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Effects of therapy with soluble tumour necrosis factor receptor fusion protein on pulmonary cytokine expression and lung injury following haemorrhage and resuscitation

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SUMMARY

Acute lung injury frequently develops following haemorrhage, and is characterized by increased proinflammatory cytokine levels and massive neutrophil accumulation in the lung. Blood loss produces rapid increases in tumour necrosis factor-alpha (TNF-α) mRNA expression among pulmonary cell populations which precede the development of lung injury. In order to examine the role of TNF-α in producing acute inflammatory lung injury, we treated mice following haemorrhage and resuscitation with a TNF antagonist, composed of soluble dimeric human p80 TNF receptor linked to the Fc region of human IgG1 (sTNFR:Fc). Therapy with sTNFR:Fc prevented the post-haemorrhage increases in circulating and pulmonary TNF-α levels normally found following blood loss. Administration of sTNFR:Fc also diminished the increase in IL-1β, IL-6, TNF-α and interferon-gamma (IFN-γ) mRNA normally found in the lungs following haemorrhage. However, therapy with sTNFR:Fc was not associated with improvement in the histologic parameters of post-haemorrhage lung injury, such as neutrophil infiltration and interstitial oedema. In contrast to the effects of sTNFR:Fc on cytokine mRNA levels among intraparenchymal pulmonary mononuclear cells, such therapy following haemorrhage was associated with increased amounts of mRNA for TNF-α among peripheral blood mononuclear cells, as well as increased IFN-γ titres in serum and bronchoalveolar lavage (BAL) specimens. These results indicate that therapy with sTNFR:Fc in the post-haemorrhage period, although capable of decreasing proinflammatory cytokine expression in the lungs, does not prevent the development of acute lung injury in this setting.

Keywords cytokines acute lung injury haemorrhage TNF-α

INTRODUCTION

Acute pulmonary injury is a major cause of mortality following trauma and haemorrhage [1–4]. Elevated titres of proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and IL-1β, are a prominent feature in bronchoalveolar lavages (BAL) from patients with acute lung injury (ARDS), and have been proposed to be an important mechanism contributing to the development of inflammatory lung injury [5,6]. In mice subjected to haemorrhage and resuscitation, expression of mRNA for multiple proinflammatory cytokines, including TNF-α, interferon-gamma (IFN-γ) and IL-1β, is increased among pulmonary cellular populations as early as 1 h following blood loss [7]. Lungs from mice subjected to haemorrhage and resuscitation 3 days previously demonstrate acute lung injury, with neutrophil infiltrates, oedema, intra-alveolar haemorrhage, and fibrin generation [8]. However, despite the apparent association between increased cytokine expression and inflammatory lung injury, the relative importance and roles of the cytokines implicated in producing acute lung injury remain incompletely defined.

Soluble, extracellular portions of cytokine receptors occur naturally and are capable of binding to and regulating the effects of cytokines. A recombinant dimeric molecule consisting of two soluble 80-kD (p80) TNF receptors linked to the Fc region of human IgG1 has recently been described [9]. The dimeric sTNFR:Fc construct appeared to be an effective inhibitor of the actions of TNF in a murine endotoxaemia model, ablating the rise in serum TNF bioactivity that normally

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follows the administration of lipopolysaccharide (LPS) and resulting in improved survival of mice even when treated with sTNFR:Fc 3 h after a lethal endotoxin injection. We therefore used this sTNFR:Fc fusion protein to investigate the role of TNF-α in producing acute lung injury following haemorrhage and resuscitation.

MATERIALS AND METHODS

Animals

Male BALB/c mice, 8–12 weeks of age, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were kept on a 12 h light/dark cycle with free access to food and water.

