TNF Increases Expression of IL-4 and PARs in Mast Cells

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Key Words

TNF • IL-4 • Mast cell • Protease activated receptor
• Trypsinase

Abstract

Tumor necrosis factor (TNF) is a proinflammatory cytokine which has been shown to be actively involved in the pathogenesis of allergic inflammation. However, little is known of the effects of TNF on cytokine secretion and protease activated receptor (PAR) expression in mast cells. In the present study, we examined potential influence of TNF on IL-4 and IL-12 release from P815 cells and PAR expression in P815 cells by using flow cytometry analysis, quantitative real time PCR, ELISA and cellular activation of signaling ELISA (CASE) techniques. The results showed that TNF induced up to 2.7-fold increase in IL-4, but not IL-12 release from P815 cells, and PAR-2 antagonist peptide FSLLRY-NH₂ and PAR-4 antagonist peptide trans-cinnamoyl (tc)-YPGKF-NH₂ did not affect TNF induced IL-4 release. Approximately up to 2.4 and 2.3 fold increases in expression of PAR-2 and PAR-4 were observed when cells were incubated with TNF. Pretreatment of cells with TNF for 60 min enhanced trypsin and trypsinase induced

PAR-2 expression by 2.4 and 2.3 fold; PAR-3 expression by 1.6 and 1.7 fold and PAR-4 expression by 1.6 and 1.7 fold, respectively. LY294002, an inhibitor PI3K abolished TNF induced IL-4 release and phosphorylation of Akt in P815 cells, indicating Akt cell signalling pathway is involved in the event. In conclusion, TNF can stimulate IL-4 release from mast cells through an Akt cell signalling pathway dependent, but PAR independent mechanism. TNF may serve as a regulator for IL-4 production and PAR expression, and through which participates in the mast cell related inflammation.

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Introduction

Protease activated receptors (PARs) named PAR-1, 2, 3 and 4, have been identified as receptors for serine proteases. Among them, PAR-1 is a receptor of thrombin and trypsin [1]; PAR-2 is a receptor of trypsin, trypsinase [2] and elastase [3]; PAR-3 and PAR-4 are receptors of thrombin [4, 5]. Recently, activation of proteinase-activated receptors (PARs) has been suggested to be

involved in the pathogenesis of inflammation. For example, upregulation of PAR-2 expression has been found in the airways of asthma [6] and allergic rhinitis [7]. Using PAR-2 wild-type and knockout mice, it was found that PAR2 signalling promotes DC trafficking to the lymph nodes and subsequent T-cell activation, which provides an explanation for the pro-inflammatory effect of PAR2 in animal models of inflammation [8]. Protease activity of whole mite culture extract, rDer f 1 and rDer p 1 induced the release of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) from primary human keratinocytes [9]. Trypsin has been reported to stimulate IL-6 release from peripheral blood T cells [10] and monocytes [11] through PAR related mechanisms.

TNF is a major proinflammatory cytokine that is thought to be important in the pathogenesis of asthma [12], food allergy [13, 14], ocular allergy [15], and atopic dermatitis [16]. It has been reported that increased number of TNF expressing cells and levels of TNF are observed in the bronchoalveolar lavage (BAL) and in the airways of asthmatics [17], respectively. It has also been discovered that TNF can induce adhesion of mast cells to skin endothelial cells [18], and that mast cells can produce and release TNF [15]. These findings implicate further that TNF is involved in allergic inflammation. However, little is known of the influence of TNF on mast cell cytokine release and expression of PARs.

IL-4 is a multifunctional cytokine that plays an important role in the pathogenesis of allergic disease [19], and it is the key cytokine in the development of Th2 cell responses [20]. It has been demonstrated that IgE-dependent stimulation of purified human lung mast cells leads to the rapid release of IL-4 into the extracellular environment [19], and IL-4 can enhance the adhesion of murine bone marrow-derived cultured mast cells to skin endothelial cells [18]. We have found previously that P815 mast cells can release IL-4 in response to GM-CSF [21] and IL-12 [22]. Since IL-4 can be released from mast cells and TNF is a crucial cytokine in allergy, we investigated the effect of TNF on IL-4 release from P815 cells in the present study.

