The kinase TBK1 functions in dendritic cells to regulate T cell homeostasis, autoimmunity, and antitumor immunity

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Dendritic cells (DCs) are crucial for mediating immune responses but, when deregulated, also contribute to immunological disorders, such as autoimmunity. The molecular mechanism underlying the function of DCs is incompletely understood. In this study, we have identified TANK-binding kinase 1 (TBK1), a master innate immune kinase, as an important regulator of DC function. DC-specific deletion of Tbk1 causes T cell activation and autoimmune symptoms and also enhances antitumor immunity in animal models of cancer immunotherapy. The TBK1-deficient DCs have up-regulated expression of co-stimulatory molecules and increased T cell–priming activity. We further demonstrate that TBK1 negatively regulates the induction of a subset of genes by type I interferon receptor (IFNAR). Deletion of IFNAR1 could largely prevent aberrant T cell activation and autoimmunity in DC conditional Tbk1 knockout mice. These findings identify a DC-specific function of TBK1 in the maintenance of immune homeostasis and tolerance.

INTRODUCTION

The immune system is capable of launching robust responses against invading pathogens while maintaining tolerance to self-antigens. As the primary antigen-presenting cells, DCs are crucial for both stimulating T cell responses to foreign antigens and maintaining immune tolerance to self-antigens (Steinman et al., 2003; Mayer et al., 2012; Hammer and Ma, 2013). Although the tolerant function of DCs is important for preventing autoimmune diseases, it also poses a major obstacle for immune responses against cancer, and a general principal of cancer immunotherapy is to break immune tolerance (Sharma et al., 2011; Maueroerder et al., 2014). DCs sense the environment largely via pattern-recognition receptors (PRRs), which recognize diverse molecular patterns associated with pathogens and commensal microorganisms, as well as self-ligands such as DNAs released from dying cells (van Vliet et al., 2007; Seya et al., 2010; Ahn and Barber, 2014).

During an infection, DCs are stimulated for maturation by pathogen-associated molecular patterns, acquiring the ability to stimulate T cells for immune activation (Dudek et al., 2013; Hammer and Ma, 2013). However, in steady state, DCs undergo partial or homeostatic maturation, characterized by low surface expression of co-stimulatory molecules (e.g., CD80 and CD86), which is important for maintaining peripheral immune tolerance by inducing T cell anergy and promoting regulatory T cell (Treg cell) production (Dhodapkar et al., 2001; Hawiger et al., 2001; Mahnke et al., 2002; Dalod et al., 2014). The signaling network that mediates the tolerant function of DCs has been poorly defined.

TANK-binding kinase 1 (TBK1) along with its homologue IKKε kinase epsilon (IKKe; also known as IKKε) are innate immune kinases that activate the transcription factor IRF3 and, thereby, mediate induction of type I IFNs by various PRR ligands during viral infections (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; McWhirter et al., 2004; Hiscott, 2007). TBK1 and IKKe display redundant or unique functions in IFN induction depending on the cell...
The present study. We demonstrated that DC-specific deletion of Tbk1 in the conventional Tbk1 KO mice (Bonnard et al., 2000). Recent studies demonstrate that Tbk1 can be activated by a large variety of stimulators, including PRR ligands, inflammatory cytokines, and the TNF superfamily of co-stimulatory factors (Clark et al., 2009; Jin et al., 2012; Liu et al., 2015; Yu et al., 2015). Interestingly, Tbk1 activation is insufficient for triggering IFN-γ production and neutralizes the IFN-γ response against tumor challenge. We provide evidence that Tbk1 is dispensable for regulating DC development.

To study the function of Tbk1 in DCs, we generated DC-conditional Tbk1 KO (hereafter called DKO) mice in the present study. We demonstrated that DC-specific deletion of Tbk1 causes aberrant activation of T cells coupled with autoimmune symptoms, including splenomegaly and lymphadenopathy as well as tissue infiltration with lymphocytes. The Tbk1-DKO mice also mounted stronger immune responses against tumor challenge. We provide evidence that Tbk1 has a role in regulating type I IFN receptor (IFNAR) signaling. These findings suggest that Tbk1 is important for DC functions in the maintenance of T cell homeostasis and prevention of autoimmune responses. Our data also suggest that targeting Tbk1 in DCs may be an approach to stimulate antitumor immunity.

RESULTS

Tbk1 is dispensable for DC development

To assess the function of Tbk1 in DCs, we analyzed the activation of Tbk1 in DCs. Phospho-immunoblotting analyses revealed that although BMDCs lacked detectable Tbk1 activation unless stimulated with the TLR4 ligand LPS, splenic DCs displayed a basal level of Tbk1 activation that became even more prominent in older mice, suggesting in vivo activation of Tbk1 under homeostatic conditions (Fig. 1 A). To directly examine the functions of Tbk1 in DCs, we generated Tbk1-DKO mice by crossing the Tbk1-flox mice (Jin et al., 2012) with CD11c-Cre mice (Fig. 1 B). The Tbk1-DKO and WT control mice had similar frequencies of DCs, macrophages, and neutrophils in the bone marrow and spleen, suggesting a dispensable role for DC-specific Tbk1 in the development of these myeloid cells (Fig. 1 C). The frequency of conventional DCs, plasmacytoid DCs, CD8α+ DCs, and the resident and migratory DCs in the cutaneous lymph nodes was also comparable between the WT and Tbk1-DKO mice (Fig. 1, D–G). These data suggested a dispensable role for Tbk1 in regulating DC development.