Haemorrhage and resuscitation

The murine haemorrhage and resuscitation model to be used was developed in our laboratory and reported previously [7,8,10]. Male BALB/c mice, 8–14 weeks of age, were used in this procedure. With this model, 30% of the calculated blood volume (approximately 0·55 ml for a 20-g mouse) was withdrawn from a methoxyfluorane anaesthetized mouse by cardiac puncture over a 60-s period. Blood was collected into a heparinized syringe (5 U heparin), kept at 37°C for 1 h, then reinfused into the reanaesthetized mouse through a retroorbital plexus injection. Using this method, we are able to resuscitate all haemorrhaged mice, without complication. The total period of anaesthesia is less than 2 min in all cases. The mortality rate with this haemorrhage protocol is approximately 12%, with all deaths occurring over the 24 h post-haemorrhage, and most deaths occurring within the 1 h post-haemorrhage. Control mice were subjected to anaesthesia and cardiac puncture, but no blood withdrawal, and had retroorbital injection of 5 U of heparin in 0·1 ml PBS 1 h following cardiac puncture.

With this haemorrhage model, anaesthesia and cardiac puncture without blood withdrawal produces no changes in mitogen-induced lymphocyte proliferation, IL-2 receptor expression, phenotypic characteristics (CD3, CD4, CD8, B220, u, Ly-1 expression) of B or T lymphocytes, cytokine (IL-2, IL-3, IL-4, IL-5, IL-10, IFN-γ) release, cytokine (IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-10, TNF-α, IFN-γ, transforming growth factor-beta (TGF-β)) mRNA levels, nor splenic, intestinal, or pulmonary B cell clonal precursor frequencies [7,8,10–12]. No evidence of haemothorax, bleeding into the pericardial space, lung or cardiac contusion has been found in surviving mice with this method of haemorrhage.

sTNFR:Fc treatment

sTNFR:Fc was a gift of Jan Agosti and Michael B. Widmer (Immunex, Seattle, WA). Mice were treated intravenously with 100 μg sTNFR:Fc or human albumin (Sigma, St Louis, MO) in 0·2 ml PBS 1 h following haemorrhage or following anaesthesia/cardiac puncture (in sham haemorrhage, control mice).

Cell suspensions

Three days after haemorrhage and resuscitation in experimental groups, and after anaesthesia and cardiac puncture, but no blood withdrawal, followed 1 h later by retroorbital injection of 0·1 ml PBS containing 5 U heparin in control groups, mice were anaesthetized with methoxyfluorane and killed by exsanguination through cardiac puncture into a heparinized syringe. Additional control mice, not subjected to anaesthesia or cardiac puncture, and killed following anaesthesia and exsanguination, were included.

BAL specimens, using 1·0 ml PBS injected and aspirated three times into the trachea and lungs, were obtained as previously described by our laboratory [13]. Alveolar macrophages were collected by centrifugation of BAL specimens. After centrifugation, supernatants from BAL were stored at −70°C. As determined by cytology, there was less than 3% contamination of alveolar macrophages with lymphocytes, neutrophils, or other mononuclear cell populations when BAL specimens were collected in normal or control, sham haemorrhage mice. In mice subjected to haemorrhage and resuscitation 3 days previously, approximately 14% of the cells isolated from BAL are neutrophils.

Intraparenchymal pulmonary mononuclear cells were isolated by collagenase digestion and Percoll gradient purification, using techniques previously described by our laboratory [14]. In brief, after the mouse was killed by exsanguination, the chest was opened and the lung vascular bed was flushed by injecting 3–5 ml of chilled (4°C) PBS into the right ventricle. The lungs were then excised, avoiding the paratracheal lymph nodes, and washed twice in RPMI 1640. The lungs were minced finely, and the tissue pieces placed in RPMI 1640 with 5% fetal calf serum (FCS), penicillin/streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol (2-ME), 20 mM L-glutamine, containing 20 U/ml collagenase and 1 μg/ml DNase. Following incubation for 60 min at 37°C, any remaining intact tissue was disrupted by passage through a 21 G needle. Tissue fragments and most dead cells were removed by rapid filtration through a glass wool column, and cells collected by centrifugation. The cell pellet was suspended in 4 ml of 40% Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% Percoll. After centrifugation at 600 g for 20 min at 15°C, the cells at the interface were collected, washed in RPMI 1640, and counted. Viability as determined by trypan blue exclusion was consistently greater than 98%.

