

## RESEARCH ARTICLE

# TNFR2 expression on non-bone marrow-derived cells is crucial for lipopolysaccharide-induced septic shock and downregulation of soluble TNFR2 level in serum

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Persistently high serum levels of soluble tumor-necrosis factor (TNF) receptor 2 (sTNFR2) have been observed in septic shock and many inflammatory diseases. However, its origin and regulation during these pathological processes are still largely unknown. In this study, murine bone marrow (BM) chimeras selectively expressing TNFR2 on either BM-derived or non-BM-derived cells were generated and challenged with lipopolysaccharide (LPS). The results show that TNFR2 expression on non-BM-derived cells is crucial for both the sensitivity of mice to LPS and the downregulation of sTNFR2 in serum. Most importantly, sTNFR2 was released from both BM- and non-BM-derived cells. Non-BM TNFR1 expression influenced the sensitivity of mice to LPS challenge but not the level of serum sTNFR2. These results provide the first *in vivo* evidence for the origin and regulation of sTNFR2 in serum and could aid in the development of novel anti-TNF strategies against septic shock.

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## INTRODUCTION

Tumor-necrosis factor (TNF) is a pleiotropic cytokine that plays a critical role in triggering the pro-inflammatory cascade during host responses to various infections and cancers.<sup>1–4</sup> High levels of TNF have been detected in patients with serious bacterial infections, endotoxic shock and cancer-related inflammation.<sup>5–8</sup> Furthermore, the concentration of TNF often correlates with the severity of infectious diseases. Inhibition of TNF improves organ function and survival rates in animal sepsis models.<sup>9</sup> However, for unknown reasons, the results of clinical trials using anti-TNF strategies to treat sepsis remain disappointing.<sup>10</sup>

Previous studies have suggested that TNF receptor 1 (TNFR1), which can be expressed on all nucleated cells, triggers the majority of TNF activities. However, TNFR2 has also been reported to play an important role in inflammatory processes in recent years. For example, collecting TNF and ligating it to TNFR1, termed ‘ligand passing’,<sup>7,11</sup> mediates the anti-tumor effects of TNF when expressed on host innate immune cells,<sup>1</sup> activating different subgroups of T cells during inflammation, tumor growth and autoimmune diseases.<sup>12–15</sup> Interestingly, TNFR2 expression has also been reported on non-bone marrow (BM)-derived cells, such as vascular endothelial cells.<sup>16</sup> Non-BM TNFR2 expression can function through inhibiting collagen degradation and stimulating the proliferation of intestinal myofibroblasts.<sup>17</sup> Compared to TNFR1, the function of TNFR2 during inflammation and immune responses is poorly understood.

The extracellular domain of TNFR2 also exists as a soluble molecule formed *via* shedding from the cell surface.<sup>18</sup> Similar to TNF, elevated levels of soluble TNFR2 (sTNFR2) in serum or other body fluids has been recognized as a predictor of pathological conditions, such as sepsis, infections, hepatitis, organ fibrosis, cancer development and other inflammatory diseases.<sup>19,20</sup> *In vitro* experiments demonstrated that sTNFR2 can be rapidly shed from activated BM-derived cells, such as neutrophils and cultured mononuclear cells, upon TNF, lipopolysaccharide (LPS) or IL-10 stimulation.<sup>21,22</sup> The origin of non-BM-derived sTNFR2 in serum has not yet been formally demonstrated, especially during inflammatory diseases.

LPS-induced septic shock is a complicated inflammatory process involving a variety of cytokines and their receptors.<sup>15,23–25</sup> Controversially, the pool of sTNFR2 can act both as an antagonist scavenging soluble TNF and as an agonist protecting circulating TNF from degradation.<sup>26,27</sup> To understand the effect of TNFR2 signaling in LPS-induced septic shock, we generated BM-chimeric mice selectively expressing TNFR2 on either BM- or non-BM-derived cells and challenged these mice with LPS. Our results show that TNFR2 expression on non-BM-derived cells, but not on BM-derived cells, is critical to the sensitivity of mice to LPS. Both BM- and non-BM-derived cells contributed to serum sTNFR2; however, TNFR2 expression on non-BM-derived cells was also necessary for its downregulation upon LPS challenge.

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## MATERIALS AND METHODS

### Mice

Sex- and age (6–8 weeks)-matched wild-type (WT) C57BL/6 mice were purchased from Vital River Animal Company (Beijing, China). TNFR1- and TNFR2-deficient (TNFR1<sup>-/-</sup> and TNFR2<sup>-/-</sup>) mice with a C57BL/6 background were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and confirmed as previously described.<sup>1</sup> All mice were housed under specific pathogen-free conditions at the animal facility of the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). Animal studies were conducted with the approval of the corresponding authorities.

### Generation of BM-chimeric mice

BM-chimeric mice were generated as described.<sup>28</sup> For R2<sup>-/-</sup>>R2<sup>-/-</sup> mice, freshly prepared BM cells obtained from the femurs of non-irradiated TNFR2<sup>-/-</sup> mice were intravenously injected into lethally irradiated (10.2 Gy) TNFR2<sup>-/-</sup> recipient mice (5×10<sup>6</sup> cells/mice). Similar chimeric constructs are shown in Table 1. Successful reconstitution of the hematopoietic system was determined by flow cytometric analysis of peripheral blood mononuclear cells for TNFR2 expression. Additionally, chimerism was confirmed by PCR analysis of the TNFR alleles using genomic DNA from blood (containing BM-derived cells) and tail (containing both BM- and non-BM-derived cells) samples.

