Activated Macrophage Survival Is Coordinated by TAK1 Binding Proteins

September R. Mihaly1, Sho Morioka1, Jun Ninomiya-Tsujii1*, Giichi Takaesu1,2*

1 Department of Biological Sciences, Environmental and Molecular Toxicology, North Carolina State University, Raleigh, North Carolina, United States of America, 2 Center for Integrated Medical Research, Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan

Abstract

Macrophages play diverse roles in tissue homeostasis and immunity, and canonically activated macrophages are critically associated with acute inflammatory responses. It is known that activated macrophages undergo cell death after transient activation in some settings, and the viability of macrophages impacts on inflammatory status. Here we report that TGFβ-activated kinase (TAK1) activators, TAK1-binding protein 1 (TAB1) and TAK1-binding protein 2 (TAB2), are critical molecules in the regulation of activated macrophage survival. While deletion of Tak1 induced cell death in bone marrow derived macrophages even without activation, Tab1 or Tab2 deletion alone did not profoundly affect survival of naive macrophages. However, in lipopolysaccharide (LPS)-activated macrophages, even single deletion of Tab1 or Tab2 resulted in macrophage death with both necrotic and apoptotic features. We show that TAB1 and TAB2 were redundantly involved in LPS-induced TAK1 activation in macrophages. These results demonstrate that TAK1 activity is the key to activated macrophage survival. Finally, in an in vivo setting, Tab1 deficiency impaired increase of peritoneal macrophages upon LPS challenge, suggesting that TAK1 complex regulation of macrophages may participate in in vivo macrophage homeostasis. Our results demonstrate that TAB1 and TAB2 are required for activated macrophages, making TAB1 and TAB2 effective targets to control inflammation by modulating macrophage survival.

Citation: Mihaly SR, Morioka S, Ninomiya-Tsujii J, Takaesu G (2014) Activated Macrophage Survival Is Coordinated by TAK1 Binding Proteins. PLoS ONE 9(4): e94982. doi:10.1371/journal.pone.0094982

Editor: Marcello G. Bonini, University of Illinois at Chicago, United States of America

Received January 16, 2014; Accepted March 20, 2014; Published April 15, 2014

Copyright: © 2014 Mihaly et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by “Keio Kanrinmaru Project,” the Promotion of Environmental Improvement for Independence of Young Researchers under the Special Coordination Funds for Promoting Science and Technology (JST, Japan) (to G. T.), and National Institutes of Health Grant GM068812 (to J. N-T.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Jun_Tsuji@ncsu.edu (JNT); takaesug@med.u-ryukyu.ac.jp (GT)

Introduction

Macrophages are characterized by phagocytic activity, and play diverse roles in different tissue types. While resident macrophages participate in morphogenesis and tissue homeostasis, resident and recruited macrophages also play a major role in acute inflammatory responses [1]. Upon tissue injury or invasion by microorganisms, circulating inflammatory monocytes are recruited and differentiated toward mature macrophages. These macrophages are canonically activated by necrotic debris and bacterial moieties through Toll-like receptor signaling pathway, developing into so-called M1 polarized macrophages [2]. Activated macrophages clean dead cells and microorganisms by phagocytosis and produce inflammatory cytokines resulting in amplification of inflammation. Subsequently, these activated macrophages are deactivated or killed to terminate inflammatory conditions. In some experimental settings, it is known that lipopolysaccharide (LPS)-induced activation of macrophages reduces macrophage viability [3–5]. However, the mechanism by which activated macrophages undergo cell death is still largely elusive.

TGFβ-activated kinase (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, and is an indispensable intermediate of cytokine and Toll-like receptor pathways [6–8]. TAK1 is recruited to and activated by the receptor proximal complex of TNF, IL-1, and Toll-like receptors through a poly-ubiquitin chain-mediated mechanism [9]. TAK1-binding protein 2 (TAB2) and its closely related protein, TAK1-binding protein 3 (TAB3), have ubiquitin binding domains and tether between TAK1 and the poly-ubiquitin chain resulting in is involved in stress-dependent TAK1 activation [19] and activity of TAK1 in epithelial tissues [20]. Major known downstream molecules of TAK1 are IκB-kinases (IKKs) and mitogen-activated protein kinases (MAPKs) including p38 and JNK, which in turn activate transcription factors NF-κB and AP-1, respectively.

In vivo, TAK1 signaling is found to be important for immune responses in T and B cells through regulation of NF-κB and MAPK pathways in a mouse model [21–24], which is anticipated from the results in the tissue culture system described above. However, unexpectedly, the most overt phenotype caused by Tak1 deficiency in vivo is tissue damage associated with cell death in the epidermis, intestinal epithelium and liver [25–31]. Since Tak1
deficiency does not cause cell death in primary culture fibroblasts or keratinocytes, the cell death must be induced depending on the in vivo environment. TAK1 has been found to be integral to prevent tissue-derived TNF-induced cell death in vivo, which is evidenced by the fact that Tnfr1 deletion can rescue cell death and tissue damage in these tissues [26,27,29]. Single deletion of Tab1 or Tab2 does not cause any abnormalities in the epidermis and intestinal epithelium but double deletion of Tab1 and Tab2 phenocopies Taki deficiency [20], suggesting that TAB1 and TAB2 redundantly function in TAK1 regulation in these tissues. However, the specific roles of TAB1 and TAB2 in adult tissues are still largely elusive.

