

TWEAK Attenuates the Transition from Innate to Adaptive Immunity

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SUMMARY

Innate immunity is the first line of defense against infection, protecting the host during the development of adaptive immunity and critically affecting the nature of the adaptive response. We show that, in contrast to tumor necrosis factor α (TNF- α), the related protein TWEAK attenuates the transition from innate to adaptive mechanisms. *TWEAK*^{-/-} mice had overabundant natural killer (NK) cells and displayed hypersensitivity to bacterial endotoxin, with their innate immune cells producing excess interferon (IFN)- γ and interleukin (IL)-12. TWEAK inhibited stimulation of the transcriptional activator STAT-1 and induced p65 nuclear factor (NF)- κ B association with histone deacetylase 1, repressing cytokine production. *TWEAK*^{-/-} mice developed oversized spleens with expanded memory and T helper 1 (T_H1) subtype cells upon aging and mounted stronger innate and adaptive T_H1-based responses against tumor challenge. Thus, TWEAK suppresses production of IFN- γ and IL-12, curtailing the innate response and its transition to adaptive T_H1 immunity.

INTRODUCTION

Host defense against infection requires integrated function of the innate and adaptive immune systems. The innate system, which is based on NK cells, dendritic cells, macrophages, and neutrophils, plays a crucial role not only in the early response to infection but also in guiding the transition to a T and B cell-based adaptive immunity (Diefenbach

and Raulet, 2002). Innate immune cells mediate direct killing and elimination of infected cells; subsequently, they provide active support for development of adaptive functions through physical interactions with dendritic cells and consequent secretion of specific cytokines (Diefenbach and Raulet, 2001; Fernandez et al., 2002; Ikeda et al., 2002). IFN- γ and IL-12 polarize the development of helper CD4⁺ T cells toward the T helper 1 (T_H1) subtype, which activates CD8⁺ effector T cell responses, while IL-4 induces the T_H2 class, which stimulates B cell-mediated antibody responses (Diefenbach and Raulet, 2002; Fernandez et al., 2002; Ikeda et al., 2002). Few other cytokines that mediate the influence of the innate system on the subsequent adaptive response are known.

Various members of the tumor necrosis factor (TNF) cytokine superfamily regulate development, homeostasis, and function of the immune system (Locksley et al., 2001). TWEAK (TNF-related weak inducer of apoptosis, also known as Apo3L or TNFSF12) was first described as an inducer of apoptosis in transformed cell lines (Chicheportiche et al., 1997; Marsters et al., 1998). Several hematopoietic tissues express the *TWEAK* mRNA (Chicheportiche et al., 1997; Marsters et al., 1998). Human peripheral-blood monocytes express TWEAK protein and upregulate its production following IFN- γ stimulation (Nakayama et al., 2000). TWEAK binds with high affinity to a receptor known as fibroblast growth factor-inducible 14 kDa protein (FN14, also called TWEAK-R), which is distantly related to the TNF receptor (TNFR) superfamily (Wiley et al., 2001). FN14 contains a single cysteine-rich domain in its extracellular region and a TNFR-associated factor binding motif in its intracellular portion. TWEAK promotes the nuclear translocation of both classical and alternative NF- κ B pathway subunits (Chicheportiche et al., 1997; Marsters et al., 1998; Saitoh et al., 2003). TWEAK also stimulates endothelial cell growth and angiogenesis in certain model systems (Jakubowski et al., 2002; Lynch et al., 1999). To investigate TWEAK's biological role in immunity, we generated *TWEAK* gene knockout mice and studied their innate and adaptive responses. Our results suggest that TWEAK controls the innate inflammatory response as well as the transition to T_H1-based adaptive immunity.

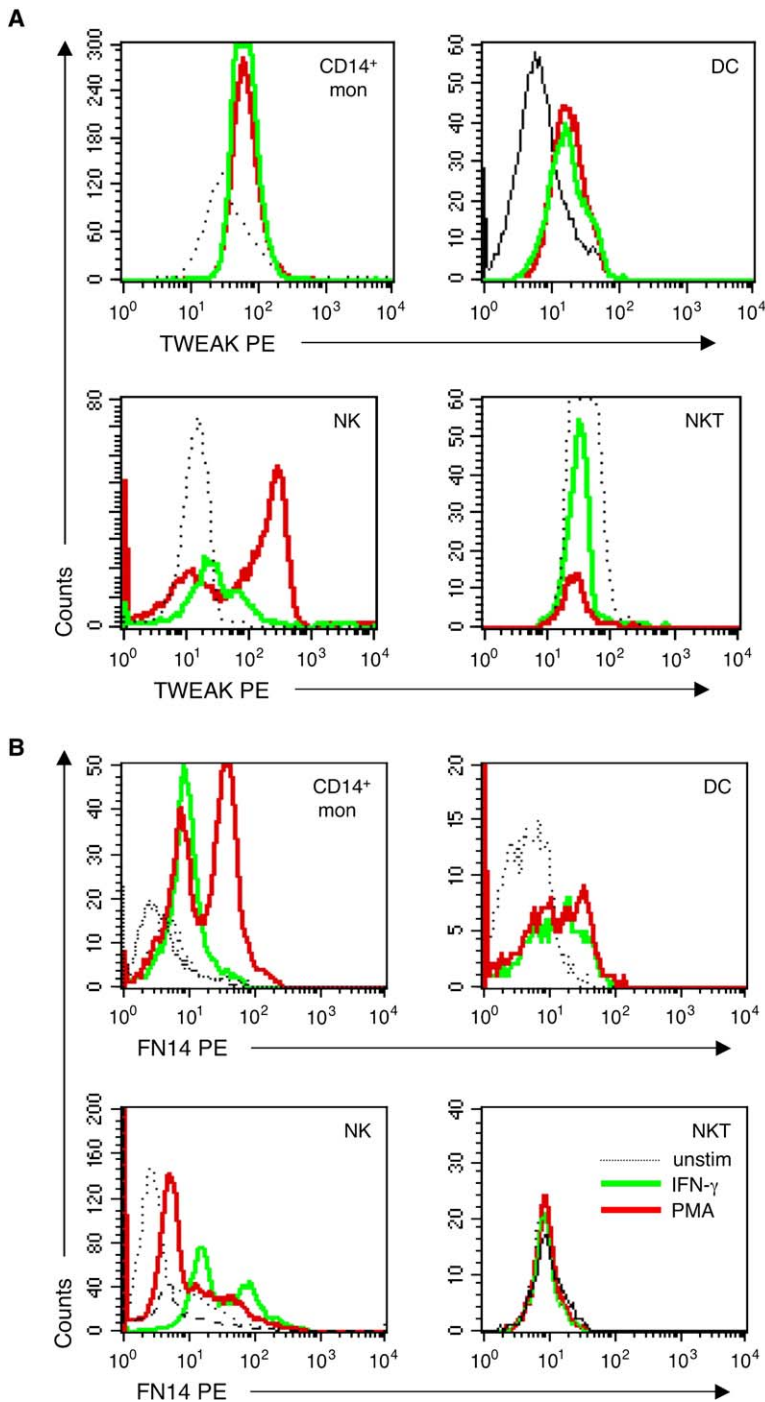


Figure 1. Cells of the Innate Immune System Express TWEAK and Its Receptor FN14

(A) Resting human PBMC or PBMC activated for 12 hr with IFN- γ or PMA were surface stained with antibodies to lymphocyte lineage markers, permeabilized, stained with TWEAK antibody, and analyzed by FACS.

(B) Resting or stimulated human PBMC were surface stained for lineage markers as well as with FN14 antibody and analyzed by FACS. Dendritic cells were defined as HLA-DR⁺/CD11c⁺/LIN-1⁻ cells.

RESULTS

TWEAK Inhibits the Innate Inflammatory Response by Supporting NK AICD and Repressing Production of IFN- γ and IL-12

To explore potential immune-cell sources and targets of TWEAK, we analyzed expression of this ligand and its receptor in lymphoid cells (Figure 1). NK cells, macrophages, and dendritic cells expressed TWEAK and FN14, both of which

were upregulated by stimulation with IFN- γ or phorbol myristate acetate (PMA). NKT cells expressed TWEAK but not FN14 and did not upregulate either protein in response to IFN- γ or PMA. Other lymphoid cells, including T and B cells, did not express significant levels of TWEAK or FN14 (data not shown).