Tbk1 ablation in DCs perturbs T cell homeostasis and causes spontaneous autoimmunity

DCs play a crucial role in regulating T cell homeostasis, tolerance, and activation. Thus, we analyzed the effect of DC-specific deletion of Tbk1 on T cell homeostasis and activation. The Tbk1 deficiency did not influence T cell development in the thymus, as the Tbk1-DKO and WT control mice had a similar frequency of double-negative and double-positive as well as CD4+ and CD8+ single-positive thymocyte populations (Fig. 2 A). At a young age (10 wk), the Tbk1-DKO and WT control mice also had a similar frequency of total CD4+ and CD8+ T cells in the spleen (not depicted); however, the Tbk1-DKO mice had an increased frequency of activated or memory-like CD4+ and CD8+ T cells displaying CD44loCD62Lhi or CD44hi surface markers (Fig. 2 B). This phenotype became more profound in older Tbk1-DKO mice (8 mo old; Fig. 2 C), coupled with strikingly elevated IFNγ-producing CD4+ and CD8+ effector T cells in the spleen (Fig. 2 D), although the control and Tbk1-DKO mice had a similar frequency of Foxp3+ Treg cells in the thymus, spleen, and lymph nodes (Fig. 2 E). Consistent with their perturbed T cell homeostasis, the older Tbk1-DKO mice displayed autoimmune symptoms characterized by enlargement of spleen and lymph nodes, increased cellularity in these lymphoid organs, and infiltration of lymphocytes to the liver and lung (Fig. 2, F–H). Collectively, these results demonstrated an important role for DC-specific Tbk1 in maintaining T cell homeostasis and preventing autoimmune responses at steady state.

Tbk1 deficiency in DCs sensitizes mice to experimental autoimmunity

To further investigate the role of Tbk1 in regulating immune responses, we examined the effect of DC-specific Tbk1 deletion on tissue-specific autoimmunity using experimental autoimmune encephalomyelitis (EAE), an animal model of the autoimmune neuroinflammatory disease multiple sclerosis (Simmons et al., 2013). The pathogenesis of EAE involves priming of autoimmune T helper type 1 inflammatory T cells (Th1 cells) and Th17 inflammatory T cells in the periphery and their subsequent migration into the central nervous system (CNS). As expected, immunization of WT young adult (8 wk old) mice with a CNS-specific autoantigen, myelin oligodendrocyte glycoprotein (MOG) peptide, induced EAE disease (Fig. 3 A) and CNS infiltration of the IFN-γ–producing Th1 cell and IL-17–producing Th17 cells (Fig. 3, B–E). Importantly, the age-matched Tbk1-DKO mice were more sensitive to EAE induction, exhibiting earlier onset and increased severity of EAE disease (Fig. 3 A). This clinical phenotype was associated with a higher level of CNS infiltration with both total CD4+ and CD8+ T cells (Fig. 3, B and C) and Th1 and Th17 inflammatory effector cells (Fig. 3, D and E) in Tbk1-DKO mice. In addition, the spleen of the EAE-challenged Tbk1-DKO mice had a higher frequency and number of activated T cells, displaying the CD62LloCD44hi and CD69+ surface markers, than that of the WT control mice (Fig. 3, F and G), whereas the frequency of Treg cells was similar between the two genotypes (Fig. 3 H). The draining lymph nodes of the Tbk1-DKO mice also contained a higher frequency and number of Th1 and Th17 inflammatory effector cells (Fig. 3, I and J) and antigen–specific T cells respond-
According to MOG peptide stimulation in vitro (Fig. 3 K). Thus, DC-specific TBK1 negatively regulates induction of EAE by the CNS-specific autoantigen MOG.

**TBK1 regulates antitumor immunity**

A hallmark of cancer is the induction of immune tolerance (Maueröder et al., 2014). Although immune tolerance prevents the development of autoimmunity, it is a major mechanism by which cancer escapes from immune destruction (Maueröder et al., 2014). In fact, the same immune mechanisms that mediate autoimmunity also contribute to the destruction of tumors, with tumor immunotherapy often being viewed as intentional induction of autoimmunity and immune homeostasis raised the intriguing question of whether TBK1 promotes or suppresses the immunostimulatory function of DCs in antitumor immunity. We used an animal model of tumor immunity that involves challenge of mice with mouse B16 melanoma cells. The Tbk1-DKO mice displayed a significantly stronger ability to suppress tumor growth, coupled with increased survival rate upon tumor challenge, compared with the WT control mice (Fig. 4, A and B). Consistently, the Tbk1-DKO mice had increased frequency of tumor-infiltrating CD4+ and CD8+ effector T cells producing IFN-γ (Fig. 4, C and D). The draining lymph nodes of the tumor-bearing Tbk1-DKO mice also contained an increased frequency and number of IFN-γ-positive CD4+ and CD8+ effector T cells compared with those of the WT mice (Fig. 4 E), whereas the frequency of T reg cells was not increased (Fig. 4 F). Parallel studies revealed that the Tbk1-DKO mice also displayed enhanced antitumor immunity in two other tumor models, induced with the EG7-OVA (Fig. 4, G and H) and EL4 (Fig. 4, I and J) thymoma cells, respectively.

