Pathogenetic role of tissue factor in graft-versus-host disease

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Summary:

Graft-versus-host disease (GVHD) is a serious complication after allogeneic stem cell transplantation, the mechanism of it is still not elucidated. Recent findings suggest that host endothelial cells are a target of alloreactive donor cytotoxic T lymphocytes in GVHD and tissue factor(TF) plays an important role not only in coagulation-inflammation cycle, but also in transplant immunology. We postulate TF expression in vascular endothelial cells(VEC) may play an pivotal role in the pathogenesis of GVHD. TF gene andprotein expression in target organs of GVHD in aGVHD mice was significantly elevated compared to that of controls as determined by real-time PCR and Western blotting. Allogeneic $CD4^+$T cell and $CD8^+$T cells enhanced TF, VCAM-1, TNF-α, IFN-γ and IL-6 expression in TNF-α prestimulated HUVECs compared to controls as determined by flowcytometry and real-time PCR. JNK and p38MAPK mediated allogeneic T cells-induced TF expression in HUVECs. These effects were largely prevented by monoclonal antibody against TF, SB203580 and SP600125. In concert, these data provide strong evidence that upregulated TF expression is related to tissue damage caused by GVHD, TF isthe key factor in GVHD mediated by endothelial cells and allogeneic T cells-induced TF and consecutive proinflammatory cytokines expression in VEC contribute to the pathogenesis of GVHD.

Keyword: Graft-versus-host disease, Tissue factor, Vascular endothelial cell, Allogeneic stem cell transplantation, Mitogen-activated protein kinase

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Introduction

Graft-versus-host disease (GVHD) is a serious complication after allogeneicstem cell transplantation(SCT). It is also a major cause of early and late morbidity and mortality in allogeneic SCT recipients[1]. Although the molecular pathogenesis of GVHD remains to be uncovered, there is a general agreement that infiltrating T lymphocytes play a central role and proinflammatory cytokines including IFN-γ,IL-6 and TNF-α are important mediators and regulators of GVHD [2,3,4].

After allogeneic SCT, vascular endothelial cells are the first allogeneic recipient cells encountered by circulating immunocompetent donor T cells. Host endothelial cells are a target of alloreactive donor cytotoxic T lymphocytes in GVHD[5,6]. Vascular injury has been described in patients with acute and chronicGVHD. The markers of vascular injury including vWF, endothelial adhesion molecules (VCAM-1, E-selectin and ICAM-1), vascular growth factor, endothelial cell in circulation are proved to be highly related to GVHD[7,8,9].

Allogeneic T cell can induce tissue factor(TF) expression in vascular endothelial cell(VEC)[10,11]. TF is a transmembrane protein present on the surface of activated cells and a member of the class II cytokine and hematopoietic growth factor receptor family[12]. Much recent data shows TF activity is important not only in coagulation-inflammation cycle, but also in regulating the release ofproinflammatory cytokines during the course of inflammation through intracellular signal transduction pathway. TF expression in VEC is upregulated post-injury and activation. And VEC is the main source of TF in vivo[13].

We postulate enhanced TF expression in VEC may play an pivotal role inthe pathogenesis of GVHD. To ascertain this speculation, we investigated TF mRNA and protein expression in organs of aGVHD mice and controls and theeffect of allogeneic T cells on TF,VCAM-1,TNF-α,IFN-γ and IL-6 expression and activation of MAPKs in HUVECs.

Materials and methods

Mice

Eight- to 12-week-old female BALB/c(H2 - K^d) and male C57BL/6 (H-2^b) wild-type mice were purchased from Beijing Vital River Laboratory Animal Co.These studies were conducted in accordance with principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

Reagent

FITC conjugated H-$2K^{b+}$ monoclonal antibody and PE conjugated H-$2K^{d+}$ monoclonal antibody were from BD Pharmingen(San Jose ,CA); Protein assay, SYBR

Green I and AmpliTaq DNA Polymerase were from BioRad (CA, USA);M-MLV Reverse Transcriptase were from Invitrogen (Carlsbad, CA); FITC conjugated TF,VCAM-1,IgG_{2a} monoclonal antibody(human) were from American Diagnostica Inc.; goat anti-mice TF monoclonal antibody, p38MAPK and phospho-p38MAPK monoclonal antibody, JNK and phospho - JNK monoclonal antibody,ERK and phospho-ERK monoclonal antibody, TNF-α(Santa Cruz, CA, USA); inhibitor of p38MAPK(SB203580) and inhibitor of JNK(SP600125) were from Alexis Biochemicals(CA, USA).

Induction of acute GVHD

After lethally irradiated with 8.5 Gy total body irradiation (TBI) using an X ray source, female BALB/c recipient mice (15 mice in each group) were intravenously injected (IV) with 1×10^7 BM cells and 1×10^7 splenic T cells from BALB/c (syngeneic) and C57BL/6(allogeneic) donors on day 0 respectively. Mice were kept on antibiotic water (250 μg/ml erythromycin and 320 μg/ml gentamycin;) for the first 28 days and 5 days before transplantation. The survival and appearance of mice were monitored daily after SCT. Donor cell engraftment was determined by flow cytometric analysis of the percentage of H-$2K^{b+}$ and H-$2K^{d+}$ cells with the relevant monoclonal antibodies in the spleen cells on 14 days and 21 days after SCT. The degree of clinical GVHD was assessed bya scoring system described by Cooke et al[14]. that sums changes in five clinical parameters: weight loss, posture, activity, fur texture and skin integrity (maximum index=10).