To examine TWEAK's role in vivo, we generated TWEAK knockout mice (see Figure S1 in the Supplemental Data available with this article online). Detailed anatomical and

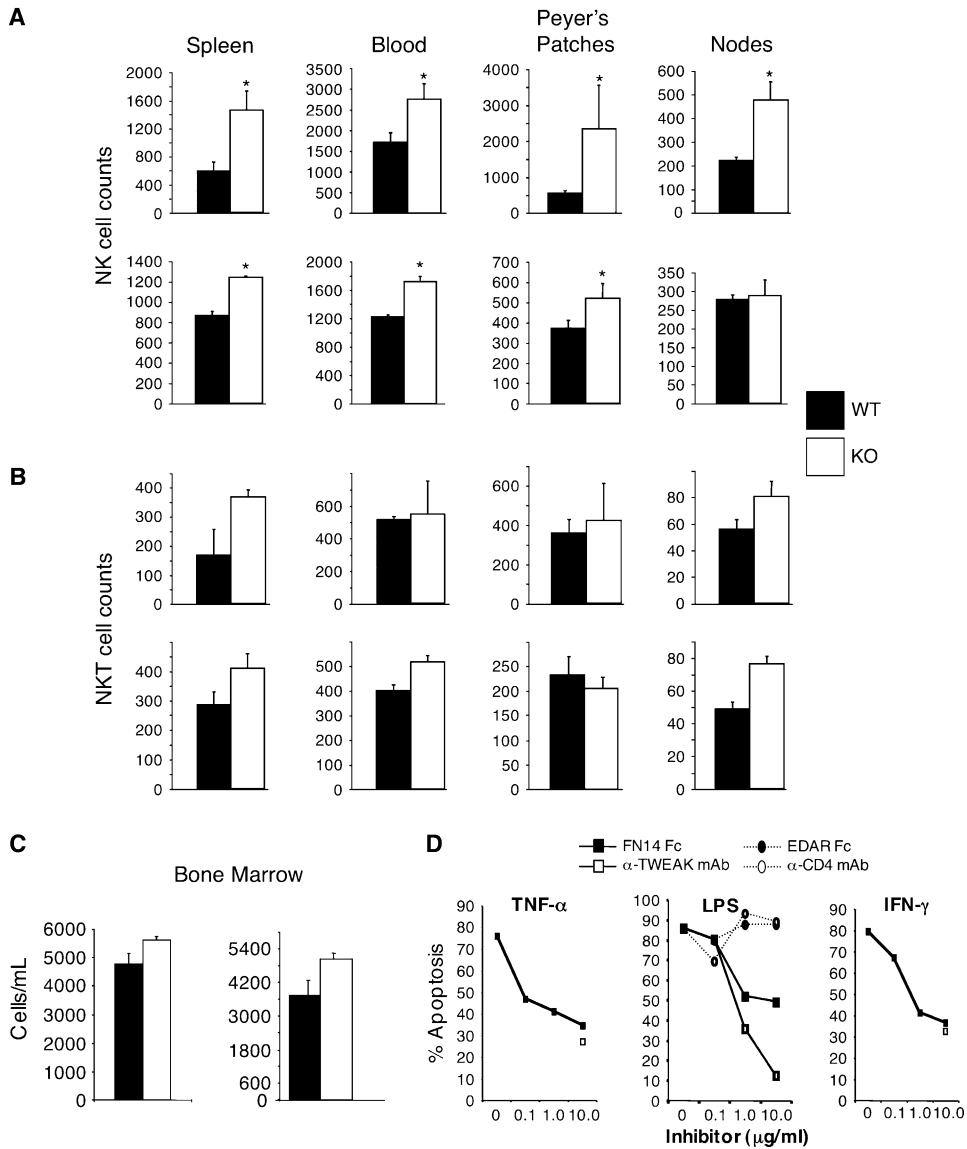


Figure 2. *TWEAK*^{-/-} Mice Have More NK Cells in Secondary Hematopoietic Tissues

(A and B) The spleen, peripheral blood, Peyer's patches, and lymph nodes were isolated from 2-month-old *TWEAK*^{+/+} (black bars) or *TWEAK*^{-/-} (white bars) mice (n = 6 per group). Dissociated NK cells (A) or NKT cells (B) were quantified by FACS (top graphs, males; bottom graphs, females). Error bars represent SEM.

(C) The bone marrow (0.5 ml) was aspirated from the right femurs of *TWEAK*^{+/+} (black bars) or *TWEAK*^{-/-} (white bars) mice (n = 6 per group) (left graph, males; right graph, females), and NK cells were quantified.

(D) Human PBMC were subjected to activation-induced cell death by stimulation with TNF-α, LPS, or IFN-γ in the presence of various concentrations (Inhibitor) of FN14 Fc (closed squares), anti-TWEAK mAb (open squares), EDAR Fc (closed circles), or anti-CD4 mAb (open circles). NK cells were then isolated and stained for their sub-G1 DNA content.

histological analysis did not suggest significant abnormalities in nonlymphoid tissues (Table S1). However, analysis of hematopoietic tissues revealed that *TWEAK*^{-/-} mice had significantly more NK cells than did age-matched wild-type (wt) littermates (Figure 2A). This increase was apparent in secondary lymphoid organs, including spleen, Peyer's patches, lymph nodes, and peripheral blood, and was greater in males than in females (Figure 2A top and bottom). In contrast, NKT

cell counts did not differ statistically between *TWEAK*^{-/-} and wt littermates (Figure 2B), nor did the numbers of CD4⁺ or CD8⁺ T cells, B cells, macrophages, dendritic cells, or granulocytes (data not shown). NK cell counts in the bone marrow of *TWEAK*^{-/-} and wt mice also did not vary significantly (Figure 2C), suggesting that the elevation in NK numbers was not the result of differences in NK generation (Kim et al., 2002). We hypothesized alternatively that impaired elimination by

activation-induced cell death (AICD) might lead to NK accumulation in TWEAK's absence. To test this, we examined the effect of TWEAK neutralization on sensitivity of human peripheral-blood NK cells to AICD (Figure 2D). TWEAK inhibition by FN14-Fc decoy or a TWEAK-blocking antibody markedly protected against NK AICD stimulation by TNF- α , bacterial endotoxin (LPS), or IFN- γ , indirectly supporting the possibility that NK cells accumulate in *TWEAK*^{-/-} mice because of insufficient AICD.

To determine the importance of TWEAK for innate immune responses in vivo, we used an established model of systemic challenge with lethal doses of LPS (Figure 3A). *TWEAK*^{-/-} mice were consistently more susceptible to LPS-induced death than wt controls, suggesting a stronger innate inflammatory response in the absence of TWEAK. *TWEAK*^{-/-} NK cells and macrophages from peripheral blood and spleens of LPS-injected mice produced more IFN- γ and IL-12 and less IL-10 as compared to wt cells (Figure 3B). Similarly, antibody neutralization of TWEAK augmented LPS-induced IFN- γ and IL-12 production by human peripheral-blood NK cells and CD14⁺ monocytes (Figure 3C). Thus, *TWEAK*^{-/-} mice are hypersensitive to LPS not only because they have more NK cells but probably also since their NK cells and macrophages produce more IFN- γ and IL-12 and less IL-10, further promoting systemic inflammation (D'Andrea et al., 1993; Emoto et al., 2002; Heremans et al., 1994). These results suggest that TWEAK functions to attenuate the innate inflammatory response.

TWEAK Inhibits STAT-1 Activation and Promotes p65 NF- κ B Association with HDAC-1

To investigate how TWEAK deletion might promote the production of IFN- γ and IL-12, we examined the activity of the signal transducer and activator of transcription (STAT)-1, which is key to pathogen-induced expression of IFN- γ in NK cells and of IL-12 in macrophages (Marodi et al., 2001; Morrison et al., 2004; Nelson et al., 1996; Varma et al., 2002). TWEAK neutralization increased basal STAT-1 phosphorylation in human NK cells and macrophages and further enhanced STAT-1 stimulation by LPS (Figure 4A). Likewise, macrophages from *TWEAK*^{-/-} mice had elevated basal levels of phospho-STAT-1 and showed stronger LPS-induced STAT-1 phosphorylation as compared to wt controls, while phospho-STAT-3 and total STAT-1 or STAT-3 levels appeared unaltered (Figure 4B). Suppressor of cytokine signaling (SOCS) proteins act as negative-feedback regulators of STAT activation (Alexander, 2002). As compared to wt controls, *TWEAK*^{-/-} macrophages showed weaker mRNA induction of SOCS-1—a protein that directly inhibits Janus kinase (JAK) activity (Alexander, 2002)—in response to LPS (Figure 4C), while SOCS-3 mRNA elevation was not significantly altered (data not shown). Thus, one mechanism contributing to TWEAK's repression of IFN- γ and IL-12 production may be attenuation of JAK-dependent STAT-1 activation through SOCS-1 induction.