### LPS-induced septic shock

Fifteen weeks after BM reconstitution, that is, when more than 95% of hematopoietic cells in the recipients were donor-derived, mice were challenged with LPS. Mice were injected intraperitoneally (i.p.) with 10 μg LPS (Sigma, St. Louis, MO, USA) and 8 mg D-gal (Sigma), and survival rates were monitored every hour. To detect serum sTNFR2 levels, sera of BM chimeric mice were collected at 0, 1.5, 6 and 48 h after LPS challenge, and sTNFR2 concentration was quantified with a sTNFR2 ELISA Kit (R&D Systems, Minneapolis, MN, USA), which detects both free and TNF-binding forms of sTNFR2.

### Isolation of lung endothelial cells and fibroblasts

Murine lungs were mechanically minced and enzymatically digested with collagenase A as previously described.<sup>29</sup> Cell suspensions were incubated with an allophycocyanin-conjugated rat anti-mouse CD31 antibody (1:300; BioLegend, San Diego, CA, USA). Pure endothelial cell populations were finally obtained by magnetic bead separation using anti-allophycocyanin microbeads (Miltenyi Biotech, Auburn, CA, USA) and cultured in Dulbecco's modified Eagle's medium

supplemented with 20% fetal bovine serum to grow as a 'cobblestone' monolayer. When isolated lung endothelial cells grew to a monolayer, they were stimulated with 10 μg/ml LPS (Sigma) or 20 ng/ml mouse TNF-α (Pepro Tech Inc., Rocky Hill, NJ, USA) and the levels of sTNFR2 in supernatants were determined by ELISA. To detect TNFR1 expression, mouse embryonic fibroblasts (MEFs) were also prepared as previously described<sup>30</sup> from both WT and TNFR2<sup>-/-</sup> mice.

### Flow cytometry

To identify lung endothelial cells, cells were harvested and subjected to cell surface staining with anti-CD31 antibody as described above.<sup>31</sup> To determine whether TNFR1 expression was influenced in TNFR2<sup>-/-</sup> mice, isolated splenocytes, lung endothelial cells and MEFs were stained with Phycoerythrin-conjugated hamster anti-mouse CD120a (TNFR1) antibody (BioLegend). Phycoerythrin-conjugated hamster IgG (eBioscience, San Diego, CA, USA) was used as an isotype control. Samples were analyzed with a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

### Histological analysis

BM-chimeric mice were injected i.p. with LPS/D-gal and sacrificed at 48 h after challenge. Paraffin-embedded sections (5 μm thick) of liver and lung tissues from mice were stained with hematoxylin and eosin using standard procedures. Frozen liver tissues were also prepared with OCT-freeze medium and immunohistochemistry was performed with rat anti-mouse Gr-1 antibody (1:500; BD Biosciences) as previously described.<sup>32</sup>

### PCR

To determine the quality of adoptive cell transfer, PCR was performed on genomic DNA derived from leukocytes of reconstituted mice to test for the presence or absence of the *TNFR2* allele. Genomic DNA was isolated from approximately 200 μl of whole peripheral blood using a Qiamp Blood Kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The primers used were T2a: 5'-AGCTCCAGGCACAAGGGCGGG-3' and T2b: 5'-CCTCTCATGCTGTCCCGGAAT-3' for the TNFR2 allele; and T2a: 5'-AGCTCCAGGCACAAGGGCGGG-3' and neo: 5'-ATCCTCGCCGTCGGGCATGC-3' for the *neo* gene. Using the former pair of primers, a 200-bp fragment of the WT *TNFR2* allele was amplified. Using the latter pair of primers, a 1-kb fragment of the mutated *TNFR2* allele was amplified. The annealing temperature for the primers used was 63 °C (0.5 min) and 72 °C (1.5 min) was used for the elongation reaction.

### Statistical analysis

Data were analyzed by the Wilcoxon signed-rank test, Student's *t*-test or one-way analysis of variance and differences were considered significant at *P*<0.05.

## RESULTS

### TNFR2 on non-BM derived cells is crucial for LPS-induced septic shock

TNFR2 deficiency leads to resistance to LPS-induced septic shock.<sup>33</sup> However, it is not known whether TNFR2 on BM- or non-BM-derived cells are responsible for this phenomenon. Lethally irradiated TNFR2<sup>-/-</sup> mice were reconstituted with BM cells from either wild type (WT>R2<sup>-/-</sup>) or TNFR2<sup>-/-</sup> mice (R2<sup>-/-</sup>>R2<sup>-/-</sup>), and as controls, WT mice were also irradiated and reconstituted (WT>WT and

**Table 1** BM chimeric mice used in this study

Designation	Donor	Recipient	Description
R2 <sup>-/-</sup> >R2 <sup>-/-</sup>	TNFR2 <sup>-/-</sup>	TNFR2 <sup>-/-</sup>	All cells are TNFR2-deficient
R2 <sup>-/-</sup> >WT	TNFR2 <sup>-/-</sup>	WT	BM-derived cells are TNFR2-deficient
WT>R2 <sup>-/-</sup>	WT	TNFR2 <sup>-/-</sup>	Non-BM-derived cells are TNFR2-deficient
R1 <sup>-/-</sup> >R1 <sup>-/-</sup>	TNFR1 <sup>-/-</sup>	TNFR1 <sup>-/-</sup>	All cells are TNFR1-deficient
R1 <sup>-/-</sup> >WT	TNFR1 <sup>-/-</sup>	WT	BM-derived cells are TNFR1-deficient
WT>R1 <sup>-/-</sup>	WT	TNFR1 <sup>-/-</sup>	Non-BM-derived cells are TNFR1-deficient
WT>WT	WT	WT	All cells are TNFR1- and TNFR2-competent