Recent studies have demonstrated that Tak1 deficiency in myeloid cells results in hyper-proliferation of neutrophils and increased inflammatory conditions [32,33]. Bone marrow derived macrophages (BMDMs) generated from myeloid-specific Tak1-deficient mice have been reported to undergo spontaneous cell death under normal culture conditions [32,34]. These studies have determined that Tak1 is required for proper differentiation of myeloid lineage and survival of macrophage precursors and/or mature macrophages. However, it is not clear whether Tak1 is important for maintenance of progenitors or mature macrophages and what the role of Tak1 is in activation of macrophages. Here we investigate the role of Tak1 and its binding partners, TAB1 and TAB2, in both mature naive and activated macrophages, and have determined that, TAB1 and TAB2 are essential modulators of Tak1 activity and cell survival in LPS-activated macrophages.

Materials and Methods

Bone marrow cell isolation and macrophage differentiation

Tak1flox/flox, Tab1flox/flox, and Tab2flox/flox C57BL/6 mice were described previously [15,22,35]. Rosa26.CreERT and Tnfr1-/- mice were purchased from Jackson Laboratories, and bred in our lab to produce the indicated genotypes [36–38]. Experiments performed in vitro required isolating bone marrow cells from Tak1, Tab1 and Tab2 mutant mice with flox/+ Rosa26.CreERT (F+Cre) or flox/flox Rosa26.CreERT (iKO). Bone marrow derived macrophages (BMDMs) were generated by the standard procedure culturing bone marrow cells in 30% L929 cell-conditioned medium. To achieve gene deletion, cells were treated with 0.3 μM 4-hydroxytamoxifen (4-OHT) for 4 days. All animal experiments including in vivo treatment described later were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee (IACUC protocol # 11-138B). All efforts were made to minimize animal suffering.

Crystal violet assay

BMDMs were plated on 12-well plates at a concentration of 2 x 10⁶ cells per well and treated with 0.3 μM 4-OHT. In some experiments, BMDMs were subsequently treated with LPS (1 μg/ml) for 1 and 3 days. Cells were fixed using 10% formalin, and stained with 0.1% crystal violet. The dye was eluted and analyzed at 595 nm.

Reagents and antibodies

Specific monoclonal and polyclonal antibodies against the following antigens were used: CD3e (145-2C11), CD11b (M1/70), and B220 (RA3-6B2) (BioLegend); F4/80 (BM8) (BioLegend), phospho-TAK1, TAK1, TAB1, and TAB2 described previously; phospho-IκB, phospho-p38, IκB, and p38 (Cell Signaling). Necrostatin-1 (Nec-1) was purchased from Santa Cruz and applied to the culture at the final concentration of 50 μM. LPS is derived from source strain Salmonella minnesota ATCC 9700 (Sigma-Aldrich, catalog number L6261).

Flow cytometry

BMDMs were detached from culture dishes and incubated with annexin V-Pacific Blue (BioLegend) and Fixable Viability Dye eFluor 780 (eBioscience) for cell death analysis. Stained cells were analyzed on flow cytometer (BD Biosciences LSRII), and data were analyzed using FlowJo software (Tree Star). Events were gated to exclude debris based on forward scatter (FSC) and side scatter (SSC) profile, then gated on Pacific Blue (annexin V) or APC-Cy7 (fixable viability dye) when compared to unstained control.

Western blotting

BMDMs were lysed in extraction buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 20 μM aprotinin, 0.5% Triton X-100) in ice for 15 minutes. Cells and debris were then pelleted by centrifugation at 20,000 G for 10 at 4°C. Cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Mouse model (in vivo)

For in vivo experiments, deletion of Tab1 was achieved in Tab1+/+ mice by intraperitoneal injection of 50 mg per kg tamoxifen on 3 consecutive days. After a period of 3-5 weeks, to reduce effects of Cre toxicity, whole blood was isolated and gene deletion was verified by Western Blot. To exclude the effect of Cre toxicity, we included F+Cre (Rosa26.CreERT2 Tab1flox/+ ) mice as controls. Mice were intraperitoneally injected with 8 mg/kg LPS, and were euthanized at 72 hours and dissected. Peritoneal leukocytes were collected by peritoneal lavage and collected in phosphate buffered saline (PBS), and splenocytes were harvested and prepared in a single cell suspension. Red blood cells were lysed by suspending cells in 0.83% NH₄Cl lysis buffer and washed once with PBS. Cells were incubated with anti-CD16/32 in ice for 20 minutes to block FcRI/III, followed by incubation with fluorophore-conjugated monoclonal antibodies (CD11b, B220 CD3e, and F4/80) to evaluate cell type. Cells were washed in PBS and characterized on a BD LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Statistical analysis

Cell counts were normalized to control and compared using a two-tailed Student’s t-test. Values shown are mean ± standard deviation with results considered significant if a probability of Type I error was <.05.