TNF- α , a crucial stimulator of the innate inflammatory response, induces expression of IFN- γ and IL-12 (as well as other immunomodulatory genes) through activation of the

classical NF- κ B pathway (Bonizzi and Karin, 2004; Chen and Greene, 2004; Chen et al., 2001; D'Andrea et al., 1993; Zhong et al., 2002). TNF- α induces transient phosphorylation of the p65/RelA NF- κ B subunit, leading to its association with the p50 subunit and to nuclear translocation of the resulting heteromeric complex. In the nucleus, the p65/p50 heterodimer transactivates downstream target genes through association with the p300/CBP transcriptional coactivator (Chen and Greene, 2004; Chen et al., 2001, 2002; Greene and Chen, 2004; Kiernan et al., 2003; Zhong et al., 2002). Alternatively, p65 may interact with histone deacetylases (HDACs), which cause transcriptional repression (Ashburner et al., 2001; Kiernan et al., 2003; Quivy and Van Lint, 2004; Rahman et al., 2004; Zhong et al., 2002). To assess whether TWEAK might further affect gene expression by modulating transcriptional interactions of NF- κ B, we examined the phosphorylation of p65 in human splenic NK cells and macrophages. We focused on serine 536 of p65, which has been linked to transcriptional repression of IL-12 (Lawrence et al., 2005). Whereas TNF- α caused transient p65 phosphorylation peaking at 0.5 hr, TWEAK induced prolonged p65 phosphorylation, starting at 0.25 hr and lasting for 3–8 hr (Figure 4D). Next, we immunoprecipitated p65 from stimulated cells and probed by immunoblot for p65 association with p300 or HDAC-1 (Figure 4E). TNF- α induced strong interaction of p65 with p300 but not with HDAC-1, while TWEAK induced robust association of p65 with HDAC-1 but not with p300. The HDAC inhibitor trichostatin A (TSA) reversed the inhibitory effect of TWEAK on production of IFN- γ and IL-12 by NK cells and macrophages but did not alter induction of these cytokines by TNF- α (Figure 4F). We further tested the effect of TWEAK on DNA binding of nuclear NF- κ B complexes in murine macrophages by electrophoretic mobility supershift assays (Figure S2). Whereas TNF- α promoted DNA binding by p65 and p50 that was detected at 0.25–1 hr, TWEAK stimulated more prolonged binding, lasting up to 3 hr, as well as detectable, although weak, DNA binding of p52. Thus, TWEAK may block the production of IFN- γ and IL-12 not only by inhibiting STAT-1 but also through HDAC-1-mediated transcriptional repression.

TWEAK Suppresses Development of a T_H1 Immune Profile

To investigate whether *TWEAK* deficiency affects immune-system development, we compared the lymphoid tissues of *TWEAK*^{-/-} mice and wt littermates at 3, 6, and 12 months of age (Figure 5). By 6 months, *TWEAK*^{-/-} mice showed notable spleen and lymph-node enlargement as compared to controls (Figures 5A and 5B), while their thymus and liver did not differ (Table S1). Histological evaluation indicated that *TWEAK*^{-/-} spleens or lymph nodes formed normal germinal centers and were free of malignancy (Figure 5C and data not shown). However, anti-CD3 antibody staining was stronger in spleens from 12-month-old *TWEAK*^{-/-} mice versus wt littermates (Figure 5C), suggesting expansion of the T cell compartment. FACS analysis confirmed that both CD4⁺ and CD8⁺ T cells were significantly more abundant in aged *TWEAK*^{-/-} mice (Figure 5D). Splenic NK cell numbers also

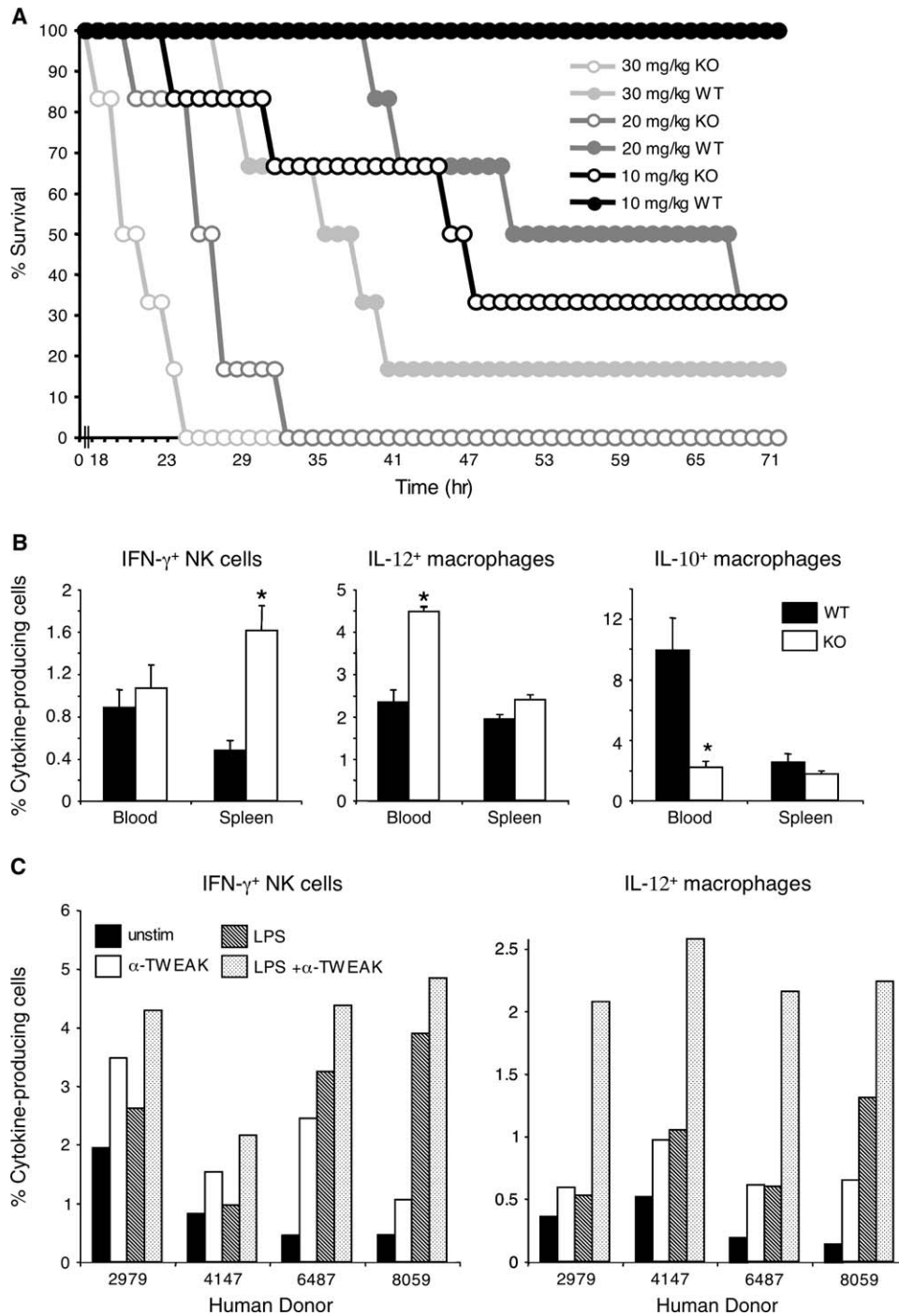


Figure 3. TWEAK Ablation or Inhibition Augments the Innate Inflammatory Response to Endotoxin

(A) *TWEAK*^{+/+} and *TWEAK*^{-/-} mice (n = 10 per group) were injected i.p. with the indicated doses of LPS, and survival was monitored over 5 days.

(B) NK cells and macrophages were isolated from the peripheral blood and spleen of *TWEAK*^{+/+} and *TWEAK*^{-/-} mice 24 hr after in vivo challenge with LPS (30 mg/kg) and stained for intracellular levels of IFN- γ , IL-12, and IL-10. Error bars represent SEM of 500,000 events/mouse based upon a 99% confidence interval for % responses (see <http://www.maeckerlab.org/CFCTools.html>).

(C) PBMC from four human donors were stimulated for 24 hr with LPS. Subsequently, marker-identified NK cells or CD14⁺ monocytes were stained and analyzed by FACS for intracellular levels of IFN- γ and IL-12, respectively.