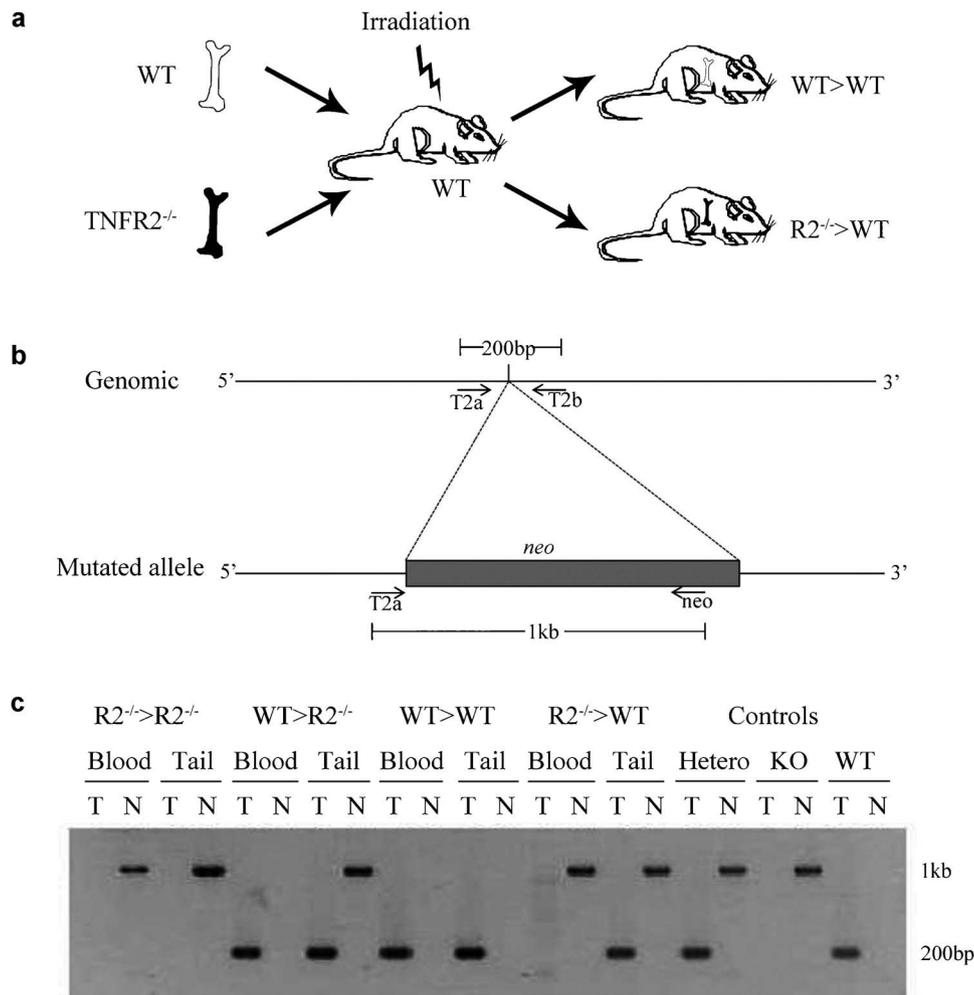
Abbreviations: BM, bone marrow; TNFR, tumor-necrosis factor receptor; WT, wild type.

$R2^{-/-}$ >WT). Successful reconstitution was confirmed 15 weeks later by PCR analysis of blood leukocytes and tail cells (Figure 1).