Mast cell has a long history of being recognized as an important mediator- secreting cell in allergic inflammation. As the primary effector cell, mast cell is actively involved in the pathogenesis of both acute and chronic allergic diseases. Functional expression of PAR-2 has been found in cutaneous mast cells [23], in rat peritoneal mast cells [24] and in P815 mast cells [22], indicating that this cell type is most likely involved in the pathogenesis of allergic inflammation through activation

of PARs. However, the regulatory mechanisms of expression of PARs on mast cells and cytokine release from mast cells remain obscure. We therefore investigate the effects of TNF on expression of PARs and cytokine production in P815 cells in the present study.

Materials and Methods

Reagents and cells

Trypsin, leupeptin, paraformaldehyde and bovine serum albumin (BSA, fraction V) and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were from Sigma Inc. (St. Louis, MO, USA). Recombinant human lung β -tryptase was obtained from Promega (Madison, WI, USA). Recombinant mouse TNF, anti-mouse TNF monoclonal antibody, anti-mouse TNF receptor I (RI) monoclonal antibody (clone 55R-170) and mouse IL-4 ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). FSLRY-NH₂ and tc-YPGKF-NH₂ were synthesized by CL Bio-Scientific Inc (Xi An, China). Tissue culture reagents including Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). Mouse IL-12 ELISA kits were obtained from Pierce Biotech Inc. (Rockford, IL, USA). TRIzol Reagent was obtained from Invitrogen (Carlsbad, CA, USA). Tyrphostin (AG490), 2-(2-diamino)-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126), 1,4-Diamino-2,3-dicyano-1,4-bis(methylthio) butadiene (U0124) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were obtained from Cell Signaling Technology (Beverly, MA, USA). ExScript™ RT reagent kit and SYBR® *Premix Ex Taq*™ (perfect real time) was obtained from TaKaRa Biotechnology Co. Ltd. (DaLian, China). Oligonucleotide primers for real time PCR were synthesized by Invitrogen Biotechnology Co. (Shanghai, China). FITC-conjugated goat anti-mouse PAR-2 monoclonal antibody; rabbit anti-mouse PAR-1, rabbit anti-mouse PAR-3 and rabbit anti-mouse PAR-4 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated goat anti-rabbit polyclonal antibody was obtained from BD Pharmingen (San Jose, CA, USA). Cellular activation of signaling ELISA CASE kits for Akt, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and signal transducer and activators of transcription (STAT)3 were obtained from SuperArray Bioscience Corporation (Frederick, MD, USA). The mouse mastocytoma cell line P815 and mouse mast cell line MC/9 were obtained from the American Type Culture Collection (Manassas, VA, USA). Most of other reagents such as salt and buffer components were analytical grade and obtained from Sigma.

Mast cell culture and challenge

P815 cells and MC/9 cells were cultured with ATCC complete growth medium and with ATCC complete medium at 37°C in a 5% (v/v) CO₂, respectively as described previously [21].

Primer	Sequence	Size of product (bp)
PAR-1 sense	5'-GTTGATCGTTTCCACGGTCT-3'	225
antisense	5'-ACGCAGAGGAGGTAAGCAAA-3'	
PAR-2 sense	5'-CACCTGGCAAGAAGGCTAAG-3'	298
antisense	5'-CCCAGGGTTACTGACGCTAA-3'	
PAR-3 sense	5'-TCAATGGCAACAACCTGGGTA-3'	205
antisense	5'-AAAACCATGACCCACACCAT-3'	
PAR-4 sense	5'-GCAGACCTTCCGATTAGCTG-3'	292
antisense	5'-AGGGCTCGGGTTTGAATAGT-3'	
β -actin sense	5'-GCTACAGCTTCACCACCACAG-3'	288
antisense	5'-GGTCTTTACGGATGTCAACGTC-3'	