Peripheral blood mononuclear cells (PBMC) and plasma were isolated from heparinized blood. After centrifugation, plasma was stored at −70°C until tested in ELISA. To purify PBMC, heparinized blood was diluted 2 : 1 with PBS pH 7·3, and then layered onto 4 ml of Lympholyte M (Accurate Chemical and Scientific Corporation, Westbury, NY). After centrifugation at 600 *g* for 20 min at 15°C, the cells at the interface were collected, washed in RPMI 1640, and counted. Viability as determined by trypan blue exclusion was consistently greater than 98%.

Semiquantitative polymerase chain reaction

mRNA was extracted from isolated cellular populations using oligo-dT microcolumns (Micro-FastTrack; Invitrogen, San Diego, CA). cDNA was synthesized from the mRNA of 100 000 cells for all cell populations except alveolar macrophages, where 20 000 cells were used, using Moloney murine leukaemia virus reverse transcriptase and random hexamer oligonucleotides according to the procedure of Kawasaki [15] and as previously [7,8] described by our laboratory.

After a 2-min, 94°C denaturation step, between 26 and 36 cycles of polymerase chain reaction (PCR) were carried out (45 s, 94°C denaturation; 45 s, 60°C annealing; and 2 min, 72°C extension) on cDNA from 10 000 cells (1000 cells from alveolar

macrophage populations). All cDNA samples for every time point after haemorrhage were mixed with aliquots of the same PCR master mix. We used cytokine MIMICs [8,16] (Clontech, Palo Alto, CA) for IL-1β, IL-6, TNF-α and IFN-γ, as well as the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (G3PDH) as internal controls for standardization of PCR product. DNA was amplified simultaneously for each organ, using the G3PDH or cytokine MIMIC primer, and the cytokine primer of interest. The primers were used at 0·4 μM each, and were purchased from Clontech.

To detect amplified cDNA, the PCR product was analysed by agarose gel electrophoresis. The number of PCR cycles were selected for each cytokine product from each cellular population so that most of the ethidium bromide-stained amplified DNA products were between barely detectable and below saturation. The gel was then captured using a computerized imaging system, which then permitted densitometry analysis of the image (UVP 5000 ImageStore System; UVP, San Bernadino, CA). Cytokine densitometry results were normalized to those for G3PDH or the cytokine MIMIC.

ELISA assay of cytokine activity

ELISA for TNF-α and IFN-γ content in BAL supernatants and plasma used paired anti-mouse TNF-α (MP6-XT3 and biotinylated MP6-XT22) and IFN-γ (R4-6A2 and biotinylated XMG1.2) MoAbs (Pharmingen, San Diego, CA), and alkaline phosphatase-conjugated strepavidin (Southern Biotechnology, Birmingham, AL), with recombinant mouse TNF-α and IFN-γ as standards. The sensitivity of the ELISA was 10 pg/ml.

Histopathology

At 3 days following haemorrhage and resuscitation in experimental groups, or following cardiac puncture in sham haemorrhage in control groups, mice were killed and the pulmonary circulation flushed with chilled PBS injected into the right ventricle, as described above. The lungs were inflated with formalin injected through the trachea, then removed *en bloc* with the heart and placed into formalin. Haematoxylin and eosin sections of the lungs were prepared, and examined in a blinded fashion by a pulmonary pathologist (W.F.C.) who was unaware of the group to which the individual animal belonged. Lungs from normal, unmanipulated BALB/c mice also were included as additional controls.