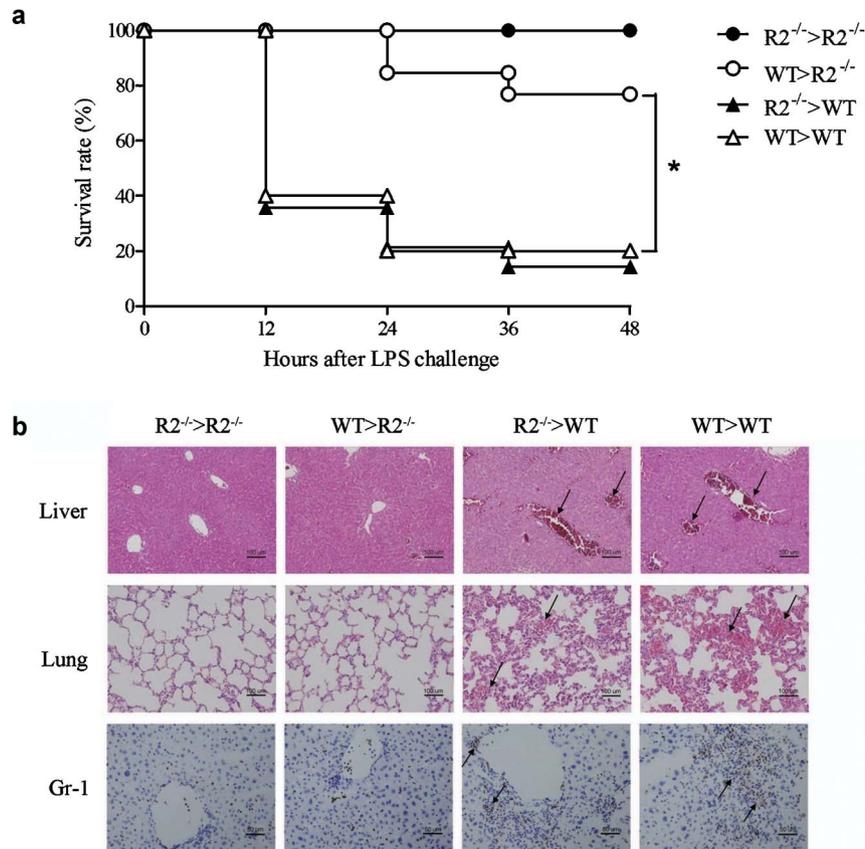
The TNFR2 chimeras were then challenged with LPS and D-gal to induce septic shock. As expected, TNFR2 was required for LPS sensitivity, with almost all  $R2^{-/-}$ > $R2^{-/-}$  mice being resistant to the dose of LPS administered (Figure 2a). Surprisingly, when TNFR2 was present only on BM-derived, but not on non-BM-derived cells (WT> $R2^{-/-}$ ), about 77% (10 of 13 mice) survived septic shock. In contrast, when TNFR2 was present on non-BM-derived, but not on BM-derived cells ( $R2^{-/-}$ >WT), only 14% (2 of 14 mice) survived LPS challenge, similar to the survival rate of WT>WT mice (20%). These results suggest that TNFR2 expression on BM-derived cells contributes to LPS-induced septic shock to a much lesser extent than expected and that TNFR2 expression on non-BM-derived cells is crucial for the sensitivity of mice to LPS-induced shock. Irradiation of mice did not significantly affect the results because irradiated WT>WT mice showed responses that were indistinguishable from that of non-irradiated WT mice (data not shown). Taken together, the above results clearly demonstrate that TNFR2 signaling in BM-

derived cells contributes to LPS-induced septic shock to a much lesser extent than expected and LPS toxicity requires TNFR2 signaling in non-BM-derived cells. This is quite surprising, considering the general concept that the TNFR2 is mainly expressed on BM-derived cells, such as macrophages and other inflammatory cells.<sup>34,35</sup>

To further investigate why TNFR2 expression on non-BM-derived cells is responsible for LPS-induced death, a series of immunohistological stains were carried out. As shown in Figure 2b, at 48 h following LPS injection, the livers of WT>WT and  $R2^{-/-}$ >WT mice displayed typical signs of severe fulminant hepatitis, with massive hemorrhage and hepatic necrosis, in accordance with the observed low survival rates of these groups of mice (Figure 2a). Among infiltrating cell populations, large numbers of Gr-1-positive granulocytes were observed around the sinus in  $R2^{-/-}$ >WT and WT>WT mice, but not in  $R2^{-/-}$ > $R2^{-/-}$  and WT> $R2^{-/-}$  mice (Figure 2b). Together, these results demonstrate that TNFR2 expression on non-BM-derived cells contributes to observed fulminant hepatitis to a great extent and, therefore, also to the manifestation of septic shock.



**Figure 1** Establishment of TNFR2 BM chimeric mice. (a) Illustration of BM reconstitution of WT C57BL/6 mice with WT and TNFR2-deficient BM cells. (b) Localization of primers for genotyping of BM chimeric mice. (c) Genotyping of chimeric mice. Chimerism of mice was confirmed by TNFR2-specific PCR analysis using genomic DNA from blood (containing only BM-derived cells) or tail (containing both BM- and non-BM-derived cells) samples. A band at 200 bp is the PCR product of the WT *TNFR2* allele and the band at 1 kb is the product of the *neo*-mutated *TNFR2* allele. BM, bone marrow; KO, knockout; TNFR, tumor-necrosis factor receptor; WT, wild type.



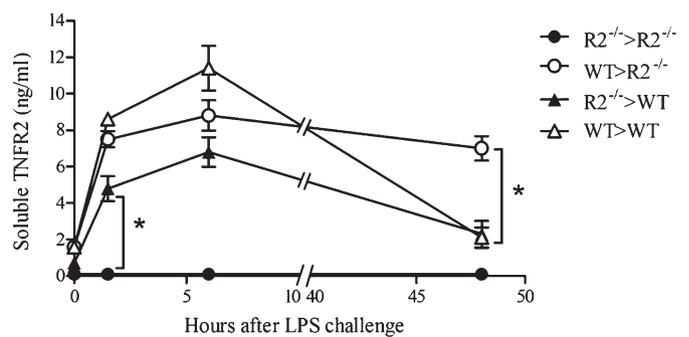
**Figure 2** TNFR2 expression on non-BM-derived cells is crucial for LPS-induced septic shock. **(a)** Survival rate of TNFR2 BM-chimeric mice in response to LPS challenge. Lethally irradiated TNFR2<sup>-/-</sup> and WT mice (8–14 mice/group) were reconstituted with TNFR2<sup>-/-</sup> (R2<sup>-/-</sup>>R2<sup>-/-</sup>, ● and R2<sup>-/-</sup>>WT, ▲) or WT BM cells (WT>R2<sup>-/-</sup>, ○ and WT>WT, △). Fifteen weeks after reconstitution, mice were i.p. challenged with LPS as described in the section on ‘Materials and methods’. The survival rates of four groups of mice at different times after LPS challenge are shown. \**P*<0.05. **(b)** Immunostaining of liver and lung sections. At 48 h after LPS challenge, liver (upper) and lung (middle) tissues of the chimeric mice were fixed with paraffin and stained with hematoxylin and eosin as indicated. Arrows indicate areas of massive hemorrhage and hepatic necrosis. Frozen liver sections (lower) were stained with anti-Gr-1 antibody. Arrows indicate the area around the sinus where granulocytes accumulated. Typical results from 3–5 mice are shown. BM, bone marrow; i.p., intraperitoneally; LPS, lipopolysaccharide; TNFR, tumor-necrosis factor receptor; WT, wild type.