Results

Deletion of Tak1 or double deletion of Tab1 or Tab2 spontaneously kills bone marrow derived macrophages

We characterized bone marrow derived macrophages (BMDMs) in adult mice having deletions of Tak1, Tab1 or Tab2 single or Tab1 and Tab2 double gene using the ubiquitously-expressed inducible Cre recombinase system, Rosa26.CreERT

Macrophage Survival Is Regulated by TAB1 and TAB2
Rosa26.CreERT Tak1flox/flox (referred to as Tak1iKO), Rosa26.CreERT Tab1flox/flox (referred to as Tab1iKO), Rosa26.CreERT Tab2flox/flox (referred to as Tab2iKO), and Rosa26.CreERT Tab1flox/flox Tab2flox/flox (referred to as diKO) were compared with littermate or age matched controls, no-Cre flox/flox (referred to as WT or control). In some experiments, mice having heterozygous gene deletion, Rosa26.CreERT flox/+ were also used as a control, which did not show any abnormality nor did WT. Bone marrow cells were differentiated to macrophages and gene deletion was subsequently induced by treatment of 4-hydroxytamoxifen (4-OHT) in macrophage culture medium, and vehicle (ethanol) was treated as control. The amounts of TAK1, TAB1 and TAB2 proteins were determined by immunoblotting at 4 days with 4-OHT or vehicle treatment. For Tak1iKO and diKO macrophages, we additionally treated with necrostatin-1 (Nec-1), an inhibitor of receptor interacting protein 1 (RIP1), which is known to block Tak1-deficient macrophage death [34]. Nec-1 blocked cell death and allowed us to recover sufficient protein for Western blotting.

We observed fluctuations in the amount of proteins among different litters and with Nec-1 treatment. Nonetheless, Tak1iKO, Tab1iKO, and Tab2iKO were greatly reduced within 4 days with 4-OHT treatment in Tak1iKO macrophages (Figure 1A). Reduction of TAB1 and TAB2 in Tak1iKO macrophages is presumably due to destabilization of unbound TAB1 and TAB2. In contrast, TAB1 or TAB2 but not TAK1 was reduced in Tab1iKO or Tab2iKO macrophages, respectively. Both TAB1 and TAB2 but not TAK1 were reduced in diKO macrophages. These indicate that TAK1 is intact in Tab1iKO, Tab2iKO, and diKO macrophages, which allow us to investigate the specific roles of TAB1 and TAB2 without altering the protein level of TAK1 in these macrophages. The number of macrophages declined at 8 days with 4-OHT treatment in Tak1iKO macrophages, whereas the number of control macrophages, including WT with 4-OHT and Tak1iKO with vehicle treatment, were not altered during the period of experiment (Figure 1B). Thus, Tak1 is essential for cultured macrophage integrity. These results are consistent with the previously reported

**Figure 1. TAK1 is required for macrophage survival.** (A) Western blotting analysis of TAK1, TAB1 and TAB2 in control, Tak1iKO, Tab1iKO, Tab2iKO and diKO BMDMs. Bone marrow cells were cultured in macrophage medium and treated with 0.3 μM 4-OHT or vehicle (ethanol) for 4 days. Tak1iKO and diKO BMDMs were additionally treated with 50 μM Necrostatin-1 (Nec-1). Anti-β-actin Western blotting was used as a loading control. The numbers beside each panel denote the size and the position of molecular weight markers. (B) Viability of WT, Tak1iKO, Tab1iKO, Tab2iKO, and inducible double-deficient (diKO) BMDMs. Cells were cultured for 8 days with 0.3 μM 4-OHT and stained with 0.1% Crystal Violet. Data are mean percentages of attached macrophages compared to ethanol-treated +/- SD for 3 independent experiments. Asterisks indicate p-values: ** = P<0.005; *** = p<0.0005.

doi:10.1371/journal.pone.0094982.g001
results in myeloid-specific deletion of Tak1 [34]. Thus, TAK1 seems to be important for survival of not only precursors but also mature naïve macrophages. In contrast to Tak1 deletion, either Tab1 or Tab2 single deletion only moderately or slightly decreased the number of macrophages (Figure 1B). This indicates that TAB1 or TAB2 may partly contribute to but is not required for naïve macrophage survival. Importantly, we found that double deletion of Tab1 and Tab2 reduced the number of macrophages to a level similar to Tak1 deletion (Figure 1B). Collectively, these results suggest that TAB1 and TAB2 may redundantly function to maintain TAK1 activity in naïve macrophages and TAK1 basal activity is required for survival of naïve macrophages.