Table 1. Primer sequences for mouse PARs used in real time PCR

Cultured P815 cells at a density of 1×10^6 cells/ml were incubated with the serum-free basal medium for 6 h before challenge. For challenge experiments, cells were exposed to various concentrations of TNF with or without its blocking antibody or antagonists of PARs; tryptase ($1 \mu\text{g/ml} = 7.4 \text{ nM}$) or trypsin ($1 \mu\text{g/ml} = 42 \text{ nM}$) with or without FSLLRY-NH₂ ($400 \mu\text{M}$) and tc-YPGKF-NH₂, respectively. At 2, 6 or 16 h following incubation, the culture supernatants (5 ml) were collected, and the cell pellet containing approximately 5×10^6 cells were resuspended for further analysis. For priming experiments, cells were preincubated with 1 ng/ml of TNF for 60 min and washed 3 times before adding tryptase or trypsin for 6 h. For cell signaling experiments, cultured cells at a density of 1.5×10^6 cells/ml were treated with the inhibitors of cell signalling pathways including PD98059 ($50 \mu\text{M}$), U0126 ($5 \mu\text{M}$), U0124 ($5 \mu\text{M}$), SB203580 ($20 \mu\text{M}$), LY294002 ($20 \mu\text{M}$) and AG490 ($40 \mu\text{M}$) for 30 min before being challenged with TNF (10 and 100 ng/ml) for 6 h. For the experiment with anti-mouse TNF RI antibody, cells were preincubated with 100 $\mu\text{g/ml}$ of the antibody for 60 min before adding 100 ng/ml of TNF for 2, 6 or 16 h. For challenge experiments, MC/9 cells were exposed to 10 and 100 ng/ml of TNF for 6 h before culture medium being collected for analysis.

Quantitative real-time PCR

Total RNA was extracted from P815 cells as described previously [22]. Briefly, after synthesizing cDNA from total RNA by using ExScriptTM RT reagent kit, real-time PCR was performed by using SYBR[®] Premix Ex TaqTM kit on the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Systems, Foster City, CA, USA). Each reaction contains 12.5 μl of 2 $\times$ SYBR green Master Mix, 300 nM oligonucleotide primers (Table 1), 10 μl of the cDNA or plasmid DNA. The thermal cycling conditions included an initial denaturation step at 95°C for 2 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Consequently, at the end of the PCR cycles, the real-time PCR products were immediately analyzed using a ramping rate of 0.03°C/s from 60 to 95°C in order to calculate the dissociation curve to verify the correctness of the amplicons. None-template

controls for each primer pair were also included to examine potential external DNA contamination. The gene specific threshold cycle (Ct) for each sample was corrected by subtracting the Ct for the housekeeping gene β -actin (ΔCt). Untreated controls were chosen as the reference samples, and the ΔCt for all experimental samples were subtracted by the ΔCt for the control samples ($\Delta\Delta\text{Ct}$). The magnitude change of test gene mRNA was expressed as $2^{-\Delta\Delta\text{Ct}}$. Each measurement of a sample was conducted in duplicate.

Flow cytometry analysis

P815 cells were pelleted by centrifugation at 450 g for 10 min, and then fixed in 2% paraformaldehyde for 30 min. After washing, the cells were re-suspended in PBS. For PAR-2 staining, cells were incubated with FITC-conjugated goat anti-mouse PAR-2 monoclonal antibody or isotype control (at a final concentration 4 $\mu\text{g/ml}$) at 4°C for 30 min. For PAR-1, PAR-3 and PAR-4 staining, cells were incubated with rabbit anti-mouse PAR-1, PAR-3, PAR-4 polyclonal antibodies or normal rabbit IgG, respectively (at a final concentration 2 $\mu\text{g/ml}$) at 4°C for 30 min, followed by 1 $\mu\text{g/ml}$ of FITC-conjugated goat anti-rabbit polyclonal antibody at 4°C for 30 min. Cells were finally re-suspended in PBS and analyzed on a FACS Calibur flow cytometer with CellQuest software (BD Biosciences).