### Serum sTNFR2 is derived from both BM- and non-BM-derived cells

High levels of sTNFR2 in serum have been observed in LPS-induced septic shock and other inflammatory diseases. The origin and function of this molecule are still not clear, although its presence correlates with an unfavorable prognosis in some cases.<sup>19,20,36</sup> To answer this question, dynamic changes in levels of sTNFR2 in serum in the different groups of mice described above were studied at 0, 1.5, 6 and 48 h after LPS injection. Without LPS stimulation, serum sTNFR2 concentrations in WT>WT chimera were similar to those of age-matched control WT mice (data not shown), indicating that irradiation and BM reconstitution did not significantly affect sTNFR2 release.

After LPS challenge, serum sTNFR2 levels in R2<sup>-/-</sup>>R2<sup>-/-</sup> chimeras were undetectable (Figure 3). A dramatic increase in serum sTNFR2 was, however, observed in both WT>R2<sup>-/-</sup> (7.5±0.45 ng/ml) and WT>WT chimeras (8.6±0.61 ng/ml) within 1.5 h after LPS challenge. This suggests that BM-derived cells are the primary contributors to the accumulation of sTNFR2 in serum, especially in the early phase. The fact that a significant increase in sTNFR2 levels at 1.5 h (4.8±0.18 ng/ml) in comparison to those at 0 h (0.2±0.11 ng/ml) after LPS challenge was observed in R2<sup>-/-</sup>>WT mice demonstrates that non-BM-derived cells also release sTNFR2 (Figure 3). These results demonstrate that both BM- and non-BM-derived cells contribute to the accumulation of circulating sTNFR2

in serum upon LPS challenge, although the former is the major source during the early phase.



**Figure 3** sTNFR2 in serum is derived from both BM- and non-BM-derived cells. Sera were collected from mice as described in Figure 1 at 0, 1.5, 6, 48 h after injection of LPS and sTNFR2 levels were determined by ELISA. Each data point represents the results derived from 3–5 mice. The ELISA detected both free and TNF-binding sTNFR2 forms. BM, bone marrow; LPS, lipopolysaccharide; sTNFR2, soluble tumor-necrosis factor receptor 2; TNF, tumor-necrosis factor; WT, wild type.

### Endothelial cells secrete sTNFR2 both spontaneously and upon stimulation

Endothelial cells, as well as fibroblasts, might be the major sTNFR2 producing cells among resident non-BM-derived cells. To further investigate the origin of sTNFR2 in serum, we isolated different non-BM derived cells, such as lung endothelial cells, from WT mice and cultured them *in vitro* with or without stimulation. The purity of the isolated endothelial cells was determined by cell morphology and flow cytometry using anti-CD31 antibody (Figure 4a). Indeed, these cells spontaneously secreted sTNFR2 into the culture medium. Upon stimulation with either LPS or TNF- $\alpha$ , sTNFR2 secretion was drastically increased. As shown in Figure 4b, the addition of 10  $\mu$ g/ml LPS led to a release of  $1.8 \pm 0.19$  ng/ml sTNFR2 at 6 h and  $7.0 \pm 1.05$  ng/ml at 48 h after stimulation. Stimulation with 20 ng/ml of mouse recombinant TNF- $\alpha$ , which binds to both TNFR1 and TNFR2, showed a similar effect on sTNFR2 production in culture.