Programmed death 1 (PD1) is a tolerance molecule that is expressed on effector T cells and mediates inhibition of antitumor T cell responses, and an anti-PD1 neutralizing antibody has demonstrated promise in cancer immunotherapy (Zou et al., 2016). Because TBK1 functions in DCs to regulate T cell tolerance, we tested whether TBK1 deletion...
Figure 2. **TBK1 deficiency in DCs impairs T cell homeostasis and causes autoimmunity.** (A) Flow cytometric analysis of thymocytes in Tbk1-DKO and WT control mice, with the percentage of double-negative (DN), double-positive (DP), and CD4+ and CD8+ single-positive (SP) populations summarized based on three WT and three Tbk1-DKO mice (8 wk old). Data are presented as means ± SD. (B and C) Flow cytometric analysis of the frequency and absolute number of naive (CD44loCD62Lhi) and memory-like (CD44hiCD62Llo for CD4+ and CD44hi for CD8+ T cells) CD4+ and CD8+ T cells in total splenocytes from WT and Tbk1-DKO mice (10 wk and 8 mo old). Data are presented as representative plots (left) and summary graphs (right). (D) Flow cytometric analysis of the percentage of IFN-γ–producing and IL-17–producing CD4+ and CD8+ T cells in the spleen of 8-mo-old WT and Tbk1-DKO mice. (E) Frequency of CD4+Foxp3+ T reg cells in the thymus (Thy), spleen (Spl), and inguinal lymph nodes (ILN) of 10-wk-old WT and Tbk1-DKO mice, presented as a representative FACS plot (left) and summary graph based on multiple mice (right). (F and G) Representative images (F) and total cell number (G) of spleen, peripheral lymph nodes (pLN), and mesentery lymph nodes (mLN) of WT and Tbk1-DKO mice (8 mo old). (H) Hematoxylin-eosin staining of the indicated tissue sections from 8-mo-old WT and Tbk1-DKO mice, showing immune cell infiltrations in the Tbk1-DKO tissues (arrows). Bars, 100 µm. Data are representative of three or more independent experiments. *, P < 0.05; **, P < 0.01.
could synergize with the action of anti-PD1 in the induction of tumor rejection using the B16 melanoma model. In this experiment, we used B16F10 melanoma cells (lacking the surrogate antigen OVA), which are known as a low immunogenicity tumor model. Under these conditions, the DC-specific TBK1 deletion promoted tumor rejection to an extent that was similar to that caused by anti-PD1 injection (Fig. 4 K). Remarkably, the combination of anti-PD1 treatment and TBK1 deletion resulted in a strong synergistic effect leading to profoundly reduced tumor growth and increased mouse survival (Fig. 4, K and L).
Figure 4. **TBK1 deficiency in DCs promotes antitumor immunity.** (A and B) Tumor growth (A) and survival (B) curves of WT and Tbk1 DKO mice injected s.c. with B16-OVA melanoma cells. n = 10. (C and D) Flow cytometric analysis of the frequency (C) and absolute cell numbers (D) of IFN-γ–producing CD4+ and CD8+ T cells in tumors of WT and Tbk1-DKO mice injected s.c. with B16-OVA melanoma cells (day 16 after injection). (E and F) Flow cytometric analysis of the percentage and absolute cell numbers of IFN-γ–producing CD4+ and CD8+ T cells (E) or percentage of T reg cells (F) in draining lymph nodes of WT and Tbk1-DKO mice injected s.c. with B16-OVA melanoma cells (day 16 after injection). Cells were stimulated with OVA peptide for 5 h after intracellular staining. (G–J) Tumor growth (G and I) and flow cytometric analysis of IFN-γ–producing CD4+ and CD8+ T cells in draining lymph nodes (H and J) of WT and Tbk1-DKO mice injected s.c. with EG7-OVA (G and H) or EL4 (I and J) thymoma cells (day 22 for EG7-OVA and day 16 for EL4 after injection). (K and L) Tumor growth (K) and survival (L) curves of WT and Tbk1-DKO mice injected s.c. with B16 melanoma cells without the surrogate antigen OVA (n = 10) followed by i.p. injection with PD-1 antibody on days 7, 10, and 13. Ctrl, control. (M) Tumor growth curve of WT mice injected s.c. with B16-OVA melanoma cells and then treated (on day 7) i.v. with WT or Tbk1-DKO BMDCs that were pulsed with OVA peptide and matured with LPS. (N) Flow cytometric analysis of the ab-
WT and TBK1-deficient DCs displayed similar homing patterns (Fig. 4, O and P). Collectively, these results suggest that targeting TBK1 in DCs may be an approach to promote antitumor immunity.

**TBK1 deficiency promotes the immunostimulatory functions of DCs**

To understand the mechanism by which TBK1 regulates DC functions in antitumor immunity, first, we examined TBK1 activation in DCs of tumor-bearing mice based on its phosphorylation. Consistent with the phospho-immunoblot assays (Fig. 1 A), flow cytometry detected TBK1 phosphorylation in lymph node DCs of untreated mice, and the level of TBK1 phosphorylation was elevated in draining lymph node DCs of B16 tumor-bearing mice (Fig. 5 A). Interestingly, TBK1 deficiency caused an increase in the expression of the co-stimulatory molecules CD80 and CD86 in splenic DCs of untreated mice, and this phenotype was more profound in DCs isolated from tumors of B16 tumor-bearing mice (Fig. 5, B and C). This finding prompted us to examine whether the TBK1 deficiency promoted the T cell–priming function of DCs. We used an in vitro model involving activation of OTII T cells with DCs pulsed with a specific peptide, chicken OVA 323–339. For measuring cell proliferation, the OTII T cells were labeled with CFSE. Compared with WT DCs, the TBK1-deficient DCs stimulated stronger proliferation of OTII T cells, as measured based on CFSE dilution (Fig. 5 D). These results suggest that TBK1 deficiency promotes the immunostimulatory function of DCs.

**TBK1 regulates a subset of IFN-responsive genes in DCs**

To further elucidate the molecular mechanism by which TBK1 regulates DC functions, we examined the effect of TBK1 deficiency on gene induction by the TLR4 ligand LPS. As expected, LPS-stimulated expression of Ifna and Ifnb genes was impaired in TBK1-deficient DCs (Fig. 5 E). Moreover, the TBK1 deficiency inhibited the induction of the immunosuppressive cytokine IL-10 and promoted the induction of several immunostimulatory cytokines, including IL-1β, IL-12, and IL-23 (Fig. 5 E).