Statistical analysis

For each experimental condition, the whole group of mice ($n = 6$) was prepared and then studied at the same time. Cells, serum, and mRNA were obtained individually from each animal, and were then analysed individually before calculating group data. Data are presented as mean $\pm$ s.e.m. for each experimental group. One-way analysis of variance and the Tukey test were used for comparisons between post-haemorrhage, control, and normal, unhaemorrhaged data groups. $P < 0{\cdot}05$ was considered significant.

RESULTS

Serum and BAL TNF-α and IFN-γ levels

No detectable TNF-α or IFN-γ was present in serum or BAL samples obtained from normal or control mice subjected to anaesthesia/cardiac puncture without blood withdrawal, followed by retroorbital injection of heparin 3 days previously. TNF-α concentrations of 2111 ± 373 pg/ml were found in BAL samples obtained 3 days following haemorrhage and resuscitation ($P < 0{\cdot}001$ *versus* control). Elevated levels of TNF-α also were detected in serum from mice that had been haemorrhaged 3 days previously, averaging 1276 ± 953 pg/ml ($P < 0{\cdot}05$ *versus* control). In contrast to the increased titres of TNF-α found in BAL and serum samples from haemorrhaged mice, no detectable TNF-α was present in BAL or serum specimens obtained 3 days following haemorrhage/resuscitation in animals treated with sTNFR:Fc during the resuscitation phase, 1 h following haemorrhage.

Significant increases in IFN-γ titres, compared with control or normal mice, were present in BAL obtained from mice which had been haemorrhaged 3 days previously. In the haemorrhaged mice, IFN-γ titres in BAL were 47 ± 16 pg/ml ($P < 0{\cdot}05$ *versus* control). No IFN-γ was detected in serum from haemorrhaged mice. IFN-γ levels in BAL and serum collected 3 days following haemorrhage and treatment with sTNFR:Fc were significantly increased compared with those found in haemorrhaged mice, not treated with sTNFR:Fc. In the haemorrhaged mice treated with sTNFR:Fc, IFN-γ concentrations in BAL were 132 ± 17 pg/ml ($P < 0{\cdot}05$ *versus* haemorrhaged, untreated) and in serum were 302 ± 67 pg/ml ($P < 0{\cdot}01$ *versus* haemorrhaged, untreated).

Cytokine gene expression

Among intraparenchymal pulmonary mononuclear cells collected 72 h following haemorrhage and resuscitation, levels of mRNA for IL-1β, IL-6, TNF-α, and IFN-γ were significantly increased compared with those found in cells isolated from normal or control, sham haemorrhage mice (Figs 1 and 2). Treatment with sTNFR:Fc following haemorrhage was associated with significant decreases, compared with untreated haemorrhage/resuscitation mice, in the amount of mRNA for IL-1β, IL-6, TNF-α, and IFN-γ. Following treatment with sTNFR:Fc, the mRNA levels for IL-1β, IL-6, and TNF-α were

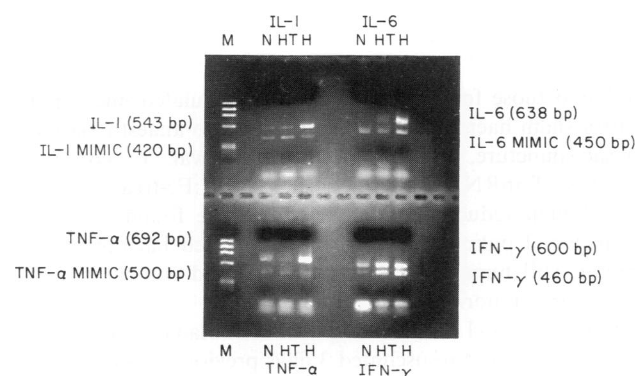

Fig. 1. Effects of therapy with sTNFR:Fc on cytokine mRNA expression among intraparenchymal pulmonary mononuclear cells obtained from normal, unhaemorrhaged mice (N), from haemorrhaged mice treated with 100 μg sTNFR:Fc 1 h following haemorrhage and immediately after resuscitation (HT), and from haemorrhaged mice given 100 μg human albumin 1 h following haemorrhage and immediately after resuscitation (H). Results from representative experiments are shown. Molecular weight markers (M) and cytokine MIMICS are included on the gels.