Analysis of signal-transduction pathways and determination of levels of cytokines

The phosphorylation of Akt, ERK, p38 and STAT3 was analyzed by using CASE kits as described previously [22]. IL-4 and IL-12 levels were measured by using ELISA kits according to the manufacturer's instruction.

Statistics

Data are expressed as mean $\pm$ SEM for the indicated number of independently performed duplicated experiments. Statistical significance between means was analyzed by one-way ANOVA or the Student's *t* test utilizing the SPSS 13.0 version. $P < 0.05$ was taken as statistically significant.

Fig. 1. Induction of IL-4 release from P815 cells by (A) TNF or (B) proteases. In (A), cells were incubated with various concentrations of TNF (ng/ml) or TNF with its blocking antibody (Ab, $\mu\text{g/ml}$) or TNF receptor I blocking antibody (Anti-TNF RI, 100 $\mu\text{g/ml}$), or with protease activated receptor (PAR)-2 antagonist peptide FSLLRV-NH₂ (400 μM) and PAR-4 antagonist peptide tc-YPGKF-NH₂ (100 μM) at 37°C for 16 h. In (B), cells were treated with or without TNF (1 ng/ml) for 60 min before being challenged with various concentrations of trypsin ($\mu\text{g/ml}$) or tryptase ($\mu\text{g/ml}$) at 37°C for 16 h. Where needed, trypsin or tryptase were mixed with FSLLRV-NH₂ (400 μM) or tc-YPGKF-NH₂ (100 μM) before being added to cells at 37°C for 16 h. Values shown are Mean $\pm$ SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to the corresponding uninhibited control. CI = calcium ionophore (100 ng/ml); LY = LY294002 (20 μM).

Results

Influence of TNF on IL-4 release from mast cells

As one of the major proinflammatory cytokines, TNF is actively involved in the pathogenesis of allergic inflammation. However, little is known of the influence of TNF on IL-4 and IL-12 release from mast cells. At the concentrations of 0.1, 1.0, 10 and 100 ng/ml, TNF induced concentration dependent release of IL-4 from P815 cells following 16 h incubation. Approximately up to 2.7-fold increase in IL-4 release was achieved when cells was challenged with TNF. Administration of TNF blocking antibody, TNF receptor I blocking antibody or LY294002 abolished TNF induced IL-4 release from P815 cells. However, FSLLRV-NH₂ and tc-YPGKF-NH₂ had little effect on TNF induced IL-4 release (Fig 1A). Similarly, U0126, PD98059, AG490, SB203580 and U0124 had little influence on TNF induced IL-4 release (data not shown). All inhibitors tested did not significantly affect

basal IL-4 release ability of P815 cells. At 6 h following incubation, TNF also induced significant release of IL-4 from P815 cells (Fig 1A), but not from MC/9 cells (TNF at 100 ng/ml vs. medium alone = 7.5 ± 1.2 vs. 6.8 ± 0.7 , $n = 4$). TNF at the concentrations tested did not elicit significant IL-12 release from P815 cells following 16 h incubation period (data not shown).

We have reported previously that tryptase can induce release of IL-13 from P815 cells and that pretreatment of P815 cells with IL-6 can significantly reduce tryptase induced IL-13 secretion [25]. We therefore anticipate that TNF may also affect the actions of tryptic proteases on P815 cells. It was found that TNF at 1 ng/ml reduced trypsin and tryptase induced IL-4 release from P815 cells by up to 65.4% and 56% respectively following 16 h incubation (Fig 1B). Furthermore, trypsin and tryptase induced IL-4 secretion can be abolished by the antagonist peptide of PAR-2, FSLLRV-NH₂ (Fig 1B), but not tc-YPGKF-NH₂ (data not shown).