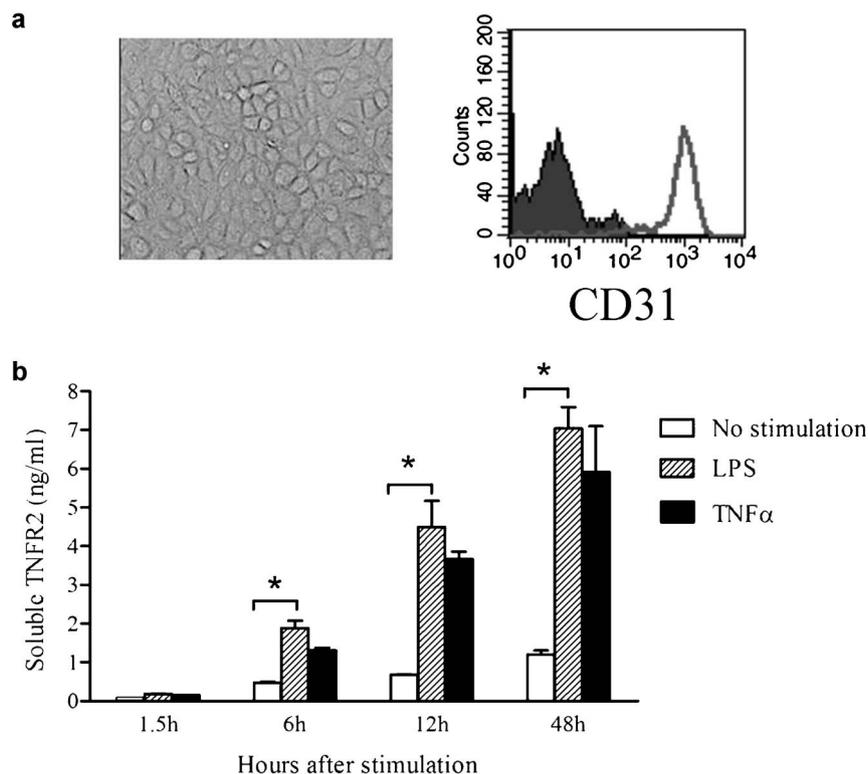
In further experiments, lung fibroblasts were isolated and sTNFR2 concentrations in culture medium were determined upon stimulation with 10  $\mu$ g/ml LPS or 20 ng/ml TNF- $\alpha$ . Compared to endothelial cells, the same number of fibroblasts secreted only about half as much sTNFR2 (data not shown). Similar experiments were performed using endothelial cells and fibroblasts isolated from TNFR1<sup>-/-</sup> mice. The results showed that, in the absence of TNFR1, sTNFR2 shedding from non-BM-derived cells increased by 1.4–1.7 fold at 48 h after LPS stimulation (data not shown). The results above indicate that, upon stimulation with LPS and TNF, non-BM-derived cells, such as endothelial

cells and fibroblasts, may release large amounts of sTNFR2 into the serum not only *in vitro*, but also *in vivo*. However, determining which cell types among non-BM-derived cells are the major sources of serum sTNFR2 *in vivo* will require further investigation.

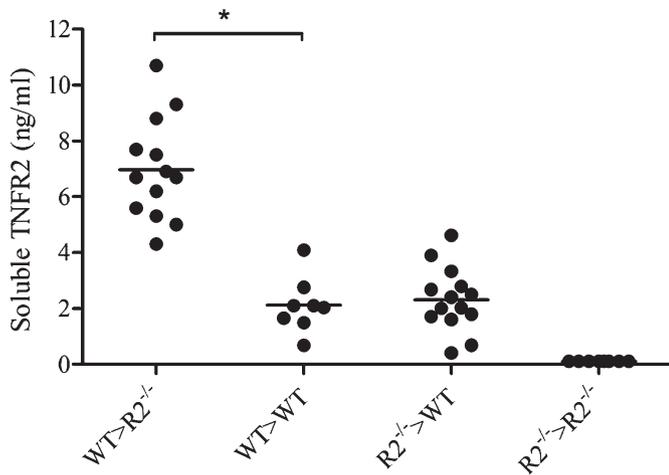
### Downregulation of sTNFR2 in serum requires TNFR2 on non-BM-derived cells

Inflammatory reactions as well as pro-inflammatory mediators are normally strictly controlled in the host. We investigated how sTNFR2 levels in serum are regulated in LPS-induced septic shock. As shown in Figure 3, sTNFR2 levels fell to baseline in both WT>WT and R2<sup>-/-</sup>>WT mice at 48 h after LPS challenge. However, a high concentration of serum sTNFR2 could still be detected at this time point in WT>R2<sup>-/-</sup> mice ( $7.0 \pm 0.65$  ng/ml).

Figure 5 shows the individual values of serum sTNFR2 in mice from different groups of BM chimeras during the recovery stage of LPS-induced septic shock. Comparison between sTNFR2 concentrations in WT>R2<sup>-/-</sup> ( $7.0 \pm 0.65$  ng/ml) and WT>WT mice ( $2.11 \pm 0.56$  ng/ml) demonstrated that TNFR2 expression on non-BM-derived cells was again important for the downregulation of sTNFR2. Interestingly, although the level of sTNFR2 in WT>R2<sup>-/-</sup> mice was significantly higher than that of R2<sup>-/-</sup>>R2<sup>-/-</sup> mice 2 days after LPS challenge, the difference in the death rate between these two groups was not significant ( $P=0.68$ ). This indicates that factors or soluble receptors other than sTNFR2 may also play a role in regulating inflammation.



**Figure 4** Endothelial cells secrete sTNFR2 both spontaneously and upon stimulation. (a) Isolation of lung endothelial cells. Cells were isolated and cultured as described in the section on 'Materials and methods'. Shown on the left are endothelial cells characterized by their 'cobblestone' morphology and on the right are cells stained with APC-conjugated anti-CD31 antibody (open histogram) or with isotype control (filled histogram). (b) Endothelial cells release sTNFR2 upon stimulation. Cells were cultured without any stimulation (open bars) or with 10  $\mu$ g/ml LPS (hatched bars) or 20 ng/ml TNF- $\alpha$  (closed bars). Levels of sTNFR2 in supernatants collected at the indicated time points are shown. Data are given as the mean  $\pm$  s.d. of triplicate samples. \* $P < 0.05$ . APC, allophycocyanin; BM, bone marrow; LPS, lipopolysaccharide; sTNFR2, soluble tumor-necrosis factor receptor 2; TNF, tumor-necrosis factor; TNFR2, tumor-necrosis factor receptor 2.