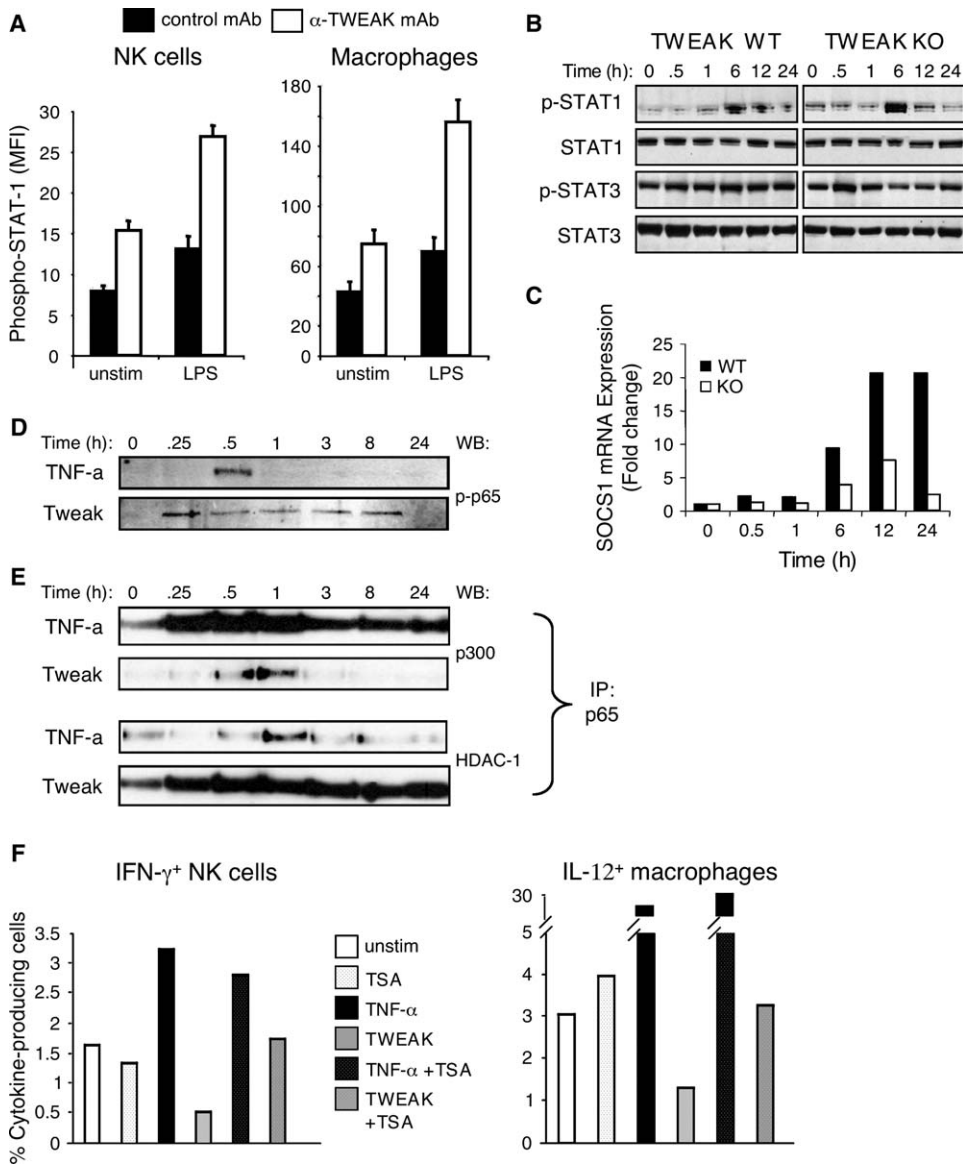


Figure 4. Involvement of TWEAK in Modulation of STAT-1 and NF-κB

(A) Human NK cells and macrophages were stimulated for 12 hr in vitro with LPS (1 μg/ml), surface stained for lineage markers, permeabilized, and stained for intracellular levels of phosphorylated STAT-1. 500,000 events/mouse were collected; error bars represent SEM.

(B) Murine macrophages from *TWEAK*^{+/+} and *TWEAK*^{-/-} mice were stimulated for 24 hr in vitro with LPS (1 μg/ml), and cellular lysates were analyzed for phosphorylated STAT-1 and STAT-3 as well as total STAT-1 and STAT-3 by immunoblot.

(C) Macrophages from *TWEAK*^{-/-} or wt mice were stimulated with LPS, and the induction of *SOCS-1* mRNA was analyzed by quantitative PCR. The data is normalized to *RPL19* control levels and depicted as fold induction over values at t = 0.

(D) Splenic human NK cells and macrophages were stimulated with TWEAK or TNF-α (100 ng/ml) over 24 hr, and cell lysates were analyzed for phosphorylated serine 536 on p65 by immunoblot.

(E) p65 was immunoprecipitated from cell lysates after stimulation by TWEAK or TNF-α, and association with p300 and HDAC-1 was analyzed by immunoblot.

(F) Human macrophages or NK cells were stimulated with TNF-α or TWEAK for 12 hr in the presence or absence of TSA. Innate immune cells were surface stained for lineage markers and then stained for intracellular levels of IFN-γ and IL-12.

were increased, while abundance of B cells, macrophages, granulocytes, or platelets was similar (data not shown). Given that NK cells comprise a small percentage of splenocytes, it is likely that T cell expansion contributes to the larger spleen size in *TWEAK*^{-/-} mice, although other compart-

ments we did not test, such as erythrocytes or stromal cells, also may contribute. Further analysis of *TWEAK*^{-/-} mice demonstrated a marked increase in memory T cells and in T cells positive for expression of the T_H1-specific transcription factor T-bet (Hwang et al., 2005; Szabo et al., 2002)

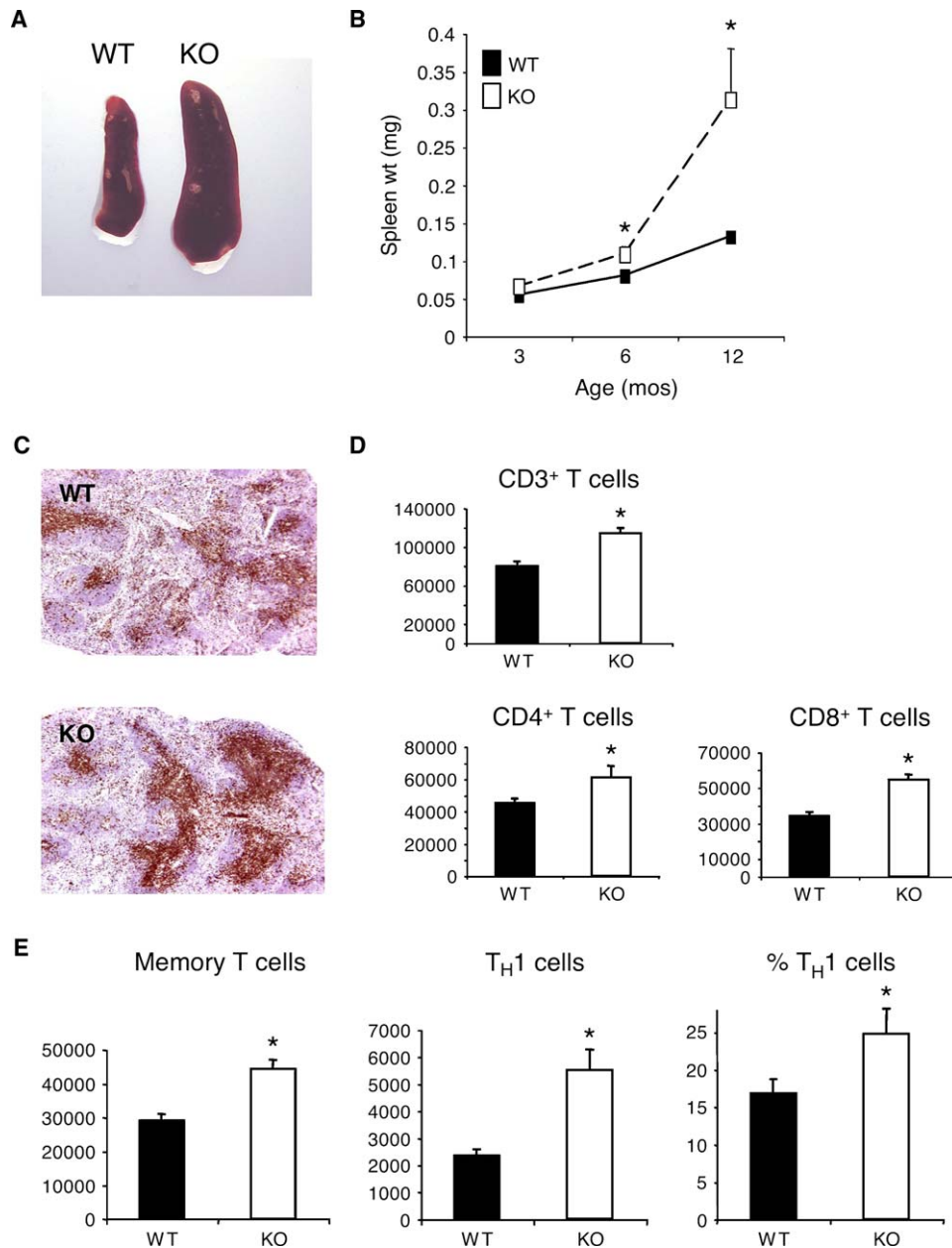


Figure 5. Aged *TWEAK*^{-/-} Mice Have Larger Spleens with Expanded Memory and T_H1 T Cell Compartments

