomycin, followed by immunoprecipitation of chromatin with antibodies specific for c-Jun, NF κ B p50, p65 or control IgG. Quantitative PCR analysis of binding of c-Jun to the 5' *TNFSF15* UTR or -210 bp NF κ B site was carried out. Results are expressed as the fraction of the input DNA calculated from a standard curve. Data represent mean $\pm$ standard error of the mean (SEM) from at least three independent experiments.

Fig. 5.

Mutation of *TNFSF15* promoter AP-1 and NFκB binding sites modified promoter activation in PBMC. Wild type or mutated -74 bp or -330 bp *TNFSF15* promoter reporter construct were transfected into human PBMC, followed by stimulation with PMA/ionomycin for 4 h. Constructs -74 AP, -330 AP or -330 NFκB were mutated at AP-1 or NFκB sites, respectively. -330 NF/AP has been mutated at both the AP-1 and NFκB sites. Data represents mean $\pm$ SEM for four experiments.

Fig. 6.

Inhibition of AP-1 reduced myeloid expression of TL1A. (A) Monocytes were stimulated with PMA/ionomycin for 18 h in presence of indicated concentrations of TAT-TI-JIP. TL1A mRNA was measured by real-time RT-PCR (expressed as a percentage of housekeeping gene EF1 α) and supernatants were analyzed for secreted TL1A. (B) Monocytes were transfected with siRNAs for c-Jun, TL1A or nontarget scramble control siRNA. Cells were activated with PMA/ionomycin and supernatants were analyzed for TL1A mRNA by real-time RT-PCR. (Average TL1A mRNA level was 5% of EF1 α and 320 pg/ml for secreted TL1A protein) Data represents mean of two similar experiments for TAT-TI-JIP and five experiments for siRNA.

Fig. 7.

Inhibition of NF κ B reduces myeloid expression of TL1A. (A) SN50 or control peptide or (B) MG132 were added to monocytes at the time of PMA/ionomycin stimulation. *TNFSF15* mRNA was measured by real-time RT-PCR, secreted TL1A was measure by ELISA and TL1A membrane expression was measured by flow cytometry. (Average TL1A mRNA level was 11% of EF1 α and 2.8 ng/ml for secreted TL1A protein). Data represents mean of three similar experiments for TL1A protein, two similar experiments for mRNA and a representative histogram of five similar experiments for flow cytometry.