Although the in vitro studies provided insight into the role of TBK1 in regulating TLR-stimulated gene expression, it was still unclear how TBK1 regulated the DC gene expression profile under homeostatic conditions in vivo. To this end, we analyzed the gene expression profile of WT and TBK1-deficient splenic DCs by RNA sequencing. This experiment, based on three independent samples, revealed significant alterations in the expression of several genes (Fig. 5, F and G), most notably the enhanced expression of a large subset of IFN-responsive genes in the TBK1-deficient DCs (Fig. 5 H). As a previous study identified a DC-specific role for the NF-κB–activating kinase IKKβ in regulating immune homeostasis and tolerance (Baratin et al., 2015), we examined the possible connection of TBK1 with the NF-κB pathway. We analyzed the RNA sequencing data for NF-κB gene signature and included the IFN-responsive gene Ifi202b as a control. Unlike Ifi202b, the NF-κB signature genes were not substantially affected by the TBK1 deficiency (Fig. 5 I). The TBK1 deficiency also did not inhibit TLR-stimulated activation of NF-κB or mitogen-activated protein kinases (not depicted). To further confirm the role of TBK1 in regulating IFN-responsive gene expression, we performed quantitative RT-PCR (qRT-PCR) assays using freshly isolated WT and TBK1-deficient spleen DCs. Several, although not all, of the IFN-responsive genes tested were found to be up-regulated in the TBK1-deficient DCs (Fig. 5 J). We found that the TBK1 deficiency did not significantly affect the homeostatic expression level of Ifna and Ifnb genes (Fig. 5 J), suggesting a novel function of TBK1 in regulating IFN receptor signaling. Indeed, upon in vitro starvation and restimulation with IFN-β, the TBK1-deficient DCs also expressed profoundly higher levels of IFN-responsive genes than the WT control DCs (Fig. 5 K). These results are intriguing as type I IFN signaling is known to promote antitumor immunity and mediate systemic autoimmunity (Hall and Rosen, 2010; Fuertes et al., 2013).

**Deletion of IFNAR1 prevents aberrant T cell activation and autoimmunity in TBK1 DKO mice**

To examine whether TBK1 functions in conjunction with type I IFN signaling in the regulation of immune tolerance, we crossed the Tbk1-DKO mice with Ifnar1-KO mice. As expected, DC-specific deletion of TBK1 in IFNAR-WT genetic background caused a drastic increase in the frequency of CD4+CD62Lhi effector/memory-like CD4+ T cells producing the effector cytokine IFN-γ (Fig. 6, A and B). Deletion of IFNAR1 did not substantially alter the frequency of effector/memory-like CD4+ T cells or IFN-γ–producing CD4+ T cells. However, the IFNAR1 ablation largely corrected the aberrant T cell activation phenotype of the Tbk1-DKO mice (Fig. 6, A and B). The IFNAR1 deficiency also suppressed the autoimmune symptoms of the Tbk1-DKO mice, including splenomegaly and lymphadenopathy (Fig. 6 C) and immune cell infiltration into the liver and lung (Fig. 6 D).

We also examined the role of IFN signaling in the antitumor immunity of TBK1-deficient DCs by performing DC-based tumor immunotherapy. Under these conditions, the IFNAR1 deficiency only moderately reduced the tumor
suppression function of the DCs, whereas TBK1 deficiency greatly enhanced the tumor-suppression function of the DCs (Fig. 6 E). Moreover, in the *Ifnar1*-null background, the *Tbk1*-DKO and WT mice no longer had differences in mediating tumor rejection (Fig. 6 E). Collectively, these results suggest that the DC-specific functions of TBK1 involve modulation of IFNα/β signaling.

**TBK1 mediates STAT3 serine phosphorylation**

A major signaling event induced by type I IFNs is activation of STAT1 via its phosphorylation at tyrosine 701 (Y701) and serine 727 (S727; Ivashkiv and Donlin, 2014). Under homeostatic conditions, STAT1 was constitutively, although moderately, phosphorylated at Y701, which appeared to be dependent on type I IFNs, as the STAT1 Y701 phosphorylation was abolished in IFNα-deficient DCs (Fig. 7 A, left). Moreover, TBK1 deficiency caused an increase in STAT1 Y701 phosphorylation, but it did not affect the S727 phosphorylation of STAT1 (Fig. 7 B, left). Because TBK1 is a serine/threonine kinase, the enhanced STAT1 Y701 phosphorylation was obviously caused by indirect effect. In this regard, STAT3 is known to be activated by type I IFNs and inflammatory cytokines and be involved in negative regulation of STAT1 activation and type I IFN responses (Wang et al., 2011; Ivashkiv and Donlin, 2014).
Furthermore, STAT3 is crucial for the tolerant function of DCs, and DC-conditional deletion of STAT3 in mice results in T cell activation and autoimmunity (Cheng et al., 2003). Thus, we examined STAT3 activation based on its phosphorylation at Y705 and S727. Interestingly, DCs displayed a high level of S727 phosphorylation, although only moderate Y705 phosphorylation, and of STAT3, and the STAT3 S727 phosphorylation was partially inhibited in IFNAR1-deficient DCs (Fig. 7 A, right). Moreover, the TBK1 deficiency severely attenuated the S727 phosphorylation of STAT3 in DCs (Fig. 7 B, right). This intriguing result prompted us to determine the role of DC-specific STAT3 in regulating IFN-responsive genes and immune tolerance using DC-conditional Stat3 KO (Stat3-DKO) mice. Similar to the TBK1-deficient DCs, the STAT3-deficient DCs had up-regulated expression of several IFN-responsive genes (Fig. 7 C). Furthermore, as seen with the Tbk1-DKO mice, the Stat3-DKO mice had increased frequency of CD4+ and CD8+ T cells with activated phenotype (Fig. 7 D).