TWEAK^{+/+} and *TWEAK*^{-/-} male mouse littermates were grown to 3, 6, or 12 months of age, and their spleens and lymph nodes were examined.

(A) Representative images of spleens from 12-month-old *TWEAK*^{+/+} or *TWEAK*^{-/-} mice.

(B) Mean \pm SEM spleen weights as a function of age ($n = 6$ per group).

(C) Representative images of spleen sections from 12-month-old *TWEAK*^{+/+} or *TWEAK*^{-/-} mice stained with CD3 antibody.

(D and E) Splenocytes of 12-month-old *TWEAK*^{+/+} mice and *TWEAK*^{-/-} littermates were analyzed by FACS to determine numbers of CD3⁺, CD4⁺, and CD8⁺ T cells (D) and memory (CD3⁺, CD44^{high}, CD62L^{low}, CD45⁺) and T_H1 (CD3⁺, CD4⁺, CD45⁺, T-bet⁺) T cells (E). 500,000 events/mouse were collected; error bars represent SEM.

(Figure 5E). These results suggest that TWEAK function inhibits the development of an adaptive T_H1 immune profile.

TWEAK Attenuates Adaptive T_H1 Antitumor Immunity

To study the involvement of TWEAK in modulating the transition to an adaptive response, we turned to an established

model of antitumor immunity, based on syngeneic mouse C57 black 6 B16 melanoma cells; in this model, both NK cells and effector T cells are important for immune-based tumor rejection (Prevost-Blondel et al., 2000; Turk et al., 2004; Yang et al., 1997, 2003; Yei et al., 2002). First, we challenged mice with the moderately aggressive B16.F10

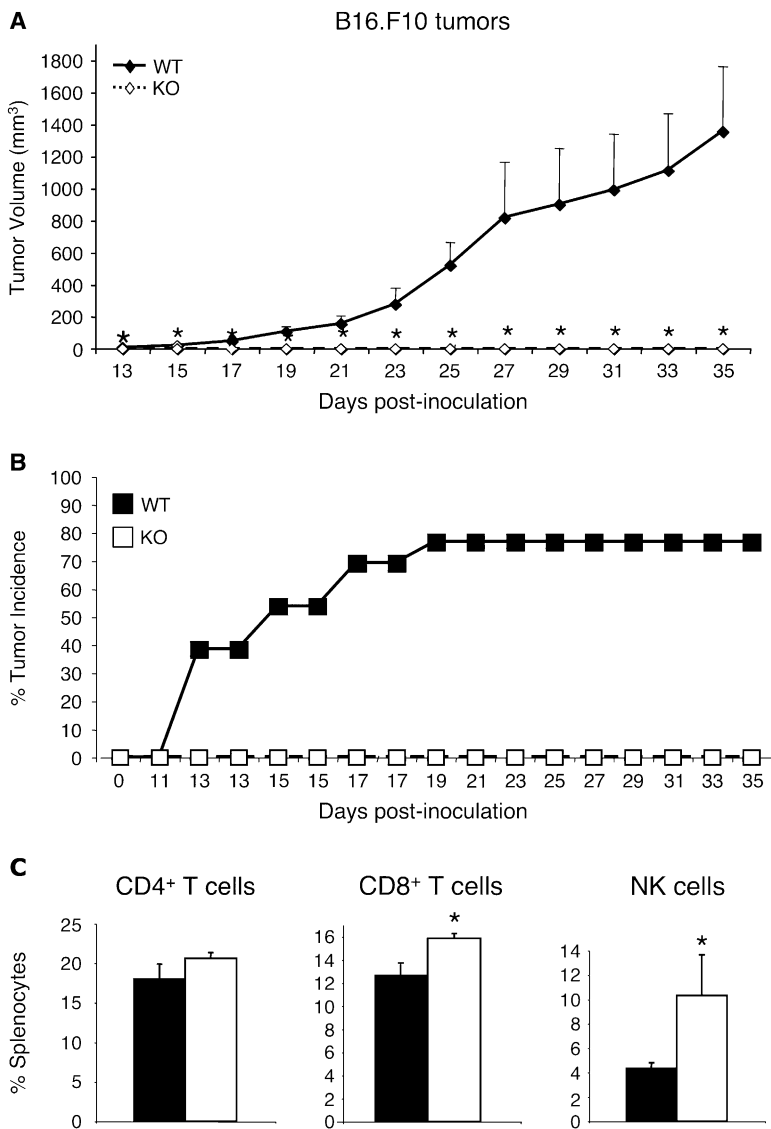


Figure 6. TWEAK Deletion Inhibits Establishment and Growth of B16.F10 Melanomas and Promotes Expansion of NK Cells and Adaptive CD8⁺ T Cells

TWEAK^{+/+} and *TWEAK*^{-/-} mice were injected s.c. with B16.F10 cells (10⁵/mouse), and tumor growth (A) and incidence (B) were monitored; n = 10; error bars represent SEM. At study termination, spleens were harvested and analyzed for the indicated lymphocyte subsets (C). 500,000 events/mouse were collected; error bars represent SEM.

subclone of the B16 cell line (Figure 6). *TWEAK*^{-/-} mice completely resisted the establishment and growth of B16.F10 tumors, whereas wt controls succumbed to tumor growth as previously reported (Figures 6A and 6B) (Yei et al., 2002). To define which immunological differences might have caused this marked disparity in tumor rejection, we analyzed the splenic lymphocyte populations of the B16.F10-injected mice (Figure 6C). Consistent with our other findings, *TWEAK*^{-/-} mice had more splenic NK cells than the wt controls. Surprisingly, despite their lack of detectable tumors and hence absence of abundant tumor-associated antigens, B16.F10-injected *TWEAK*^{-/-} mice displayed a significant expansion of CD8⁺ T cells relative to controls. Taking this finding together with the increase in memory T cells in aged *TWEAK*^{-/-} mice, we postulated that the absence of TWEAK might facilitate an enhanced tumor-induced memory response, perhaps through stronger T cell priming in the presence of higher IFN- γ and IL-12 levels.

To test this latter hypothesis, we injected mice with a more aggressive B16 melanoma subclone, B16.BL6; this ensured tumor implantation, while tumor growth was significantly attenuated in *TWEAK*^{-/-} mice compared to wt controls (Figure 7A). Tumors from *TWEAK*^{-/-} mice exhibited greatly increased lymphocytic infiltration, with 2- to 8-fold more T and NK cells (Figure S3). Tumor-bearing *TWEAK*^{-/-} mice also had larger spleens than controls (Figure 7B), with expanded NK and T cell populations (Figure 7C). To verify whether these bigger lymphocytic populations harbored specific antitumor activity, we isolated splenocytes from tumor-bearing mice and measured their responsiveness to ex vivo rechallenge with B16.BL6 cells (Figure 7D). *TWEAK*^{-/-} CD8⁺ T cells and NK cells produced significantly more IFN- γ , while macrophages generated more IL-12 upon tumor rechallenge than did corresponding wt controls. Together, these studies demonstrate that TWEAK's absence augments innate as well as adaptive antitumor immunity,

suggesting that TWEAK acts physiologically to repress both functions.