The allogeneic SCT mice were killed when they showed typical GVHD signs(GVHD score≥8), syngeneic SCT mice were killed 3 or 4 weeks after SCT, and the ear skin, liver, small intestine, stomach, lung, kidney were subjectedto histopathological examination, quantitative real-time PCR and Western blotting analysis.

Histologic examination

The organs were fixed with 4% neutral buffered formalin, and embedded in paraffin. After the thin tissue sections had been deparaffinized on glass slides, samples were stained with hematoxylin and eosin.

Cell isolation and cell cultures

Human peripheral blood mononuclear cells(PBMC) were separated from healthy volunteers using Ficoll-Hypaque gradients. Monocytes were separated by plastic adhesion; $CD4^+$T cells or $CD8^+$T cells were isolated from PBMC by positive selection using magnetic beads coated with anti-$CD4^+$ or anti-$CD8^+$ antibody(Miltenyi Biotech,German) according to the manufacturer's instructions. Samples of the resulting populations were immunostained to check the purity of thepopulation; the CD4 and CD8 populations were at least 95% CD4 or CD8 positive.

Human umbilical vein endothelial cell(HUVEC) and endothelial complete medium(ECM) were purchased from Sciencecell Research Laborotory（CA，USA）and grown according to the supplier's directions. Cells in passage 3 and 8 were used

in all experiments. Eight hours before starting an experiment ECM was removed and replaced by the same medium but without FCS, growth factors. Every test used HUVECs from at least two different persons and was conducted for three times.

Coculturing of HUVECs and allogeneic T cells

1×10^4 HUVECs were seeded on six-well plates in triplicate and were presimulated by TNF-α(1ng/ml) for 4 hours. After thorough washing with ECM, 1×10^5 allogeneic $CD4^+$T cells or $CD8^+$T cells were added to HUVECs(ratio 10:1). The cells were grown at 37□ in a humidified atmosphere containing 5% CO_2. To detect the effect of TF antibody, SB203580(inhibitor of p38MAPK), SP600125(inhibitor of JNK) on allogeneic T cell-induced HUVECs, HUVECs were pretreated with TF antibody(20μg/ml), SB203580(10μM), SP600125(30μM) seperatedly 30 minutes before coculturing.

Measurements of TF and VCAM-1 expression on HUVEC membrane by flowcytometry

After coculturing with allogeneic CD4+T cells or CD8+T cells for 0,3,6,12hours, HUVECs were removed from the wells with non-enzymatic Cellstripper after being washed with cold PBS, and labeled with FITC-TF, FITC-VCAM-1,FITC-IgG_{2a} respectively according to manufacturer's instructions. The percentageof TF and VCAM-1 expression was analyzed by flowcytometry.

Quantitative real-time PCR

Freshly isolated organs(n =10) were snap-frozen in liquid nitrogen and stored at -80□ until total RNA extraction. Total RNA of the organs and HUVECs was extracted by using TRIzol reagent according to the protocol provided bythe manufacturer. Two micrograms of total RNA each probe were reverse transcribed to single-strand cDNA using oligo(dT) primer and 20 U MMLV.

Quantitative real-time PCR was then used to detect TF mRNA in organs of aGVHD mice and the controls. TF, TNF-α, IFN-γ, IL-6 mRNA of HUVECswas also detected. With 2.5μl of cDNA being incubated in 25μl IQ Supermix containing 500nM of respective primers, or 50nM of 18S primers used for sample normalization, and fluorescence dye SYBR Green Ⅰ. The sequence of primers and condition of real-time PCR was given in table 1. The specificity of the amplified products was monitoredby means of melting curves at the end of each amplification. Real-time RT-PCR was carried out in triplicate for each sample using a 7500 Real-Time PCR System(Applied Biosystems, Foster City, CA, USA).

Assessments of TF expression in the organs and MAPKs activation of HUVEC by Western blotting

After coculturing with allogeneic $CD4^+$T cells or $CD8^+$T cells for 0,15,30,60 minutes, HUVECs were lysed with nonionic detergent-containing buffer, the lysed cells were scraped off the dish using a rubber policeman, the tissues were also lysed with nonionic detergent-containing buffer, then transferred to microcentrifuge tubes, and vortexed for 10 s. The cell and tissue lysates were centrifuged to remove insoluble materials and the protein concentration of each sample was measured. Approximately 50μg of supernatant protein from each samplewas used for gel

electrophoresis analysis on a 10% SDS-polyacrylamide gel. After electrophoresis, the fractionated proteins on gel were transferred to PVDF membranes. After blocking with 5% milk overnight, the membrane was incubated overnight with the primary antibody separately, then the membranes were extensively washed with TBST and incubated for 60 min with the secondary antibody. After extensive washing with TBST, the immune complexes were detected by ECL kit (Pierce Biotechnology,USA).

Statistical analysis

Data are shown as means±SD of three independent experiments. Data were analyzed by two-way analysis of variances(ANOVA) using the Statistical Analysis System(SAS, Cary, NC). A P-value less than 0.05 was considered significant.