Figure 6. Deletion of IFNAR1 prevents autoimmunity and suppresses antitumor immunity in Tbk1-DKO mice. (A) Frequency of naive (CD44loCD62Lhi) and memory (CD44hiCD62Llo) CD4+ T cells and IFN-γ-producing CD4+ T cells in the spleen of age- and sex-matched mice with the indicated genotype (4 mo old). (B) Summary data of flow cytometric analysis of memory CD4+ (CD4+CD44hiCD62Llo) and CD8+ (CD8+CD44hi) T cells in the spleen described in A. (C) Representative spleen and peripheral lymph node (pLN) images of age- and sex-matched mice with the indicated genotype (4 mo old). (D) Hematoxylin-eosin staining of the indicated tissue sections from age- and sex-matched mice (5 mo old), showing immune cell infiltrations in the Tbk1-DKO tissues (arrows). Bars, 100 µm. (E) Growth curve of tumors (n = 5) of WT mice that were injected s.c. with B16-OVA melanoma cells and then treated i.v. by BMDCs with the indicated genotype that were pulsed with OVA peptide and matured with LPS. Data are representative of at least three independent experiments and are presented as means ± SD. *, P < 0.05; **, P < 0.01.
Figure 7. **TBK1 mediates STAT3 phosphorylation in DCs.** (A and B) Flow cytometric analysis of tyrosine (Y701) and serine (S727) phosphorylation of STAT1 and STAT3 in freshly isolated splenic CD11c+ DCs from 8-wk-old Ifnar+/+ and Ifnar−/− mice (A) or 8-wk-old WT and Tbk1-DKO mice (B). (C) qRT-PCR analysis of the indicated genes in freshly isolated splenic CD11c+ DCs from age- and sex-matched WT and Stat3-DKO mice (8 wk old). (D) Flow cytometric analysis of naive (CD44loCD62Lhi) and memory-like (CD44hiCD62Llo) CD4+ and CD8+ T cells in splenocytes from age- and sex-matched WT and Stat3-DKO mice (3 mo old). (E) Sequence alignment of the S727 phosphorylation site of STAT3 with the phosphorylation site of several known TBK1 substrate proteins. Conserved residues are shown in red, and the phosphorylation serine is underlined. MAVS, mitochondrial antiviral-signaling protein. (F) Lysates of WT or Tbk1-DKO splenic DCs were subjected to IP using anti-TBK1 or a control Ig (Ctrl); TBK1 and TBK1-associated STAT3 were detected by immunoblotting (IB). (G) Immunoblot analysis of STAT3 phosphorylation in HEK293T cells transfected with STAT3 along with either TBK1 or a catalytically inactive TBK1 mutant (TBK1M). (H) Immunoblot analysis of S727 phosphorylated STAT3, total STAT3, and loading control HSP60 in BMDCs prepared from STAT3-WT, Stat3-DKO, or STAT3-S727A mutant (STAT3-SA) mice, either not treated (NT) or stimulated with LPS. (I) Tumor growth curve of WT mice injected s.c. with B16-OVA melanoma cells and then injected i.v. (on day 7) with BMDCs described in H that were pulsed with OVA peptide and matured with LPS. (J and K) Tumor-infiltrating CD4+ (J) and CD8+ (K) T cells from the tumor-bearing mice described in I, presented as representative FACS plots (left) and summary graphs based on multiple mice (right). Data are representative of three or more independent experiments and are presented as means ± SD. *, P < 0.05; **, P < 0.01.
proteins (Liu et al., 2015). Interestingly, the serine phosphorylation site (S727) of STAT3 also has such a conserved sequence motif (Fig. 7 E). TBK1 physically interacted with STAT3, as shown by a communoprecipitation (co-IP) assay (Fig. 7 F). Furthermore, TBK1, but not a catalytically inactive TBK1 mutant, potently phosphorylated S727 of STAT3 (Fig. 7 G). Using a DC-based tumor therapy model, we found that DCs deficient in STAT3 or expressing S727A mutant of STAT3 displayed significantly higher tumor-suppressing activity (Fig. 7, H and I). The STAT3-KO and STAT3-5A DCs also induced a significantly higher level of tumor-infiltrating CD4+ and CD8+ T cells producing IFN-γ compared with the WT DCs (Fig. 7, J and K). These results indicated that the role of TBK1 in regulating DC functions involves phosphorylation of STAT3, although the possibility for TBK1 to target additional signaling factors in DCs cannot be excluded.

**DISCUSSION**

TBK1 and IKKe are known as kinases that mediate induction of type I IFNs in response to various PRR signals (Hiscott, 2007). In addition to the PRR ligands, many other agents, such as inflammatory cytokines, the TNF superfamily of co-stimulatory factors, and T cell receptor stimuli, stimulate TBK1 activation, although not all of these agents induce type I IFN expression (Clark et al., 2009; Jin et al., 2012; Liu et al., 2015; Yu et al., 2015). Emerging evidence suggests that TBK1 has additional roles in the regulation of immune system functions (Jin et al., 2012; Yu et al., 2015). Our present study demonstrated a DC-specific function of TBK1 in the regulation of immune homeostasis and tolerance. DC-specific deletion of TBK1 caused aberrant activation of T cells, a phenotype that became particularly striking in older mice. Consistently, the older Tbk1-DKO mice displayed overt autoimmune symptoms, and the young Tbk1-DKO mice were also more sensitive to the induction of a T cell–dependent experimental autoimmune disease, EAE.