To investigate whether TWEAK affects the adaptive response through direct action on T cells or indirectly through modulation of the innate-to-adaptive interface, we generated T cell-chimeric mice by adoptive transfer of *TWEAK*^{+/+} or *TWEAK*^{-/-} T cells into *TWEAK*^{+/+} or *TWEAK*^{-/-} congenic recipients (Figure S4A). B16.BL6 tumors grew equally well in *TWEAK*^{+/+} recipients with transferred *TWEAK*^{+/+} or *TWEAK*^{-/-} T cells; however, tumor growth in *TWEAK*^{-/-} mice with transferred *TWEAK*^{+/+} T cells was greatly attenuated, suggesting indirect T cell modulation by TWEAK (Figure S4B). Moreover, upon ex vivo rechallenge, both wt (Thy1.1) and knockout (Thy1.2) T cells showed much stronger IFN- γ production after conditioning in *TWEAK*^{-/-} compared to *TWEAK*^{+/+} recipients (Figure 7E). These results indicate that TWEAK attenuates the priming of T cells by the innate system, suppressing development of adaptive antitumor immunity.

DISCUSSION

Innate immunity is important not only as the first line of defense against infection but also for protecting the host during the time required for development of adaptive immunity. Furthermore, the innate response critically influences the nature of adaptive mechanisms that develop in response to a given infectious challenge (Gastriconi et al., 2004; Lo et al., 1999; Palucka and Banchereau, 1999a, 1999b). Interactions of NK cells with macrophages and dendritic cells stimulate the secretion of specific cytokines that support the development of particular T and/or B cell responses (Palucka and Banchereau, 1999a, 1999b; Trinchieri, 1995). IFN- γ secretion by NK cells and IL-12 production by macrophages and dendritic cells promotes the development of an adaptive T_H1 response, leading to cytotoxic T cell effector function (Coudert et al., 2002; Fujii et al., 2003; Gerosa et al., 2002; Pan et al., 2004; Varma et al., 2002). In contrast, IL-4 production by NKT cells promotes adaptive T_H2 differentiation and consequent B cell activation (Araujo et al., 2000; Kaneko et al., 2000; Leite-de-Moraes et al., 2001).

The studies we report here implicate TWEAK as an important regulator of the innate system and its interface with adaptive immunity. Innate immune cells—namely, NK cells, macrophages, and dendritic cells—expressed TWEAK and its receptor FN14 and upregulated both molecules upon stimulation. In contrast, cells of the adaptive system, including T and B cells, did not express significant levels of TWEAK or FN14. This expression pattern is consistent with our subsequent conclusion that TWEAK directly modulates innate immune function, thereby indirectly influencing the ensuing adaptive response.

TWEAK knockout mice were viable and healthy, demonstrating that TWEAK is not crucial for normal development. However, *TWEAK*^{-/-} mice showed a significant accumulation of NK cells as compared to age-matched, wt littermates. *TWEAK* gene ablation did not significantly alter the amount of NK cells in the bone marrow, suggesting unabated NK devel-

opment. Conversely, neutralization of TWEAK protected human NK cells from apoptosis induction by TNF- α , LPS, or IFN- γ . These findings suggest that impaired AICD rather than increased generation causes NK cell accumulation in *TWEAK*^{-/-} mice. Thus, one immunomodulatory role of TWEAK may be to help prevent the potentially harmful development of an excessive innate response by supporting the deletion of activated NK cells upon immunological resolution.

TWEAK^{-/-} mice were hypersensitive to systemic LPS injection, further implicating TWEAK in curbing the innate response. Given that NK cell activity is an important component of the systemic inflammatory reaction to LPS (Emoto et al., 2002; Heremans et al., 1994), one reason for the hypersensitivity of *TWEAK*^{-/-} mice could be their elevated NK cell numbers. However, we found in addition that, after in vivo exposure to LPS, *TWEAK*^{-/-} NK cells produced more IFN- γ , while macrophages generated more IL-12 and less IL-10. Furthermore, TWEAK neutralization enhanced the production of IFN- γ and IL-12 by LPS-stimulated NK cells and macrophages. Thus, TWEAK may curtail the innate response also by repressing secretion of key proinflammatory cytokines such as IFN- γ and IL-12 and/or promoting production of anti-inflammatory cytokines such as IL-10. TWEAK therefore differs strikingly from its relative TNF- α , which stimulates the secretion of IFN- γ and IL-12, augmenting the innate inflammatory response (D'Andrea et al., 1993; Oswald et al., 1999; Wilhelm et al., 2001; Zhan and Cheers, 1998). Indeed, contrary to the LPS hypersensitivity of the *TWEAK* knockouts, *TNF- α* or *TNFR1* knockout mice are resistant to LPS-induced lethality (Pasparakis et al., 1996; Rothe et al., 1994).

STAT-1 is a key signal transducer involved in the production of IFN- γ and IL-12 in response to infection (Dupuis et al., 2000; Feinberg et al., 2004). TWEAK deletion augmented basal as well as LPS-induced STAT-1 phosphorylation and attenuated mRNA induction of SOCS-1, which can directly inhibit JAK catalytic activity and consequent STAT phosphorylation (Alexander, 2002). Thus, TWEAK may suppress the production of IFN- γ and IL-12 as well as the engagement of proinflammatory cytokine signaling cascades by inhibiting JAK-mediated STAT-1 activation through upregulating SOCS-1. This TWEAK activity contrasts with the documented enhancement of STAT-1 stimulation by TNF- α (Chen et al., 2002). Like STAT-1, NF- κ B also plays an important role in controlling cytokine gene transcription (Feinberg et al., 2004; Zhan and Cheers, 1998). In human NK cells and macrophages, TWEAK stimulated prolonged phosphorylation of p65, which was associated with strong binding of p65 to HDAC-1. In contrast, TNF- α induced transient p65 phosphorylation and binding to the transcriptional coactivator p300. Moreover, the HDAC inhibitor TSA reversed TWEAK's attenuation of the production of IFN- γ and IL-12 but did not alter the effect of TNF- α . Thus, a second mechanism contributing to TWEAK's repression of the synthesis of IFN- γ and IL-12 may involve induced association of p65 and HDAC-1. Recent work shows that LPS-stimulated phosphorylation of p65 on serine 536 by IKK α promotes proteasomal degradation of p65 (Lawrence et al., 2005),