**TAB1 and TAB2 are required for survival of LPS-stimulated macrophages**

To determine the role of TAK1 complex in activated macrophages, we treated Tab1iKO or Tab2iKO macrophages with bacterial moiety, lipopolysaccharide (LPS). Wild type macrophages treated with LPS did not exhibit reduced cell viability in our experimental setting (Figure S1). Even in this condition, we found...
that activation of macrophages by LPS treatment noticeably reduced the numbers of Tab1\textsuperscript{KO} macrophages within 3 days (Figure 2A). Tab1\textsuperscript{KO} macrophages exhibited both increased annexin V binding and loss of plasma membrane integrity (Figures 2B). The percentage of cells showing a necrotic feature, cell permeability dye positive, was greatly increased by LPS treatment (Figure 2C). Apoptotic cells characterized by annexin V binding-positive but permeability dye-negative were also increased by LPS treatments (Figure 2D). Thus, LPS-activated macrophages require TAB1 for their survival, and Tab1 deficiency causes cell death having both necrotic and apoptotic features. Similarly, we found that Tab2\textsuperscript{KO} macrophages were significantly declined upon LPS treatment (Figure 3A). In contrast to Tab1 deletion, Tab2\textsuperscript{KO} macrophage underwent cell death only one day after LPS treatment. LPS-treatment of Tab2\textsuperscript{KO} macrophages increased the frequency of annexin V-positive and cell permeability dye-positive population (Figure 3B). Similar to Tab1\textsuperscript{KO}, both necrotic and apoptotic cells were increased by LPS treatment in Tab2\textsuperscript{KO} macrophages (Figure 3C and 3D). These results demonstrate that, in contrast to naive macrophages, both TAB1 and TAB2 are important for activated macrophage survival.

Figure 3. TAB2 is required for LPS-activated macrophage survival. (A) Viability of LPS-activated Tab2-deficient macrophages. Tab2\textsuperscript{KO} MDMs were cultured for 8 days with 0.3 µM 4-OHT, then 1 µg/ml LPS was added to culture medium for 1 day. Data are mean percentages of attached macrophages compared to 8 days treated with 4-OHT alone +/− SD between 3 independent experiments as measured by Crystal Violet Assay. (B) Percentages of annexin V and viability dye positive cells. Tab2\textsuperscript{KO} and control (WT) MDMs were cultured with 0.3 µM 4-OHT for 3 days then with 1 µg/ml LPS for 1 additional day in 3 independent experiments. Cells were stained with viability dye and annexin V and analyzed by flow cytometry. Percentages of single positive and double positive cells based on unstained controls in a representative experiment are shown. (C and D) Necrotic and apoptotic LPS-activated macrophages with Tab2 deficiency. Tab2\textsuperscript{KO}, and controls including WT and F+Cre MDMs were cultured 3 days in 0.3 µM 4-OHT-containing macrophage medium, then 1 day in medium containing 0.3 µM 4-OHT and 1 µg/ml LPS. (C) Necrotic cells are shown as percentage positive for viability dye. (D) Annexin V positive but viability dye-negative cells as a percentage of total cells is shown. Graphs indicate results of four independent experiments +/− SD. doi:10.1371/journal.pone.0094982.g003
Figure 4. LPS activates TAK1 through TAB1 and TAB2. (A) Western blotting analysis of Tak1iKO and Tab1Tab2dKO and control BMDMs. Cells were cultured for 4 days with 0.3 μM 4-OHT in the presence of 50 μM Nec-1, followed by 1 μg/ml LPS treatment for the indicated period of time. Anti-β-actin was used as a loading control. Asterisks indicate non-specific bands. (B) Tab1iKO or control BMDMs were cultured with 0.3 μM 4-OHT for 3 days.
LPS activates TAK1 through TAB1 and TAB2 in macrophages

We hypothesize that TAB1 and TAB2 mediate LPS-induced TAK1 activation, which may be required for LPS-activated macrophage survival. To test this, we examined the levels of TAK1 activation and subsequent downstream events, activation of NF-κB and p38, following LPS stimulation. We note that, since Tak1 or Tab1 and Tab2 double deletion spontaneously kills macrophages, we treated macrophages with Tak1 or Tab1 and Tab2 double deletion. It is known that while RIP1 participates in NF-κB and p38 pathways, RIP1 catalytic activity is dispensable [39]. Consistent with this notion, NF-κB and p38 were activated in wild type macrophages even with Nec-1 treatment. We found that LPS activated TAK1 and its downstream IKK and p38 in macrophages (Figure 4A, middle 4 lanes), and Tak1 deficiency reduced activation of both IKK and p38 (Figure 4A, left 4 lanes). TAK1 activity was monitored by phosphorylation of Thr 187 (Figure 4A, top panel), which is known to be associated with activation of TAK1 [40]. However, non-specific bands were detected around the phosphorylated TAK1 in macrophages protein extracts, which were seen even in unstimulated macrophage (Figure 4A, asterisks). Thus, we also utilized retardation of TAK1 band on SDS-PAGE to monitor TAK1 activation, which is caused by phosphorylation of several sites associated with TAK1 activation [41,42]. While TAK1