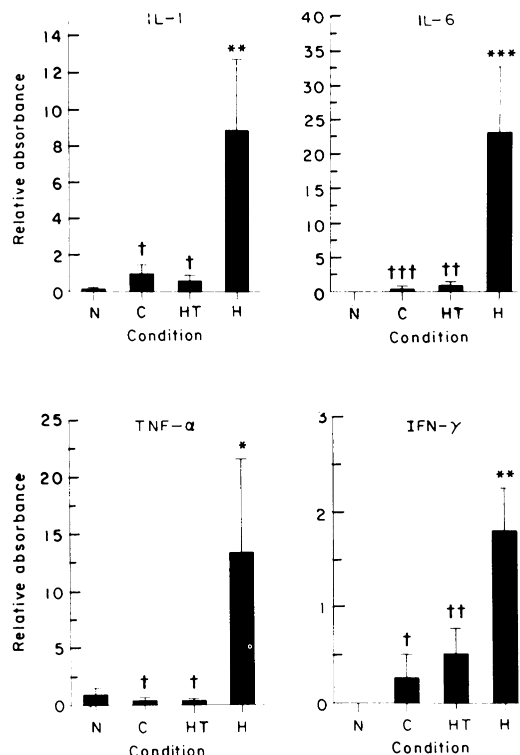

Fig. 2. Cytokine mRNA levels among intraparenchymal pulmonary mononuclear cells from normal unhaemorrhaged mice (N), from control sham haemorrhage mice subjected to anaesthesia/cardiac puncture, but no blood withdrawal, with retroorbital injection of 5 U heparin in 0·1 ml PBS 1 h following cardiac puncture (C), from haemorrhaged mice treated with 100 μg sTNFR:Fc 1 h following haemorrhage and immediately after resuscitation (HT), and from haemorrhaged mice given 100 μg human albumin 1 h following haemorrhage and immediately after resuscitation (H). Densitometry results are normalized to the appropriate cytokine MIMIC, which had been included during the PCR reaction. For each condition, groups of six mice were utilized. Results are shown as mean ± s.e.m. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ *versus* normal. †$P < 0.05$; ††$P < 0.01$; †††$P < 0.005$ *versus* haemorrhaged mice, not treated with sTNFR:Fc.