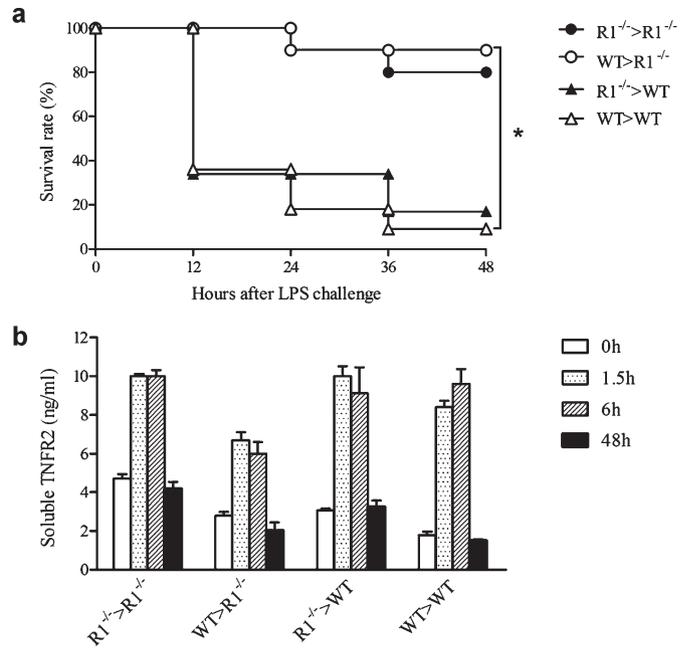


**Figure 5** Downregulation of sTNFR2 in serum requires TNFR2 on non-BM-derived cells. Sera of TNFR2 BM chimeric mice (8–14 mice/group) described above in Figure 1 were collected at 48 h after LPS challenge. Levels of sTNFR2 in serum evaluated with ELISA are shown. Each point represents the serum sTNFR2 level of one individual mouse. The difference between sTNFR2 levels of WT>R2<sup>-/-</sup> and WT>WT mice is significant. \**P*<0.05. BM, bone marrow; LPS, lipopolysaccharide; sTNFR2, soluble tumor-necrosis factor receptor 2; TNFR2, tumor-necrosis factor receptor 2; WT, wild type.

### TNFR1 on non-BM-derived cells influences the sensitivity of mice to LPS but not to sTNFR2 levels in serum

One of the functions of TNFR2 is thought to collect and present circulating TNF to TNFR1 by so called ‘ligand passing’. To investigate whether TNFR1 deficiency on BM- or non-BM-derived cells can affect TNFR2 function and sTNFR2 release in response to LPS, we generated new groups of TNFR1 BM chimeras (R1<sup>-/-</sup>>R1<sup>-/-</sup>, WT>R1<sup>-/-</sup>, R1<sup>-/-</sup>>WT and WT>WT). Each group of mice was administered 10 μg of LPS plus 8 mg of D-gal as described above. As shown in Figure 6a, WT>R1<sup>-/-</sup> mice were significantly less sensitive (survival rate: 90%) to LPS-induced shock than WT>WT counterparts (survival rate 9%), while there was no significant difference between the survival rate of R1<sup>-/-</sup>>WT mice (17%) and that of WT>WT mice (9%). This result demonstrates that TNFR1 deficiency in non-BM-derived cells is also important for LPS resistance. As shown above, both TNFR1 and TNFR2 expression on non-BM-derived cells contributed to the sensitivity of mice to LPS challenge. To determine whether TNFR1 expression had changed in cells from TNFR2<sup>-/-</sup> mice, we examined TNFR1 expression on different types of cells isolated from WT and TNFR2<sup>-/-</sup> mice by FACS staining. No significant difference in TNFR1 expression on splenocytes, lung endothelial cells or embryonic fibroblasts was detected (Supplementary Figure 1). TNFR1 expression was also examined at the mRNA level by real-time PCR and no difference was observed (data not shown).

Serum levels of sTNFR2 in different groups of chimeric mice were also determined at 0, 1.5, 6 and 48 h following LPS injection. In the R1<sup>-/-</sup>>R1<sup>-/-</sup> mice, there was 4.71±0.23 ng/ml sTNFR2 in serum even before LPS treatment (Figure 6b). Levels of sTNFR2 increased within the first 1.5 h and reached a value of 10.0±2.85 ng/ml at 6 h before falling to the baseline level (4.17±0.37 ng/ml) at 48 h after LPS challenge. Serum sTNFR2 levels in other groups showed similar dynamics. It is important to note that, in the absence of TNFR1, serum sTNFR2 levels were generally upregulated in comparison to the corresponding TNFR2 BM chimeras (Figure 3) due to a



**Figure 6** TNFR1 on non-BM-derived cells influences the sensitivity of mice to LPS but the level of sTNFR2 in serum does not. (a) Survival rate of TNFR1 BM chimeric mice in response to LPS challenge. Lethally irradiated TNFR1<sup>-/-</sup> and WT mice (10–15 mice/group) were reconstituted with TNFR1<sup>-/-</sup> (R1<sup>-/-</sup>>R1<sup>-/-</sup>, ● and R1<sup>-/-</sup>>WT, ▲) or WT BM cells (WT>R1<sup>-/-</sup>, ○ and WT>WT, △). Fifteen weeks after reconstitution, mice were challenged i.p. with LPS as described in the section on ‘Materials and methods’. The survival rate of four groups of mice at different times after LPS challenge is shown. \**P*<0.05. (b) Sera from the mice described above were collected at 0, 1.5, 6, 48 h after LPS injection and sTNFR2 levels were determined by ELISA. Each of the points represents the results derived from 3–5 mice. The results show no significant difference in serum sTNFR2 among the four groups. BM, bone marrow; i.p., intraperitoneally; LPS, lipopolysaccharide; sTNFR2, soluble tumor-necrosis factor receptor 2; TNFR, tumor-necrosis factor receptor; WT, wild type.

feedback mechanism. The fact that there was no significant difference in sTNFR2 levels among all groups indicates that non-BM TNFR1 expression has little influence on the release of sTNFR2 in this model.