Figure 5. TAB1 or TAB2 deletion causes RIP1-dependent cell death. (A) Tab1iKO or control BMDMs were cultured with 0.3 μM 4-OHT with or without 50 μM Nec-1 for 8 days then treated with 1 μM LPS for 3 days, and viability was measured by Crystal Violet Assay. * = p<.05. (B) Viability of Tab2iKO BMDMs treated with RIP1 inhibitor. Tab2iKO BMDMs were treated with 0.3 μM 4-OHT for 8 days with our without 50 μM Necrostatin-1 (Nec-1), then treated with 1 μM LPS for one day. Viability was measured by Crystal Violet Assay. * = p<.05.

doi:10.1371/journal.pone.0094982.g005
exhibited migration shift upon LPS stimulation in wild type macrophages, Tab1 and Tab2 double deficiency abolished migration shift (Figure 4A, second panel), and reduced activation of IKK and p38 (Figure 4A, right 3 lanes) suggesting that LPS-induced activation of IKK and p38 is largely mediated by TAK1 and that TAB1 and TAB2 are essential for TAK1 activation in response to LPS. Deletion of either Tab1 or Tab2 impaired LPS-induced migration shift of TAK1, suggesting some impairment of TAK1 activation in response to LPS (Figures 4B and C, top panels). However, a single deletion of either Tab1 or Tab2 had a marginal effect on the LPS-induced degradation of IkB and phosphorylation and p38, suggesting that one of these proteins can activate TAK1 sufficiently at least in the pathways leading to activation of IKK and p38. These results collectively suggest that LPS-induced activation of TAK1 is mediated by TAB1 and TAB2, and that either deletion of Tab1 or Tab2 reduces TAK1 activation. However, LPS-induced activation of NF-kB and p38 seems to require at a minimum TAB1 or TAB2.

LPS-induced cell death in Tab1- or Tab2-deficient macrophages is partially rescued by inhibition of RIP1

Tab1 deletion is reported to cause RIP1-dependent cell death upon TNF treatment in several cell types [26,27,29]. Earlier study demonstrates that Tab1-deficient naïve macrophages die in a RIP1-dependent manner [34]. We hypothesize that activated macrophages die with the mechanism similar to that in naïve macrophages due to insufficient activity of TAK1. To test this, we examined the involvement of RIP1 kinase activity in Tab1^{iKO} and Tab2^{iKO} macrophage death by using Nec-1. In Tab1^{iKO} macrophages, Nec-1 significantly and marginally reduced LPS-induced necrotic and apoptotic cells, respectively (Figure 5A and 5B). Nec-1 treatment exhibited some trends of reduction of cell death in Tab2^{iKO} macrophages, although these did not reach to a statistic significance (Figure 5C and 5D). Both necrotic and apoptotic cells were decreased. These results suggest that RIP1 kinase activity may be at least partially involved in LPS-induced cell death in Tab1^{iKO} and Tab2^{iKO} macrophages.
Tab1-deficient macrophages were reduced upon LPS injection in vivo.

We next examined the role of Tak1 complex in macrophages in vivo. We anticipate LPS injection to recruit and activate monocytes from the bone marrow and circulation to the peritoneal cavity. Germline deletion of Tak1 gene causes multiple defects in embryogenesis [43,44], and deficiency of Tak1 in adult mice also causes acute severe liver injury and mortality [25,26]. We were not able to analyze Tak1 deficient macrophages in adult mice due to these multiple acute effects. Tab2iKO mice did not exhibit overt abnormality upon gene deletion by tamoxifen injection; however, LPS stimulation caused acute liver dysfunction within 6 h (unpublished observations). Tab2-deficient macrophages were difficult to analyze in LPS-challenged mice for this reason. We then focused on Tab1iKO mice. Tab1 gene was deleted in Tab1iKO mice by tamoxifen injection for 3 consecutive days. Although germline deletion of Tab1 causes embryonic lethal phenotype [35,45], we found that deletion of Tab1 gene in adult mice did not cause overt abnormalities even though Tab1 proteins were greatly diminished (Figure 6A). To rule out the effect of Cre toxicity described previously [46,47], the experiments were performed after more than 3 weeks post-tamoxifen injections. The number of peritoneal macrophages was determined in Tab1iKO-deficient adult mice following LPS injection (Figure 6B). The frequency of CD11b+ F4/80+ macrophages in peritoneal fluid was lower in Tab1iKO mice compared to littermate controls following LPS stimulation. T-cells, B-cells and CD11b single positive cells were not significantly changed by Tab1 deficiency. Tab1 deficiency alone did not cause changes in macrophage population (Figure S2A). Importantly, the frequency of CD11b+ F4/80+ macrophages in peritoneal fluid was increased by LPS to around 30% from 10–15% under unstimulated conditions in control mice, but such increase was not observed in Tab1iKO mice. We noted that splenic CD11b+ F4/80+ macrophages were not significantly altered by LPS stimulation, and Tab1 deletion did not cause any alteration of splenic macrophages (Figure S2B), supporting the notion that participation of Tab1-dependent pathway in macrophage survival varies depending on macrophage type or activation state [48]. These suggest that Tab1 is important for activated macrophage maintenance in vivo.