Results

The manifestation of aGVHD after allogeneic SCT

Allogeneic SCT mice showed typical symptoms of aGVHD such as weightloss, diarrhea, dermal ulcer, depilate and posture change during 18~28 days after SCT and all died in the first 28 days after SCT. Typical histologic changesof aGVHD were seen in liver, skin, stomach, small intestine of them. On 14 days and 21 days after SCT flowcytometric assay demonstrated over 95% spleencells of allogeneic SCT mice were from donors. It verified successful implantation of donor hematopoietic stem cells. But there was no clinical and hitologic appearance of aGVHD in the controls.

Upregulation of TF mRNA and protein expression in target organs of aGVHD

Enhanced TF mRNA expression were seen in the skin, stomach, small intestine, liver of aGVHD mice($p<0.001$), they were respectively 15.1±2.1，5.5±1.4，9.7±2.3，14.3±2.9 times higher than controls. There were no difference in the TF mRNA expression of the lung and kidney between the two groups(figure 1). The results of TF protein expression tested by Western blotting were inaccord with the results of quantitative real-time PCR(figure 2). Protein expression of TF in skin,stomach,small intestine,liver of aGVHD mice were respectively 6.1±3.1, 7.2±2.5，8.7±2.3, 10.7±4.9 times higher than the controls($P<0.01$).

Allogeneic $CD4^+$T cells and $CD8^+$T cells enhance TF, VCAM-1, TNF-α, IFN-γ, IL-4 expression in HUVECs

Flowcytometric assay showed TF、VCAM-1 expression on TNF-α pretreated HUVEC membrane are very low. After incubated with allogeneic $CD4^+$T cells or $CD8^+$T cells, TF and VCAM-1 expression on HUVEC membrane increased significantly($p<0.01$), and reached peak value at the 6-h time-point(showed in table 2,3).

After coculturing with allogeneic $CD4^+$T cells or $CD8^+$T cells for 3,6,12 hours, TF mRNA expression in HUVECs was elevated significantly and reachedpeak value at the 3-h time-point; TNF-α,IFN-γ,IL-6 mRNA expression also wasupregulated, and reached the peak value at the 6-h time-point(figure 3).

Involvement of MAPKs in allogeneic $CD4^+$T cells and $CD8^+$T cells-inducedTF expression in HUVECs

Allogeneic $CD4^+$T cells and $CD8^+$T cells enhanced JNK and p38MAPK phosphorylation of HUVECs compared to untreated HUVECs. The increase in JNK activation and p38MAPK activation were maximal after 30 minutes of stimulation (Figure 4a,4b). The increase in JNK activation after 30 minutes was 67.3-fold for allogeneic $CD4^+$T cells($n=9$; $p<0.0001$) and 78.9-fold for allogeneic $CD8^+$T cells($n=9$;$p<0.0001$); the increase in p38MAPK activation after 30 minutes was 54.6-fold for allogeneic $CD4^+$T cells($n=9$; $p<0.0001$) and 62.3-fold for allogeneic $CD8^+$T cells($n=9$; $p<0.0001$). Expression of total JNK, p38MAPK,ERK and phosphorylated ERK were not affected.

SB203580, a specific inhibitor of p38MAPK, reduced allogeneic $CD4^+$T cells and $CD8^+$T cells-induced TF expression on HUVEC membrane by 39.9±4.3% ($p<0.05$) and 64.4±6.1% ($p<0.01$); SP600125 a specific inhibitor of JNK reduced allogeneic $CD4^+$T cells and $CD8^+$T cells–induced TF expression on HUVEC membrane by 66.1±5.2% and 6.8±3.4% ($n=9$; $P<0.01$;Table 4). Allogeneic$CD4^+$T cells and $CD8^+$T cells induced TF mRNA expression in HUVECs was also decreased by SP600125 and SB203580 ($p<0.01$). Data are expressed as fold induction relative to cytokine expression by non-induced HUVEC (Figure 4c).

The effect of monoclonal antibody against TF, SB203580, SP600125 on allogeneic T cells-induced cytokines expression of HUVECs

Mice anti-human TF monoclonal antibody(American Diagnostica Inc.) is the neutralizing antibody of TF[15]. HUVECs were pretreated with TF antibody, SP600125, SB203580 30 minutes before stimulation with T cells. At t=6h, allogeneic T cells-induced VCAM-1 expression on HUVECs membrane was decreased compared with non-treated HUVEC (Table 4), allogeneic T cells-induced TNF-α, IFN-γ, IL-6 mRNA expression in HUVECs was also downregulated by TF antibody, SB203580, SP600125 compared with non-treated HUVECs(Figure 5).

Discussion

In the present study, a model of murine aGVHD after allogeneic SCT was established. To exclude the influence of conditioning regimen, syngeneic SCTmice were used as controls. TF mRNA and protein expression were enhanced specifically in target organs of GVHD including skin, liver, stomach, smallintestine. These data indicates that upregulation of TF expression is related to tissue damage caused by GVHD. Because there are abundant vascular beds in these organs, and TF expression of VECs upregulates post injury and activation, we postulate that upregulated TF expression of VEC may contribute to tissue injury caused by GVHD.

To verify this speculation, we cocultured allogeneic $CD4^+$T cells and $CD8^+$T cells with TNF-α prestimulated HUVECs. The results demonstrate that allogeneic $CD4^+$T cells and $CD8^+$T cells lead to upregulation of TF, VCAM-1 and proinflammatory cytokines(including TNF-α，IFN-γ, IL-6) expression in HUVECs as determined by FCM and real-time PCR. And these effects can be preventedby monoclonal antibody against TF, inhibitor of p38MAPK, and inhibitor of JNK.

