Tumor necrosis factor–related apoptosis-inducing ligand induces the expression of proinflammatory cytokines in macrophages and re-educates tumor-associated macrophages to an antitumor phenotype

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ABSTRACT Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising candidate for cancer therapy, because it can induce apoptosis in various tumor cells but not in most normal cells. Although it is well known that TRAIL and its receptors are expressed in many types of normal cells, including immune cells, their immunological effects and regulatory mechanisms are still obscure. In the present study, we demonstrated that TRAIL affected the activity of NF-κB (nuclear factor-κB) and the expression of its downstream proinflammatory cytokines IL-1β (interleukin-1β), IL-6, and tumor necrosis factor α in macrophages. TRAIL also induced microRNA-146a (miR-146a) expression in an NF-κB–dependent manner. As a result, miR-146a was involved as a negative-feedback regulator in the down-regulation of proinflammatory cytokine expression. In addition, the suppression of histone deacetylase (HDAC) activities by trichostatin A improved miR-146a expression due to the up-regulation of the DNA-binding activity of NF-κB at the miR-146a promoter in TRAIL-induced macrophages, suggesting that histone acetylation was involved in the suppression of miR-146a expression. Further investigation revealed that the HDAC subtype HDAC1 directly regulated the expression of miR-146a in TRAIL-stimulated macrophages. Finally, the TRAIL-sensitive human non small cell lung carcinoma cell line NCI-H460 was used to elucidate the physiological significance of TRAIL with respect to tumor-associated macrophages (TAMs). We demonstrated that TRAIL re-educated TAMs to an M1-like phenotype and induced cytotoxic effects in the tumor cells. These data provide new evidence for TRAIL in the immune regulation of macrophages and may shed light on TRAIL-based antitumor therapy in human patients.

INTRODUCTION Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2 L) is a typical member of the tumor necrosis factor (TNF) superfamily that includes FasL and TNF-α (Wiley et al., 1995; Pitti et al., 1996). TRAIL interacts with its death receptors (DR4 and DR5 in human, mDR5 in mouse) and subsequently induces formation of a death-inducing signaling complex that ultimately triggers a caspase cascade to induce cellular apoptosis (Pan et al., 1997; Sheridan et al., 1997; Bodmer et al., 2000). Most TNF superfamily members, including FasL and TNF-α, have the ability to induce apoptosis in tumor cells and cause severe toxicity in normal tissues (Fujii et al., 2001). However, various tumor cells are susceptible to TRAIL-mediated apoptosis, whereas the majority of normal cells are resistant to
TRAIL (Ashkenazi et al., 1999). The possible mechanisms of normal cells in resisting TRAIL-induced apoptosis include the expression of decoy receptors and intracellular inhibitors (Zhang et al., 2000). Based on its tumor-specific cytotoxic activity, TRAIL is one of the most promising candidates for cancer therapy (Cretney et al., 2007; Herbst et al., 2010). Our group previously showed that human recombiant soluble TRAIL (sTRAIL) significantly suppressed human hepatocellular carcinoma and lung adenocarcinoma (Ma et al., 2005a,b; Shi et al., 2005).

A widely held consensus is that TRAIL and its receptors are expressed not only in tumor cells but also in such normal immune cell types as T-cells, natural killer cells, dendritic cells, and macrophages (Wiley et al., 1995; Fanger et al., 1999; Griffith et al., 1999). Indeed, TRAIL and its receptors are present in the spleen, prostate, ovary, heart, lung, kidney, and skeletal muscle (Chaudhary et al., 1997; Pan et al., 1997; Walczak et al., 1997). Because the majority of studies of TRAIL have focused on its potential in clinical cancer therapy, the functions of TRAIL in inflammation and immunity are much less understood. Recently, several studies revealed a pivotal role for TRAIL in innate immune responses. Our previous study reported that DR4 or DR5 overexpression dramatically activated the release of the inflammatory cytokines IL-8, TNF-α, CCL20, MIP-2, and MIP-1B in an NF-kB-dependent manner in 293T, MDA-MB-231, and HCT-116 cells (Tang et al., 2009a). In one study, increased expression of TRAIL showed cytotoxic effects at the site of inflammation in patients with inflammatory bowel disease (Brost et al., 2010).

High expression of TRAIL has been detected in serum from patients with systemic lupus erythematosus and psoriatic arthritis (Lub-de Hooge et al., 2005; Hofbauer et al., 2006). However, other studies have demonstrated an important role for TRAIL as an inhibitory immune modulator. Researchers have noted that TRAIL can be used as a novel anti-inflammatory agent in invasive infections (Hoffmann et al., 2007), and there is increasing evidence that TRAIL acts as a protective factor in autoimmunity and inflammation, especially in rheumatoid arthritis (Auffray et al., 2009). These different results indicate that TRAIL may have diverse functions in a variety of physiological conditions and cell types. Macrophages, among the most important immune cells, are potent immune regulators with essential roles in tissue homeostasis and remodeling (Gordon, 2003; Gordon and Taylor, 2005; Traves et al., 2012). However, the function of TRAIL in these cells has been little reported. In the present study, we focused on the TRAIL-induced innate immune response and its molecular mechanisms in macrophages.