A major phenotype of the Tbk1-DKO mice was the perturbation of T cell homeostasis and spontaneous development of autoimmune at older ages. The TBK1 deficiency in DCs did not alter the frequency of T reg cells in the thymus, spleen, and lymph nodes. Although it remains to be examined whether T reg function is impaired in the Tbk1-DKO mice, DCs are known to mediate immune tolerance by both promoting T reg cell induction and inducing T cell anergy (Mahnke et al., 2002). It is thought that DCs with immature or semi-mature phenotypes, characterized by low surface expression of co-stimulatory molecules and MHC class II, are important for maintaining immune tolerance (Mahnke et al., 2002; Dalod et al., 2014). We found that TBK1-deficient DCs had elevated levels of CD80 and CD86 under both homeostatic and tumor-challenged conditions and displayed stronger ability to prime T cells in vitro. The perturbed immune homeostasis may also contribute to the enhanced sensitivity of the Tbk1-DKO mice to the inducible autoimmune EAE. Similarly, because interruption of immune tolerance promotes antitumor immunity, the perturbed immune homeostasis and tolerance also likely contributed the enhanced antitumor immunity in the Tbk1-DKO mice.

A recent study suggests that the IKK/NF-κB signaling pathway functions in DCs to regulate immune tolerance (Baratin et al., 2015). By performing RNA sequencing, we demonstrated that TBK1 deficiency in DCs had no obvious effect on the expression of NF-κB signature, which was consistent with the involvement of TBK1 in IRF3 but not NF-κB signaling pathways. Under homeostatic conditions, the TBK1 deletion in DCs also did not influence the expression of type I IFNs. Interestingly, the TBK1-deficient DCs displayed a gene expression signature characterized by up-regulated expression of a subset of IFN-responsive genes. The TBK1-deficient DCs also had several down-regulated genes, most notably Adamdec1 (unpublished data), which encodes metzincin metallopeptase known to be involved in regulation of intestinal immunity and inflammation (O’Shea et al., 2016). The role of Adamdec1 in DC function remains to be further studied. Nevertheless, the RNA-sequencing results uncovered an intriguing function of TBK1 in regulating IFN-AR1 signaling. When stimulated in vitro, the TBK1-deficient DCs were also hyper-responsive to IFN-β–induced gene expression, further emphasizing a negative function of TBK1 in regulating IFNAR signaling. Moreover, deletion of IFN AR1 largely corrected the abnormalities of the Tbk1-DKO mice in T cell homeostasis and inhibited the development of spontaneous autoimmunity. These results suggest TBK1-mediated negative regulation of IFNAR signaling contributes to the maintenance of T cell homeostasis and immune tolerance.

A major signaling event resulting from the binding of type I IFNs to IFNAR is activation of the transcription factor STAT1. In splenic DCs, STAT1 is constitutively phosphorylated at Y701 in an IFNAR-dependent manner. Consistent with the up-regulated expression of IFN-responsive genes, the Tbk1-deficient DCs had enhanced phosphorylation of STAT1 at Y701. However, because TBK1 has no tyrosine kinase activity, this result was obviously caused by an indirect effect of the TBK1 deficiency. In this regard, STAT3 has been shown to negatively regulate IFN responses and attenuate the induction of a subset of STAT1-target genes (Ho and Ivashkiv, 2010; Wang et al., 2011). STAT3 deletion in DCs breaks immune tolerance and causes T cell activation in mice (Cheng et al., 2003; Melillo et al., 2010; Li et al., 2016). STAT3 is activated by inflammatory cytokines and type I IFNs via phosphorylation at both Y705 and S727 (Ng and Cantrell, 1997; Shen et al., 2004). Our data suggest that TBK1 may function as a kinase mediating S727 phosphorylation of STAT3. Loss of TBK1 in DCs reduced STAT3 S727 phosphorylation, and overexpressed TBK1 could phosphorylate STAT3 S727. Interestingly, S727 of STAT3 is located in a sequence motif that shares homology with the typical TBK1 phosphorylation sites in other proteins.

In conclusion, we demonstrated a DC-specific role for TBK1 in regulating T cell homeostasis and autoimmune
responses. Our data also suggest that targeting TBK1 in DCs promotes antitumor immunity, implicating TBK1 as a potential therapeutic target in DC-based cancer immunotherapy. These findings provide additional evidence that TBK1 is a kinase with diverse immunoregulatory functions in addition to mediating antiviral innate immunity.

**MATERIALS AND METHODS**

**Mice**

Tbk1-floxed mice (in C57BL/6–129S background) were generated using a LoxP targeting system (Taconic; Jin et al., 2012). The Tbk1-floxed mice were crossed with CD11c-Cre transgenic mice (The Jackson Laboratory) in B6 background to produce age-matched Tbk1+/+CD11cCre (termed WT) and Tbk1fl/flCD11cCre (termed Tbk1-DKO) mice for experiments. In some experiments, these mice were further crossed with the Ifnar−/− mice to generate Tbk1WTIfnarKO and Tbk1fl/flIfnarKO mice for experiments. In some experiments, these mice were further crossed with the Stat3−/− mice to generate Tbk1+/+CD11cCre (termed WT) and Tbk1fl/flCD11cCre (termed WT) and Tbk1fl/flCD11cCre (termed Stat3-DKO) mice for experiments. Mice carrying STAT3 S727A mutation (called STAT3-SA mice) were as previously described (Shen et al., 2004). Mice were maintained in a specific pathogen–free facility, and all animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