Discussion

Our current studies identify an essential role for Tak1 modulator proteins, Tab1 and Tab2, in LPS-activated macrophages, in BMDMs, and in a mouse model of inflammation (Figure 7). The roles of Tab1 and Tab2 in macrophages have not been well characterized in the published literature to date. We demonstrate that Tab1 and Tab2 are essential for LPS-activated macrophage survival but are dispensable for survival of naive macrophages. Bacterial moieties including LPS are strong activators of macrophages, which polarize macrophages toward an inflammatory, M1 phenotype. M1 macrophages mediate acute inflammation by secreting cytokines and chemokines, which play a major role in initiation of inflammatory responses [49]. These classically activated macrophages are prone to cell death, which contributes to the termination of inflammatory responses [3–5]. Chronically activated macrophages are associated with many disease conditions including obesity, autoimmune diseases, atherosclerosis and asthma [50]. In contrast, naive macrophages in tissues, so-called resident macrophages such as microglia, Kupffer cells and Langerhans cells, play indispensable roles during development and in tissue repair [51–53]. Resident unstimulated macrophages are also important for prompt responses to protect tissues from insults. Therefore, the presence of unstimulated resident macrophages is beneficial to maintain tissue integrity. Thus, limiting inflammatory conditions by controlling only an activated sub-set of macrophages could be a useful tool in treating disease. Our results demonstrate that deletion of either Tab1 or Tab2 effectively kills only LPS-activated macrophages in vitro, and that Tab1 deletion prevents increase of peritoneal macrophages upon LPS stimulation. These results indicate that Tab1 and Tab2 are potentially useful targets to selectively control the activated fraction of macrophages.

It is interesting that the Tab1 Tab2iKO shows diminished activation of IKK and p38 following LPS stimulation, which is similar to the Tab1iKO phenotype. When these genes are individually deleted, LPS-induced IKK and p38 activation are nearly normal and naive macrophages persist. However, macrophages having deletion of either Tab1 or Tab2 could not withstand LPS-activation and Tab1iKO macrophages underwent necrosis after 3–4 days, while Tab2iKO die after 1 day, despite showing early LPS-induced Tak1 activation at or near WT levels (Figures 4B and C). In light of the nearly normal activation of NF-kB and p38 in Tab1iKO and Tab2iKO in response to LPS treatment, Tab1- and Tab2-dependent activated macrophage survival may occur through signaling pathway[s] independently of NF-kB and p38, which is in contrast to previous studies [4,5].

Recapitulating our in vitro results, we found that the number of peritoneal macrophages was lesser in LPS-treated Tab1iKO mice when compared to control. Based on our in vitro data, we anticipate this disease model to produce a net increase in LPS-activated peritoneal macrophages in control but not Tab1iKO mice, however it cannot be conclusively excluded that the observed
reduction is due to a defect in recruitment or expansion independent of cell death. Importantly, other hematopoietic cell types, including T cells, B cells and granulocytes, were found to be unaffected, suggesting TAB1-dependent survival signaling that is potentially unique to macrophages. We note here that liver was deleted in all cells, such that one cannot rule out the effects from other cell types in vivo. Future studies analyzing macrophage-specific conditional knockout mice for Tab1 and Tab2 could bring insight to the roles of these genes in macrophage cell death. Further research focused on this important mechanism in macrophages could inform inflammatory disease models, particularly diseases in which microorganisms target macrophages. The data supporting that TAB1 is essential for macrophage survival in LPS-treated mice could be used to improve our understanding of the control of inflammation.

**Supporting Information**

**Figure S1** Wild-type macrophages treated with LPS do not have reduced viability under experimental conditions. Viability of LPS-treated control macrophages. Tab2flox/flox M 4-OHT followed by 3 days M 4-OHT (Tab2flox/flox M 4-OHT). Macrophages were isolated from bone marrow-derived cells. The percentage positive for CD11b and F4/80 staining was determined by flow cytometry. The mean percentages of attached macrophages compared to 8 days treated with vehicle +/− SD of 3 independently performed experiments. (TIF)

**Figure S2** TAB1-dependent survival depends on type of macrophage. (A) Peritoneal leukocytes were collected from Tab1-deficient mice treated with vehicle (PBS) at 72 hours and stained with fluorophore-conjugated antibodies. Shown is percent positive of 2 control and 3 Tab1iKO for the indicated markers. Percentages of CD11b+ F4/80+, CD11b+, CD3e+ or B220+ cells of total cells ± SD is shown. (B) Tab1iKO and control mice were intraperitoneally injected with 8 mg/kg LPS. Spleenocytes were collected and stained with fluorophore-conjugated antibodies. Shown is percent positive of 6 control and 4 Tab1iKO for CD11b+ F4/80+, CD11b+, CD3e+ or B220+ as a percentage of total cells ± SD. (TIF)

**Acknowledgments**

We thank S. Akira for Tab1-floxed and Tab2-floxed mice, and S. Elliott, L. Hester, and J. Dow for support.