Much research of recent years provide the evidence that TF is involved in transplant immunology. Naji,et al[16] pointed out that donor specific antibody can induce TF overexpression in VEC. Schmid,et al[11] reportted that after coculturingwith allogeneic $CD4^+$T cells and $CD8^+$T cells, TF mRNA and protein expression in VEC increased significantly. Significantly higher levels of microparticles (containing TF) from endothelial cells were observed in patients with acute GVHD, in contrast, neither the conditioning regimen nor infectious complications influenced the level of these microparticles[17].

It is clearly explicated that VCAM-1 expression in target organs of GVHD increased and enhanced expression of VCAM-1 is correlated to donor T cellinfiltration[18]. Much data shows TNF-α , IFN-γ, IL-6 are important markers of GVHD[19]. Enhanced TF expression in VEC is induced by proinflammatory cytokines that are secreted by T cells, which in turn contribute to cytokine generation or inflammatory cell apoptosis through intracelluar signal transduction[12,20,21]. So allogeneic T cells-induced TF and consecutive proinflammatory cytokinesexpression in VEC may contribute to the pathogenesis of GVHD. TF is the key factor in GVHD mediated by VEC, measures which block TF expression inVEC may inhibit production of proinflammatory cytokines and donor T cells infiltration contributing to GVHD and alleviate GVHD. Furthermore, there is increasing evidence of the existence of an endothelial form of graft-versus-host disease, VEC injury is closely related to tissue damage caused by GVHD[22] , measures to ameliorate VEC injury may separate GVHD from the beneficial graft-versus-leukaemia effect.

The mitogen-activated protein kinases (MAPKs), play an essential role in regulating several intracellular processes, such as gene expression, growth, cell survival, differentiation or death[23,24].To further explore the mechanisms of allogeneic T cells-induced TF expression in HUVECs, Western blotting was applied to measure the phosphorylation of three members of the MAPK family (ERK, JNK, p38 MAPK). Our results demonstrate that allogeneic T cells activate the phosphorylation of JNK and p38MAPK, and inhibition of p38 MAPK and JNK,significantly prevent allogeneic T cells-induced TF, VCAM-1 and proinflammatory cytokines expression. These data suggest targeting JNK and p38MAPK pathway may be valuable in designing new therapy modalities for treating GVHD.

In conclusion, this preliminary study indicates that enhanced TF expressionof VEC plays an important role in the pathogenesis of tissue damage caused by GVHD, and measures to block TF expression in VEC including monoclonal antibody against TF, inhibitor of p38MAPK and JNK can interrupt TF mediated inflammation, thus may ameliorate GVHD. These data lead to better understanding of the mechanisms of GVHD and may provide a new target for treatment of GVHD.

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Table 1 Sequences of primers, condition of real-time PCR

Gene	Sequence of primer	Condition of PCR
TF	Sense 5'-GGCAACCCAAACCCACCAAC -3'	95□30s
(murine)	Antisense 5'- CCCAGGTCACATCCTTCACG -3'	58□40s 72□30s
β-actin	Sense 5'- CTCTTTGATGTCACGCACGATTTC -3'	95□30s
(murine)	Antisense 5'- CTGTCCCTGTATGCCTCTGGTC -3'	57□40s 72□1min
TF	Sense 5'-CACCTTACCTGGAGACAAACC -3'	95□30s
(human)	Antisense5'- GAGGGAATCACTGCTTGAACAC -3'	58□40s 72□30s
TNF-α	Sense 5'-AACGGCATGTCTCTCAA -3'	94□30s
(human)	Antisense 5'- AGTCGGTCACCTTTCT -3'	60□40s 72□1min
IFN-γ	Sense 5'- GACAACCAGGCCATCC -3'	94□30s
(human)	Antisense 5'-CAAAACAGCACCGACT -3'	58□40s 72□30s
IL-6	Sense 5'-CTCCTATCACTGACGGT -3'	94□30s
(human)	Antisense 5'-ATTCACATTGCAGCTCT -3'	61□40s 72□40s
β-actin	Sense 5-CGAAACTACCTTCAACTCCATCA-3'	95□30s
(human)	Antisense 5'-CGGACTCGTCATACTCCTGCT -3'	58□40s 72□30s

Table 2 TF expression on HUVECs membrane

	0h(%HUVEC)	3h(% HUVEC)	6h(% HUVEC)	12h(% HUVEC)
$CD4^+$ T cells	4.2±1.4	9.3±2.6*	34.5±9.1*	15.7±3.8*
$CD8^+$ T cells	3.8±1.7	6.9±3.3	14.6±5.7*	4.6±1.8

*Statistically significant vs. non-treated HUVEC($p<0.05$)

Table 3 VCAM-1 expression on HUVECs membrane

	0h(%HUVEC)	3h(% HUVEC)	6h(%HUVEC)	12h(%HUVEC)

$CD4^+$ T cells	3.3±2.1	19.3±4.8*	67.5±10.6*	16.8±3.2*
$CD8^+$ T cells	2.8±1.4	24.9±2.4*	57.6±8.3*	14.6±4.5*

*Statistically significant vs. non-treated HUVEC($p<0.05$)

Table 4 The effect of SB203580 ,SP600125 , TF antibody on allogeneic T cells-induced TF, VCAM-1 expression on HUVEC membrane.