**Plasmids, antibodies, and reagents**

FLAG-tagged STAT3 (STAT3-Flag pRC/CMV) was obtained from Addgene. FLAG–TBK1 and its catalytically inactive mutant (K38A) were provided by C Wang (Shanghai Institutes for Biological Sciences, Shanghai, China). Antibodies for TBK1, STAT3, phospho-STAT3 (Ser727), and phospho-STAT1 (Tyr701) were purchased from Cell Signaling Technology. Antibodies used for flow cytometry were from eBioscience. Anti–IFN-γ and IL-17. Cells derived from EAE-induced mice were stimulated with the MOG35–55 peptide (MEVGWYRSPFSRVVHYLRNGK) for 16 h (monensin added in the last 4 h); cells derived from mice challenged with OVA-expressing tumor cells were stimulated for 16 h (monensin added in the last 4 h) with the MHC I–restricted OVA257–264 peptide (SIINFEKL) for analysis of CD8+ T cells or the MHC II–restricted OVA223–239 peptide (ISQAVHAAHINEAGR) for analysis of CD4+ T cells; and cells derived from other tumor-bearing mice were stimulated for 4 h with PMA plus ionomycin in the presence of monensin and then subjected to intracellular cytokine staining to detect T cells producing IFN-γ and IL-17. Cells derived from EAE-induced mice were stimulated with the MOG35–55 peptide (MEVGWYRSPFSRVVHYLRNGK) for 16 h (monensin added in the last 4 h); cells derived from mice challenged with OVA-expressing tumor cells were stimulated for 16 h (monensin added in the last 4 h) with the MHC I–restricted OVA257–264 peptide (SIINFEKL) for analysis of CD8+ T cells or the MHC II–restricted OVA223–239 peptide (ISQAVHAAHINEAGR) for analysis of CD4+ T cells; and cells derived from other tumor-bearing mice were stimulated for 4 h with PMA plus ionomycin in the presence of monensin. After permeabilization and fixation, the cells were stained with the indicated antibodies and subjected to flow cytometry analyses.

**Histology**

Organs were removed from WT or Tbk1-DKO mice, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for staining with hematoxylin and eosin.

**Induction and assessment of EAE**

For active EAE induction, age- and sex-matched mice (8–10 wk) were immunized i.v. with 200 µg MOG35–55 peptide mixed in CFA (Sigma-Aldrich) containing 5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (Difco). 200 ng pertussis toxin (List Biological Laboratories) in PBS was administered i.v. on days 0 and 2. Mice were examined daily and scored for disease severity using the standard scale: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness and incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or death. After the onset of EAE, food and water were provided on the cage floor.

**Flow cytometry**

Cell suspensions were subjected to flow cytometry analyses as previously described (Reiley et al., 2006) using an LSR II flow cytometer (BD).

**Isolation and analysis of CNS mononuclear cells**

For preparation of CNS mononuclear cells, brains and spinal cords from MOG35–55–immunized mice were excised and dissociated for 45 min at 37°C by digestion with 2 mg/ml collagenase IV (Sigma–Aldrich) and 100 µg/ml DNase I (Sigma–Aldrich) in DMEM. Dispersed cells were isolated through a Percoll density gradient and collected on the interface fraction between 37 and 70% Percoll. After intensive washing, suspensions of cells were subjected to flow cytometry.

**Intracellular cytokine staining**

Mononuclear cells were isolated from spleen, draining lymph nodes, CNS tissues, or tumor tissues of the indicated mice, stimulated in vitro with peptide antigens or PMA plus ionomycin in the presence of monensin, and then subjected to intracellular cytokine staining to detect T cells producing IFN-γ and IL-17. Cells derived from EAE-induced mice were stimulated with the MOG35–55 peptide (MEVGWYRSPFSRVVHYLRNGK) for 16 h (monensin added in the last 4 h); cells derived from mice challenged with OVA-expressing tumor cells were stimulated for 16 h (monensin added in the last 4 h) with the MHC I–restricted OVA257–264 peptide (SIINFEKL) for analysis of CD8+ T cells or the MHC II–restricted OVA223–239 peptide (ISQAVHAAHINEAGR) for analysis of CD4+ T cells; and cells derived from other tumor-bearing mice were stimulated for 4 h with PMA plus ionomycin in the presence of monensin. After permeabilization and fixation, the cells were stained with the indicated antibodies and subjected to flow cytometry analyses.

**Generation of BMDCs**

Bone marrow cells isolated from WT and Tbk1-DKO mice were cultured for 7 d in RPMI 1640 medium containing 10% FBS supplemented with 10 ng/ml GM-CSF. The differentiated BMDCs were stained with Pacific blue–conjugated anti-CD11c and isolated by flow cytometry.

**Isolation of splenic DCs**

Splenic DCs were isolated essentially as described previously (Tavernier et al., 2015). In brief, spleens from WT or Tbk1-DKO mice were digested with 0.05% collagenase D (Sigma–Aldrich) and 100 µg/ml DNase I (Sigma–Aldrich) in RPMI 1640 medium for 30 min at 37°C. The cell suspension was spun down and resuspended in a red blood cell
alysis buffer (Sigma-Aldrich) to remove red blood cells. For enrichment of DCs, the cell suspension was stained with anti-CD3 FITC and anti-CD19 FITC antibodies and then incubated with anti-FITC microbeads to eliminate non-DCs by passing through magnetic-activated cell-sorting LD columns (Miltenyi Biotec). The effluent cells (DCs) were stained with anti-CD11c Pacific blue antibody and further purified by flow cytometric cell sorting using a FACSAria II flow cytometer (BD).

In vitro T cell activation assay

Splenic DCs isolated from WT and Tbk1-DKO mice were incubated with 10 µg/ml OVA overnight and matured with 100 ng/ml LPS for 6 h. The pretreated DCs (2 × 10^3) were incubated with 2 × 10^5 CFSE-labeled naive OTII CD4+ T cells (CD44^loCD62L^hi) for 72 h and then subjected to flow cytometric analysis to measure the proliferation of OTII T cells based on CFSE dilution.