Fig. 2. Real-time PCR analysis on the expression of protease activated receptor (PAR)-1 (A), PAR-2 (B), PAR-3 (C) and PAR-4 (D) mRNAs in P815 cells modulated by TNF. Cells were treated with various concentrations of TNF or TNF with TNF receptor I blocking antibody (Anti-TNF RI, 100 ig/ml) at 37°C for 2, 6 and 16 h, respectively before total cellular RNA was isolated, reverse transcribed to cDNA. The data were normalized to the housekeeping gene (β -actin gene) and were expressed as Mean $\pm$ SE folds over control for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to corresponding medium alone control.

Effect of TNF on expression of PAR mRNAs in P815 cells

We have previously showed that GM-CSF and IL-12 can modulate expression of PARs in P815 cells [21, 22], and therefore anticipated that TNF may have similar actions. Real time PCR analysis showed that TNF induced up to 5.8, 7.7 and 2.0 fold upregulated expression of PAR-1 mRNA in P815 cells at 2, 6 and 16 h following incubation, respectively (Fig 2A). TNF induced also concentration dependent expression of PAR-2 (Fig 2B) and PAR-3 mRNAs (not at 16 h, Fig 2C). Most strikingly, up to approximately 10.3, 13.2 and 12.1 fold increases in PAR-4 mRNA expression were achieved respectively when TNF was added to P815 cells for a period of 2, 6 or 16 h (Fig 2D). Preincubation of TNF receptor I blocking antibody with cells for 60 min reduced TNF induced expression of PAR-1, PAR-2, PAR-3 and PAR-4 by up to approximately 59.2, 67.1, 70.2 and 65.4%, respectively (Fig 2).

Modulation of expression of PAR proteins by TNF

In order to further understand the influence of TNF on expression of PARs, we examined expression of PAR proteins in P815 cells by flow cytometry analysis. The results showed that increased mean fluorescence intensities of PAR-2 and PAR-4 were achieved when various concentrations of TNF were incubated with P815 cells. At the concentrations of 1.0, 10 and 100 ng/ml, TNF elicited concentration dependent increase in PAR-2 expression at 6 h following incubation. Approximately up to 2.4 fold increase in PAR-2 expression was obtained when P815 cells were incubated with 100 ng/ml of TNF for 6 h. In contrast, TNF had little effects on PAR-2 expression following 2 h incubation period (Fig 3A).

Unlike the influence on PAR-2, TNF induced concentration dependent PAR-4 expression was observed at all three time points (2, 6 and 16 h) examined. As little as 0.1 ng/ml of TNF was able to upregulate PAR-4 expression, and approximately up to 2.3 fold increase in

Fig. 3. Flow cytometry analysis on the expression of protease activated receptor (PAR)-2 (A) and PAR-4 (B) in P815 cells induced by TNF. Cells were treated with various concentrations of TNF (ng/ml) with or without its blocking antibody (Ab, $\mu\text{g/ml}$) or TNF receptor I blocking antibody (Anti-TNF RI, 100 $\mu\text{g/ml}$) at 37°C for 2, 6 and 16 h, respectively before PAR expression were examined. Values shown were Mean $\pm$ SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to the corresponding uninhibited control.

PAR-4 expression was achieved when P815 cells were incubated with TNF (Fig 3B). Administration of TNF blocking antibody or TNF receptor I blocking antibody abolished TNF induced upregulation of PAR-2 (Fig 3A) and PAR-4 protein expression (Fig 3B). TNF at the concentrations tested had little effects on PAR-1 and PAR-3 expression following 2, 6 and 16 h incubation periods (data not shown).

Modulation of protease induced PAR expression by TNF

In order to investigate whether TNF can modulate serine protease induced expression of PARs, P815 cells were preincubated with TNF at 1 ng/ml, the minimum concentration required for enhancing PAR-2 and PAR-4 expression, but had no effect on PAR-1 and PAR-3

expression, for 60 min before adding trypsin and trypsinase to cells. The results showed that approximately up to 2.4 and 2.3 fold increase in PAR-2 expression were achieved when TNF primed P815 cells were incubated with various concentrations of trypsin and trypsinase, respectively (Fig 4A). Trypsin and trypsinase induced expression of PAR-3 were also upregulated by up to 1.6 and 1.7 fold respectively when P815 cells were pretreated with TNF (Fig 4B). Similarly, pretreatment of cells with TNF enhanced trypsin and trypsinase induced PAR-4 expression by up to 1.6 and 1.7 fold, respectively (Fig 4C). Trypsin and trypsinase at the concentrations tested appeared to have little effect on the expression of PAR-2, PAR-3 and PAR-4 in TNF untreated P815 cells (Fig 4). TNF did not significantly alter the responsiveness of PAR-1 expression in P815 cells to trypsin, and trypsinase (data not shown).