	SB203580	SP600125	抗 TF 抗体	TF expression at 12h(% HUVEC)	VCAM-1 expression at 12h(% HUVEC)
$CD4^+$T cells	-	-	-	34.5±9.1	67.5±10.6
	+	-	-	20.9±2.4◆ 11.7±1.9▲	23.1±3.8◆ 29.3±4.1◆
$CD8^+$T cells	-	+	-	14.6±5.7	37.4±5.6◆
	-	-	+	4.2±0.8 ▲ 5.3±1.1 ▲	57.6±8.3
	-	-	-		35.7±2.6◆
	+	-	-		28.7±3.9◆
	-	+	-		38.2±3.9◆
	-	-	+		

◆**Statistically significant vs. non-treated HUVEC($p<0.05$)** ▲**Statistically significant vs. non-treated HUVEC($p<0.01$)**

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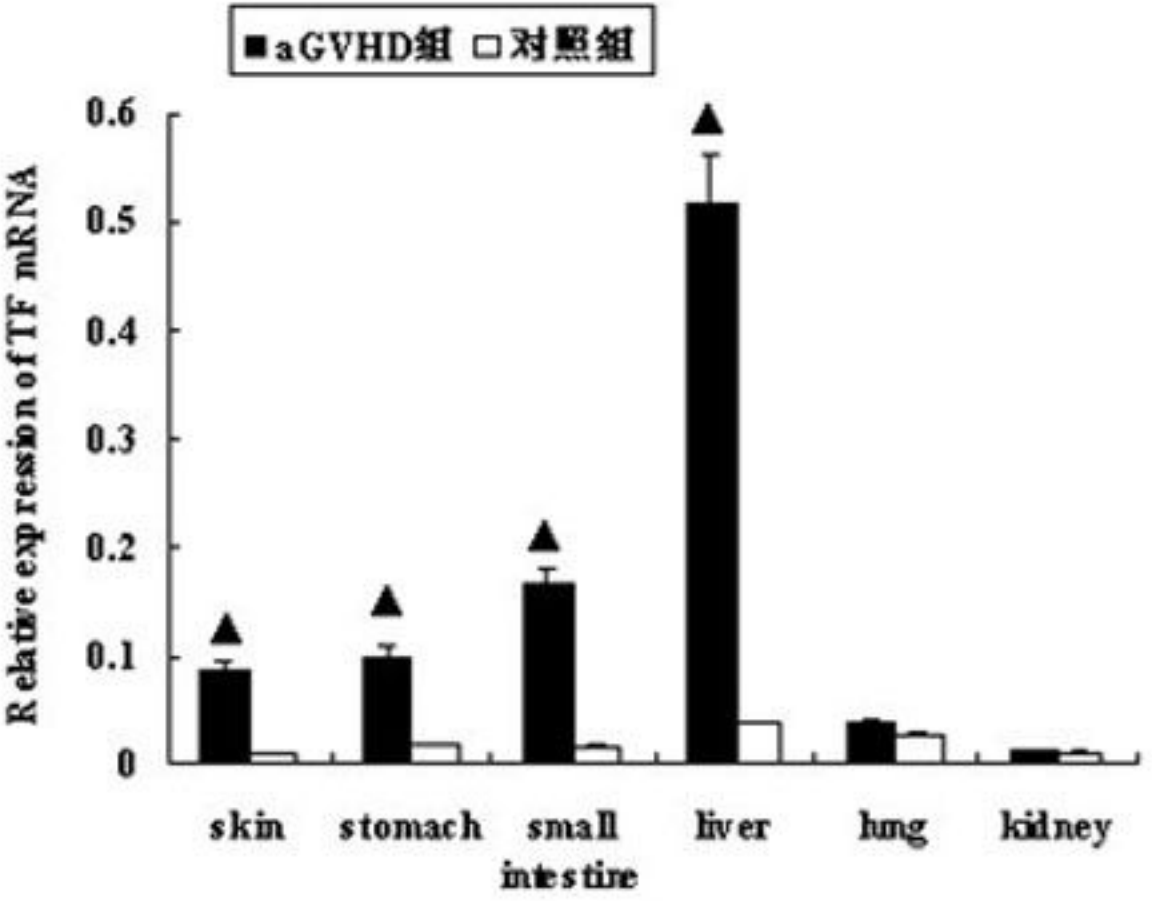
Figure 1 Expression of TF mRNA in tissues of aGVHD mice and controls.▲ $p<0.001$, aGVHD group vs. controls.