Tumor models

B16-F10 and B16-OVA (B16-expressing OVA) melanoma cells were cultured in DMEM supplemented with 10% FBS; EL4 and E.G7-OVA cells (a derivative of EL4 cells expressing OVA) were maintained in RPMI 1640 medium supplemented with 10% FBS. These tumor cells were injected s.c. into 8-wk-old WT and Tbk1-DKO mice (5 × 10^5 for B16 and B16-OVA cells and 10^5 for EL-4 and E.G7-OVA cells). The challenged mice were monitored for tumor growth, and the tumor size was expressed as tumor area. To minimize individual variations, age- and sex-matched, mostly littermate, WT, and Tbk1-DKO mice were used.

In tumor models involving DC-based therapy, B16-OVA tumor-bearing WT mice (day 7 after tumor cell inoculation) were injected via tail vein with WT or Tbk1-DKO BMDCs pulsed with 10 µg/ml OVA overnight and matured with 100 ng/ml LPS for 6 h. Tumor growth was monitored every other day. For analyzing DC homing properties, the OVA-pulsed and LPS-matured WT and Tbk1-DKO BMDCs were labeled with 1 µM CFSE and efluor450 (Thermo Fisher Scientific), respectively, washed three times, and then adoptively transferred as a 1:1 ratio mixture into the B16-OVA tumor-bearing WT mice. After 18 h, flow cytometry was performed to track the migration of WT and DKO DCs to the spleen, draining lymph nodes, and tumors.

RNA-sequencing analysis

Fresh splenic DCs were isolated from young WT and Tbk1-DKO mice (6–8 wk old), used for total RNA isolation with TRIzol (Invitrogen), and subjected to RNA-sequencing analysis. RNA sequencing was performed by the MD Anderson Cancer Center Sequencing and Microarray Facility using an Illumina sequencer. The raw reads were aligned to the mm10 reference genome (build mm10), using TopHat2 RNASeq alignment software. The mapping rate was 70% overall across all the samples in the dataset. HTseq-Count was used to quantify the gene expression counts from TopHat2 alignment files. Differential expression analysis was performed on the count data using R package DESeq2. P-values obtained from multiple binomial tests were adjusted using false discovery rate (Benjamini-Hochberg). Significant genes are defined by a Benjamini-Hochberg corrected p-value of cut-off of 0.05 and fold-change of at least two. RNA-sequencing data were deposited to Gene Expression Omnibus under accession number GSE94543.

qRT-PCR

Real-time qRT-PCR was performed as previously described (Chang et al., 2009) using gene-specific primer sets (Table 1). Gene expression was assessed in triplicate and normalized to a reference gene, Actb.

Table 1. Gene-specific primers used for quantitative PCR

| Gene | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|------|------------------------|-----------------------|
| Ifna | TGCACCTAACAGCCTGTGTATG | AAGTTTTTTCCCTCACAGCACG |
| Ifnb | AGCTCAAAGAAAGGACAGACATC | GCCCGTGAGTGAGCTGGTGGCTC |
| Ir7  | CCAGGACTGCTGTTTGAGAC  | AACGAGCTACCAATTCAGGGG |
| Ir1  | CACACACAGAGCATACAGC  | AGGCTCTTCTCAGCCGAGTTA |
| Akt1 | CCACATTTCTCAGCCAGT  | AGGATGCCTGATACAGGGTTA |
| If202b | CGTAAAAGAGTTAGCAGCACAGCT  | ATGAGAAATGCACCTAGTGGTAT |
| Slfn6 | CGGCGCAAAACACCGTTGTT | GAACAGCAAGGCTGGTTTCT |
| Oas2 | TTTACCCCAAAAAAGATTCCGCC | ATGCTGCAGTGGCTGTTAT |
| Oas3 | CCTCAAGACCTCTTCAGCC | GGTGGTTCAGTTAAAAAGAG |
| If10 | CAGAGCCACATGCTCCTTAA | GTCGTTGGTACATTCAAAGAC |
| Ifb  | TGACAAAGATGAGTGGCTGCC | CACGTTGACAGTTACAGGG |
| Tnf  | CATCTTTTTCAAAAAATTCGAGTCACA | CAGCTGTCCTCCACACTTG |
| If23a | CTTAATGCTGATGAAAGTTT | CTTAAGAGGATTAGAGAGCCT |
| If22a | AACTGACAGACTTCTCCACAAACAA | CACAGGAGCTCATAAAGAC |
| If22b | GAGACACACAGCAGAAAAAGAT | TCCGACATGACCTCAGG |
| If3b | AAGCCTCGTGCTGGCACC | TGGAGCGCAAACCGCACAGT |
**Immunoblotting and IP**

Total cell protein extracts were prepared in radio-IP assay buffer and subjected to immunoblotting and IP assays as described previously (Chang et al., 2009).

**Statistical analysis**

One-way ANOVA, where applicable, was performed to determine whether an overall statistically significant change existed before the Student’s t test to analyze the difference between any two groups. Data are presented as means ± SD. A p-value <0.05 is considered statistically significant.

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Author contributions: Y. Xiao, Q. Zou, and X. Xie designed and performed the experiments, prepared the figures, and wrote the manuscript. T. Liu, H.S. Li, Z. Jie, J. Jin, H. Hu, X. Cheng, and H. Wang contributed to the performance of the experiments. G. Manyam and L. Zhang contributed to the microarray analysis. I. Marie and D.E. Levy contributed critical reagents. S.S. Watowich was involved in supervision of H.S. Li, and S.-C. Sun supervised the work and wrote the manuscript.

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