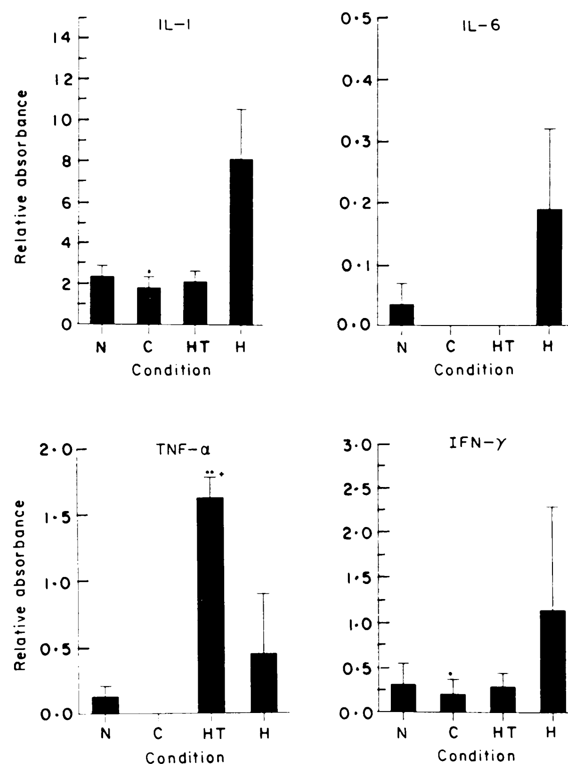

Fig. 3. Cytokine mRNA levels among peripheral blood mononuclear cells from normal unhaemorrhaged mice (N), from control sham haemorrhage mice subjected to anaesthesia/cardiac puncture, but no blood withdrawal, with retroorbital injection of 5 U heparin in 0·1 ml PBS 1 h following cardiac puncture (C), from haemorrhaged mice treated with 100 μg sTNFR:Fc 1 h following haemorrhage and immediately after resuscitation (HT), and from haemorrhaged mice given 100 μg human albumin 1 h following haemorrhage and immediately after resuscitation (H). Densitometry results are normalized to the appropriate cytokine MIMIC, which had been included during the PCR reaction. For each condition, groups of six mice were utilized. Results are shown as mean ± s.e.m. †$P < 0.05$ *versus* normal. *$P < 0.05$; **$P < 0.01$ *versus* haemorrhaged mice, not treated with sTNFR:Fc.

similar to those found in normal, unmanipulated mice or in control sham haemorrhage mice subjected to anaesthesia and cardiac puncture, but no blood withdrawal. In contrast, amounts of mRNA for IFN-γ in sTNFR:Fc-treated mice, even though reduced compared with those found in intraparenchymal pulmonary cells from untreated haemorrhaged animals, still remained significantly elevated compared with levels found in normal, unmanipulated mice.

Levels of IL-1β mRNA in PBMC obtained from mice haemorrhaged and resuscitated 3 days previously were significantly elevated compared with normal or control, sham haemorrhage mice (Fig. 3). Therapy with sTNFR:Fc during the post-haemorrhage resuscitation period resulted in IL-1β mRNA levels which were similar to those found in normal or sham haemorrhage mice. In contrast to the situation in the lung, where therapy with sTNFR:Fc following haemorrhage was associated with decreased amounts of mRNA for TNF-α, mRNA levels for TNF-α among PBMC obtained from haemorrhaged mice treated with sTNFR:Fc were significantly increased. No significant alterations in IL-6 or IFN-γ mRNA levels from PBMC were detected in haemorrhaged mice treated with sTNFR:Fc.

Histopathology

Lungs obtained 3 days following haemorrhage and resuscitation showed inflammatory changes with interstitial oedema as well as increased interstitial cellularity with neutrophil infiltrates. Grading, on a 0–3 scale (with 0 being normal and 3 being the most severe change), of the histopathologic changes in the lungs of mice ($n = 6$) subjected to haemorrhage and resuscitation 3 days previously showed interstitial neutrophil infiltration 1.5 ± 0.1 and interstitial oedema 1.5 ± 0.3. Increased cellularity and oedema were also found in haemorrhaged mice treated with sTNFR:Fc. In the mice treated with sTNFR:Fc, interstitial cellularity was graded at 1.9 ± 0.5 ($P = 0.09$ *versus* haemorrhage, not treated with sTNFR:Fc), and interstitial oedema was graded as 1.9 ± 0.3 ($P = 0.10$ *versus* haemorrhage, not treated with sTNFR:Fc). Lungs obtained 3 days following cardiac/retroorbital puncture in sham haemorrhage

control mice showed no histopathologic alterations, and had the same degree of cellularity as did lungs from normal, unmanipulated animals. There was maintenance of alveolar spaces, with no fibrin generation, injury to endothelial cells, oedema, or intra-alveolar haemorrhage.

DISCUSSION

The present results demonstrate that therapy with sTNFR:Fc in the post-haemorrhage period can effectively prevent the increases in circulating and pulmonary TNF-α levels normally found following blood loss. Post-haemorrhage administration of sTNFR:Fc also down-regulated the increase in proinflammatory cytokine mRNA normally found in the lungs following haemorrhage. In contrast to the effects of sTNFR:Fc on cytokine mRNA levels among intraparenchymal pulmonary mononuclear cells, such therapy following haemorrhage was associated with increased amounts of mRNA for TNF-α among PBMC, as well as increased IFN-γ titres in serum and BAL specimens.