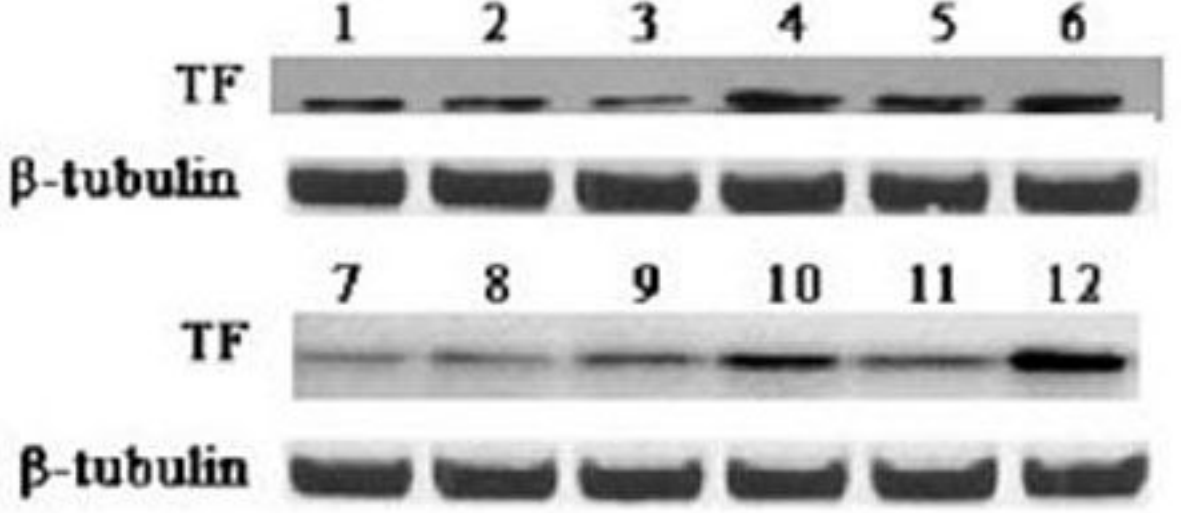
Figure 2 Protein expression of TF in the skin, small intestine, liver, lung,kidney and stomach of aGVHD mice(lane 4,5,6,10,11,12) and respective organs of controls(lane 1,2,3,7,8,9). Enhanced protein expression of TF were seenin the skin, small intestine, liver, stomach of aGVHD mice(lane 4,5,6,12)compared to the respective tissues of controls(lane 1,2,3,11),$p<0.01$.

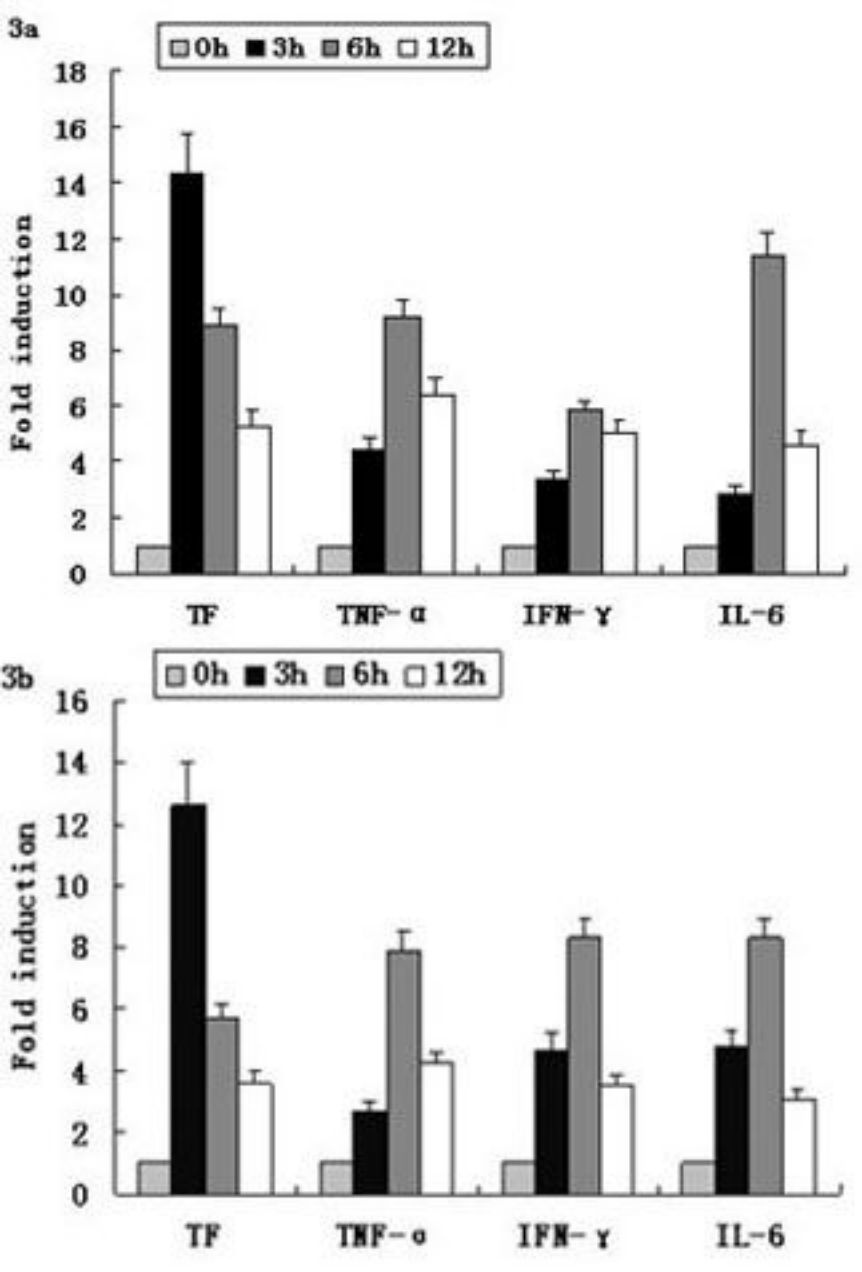
Figure 3 Effect of allogeneic $CD4^+$T cell(3a) and allogeneic $CD8^+$T cell (3b) o n TF, TNF-α , IFN-γ and IL-4 mRNA expression in HUVECs. Data are express ed as fold induction relative to cytokines expression by non-induced HUVECs. At 3,6,12h time-point, TF, TNF-α , IFN-γ and IL-4 mRNA expression in alloge neic $CD4^+$T cell and allogeneic $CD8^+$T cell-HUVECs are significantly elevated ($p<0.05$, compared to controls).