**Author Contributions**

Conceived and designed the experiments: JNT SRM SM GT. Performed the experiments: JNT SRM SM GT. Analyzed the data: SRM GT. Contributed reagents/materials/analysis tools: JNT GT. Wrote the paper: JNT SRM GT.

**References**

1. Wynn TA, Chawla A, Pollard JW (2013) Macrophage biology in development, homeostasis and disease. Nature 496: 445–455. doi:10.1038/nature12034
2. Weiger A, Johann AM, Kruehen A von, Schmidt H, Greislinger G, et al. (2006) Apoptotic cells promote macrophage survival by releasing the antiprotective mediator sphingosine-1-phosphate. Blood 108: 1635–1642. doi:10.1182/blood-2006-04-14852
3. He S, Liang Y, Shao F, Wang X (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci U S A 108: 20054–20059. doi:10.1073/pnas.1116302108
4. Park JM, Greten FR, Wong A, Westrick RJ, Arthur JS, et al. (2005) Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis: CREB and NF-kappaB as key regulators. Immunity 23: 319–329. doi:10.1016/j.immuni.2005.08.010
5. Ma Y, Temkin V, Liu H, Pope RM (2005) NF-kappaB protects macrophages from lipopolysaccharide-induced cell death: the role of caspase 8 and receptor-interacting protein. J Biol Chem 280: 41827–41834. doi:10.1074/jbc.M510049200
6. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132: 344–362. doi:10.1016/j.cell.2008.01.020
7. Ninomiya-Tsuji J, Kishimoto T, Hayaama A, Inoue J, Cao Z, et al. (1999) The kinase TAK1 can activate the NIK-kappaB as well as the MAP kinase cascade in the IL-1 signaling pathway. Nature 398: 252–256. doi:10.1038/14655
8. Takesu G, Surabhi RM, Park J, Ninomiya-Tsuji J, Matsumoto K, et al. (2003) TAK1 is critical for kappaB kinase-mediated activation of the NF-kappaB pathway. J Mol Biol 326: 165–115. doi:10.1016/S0022-2836(02)01494-5
9. Chen ZJ, Bhoj V, Seth RB (2006) Ubiquitin, TAK1 and IKK: is there a connection? CellDeath Differ 13: 687–692. doi:10.1038/sj.cdd.4401869
10. Scholz R, Sider GL, Thali RF, Wünsinger N, Cheung PC, et al. (2010) Autoactivation of transforming growth factor beta-activated kinase 1 is a sequential bimolecular process. J Biol Chem 285: 25753–25766. doi:10.1074/jbc.M109.039468
11. Inagaki M, Omori E, Kim JY, Komatsu Y, Scott G, et al. (2008) TAK1-binding protein 1, TAK1, modulates osmotic stress-induced TAK1 activation but is dispensable for TAK1-mediated cytokine signaling. J Biol Chem 283: 33086–33096. doi:10.1074/jbc.M807574200
12. Omori E, Inagaki M, Mishina Y, Matsumoto K, Ninomiya-Tsuji J (2012) Epithelial transforming growth factor beta-activated kinase 1 (TAK1) is activated through two independent mechanisms and regulates reactive oxygen species. Proc Natl Acad Sci U S A 109: 3365–3370. doi:10.1073/pnas.1116181109
13. Liu HX, Nie M, Schneider MD, Chen ZJ (2006) Essential role of TAK1 in thymocyte development and activation. Proc Natl Acad Sci U S A 103: 11677–11682. doi:10.1073/pnas.0508911010
14. Sato S, Sanjo H, Takeda K, Tsujimura T, Ninomiya-Tsuji J, Yamamoto M, et al. (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 6: 1087–1093. doi:10.1038/ni1253
15. Sato S, Sanjo H, Tsuchimura T, Ninomiya-Tsuji J, Yamamoto M, et al. (2006) TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. Int Immunol 18: 1405–1411. doi:10.1093/intimm/dsl082
16. Wang YY, Chi H, Xie M, Schneider MD, Flavell RA (2006) The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol 7: 851–858. doi:10.1038/ni1355
17. Bettermann K, Vucic M, Haybaeck J, Koppe C, Janssen J, et al. (2010) TAK1 suppresses a NEMO-dependent but NF-kappaB-independent pathway to liver cancer. Cancer Cell 17: 481–496. doi:10.1016/j.ccr.2010.03.021
18. Inokuchi S, Aoyama T, Miura K, Osterreicher CH, Kodama Y, et al. (2010) Enterocyte-derived TAK1 signaling prevents epithelium apoptosis and carcinogenesis. Proc Natl Acad Sci U S A 107: 844–849. doi:10.1073/pnas.0909791107
19. Kajino-Sakamoto R, Inagaki M, Lippert E, Akira S, Robine S, et al. (2008) Enterocyte-derived TAK1 signaling prevents epithelium apoptosis and the
Macrophage Survival Is Regulated by TAB1 and TAB2

development of ileitis and colitis. J Immunol 181: 1143–1152. PMCID: PMC3065566, NIHMSID: NIHMS279618.