Even though therapy with sTNFR:Fc was associated with decreased levels of mRNA for proinflammatory cytokines among isolated intraparenchymal pulmonary mononuclear cells, such treatment did not diminish the histologic findings of acute lung injury induced by haemorrhage. There are several possible explanations for these results. First, TNF-α may not be a primary mediator of pulmonary injury following blood loss. Second, pulmonary cytokine expression, even though significantly diminished from that seen in control haemorrhage/resuscitation mice, may still be sufficiently elevated in sTNFR:Fc-treated mice to produce lung injury. Third, sTNFR:Fc may not prevent immediate post-haemorrhage increases in proinflammatory cytokine expression, which are then able to initiate neutrophil recruitment into the lungs and subsequent inflammatory changes. Fourth, the production of proinflammatory cytokines at sites distant from the lungs, such as the intestines, may not be efficiently blocked by sTNFR:Fc, and therefore may be capable of producing lung injury.

In our previous experiments [7], TNF-α was not among the first group of cytokines to show increased expression following blood loss. mRNA levels for the proinflammatory cytokines IL-1α, IL-1β, and TGF-β were increased among pulmonary cell populations within 1 h of blood loss. Increases in IL-1β, IFN-γ, and TGF-β mRNA transcripts also were found [7] within 1 h of haemorrhage among Peyer's patch cells, and it is possible that release of these proinflammatory cytokines into the circulation could induce tissue inflammation at distant sites, such as the lungs. In contrast, the first significant elevation in TNF-α mRNA levels was present among intraparenchymal pulmonary mononuclear cells starting 2 h post-haemorrhage, and among mesenteric lymph node lymphocytes 4 h post-haemorrhage. No significant increases in TNF-α mRNA expression were found in Peyer's patch cells during the 4-h post-haemorrhage period. IL-1, TGF-β, and IFN-γ all appear to be capable of inducing pulmonary inflammation [5,6,17,18], and anti-TNF therapy may not affect the release and actions of these cytokines in the early post-haemorrhage period.

In previous studies [9], sTNFR:Fc was shown to be an effective binder and inhibitor of the actions of TNF-α. However, in those same studies, sTNFR:Fc was also demonstrated to have carrier functions which could produce persistently increased serum levels of biologically active TNF-α following stimuli, such as LPS injection. A similar situation has recently [19] been demonstrated in models of *Escherichia coli* bacteraemia, where therapy with a dimeric p80 TNF receptor:Fc construct, similar to that used in the present study, resulted in prolonged elevations of serum levels of TNF-α and increased late mortality, which was able to be eliminated when the infected mice were treated with high-affinity anti-TNF-α MoAbs. Such a mechanism of prolonged TNF-α action due to the binding characteristics of sTNFR:Fc could conceivably contribute to the development of lung injury in the present model, and would not eliminate a role for TNF-α in initiating lung injury following haemorrhage. Even though no TNF-α was detected in serum or BAL samples obtained 3 days following haemorrhage, therapy with sTNFR:Fc may have enhanced the development of lung injury through producing prolonged elevation of TNF-α levels at earlier post-haemorrhage time points.

Increased amounts of TNF-α mRNA were found in PBMC from mice treated with sTNFR:Fc following haemorrhage. Therapy with sTNFR:Fc was associated with elevations in plasma IFN-γ levels above those seen in haemorrhaged mice not treated with sTNFR:Fc. IFN-γ has been reported to increase LPS-stimulated TNF-α production *in vitro* [20,21] and *in vivo* [18]. Although factors other than LPS appear to be responsible for the increase in TNF-α mRNA levels following blood loss, IFN-γ may also enhance TNF-α expression in this setting.

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