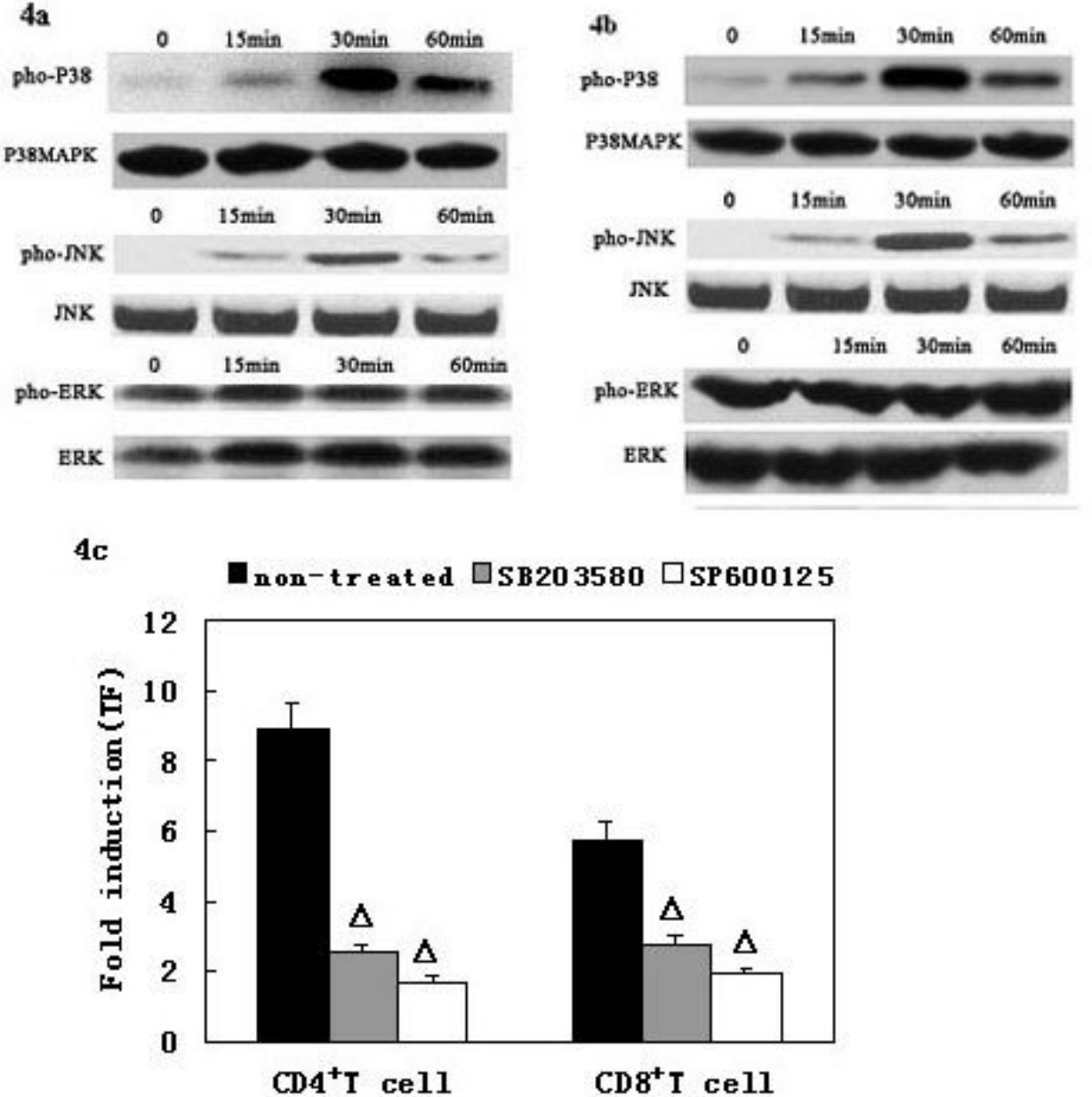
Figure 4 JNK and p38MAPK mediate the effect of allogeneic $CD4^+$T cell an d $CD8^+$T cell on TF expression. Figure 4a,4b respectively show the effect of allogeneic $CD4^+$T cell(a) and $CD8^+$T cell(b) on MAPK expression of HUVE C. Allogeneic $CD4^+$T cell and $CD8^+$T cell enhance JNK and p38MAPK pho sphorylation of HUVEC($p<0.0001$,compared to TNF-α prestimulated HUVEC). Expression of total JNK,p38MAPK,ERK and phosphorylationof ERK are not af fected. In the presence of SP600125 or SB203580(4c) , allogeneic $CD4^+$T cell a nd $CD8^+$T cell-induced TF expression are significantlyblunted.△ $p<0.01$,compare d to non-treated HUVEC.