Fig. 4. Effects of TNF on trypsin and tryptase induced expression of protease activated receptor (PAR) in P815 cells. Cells were treated with (grey bars) or without (open bars) TNF (1 ng/ml) for 60 min before being challenged with various concentrations of trypsin or tryptase at 37°C for 16 h. Values shown were Mean $\pm$ SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to the corresponding protease alone control.

Inhibition of TNF induced phosphorylation of Akt by signaling inhibitors

To confirm that TNF can activate Akt cell signaling pathways, we investigated the effect of LY294002 on phosphorylation of Akt. LY294002 diminished TNF induced phosphorylation of Akt in P815 cells by approximately 74.4% following 30 min preincubation period, and the inhibitory action of LY294002 on phosphorylation of Akt lasted at least for 2 h (Table 2). PD98059 and U0126 reduced TNF induced phosphorylation of ERK by 34.8% and 39.1%, respectively at 2 h, but not 15 min following incubation.

Discussion

Although a cell line, rather than primary mast cells was used for the study, it is found for the first time that TNF can selectively provoke IL-4, but not IL-12 release

from mast cells. Since IL-4 is a classic Th2 cytokine, which has been recognized as a crucial cytokine in regulation of allergic inflammation [26], and TNF has been found to provoke RANTES release from airway smooth muscle cells [27] and to upregulate ICAM-1 expression on bronchial epithelial cells [28], our finding may provide further evidence that TNF is a proinflammatory cytokine in allergic reactions.

We also found that tryptase and trypsin induced IL-4 release can be diminished by antagonist peptide of PAR-2, but not antagonist peptide of PAR-4, indicating tryptase and trypsin induced IL-4 release from P815 cells is likely through activation of PAR-2. PAR-2 mediated cytokine production has been reported previously. For example, trypsin has been reported to stimulate IL-6 release from peripheral blood T cells [9] and monocytes [10] through PAR-2 related mechanisms. Tryptase and trypsin can induce lactoferrin and IL-8 secretion from peripheral blood neutrophils and IL-6 release from human conjunc-

tival epithelial cells through PAR-2 activation [11, 12]. Allergen-derived proteases in German cockroach frass elicit Th2 and Th17 cytokine release from allergic airway via PAR-2 [29]. These reports may support our observation above. Pre-treatment of P815 cells with low dose of TNF reduced tryptase and trypsin induced IL-4 production, but enhanced tryptase and trypsin induced expression of PARs was little unexpected, but it may be due to different cell signaling pathways being involved in IL-4 release and expression of PARs. Obviously more detailed work is required to address this issue further. However, since tryptase is a unique product of mast cells, the finding above may implicate a novel self-regulatory mechanism of mast cell mediator production, in which TNF released from mast cells upon activation [30] may eliminate tryptase actions on IL-4 production, but enhance expression of PARs on its host or adjacent mast cells.