### DISCUSSION

The involvement of sTNFR2 in sepsis and inflammation has long been recognized and has been extensively investigated.<sup>19,20</sup> However, the origin of sTNFR2 in serum remains unclear. In the present study, we show for the first time that sTNFR2 in serum originates not only from BM-derived cells but also from non-BM-derived cells in LPS-induced septic shock. Most importantly, we show that TNFR2 signaling in non-BM-derived cells is crucial for the sensitivity of animals to LPS and for proper downregulation of sTNFR2 levels in serum during the recovery stage of shock.

Surprisingly, TNFR2 expression on non-BM-derived cells is crucial for sensitivity to LPS-induced septic shock. It is generally believed that TNFR2 is mainly expressed on BM-derived cells, such as macrophages and other inflammatory cells.<sup>37</sup> Only a few studies have reported that TNFR2 is also expressed on non-BM derived cells, such as endothelial cells. Non-BM TNFR2 can also mediate TNF-induced intestinal myofibroblast proliferation and collagen production.<sup>17</sup> However, whether TNFR2 expression on non-BM derived cells is

involved in septic shock was not clear. In this study, the survival rate of WT>R2<sup>-/-</sup> mice was significantly higher than that of WT>WT ones, while no significant difference was found between R2<sup>-/-</sup>>WT and WT>WT groups, suggesting that non-BM-derived cells with competent TNFR2 are crucial to LPS sensitivity (Figure 2a). The functional significance of TNFR2 expression on non-BM-derived cells, such as endothelial cells and fibroblasts, is currently under further investigation.

Serum sTNFR2 can originate from both BM- and non-BM-derived cells. Previous *in vitro* experiments showed that sTNFR2 can be shed from cultured neutrophils, monocytes or macrophages upon stimulation.<sup>1,18,38</sup> A previous study has also shown that TNFR2 expression on host macrophages is sufficient to arrest tumor growth.<sup>1</sup> However, less evidence has been found showing that non-BM-derived cells produce sTNFR2. The results presented here indicate that both BM- and non-BM-derived cells are sources of serum sTNFR2. Although BM-derived cells contribute more to sTNFR2 levels in serum than non-BM-derived cells in the early stage of LPS-induced septic shock, TNFR2 expression on non-BM-derived cells is crucial for the down-regulation of sTNFR2 during the recovery stage. This suggests that TNFR2 expression on different cell types may play different functions at different stages of inflammation.

It is still unclear how levels of serum sTNFR2 are regulated during LPS-induced septic shock. We showed that sTNFR2 levels in both WT>WT and R2<sup>-/-</sup>>WT chimeric mice rapidly increased within 6 h and then fell to baseline levels at 48 h after LPS challenge. However, chimeras with non-BM TNFR2 deficiency (WT>R2<sup>-/-</sup>) showed a retardation of sTNFR2 downregulation. A possible explanation is that high-serum TNF induced by LPS challenge could bind TNFR2 on non-BM-derived cells, activate a related signaling pathway and produce a negative feedback signal for sTNFR2 release. As a result, when TNFR2 was defective on non-BM-derived cells, the negative feedback was not effective and sTNFR2 was sustained at a high level during the recovery stage of LPS-induced septic shock. Persistent high serum level of sTNFR2 was reported to be correlated with poor prognosis in some cases.<sup>20</sup> However, considering the high survival rate of WT>R2<sup>-/-</sup> mice, this study does not support this conclusion.

Although TNFR1 expression on non-BM-derived cells was also critical for the sensitivity to LPS challenge, our results showed no significant difference of serum sTNFR2 levels among the groups of BM chimeras of R1<sup>-/-</sup>>R1<sup>-/-</sup>, WT>R1<sup>-/-</sup>, R1<sup>-/-</sup>>WT and WT>WT. Moreover, no significant difference in TNFR1 expression on splenocytes, lung endothelial cells and MEFs was detected between TNFR2<sup>-/-</sup> and WT mice (Supplementary Figure 1). Whether and how TNFR1 expression on non-BM-derived cells affects sTNFR2 release and its downregulation during sepsis requires further investigation.

Together, the results presented above clearly demonstrate that TNFR2 expression on non-BM derived cells is crucial for LPS-induced septic shock. The level of sTNFR2 in serum is regulated by many factors and may not always correlate with poor prognoses. The accumulation of sTNFR2 during the early stage of septic shock depends on both BM- and non-BM-derived cells; however, its downregulation in the recovery stage depends on only non-BM-derived cells. Understanding the origin and functional mechanisms of sTNFR2 is important for the development of novel strategies in treating sepsis and many inflammatory diseases.

*Note: Supplementary information is available on the Cellular & Molecular Immunology website (<http://www.nature.com/cmi/>).*

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