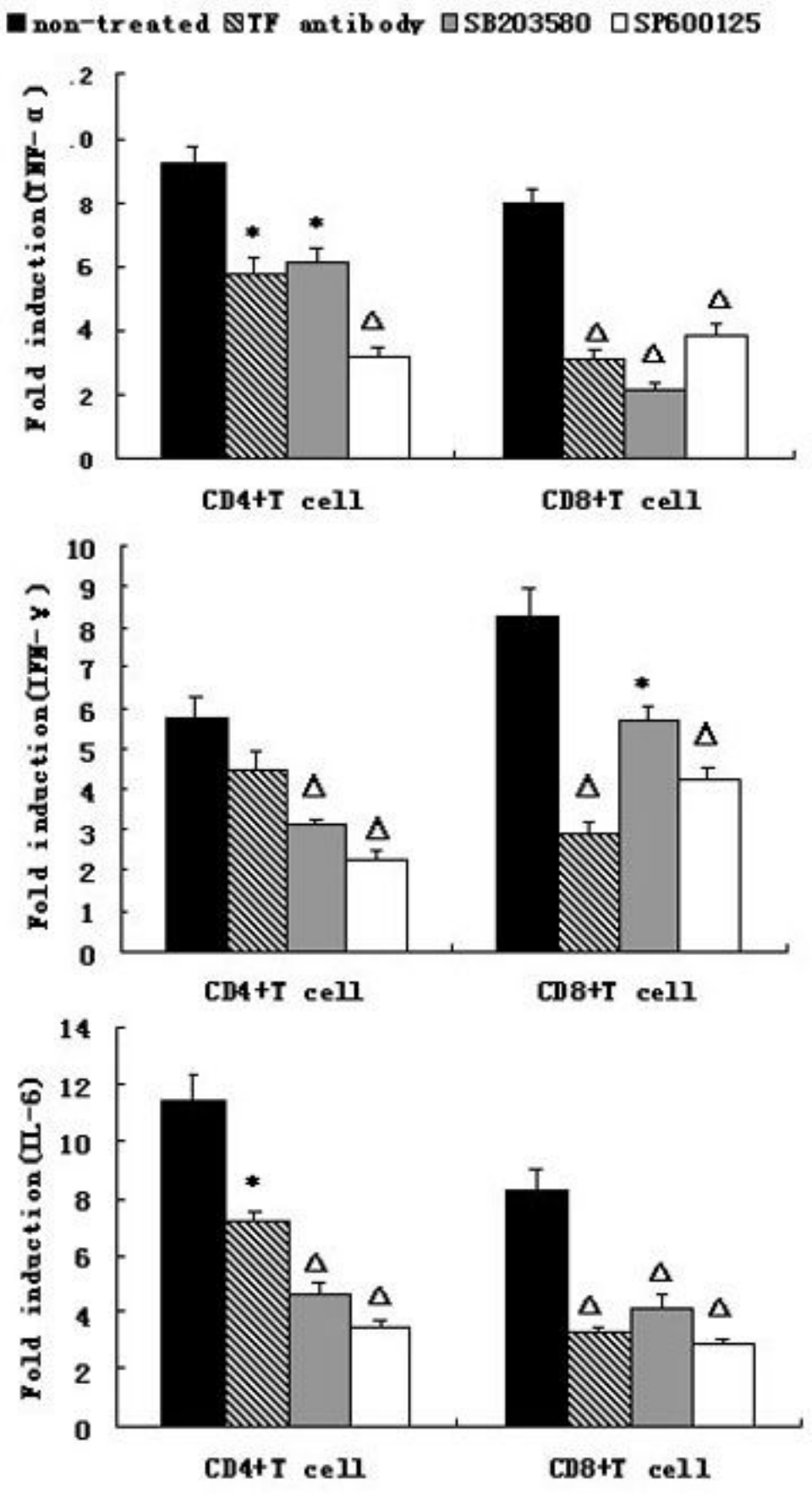
Figure 5 Effect of TF antibody, SB203580, SP600125 on allogeneic $CD4^+$T cel l and $CD8^+$T cell-induced cytokines expression in HUVECs.In the presence of TF antibody, SB203580 and SP600125, allogeneic $CD4^+$T cell and $CD8^+$T cell-induced TNF-α, IFN-γ, IL-4 expression decrease significantly. Dataare expressed as fold induction relative to cytokines expression by TNF-α prestimulated HU VEC. △$p<0.01$, compared to non-treated HUVECs *$p<0.05$, compared to non-tre ated HUVEC.


■aGVHD组 □对照组
Relative expression of TF mRNA
0.6
0.5
0.4
0.3
0.2
0.1
0
skin
stomach
small intestine
liver
lung
kidney


1
2
3
4
5
6
TF
β-tubulin
7
8
9
10
11
12
TF
β-tubulin


3a
0h 3h 6h 12h
Fold induction
18
16
14
12
10
8
6
4
2
0
TF
TNF-α
IFN-γ
IL-6
3b
0h 3h 6h 12h
Fold induction
16
14
12
10
8
6
4
2
0
TF
TNF-α
IFN-γ
IL-6


4a
0
15min
30min
60min
pho-P38
P38MAPK
pho-JNK
JNK
pho-ERK
ERK
4b
4c
non-treated
SB203580
SP600125
Fold induction(TF)
12
10
8
6
4
2
0
CD4+T cell
CD8+T cell


non-treated
TF antibody
SB203580
SP600125
Fold induction(TNF-α)
Fold induction(IFN-γ)
Fold induction(IL-6)
CD4+T cell
CD8+T cell