28. Kajino-Sakamoto R, Omori E, Nishigaki Y, Blikslager AT, Matsumoto K, et al. (2010) TGF-beta-activated kinase 1 signaling maintains intestinal integrity by preventing accumulation of reactive oxygen species in the intestinal epithelium. J Immunol 185: 4729–4737. doi:10.4049/jimmunol.0905387

29. Omori E, Matsumoto K, Sanjo H, Sato S, Akira S, et al. (2006) TAB1 is a master regulator of epithelial homeostasis involving skin inflammation and apoptosis. J Biol Chem 281: 19610–19617. doi:10.1074/jbc.M603384200

30. Omori E, Morishita S, Matsumoto K, Ninomiya-Tsuji J (2008) TAB1 regulates reactive oxygen species and cell death in keratinocytes, which is essential for skin integrity. J Biol Chem 283: 26161–26168. doi:10.1074/jbc.M804513200

31. Tang M, Wei X, Gao Y, Breslin P, Zhang S, et al. (2008) TAB1 is required for the survival of hematopoietic cells and hepatocytes in mice. J Exp Med 205: 1611–1619. doi:10.1084/jem.20080297

32. Ajibade AA, Wang Q, Cui J, Zou J, Xia X, et al. (2012) TAB1 negatively regulates NF-kappaB and p38 MAP kinase activation in Gr-1+CD11b+ neutrophils. Immunity 36: 41–54. doi:10.1016/j.immuni.2011.12.010

33. Elyha C, Karagouni N, Alexiou M, Apostolakis M, Kollas G (2012) Myeloid TAK1 [corrected] acts as a negative regulator of the LPS response and mediates resistance to endotoxemia. PLoS One 7: e31550. doi:10.1371/journal.pone.0031550

34. Lamothe B, Lai Y, Xie M, Schneider MD, Darnay BG (2013) TAK1 is essential for apoptosis and necroptosis. Mol Cell Biol 33: 582–595. doi:10.1128/MCB.01225-12

35. Inagaki M, Komatsu Y, Scott G, Yamada G, Ray M, et al. (2008) TAK1 generates a conditional mutant allele for Tab1 in mouse. Genesis 46: 431–439. doi:10.1002/dvg.20418

36. Badea TC, Wang Y, Nathans J (2003) A noninvasive genetic/pharmacologic approach to defining cell morphology and clonal relationships in the mouse. J Neurosci 23: 2314–2322. PMID: 12657690.

37. Jadrich JL, O’Connor MB, Crouchman E (2006) The TGF beta activated kinase 1 regulates vascular development in vivo. Development 133: 1529–1541. doi:10.1242/dev.02333

38. Shima JH, Xiao C, Paschal AE, Bailey ST, Rao P, et al. (2005) TAB1, but not TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19: 2668–2681. doi:10.1101/gad.136005

39. Komatsu Y, Shibuya H, Takeda N, Ninomiya-Tsuji J, Yasui T, et al. (2002) Targeted disruption of the Tab1 gene causes embryonic lethality and defects in cardiovascular and lung morphogenesis. Mech Dev 119: 239–249. doi.org/10.1016/S0925-4773(02)00391-X.

40. Higashi AY, Ibawa T, Muramatsu M, Economides AN, Niwa A, et al. (2009) Direct hematopoietic toxicity and illegitimate chromosomal recombination caused by the systemic activation of CreERT2. J Immunol 182: 5633–5640. doi:10.4049/jimmunol.0902413

41. Takaesu G, Inagaki M, Takubo K, Mishina Y, Hess PR, et al. (2009) TAB1 (MAP3K7) signaling regulates hematopoietic stem cells through TNF-dependent and -independent mechanisms. PLoS One 7: e16173. doi:10.1371/journal.pone.0016173

42. Kishimoto K, Matsumoto K, Ninomiya-Tsuji J (2000) TAB1 and TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19: 2668–2681. doi:10.1101/gad.136005

43. Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation: Sepsis and sepsis-like inflammatory conditions. J Immunol 182: 5633–5640. doi:10.4049/jimmunol.0902413

44. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, et al. (2005) TAK1, but not TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19: 2668–2681. doi:10.1101/gad.136005

45. Kohyama M, Ise W, Edelstein BT, Wilker PR, Höhler K, et al. (2009) Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. Nature 457: 318–321. doi:10.1038/nature07472

46. Miyoshi T, Sasaki T, Suzuki A, Sato Y, Sato K, et al. (2008) TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19: 2668–2681. doi:10.1101/gad.136005