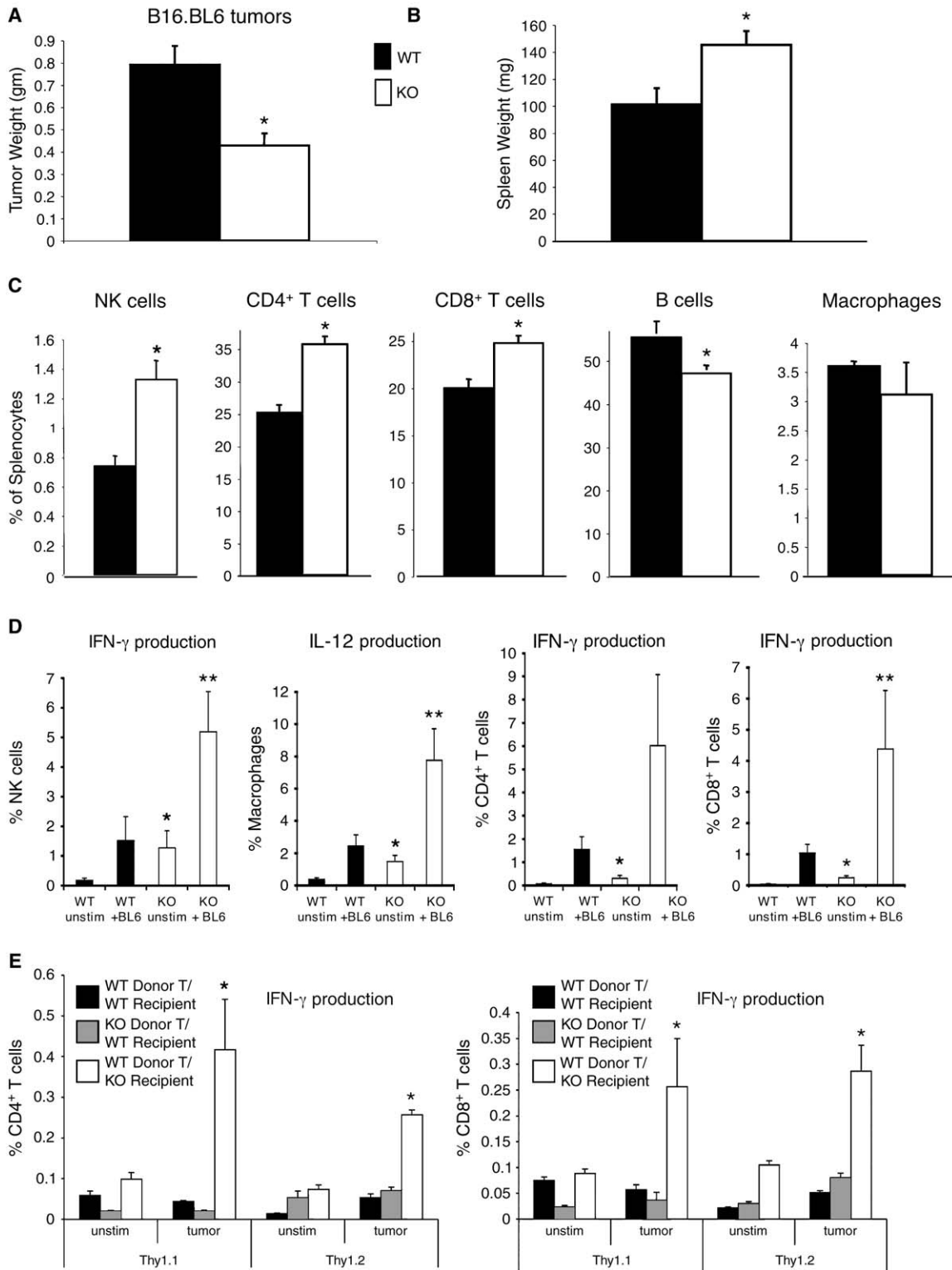


Figure 7. TWEAK Deletion Inhibits B16.BL6 Tumor Growth and Promotes Innate-to-Adaptive Antitumor Immune Priming
 (A and B) *TWEAK*^{+/+} and *TWEAK*^{-/-} mice were injected s.c. with B16.BL6 cells (5×10^5 /mouse), and tumor weights (A) and spleen weights (B) were determined at 1 month.
 (C) Splenocytes from tumor-bearing mice were stained for cell lineage and analyzed by FACS.

suggesting an additional possible mechanism for suppression of cytokine transcription through NF- κ B. TWEAK also promoted more prolonged DNA binding by p65 and p50 than did TNF- α and induced DNA binding by p52, whereas TNF- α did not. The difference between TWEAK and TNF- α in modulation of NF- κ B raises the possibility that the kinetics of p65 phosphorylation may influence the interaction of p65 with other transcriptional regulators. Indeed, there appears to be some parallel between the induction of sustained p65 phosphorylation by TWEAK and the control of the c-Jun N-terminal kinase (JNK) pathway by TNF- α , where transient versus sustained JNK phosphorylation correlates with promotion of cell survival versus cell death (Varfolomeev and Ashkenazi, 2004).

The above findings suggest that the expression of TWEAK by NK cells and macrophages in response to infection helps to curtail the innate inflammatory response; the underlying mechanisms may involve promotion of NK AICD as well as repression of IFN- γ and IL-12 production by NK cells and macrophages. Because these cytokines also influence the transition to adaptive immunity in favor of a T_H1-based cellular response, we reasoned as a corollary that TWEAK might conversely modulate the innate-to-adaptive immune interface. One piece of evidence that supports this hypothesis was the observation that in the absence of TWEAK aged mice developed enlarged spleens with increased numbers of NK cells as well as T cells of the T_H1 phenotype. A second line of supportive evidence came from experiments with the mouse B16 melanoma model. First, *TWEAK*^{-/-} mice rejected growth of the moderately aggressive B16.F10 subclone, whereas wt littermates failed to combat tumor growth. While the elevated numbers of NK cells in *TWEAK*^{-/-} mice could explain the ability to reject tumors, the antitumor response in these mice was associated also with an expansion of CD8⁺ T cells, consistent with an augmented T_H1 response. Second, *TWEAK*^{-/-} mice resisted growth of the more aggressive B16.BL6 subclone better than did wt controls, and, upon rechallenge with tumor cells ex vivo, their CD8⁺ T cells, NK cells, and macrophages produced significantly more IFN- γ and IL-12. Third, experiments with T cell-chimeric mice showed that the strength of the adaptive antitumor response was governed by TWEAK modulation of the T cell-priming innate environment rather than by direct TWEAK action on T cells. Together, these studies suggest that TWEAK curtails the innate-to-adaptive immune interface, keeping in check the development of T_H1-mediated cellular responses.

In conclusion, our studies uncover an important role for TWEAK in immune modulation, which markedly differs from the function of its structural relative, TNF- α . TNF- α plays

a key role in supporting the innate inflammatory response by promoting innate cell stimulation and proinflammatory cytokine secretion. In contrast, TWEAK is crucial for curtailing the innate response, supporting NK AICD as well as repressing the production of IFN- γ and IL-12 by NK cells and macrophages. Whereas TNF- α activates transcription of immunostimulatory genes by promoting STAT-1 activation and p65 association with p300, TWEAK represses STAT-1 activity and induces binding of p65 to HDAC-1, inhibiting proinflammatory cytokine gene transcription. Furthermore, TWEAK plays a critical role in attenuating the transition from innate to adaptive T_H1 immunity. TWEAK's function may have evolved to guard against development of potentially harmful excessive inflammatory and autoimmune responses. Therefore, TWEAK inhibition might be useful clinically for augmenting anti-infection and anticancer immunity, while TWEAK receptor activation might be useful for controlling autoimmune disease.

EXPERIMENTAL PROCEDURES

TWEAK and FN14 Expression in Human PBMC

Human peripheral-blood mononuclear cells (PBMC) were isolated from 50 ml donated whole blood with lymphocyte separation medium (ICN) according to the manufacturer's instructions. Cells were resuspended in complete Isocoves' medium in the presence of brefeldin A (5 μ g/ml) for 24 hr in the absence or presence of inflammatory stimuli. Following stimulation, Fc receptors were blocked with 2 μ g/ml Fc Block (Miltenyi Biotec, Auburn, CA) for 20 min at room temperature (RT). Cells were then surface stained with fluorescent-conjugated monoclonal antibodies to CD3, CD4, CD8, CD11b, CD11c, CD14, CD20, CD45, CD56, HLA-DR, Lin1 (BD Biosciences, San Jose, CA), or FN14 (e-Biosciences, San Diego, CA) for 30 min at RT and then treated with BD FACS Lyse solution and stored at -70°C overnight. Cells were permeabilized and then stained with TWEAK monoclonal antibody (e-Biosciences) for 30 min at RT, washed, and analyzed on a FACSCalibur (BD Biosciences).

Generation of TWEAK-Deficient Mice

A TWEAK targeting vector was constructed based on the *TNLOX1-3* vector by replacing 2.5 kb of the TWEAK gene, encompassing the first exon and all five downstream exons, with a *PGK-neo* cassette. The construct contained two DNA stretches derived from the mouse genome: a 3.1 kb fragment encompassing the sixth and the seventh exons of TWEAK and part of exon 1 of TWEAK, placed 5' of the *neo* cassette and a 4.1 kb fragment encompassing the first and second *SMT3IP1* exons placed 3' of the *PGK-neo* cassette. R1 embryonic stem cells were transfected with the linearized vector by electroporation, and G418-resistant clones were screened for the presence of the expected recombination event by Southern blot analysis with 5'- and 3'-specific DNA probes (as shown in Figure S1). Two independent *TWEAK*^{-/-} cell lines were microinjected into C57BL/6 blastocysts. Germline transmission in mice generated by crossing chimeric males with C57BL/6 females was detected by coat color and confirmed by two-step genomic PCR (Figure S1) with the following external (E) and internal (I) primer sets: E forward, TGCCCTAAGC

(D) Splenocytes isolated from tumor-bearing mice were rechallenged ex vivo with B16.BL6 cells, and the indicated cell types were analyzed for cytokine production. * denotes a significant difference in basal cytokine levels ($p < 0.01$); ** denotes a significant difference in tumor-induced cytokine responses ($p < 0.01$). For (D) and (E), 500,000 events/mouse were collected; error bars (SEM) were calculated based upon a 99% confidence interval for % responses (see <http://www.maeckerlab.org/CFCTools.html>).