Selective upregulation of expression of PAR-2 and PAR-4 in mast cells by TNF through TNF receptor I is a novel finding in the present study, which adds further evidence that TNF provokes allergic inflammation. PAR-2 has been found to regulate mast cells infiltration and T-cell activation and the Th1/inflammatory response in experimental periodontitis of mice [31], and activation of PAR-2 results in mast cell degranulation [32]. PAR-4 has been reported to participate platelet activation and microvascular inflammation in cerebral ischemia/reperfusion (I/R) injury [33], and to leads to proinflammatory changes in the knee joint [34]. These findings implicate the importance of PARs in promoting inflammation. It is difficult for us to understand why TNF induced PAR-2 expression predominantly occurred at 6 h, whereas TNF induced PAR-4 expression is a sustainable process. But the similar findings have been reported before. For instance, IL-12 down-regulated PAR-2 expression in P815 cells occurred predominantly at 6 h [22], whereas up-regulation of PAR-4 by IL-12 and GM-CSF was observed at 2 to 16 h [21]. LPS upregulated PAR-2 expression in human primary bronchial fibroblasts was found at 12, 24 and 48 h, whereas TNF upregulated PAR-2 expression mainly occurred at 12 and 24 h following incubation [35]. Nevertheless, a number of cytokines are found to be able to modulate expression of PARs, which may help to understand our above observation that TNF can upregulate expression of PAR-2 and PAR-4. Thus, IL-1 β has been found to upregulate PAR-2 expression in human airway smooth muscle cells [36]. TNF and IL-1 β can upregulate PAR-2 and PAR-4 mRNA expression in human endothelial cells [37]. TNF is able to induce selective PAR-2

Compounds	Phospho-Akt (OD450:OD595)/ Pan (OD450:OD595)	
	15 min	120 min
TNF- 10	0.18 $\pm$ 0.03	0.38 $\pm$ 0.02
TNF- 100	0.14 $\pm$ 0.03	0.43 $\pm$ 0.04
TNF- 10+ LY294002	0.07 $\pm$ 0.02 *	0.10 $\pm$ 0.01 *
TNF- 100+ LY294002	0.05 $\pm$ 0.01 *	0.11 $\pm$ 0.01 *

Table 2. Effects of LY294002 on TNF induced phosphorylation of Akt. Values shown are Mean $\pm$ SE for four separate experiments. P815 cells were treated with LY294002 (20 μ M) for 30 min before being challenged with TNF (ng/ml) for 15 min or 2 h. Phosphorylation of Akt was determined by using signalling ELISA analysis. * P < 0.05 in comparison with the corresponding uninhibited control.

upregulation in human lung fibroblasts [35].

The observation that TNF enhanced PAR-1 and PAR-3 mRNA expression, but not protein expression was a little confusing, but our previous work also showed that upregulated expression of PAR-1, PAR-2 and PAR-3 is observed at mRNA level, but not at the protein level in P815 cells [21, 22]. This could result from a rapid mRNA decay as mRNA stability involving several physiological regulators of mRNA expression and stability [38] is essential for protein synthesis [39]. However, altered function of posttranscriptional regulatory pathways [40] may also be involved. Once again, further work is required to address this issue.

It appears that TNF stimulated IL-4 release from P815 cells is most likely through receptors of TNF rather than PARs as TNF receptor I blocking antibody can block, but antagonists of PAR-2 and PAR-4 can not block the action of TNF even though TNF can enhance expression of PAR-2 and PAR-4 on P815 cells. Our results also showed that TNF-stimulated IL-4 release seems dependent upon activation of Akt and partially related to activation of ERK. Although little is known of TNF induced mast cell signalling some previous reports may support our current findings. TNF has been reported to induce CCL2 transcription through B-Raf/MEK/ERK signaling pathways in nasal polyp fibroblasts [41], and to stimulate gene expression of MMP-1 and -3 via p38 and MEK1/2 signaling pathways in synoviocytes [42]. TNF can also induce phosphorylation of Akt in cultured human fibroblast-like synoviocytes [43].

In conclusion, TNF can stimulate IL-4 release from mast cells through TNF receptor I and Akt cell signaling pathway dependent, but PAR independent mechanism.

TNF can also reduce tryptase and trypsin-induced IL-4 production, but enhance tryptase and trypsin-induced expression of PARs. By itself, TNF is able to stimulate upregulated PAR-2 and PAR-4 expression. These imply that this proinflammatory cytokine may serve as a regulator for IL-4 production and PAR expression in mast cells and through which participates in the mast cell related inflammation.

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