(E) Antitumor cytokine responses in T cells from chimeric mice. T cells from *TWEAK*^{+/+} (Thy1.1 or Thy1.2) or *TWEAK*^{-/-} (Thy1.2) mice were injected i.v. into *TWEAK*^{+/+} (Thy1.1 or Thy1.2) and *TWEAK*^{-/-} (Thy1.2) mice (as illustrated in Figure S4), and the mice were challenged with B16.BL6 melanoma cells. Four weeks later, splenocytes were isolated from tumor-bearing mice and rechallenged ex vivo with tumor cells, and T cells were analyzed for IFN- γ production. * denotes a significant difference in IFN- γ levels depicted in the white versus black bars ($p < 0.01$). T cell Thy1 genotypes are indicated at the bottom.

CAGTCTACACCCAGTATTCCTTC; E reverse, TGGCCTGAAAGAAAT GCTCACACTATCACCAAC; I forward, CTTAGAACCAGCCGTAGGAAG GATT; and I reverse, GTGCCAGGCGCTCCAGTACATACAA. *TWEAK* knockout animals were backcrossed a minimum of six times onto the C57BL/6 background. Two independent lines were generated and showed comparable results in the assays reported in Figures 2A–2C.

TWEAK, APRIL, and SMT3IP1 mRNA Expression

Analysis of several tissues by quantitative RT-PCR demonstrated that *TWEAK*^{-/-} mice did not express *TWEAK* transcripts, while mRNA expression of two nearby genes, *APRIL* and *SMT3IP1*, was unaltered in the knockouts (Varfolomeev et al., 2004; Figure S1).

Flow Cytometry

Single-cell suspensions from hematopoietic organs were obtained from 8-week-old mice by dissociation of the isolated tissues with wire mesh screens and rubber stoppers from syringes. The suspensions were incubated with Fc-blocking antibody (2 µg/ml, BD Biosciences) and stained with lineage-specific conjugated monoclonal antibodies to B220, CD3, CD4, CD8, CD11b, CD11c, CD19, CD45, DX5 (BD Biosciences), F4/80, and Gr-1 (e-Biosciences) for 30 min at RT. Following surface staining, red blood cells (RBCs) were lysed with ACK lysis buffer (Biosource International), and the remaining cells were fixed. TruCount beads (BD Biosciences) were added to the tubes for quantitation. Cell-associated fluorescence was analyzed with a FACSCalibur instrument and associated Cell Quest software (BD Biosciences).

NK Cell AICD

Human PBMC were isolated from 100 ml whole blood and stimulated for 24 hr with TNF-α (500 ng/ml), LPS (5 µg/l), or IFN-γ (500 ng/ml) in the absence or presence of anti-TWEAK mAb (CARL-1, e-Biosciences) or FN14 Fc (Genentech). Following stimulation, NK cells were isolated using Miltenyi CD56⁺ beads and stained for sub-G1 DNA content (Maecker et al., 2002b).

LPS Challenge

TWEAK^{-/-} and *TWEAK*^{+/+} mice were injected intraperitoneally (i.p.) with 10–30 mg/kg LPS (*Escherichia coli* 055:B5, Sigma) in sterile saline and monitored over 5 days. Cytokine analysis was conducted by injecting ten mice i.p. with 30 mg/kg LPS and isolating blood and spleens 24 hr later. Single-cell suspensions were incubated for 6 hr in the presence of brefeldin A (5 µg/ml). Cells were Fc blocked (2 µg/ml, BD Biosciences) for the last 20 min and stained with lineage-specific conjugated monoclonal antibodies, DX5 (to identify NK cells), CD11b, and F4/80 (to identify macrophages) as well as CD45 (common leukocyte antigen) for 30 min at RT. Following surface staining, RBCs were lysed and cells were permeabilized; stained with antibody to IFN-γ, IL-12, or IL-10; and analyzed by FACS (Maecker et al., 2002a) (BD Biosciences). Cytokine analysis was conducted by isolating PBMC from four human donors. PBMC were incubated in presence or absence of 1 µg/ml LPS for 16 hr. During the last 6 hr, brefeldin A was added at 5 µg/ml. PBMC were Fc blocked (Miltenyi) for 20 min at RT and surface stained (CD3, CD56, CD14, CD45; BD Biosciences) for 30 min at RT. RBCs were lysed and cells were fixed and permeabilized, stained with IFN-γ or IL-12 antibody, and analyzed on by FACS.

STAT-1 Activity

Human NK cells and macrophages were isolated from a donor's spleen using Miltenyi CD56⁺ and CD11b⁺ beads, respectively. 1.0 × 10⁶ NK cells/0.5 ml were cocultured with 1.0 × 10⁶ macrophages/0.5 ml macrophage-SFM medium (Invitrogen). Cells were rested in serum-free medium for 12 hr and then stimulated with 1 µg/ml LPS, TNF-α (100 ng/ml), or TWEAK (100 ng/ml) in the presence or absence of the HDAC inhibitor trichostatin A (TSA, 50 ng/ml). Six hours later, cells were surface stained for CD56 and CD11b followed by intracellular staining for phospho-STAT-1 (Perez et al., 2004) and IFN-γ and IL-12 as outlined above. Alternatively, macrophages were isolated from spleens of

TWEAK^{-/-} and *TWEAK*^{+/+} mice using Miltenyi CD11b⁺ magnetic beads, rested for 12 hr in macrophage-SFM medium, and then stimulated over 24 hr with 1 µg/ml LPS. Cellular lysates were made and analyzed by Western blot for STAT-1 or STAT-3 (Cell Signaling).

SOCS-1 mRNA Analysis

Total RNA from LPS-stimulated mouse macrophages was analyzed by quantitative real-time PCR (TaqMan). Reactions contained 50 ng of total RNA, 0.6 mM each of gene-specific forward and reverse primers, and 0.2 mM of gene-specific fluorescent probe. *SOCS-1*-specific primers were: forward, TGGGTCGGAGGGAGTGGT; reverse, AGAGGTGGGATGAGG TCTCCA; probe, AGGGTGAGATGCCTCCCACTTCTGG. Gene-specific PCR products were measured using ABI PRISM 7700 Sequence Detection System following the manufacturer's instructions (PE Corp.).

NF-κB Analysis

Human NK cells and macrophages were isolated from a donor's spleen. NK cells were cocultured with macrophages (5.0 × 10⁶ each in 5 ml macrophage-SFM medium), rested for 12 hr, and treated with TWEAK (100 ng/ml) or TNF-α (100 ng/ml). Lysates for immunoblot (20 µg total protein) and immunoprecipitates (50 µg total protein) were prepared according to antibody manufacturer's instructions (Cell Signaling, Beverly, MA).

Histology and Immunohistochemistry

Tissues of 3-, 6-, and 12-month-old male *TWEAK*^{-/-} and *TWEAK*^{+/+} mice were weighed, fixed, sectioned, and analyzed for pathological status. Hematoxylin-and-eosin-stained sections were analyzed for gross histological abnormalities. Peanut agglutinin (Vector Research, Burlingame, CA) stained frozen sections were analyzed for structure of germinal centers. Five *TWEAK*^{-/-} and *TWEAK*^{+/+} spleens from 12-month-old male mice were dissociated, stained, and quantitated for lymphocyte cellularity utilizing TruCount beads (BD Biosciences).

B16 Melanoma Experiments

Ten *TWEAK*^{-/-} and *TWEAK*^{+/+} male mice were injected subcutaneously (s.c.) with 0.1 × 10⁶ or 0.5 × 10⁶ cells/0.1 ml in sterile saline in the right hind flank. Tumor measurements were taken every other day for 4–6 weeks. At study termination, tumors were removed, weighed, and dissociated first through wire mesh screens followed by treatment with nonenzymatic cell dissociation buffer (Sigma) for 5 min to create single-cell suspensions. Splenocytes were prepared from tumor-injected mice and cocultured with either sterile saline or B16.F10 tumor cell suspensions in the presence of brefeldin A for 12 hr to measure intracellular cytokine production.

T cell-chimeric mice were generated by transferring 1 × 10⁷ T cells (isolated using Thy1.1 or Thy1.2 Miltenyi magnetic beads) from male donor into male recipient mice as illustrated in Figure S4. Chimeric mice were injected with 0.5 × 10⁶ B16.BL6 tumor cells. At 4 weeks, Thy1.1 and Thy1.2 CD4⁺ and CD8⁺ T cells from spleens of tumor-bearing mice were rechallenged ex vivo with tumor cells and analyzed for IFN-γ production.

Statistical Analysis

Statistical analysis, where appropriate, was conducted utilizing multifactor ANOVA analysis followed by Newman-Keuls post hoc tests. Significance was defined for p values < 0.05.

Supplemental Data

Supplemental Data include one table and four figures and can be found with this article online at <http://www.cell.com/cgi/content/full/123/5/931/DC1>.

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