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, small noncoding RNAs that play important regulatory roles through posttranscriptional gene repression via degradation or translational inhibition of posttarget mRNAs (Bartel, 2004; Taganov et al., 2006). This type of small RNA is critical in a wide variety of biological processes, including cell differentiation, carcinogenesis, viral infection, inflammation, and immunozy (Bartel, 2004; Bi et al., 2009; Rebane and Akdis, 2013). The miR-146 family is composed of two members: miR-146a and miR-146b. Although they are located on chromosomes 5 and 10, respectively (Williams et al., 2008), they are highly homologous and have identical seed sequences. miR-146, particularly miR-146a, is thought to modulate macrophage maturity, activation, and function in tissues (Williams et al., 2008). Taganov et al. (2006) demonstrated that miR-146a is a negative regulator of classical NF-kB activation in macrophages. They reported that IL-1 receptor–associated kinase 1 (IRAK1) and TNF receptor–associated factor 6 (TRAF6), which are key adaptors molecules in the TLR/NF-kB pathway, are direct targets of miR-146a. However, whether miR-146a is involved in the immune response to TRAIL is not clear. In the present study, we found that TRAIL induced the secretion of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in macrophages, which is negatively regulated by miR-146a.

Acetylation and deacetylation are posttranslational protein modifications that regulate many cellular processes, such as chromatin assembly and gene transcription (Quivy and Van Lint, 2004; Yang and Seto, 2007). The competition between two groups of enzymes—histone acetyltransferases and histone deacetylases (HDACs)—determines the histone-acetylation state (Yang and Seto, 2007). HDACs remove acetyl groups from specific lysine residues on histone proteins to regulate chromatin architecture and gene expression. There are 11 zinc-containing classical HDAC enzymes (HDAC1–11), divided into three distinct classes: class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), and class IV (HDAC11) (de Ruiter et al., 2003; Minucci and Pelicci, 2006). The class III HDACs (SIRT1–7) require the cofactor nicotinamide adenine dinucleotide for activity and act via a mechanism distinct from that of class I, II, and IV HDACs. Class I HDACs generally localize to the nucleus, and class II HDACs can shuttle between the nucleus and cytoplasm. HDAC11 has similarity to class I and II enzymes. It has been reported that HDACs participate in the inflammatory response of macrophages (Elsharkawy et al., 2010; Hallii et al., 2010; Chen et al., 2012; Shakespear et al., 2013). HDAC inhibitors, which were initially evaluated for their anticancer activity, have been proven to regulate TRAIL-induced apoptosis (Butler et al., 2006; Earel et al., 2006). In our previous study, the HDAC inhibitor trichostatin A (TSA) up-regulated the transcription of miR-146a by increasing the DNA-binding activity of NF-kB in lipopolysaccharide (LPS)-stimulated macrophages in both young and aged mice, suggesting that HDACs are involved in the regulation of miR-146a expression (Jiang et al., 2012). In the present study, we evaluated miR-146a levels in TRAIL-induced mouse macrophages using TSA, investigated the expression of HDACs in response to TRAIL, and analyzed the regulatory mechanism of miR-146a directly by HDAC1.

To fulfill different functions in tissues, macrophages can polarize to a myriad of phenotypes in response to multiple signals (Ruffell and Coussens, 2015). IFNγ with LPS-activated and IL-4– or IL-13–activated macrophages are two distinct subsets of macrophages. Macrophages induced by IFNγ either alone or cooperating with microbial stimuli such as LPS can guard against infection and defend against transformed cells. Transcription factors involved in induction of IFNγ or LPS-stimulated macrophages include NF-kB, STAT1, and IRF5, which interact with one another to enhance the expression of inflammatory genes. In response to Th2 cytokines (e.g., IL-4, IL-13), macrophages have high scavenging activities, produce growth factors that activate tissue repair, and suppress adaptive immune responses. STAT6, IRF4, and PPARγ have been associated with IL-4/IL-13–polarized macrophages. Actually, these two types of polarized macrophages are extremes of a continuum in a universe of functional states (Mantovani and Allavena, 2015). Tumor-associated macrophages (TAMs), which are a major component of the leukocyte infiltrate of tumors, usually display an immunosuppressive phenotype and protumorigenic properties, features that are typically associated with poor prognosis (Qian and Pollard, 2010; Hao et al., 2012). In nonprogressing or regressing tumors, however, TAMs exhibit IFNγ/LPS-polarized activation characterized by proinflammatory activity, antigen presentation, and tumor lysis (Rolny et al., 2011). Based on these findings, TAMs are attractive targets for anti-tumor interventions, and TAM re-education is one of the most important strategies. The present investigation was therefore also designed to analyze the role of TAM re-education in the antitumor activity of TRAIL.
either in vivo or in vitro.

In the present study, we demonstrated that macrophages acquire an immune-stimulating phenotype and release the proinflammatory cytokines IL-1β, IL-6, and TNF-α in response to TRAIL. The underlying regulatory mechanisms were thoroughly investigated. These data provide new evidence for TRAIL in the immune regulation of macrophages and may shed light on the utility of TRAIL-based anti-tumor therapy in human patients.

RESULTS

TRAIL-induced IL-1β, IL-6, and TNF-α expression

To investigate whether TRAIL induces the innate immune response, we challenged mice with rsTRAIL for 24 h and measured the expression of proinflammatory cytokines in TRAIL-treated mice. As shown in Figure 1A, the mice produced detectable concentrations of several proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, in serum in response to TRAIL. The supernatants from TRAIL-stimulated mice were analyzed for IL-1β, IL-6, and TNF-α using ELISA. The expression of these cytokines was detected in peritoneal macrophages from TRAIL-treated mice by q-PCR. Data are shown as mean ± SD (n = 5). (B) q-PCR analysis of IL-1β, IL-6, and TNF-α in mouse peritoneal macrophages after stimulation with rsTRAIL for the indicated times. The mRNA levels were normalized relative to the expression of β-actin. The supernatant from TRAIL-stimulated macrophages was analyzed using ELISA. Data are shown as mean ± SD (n = 5). (C) q-PCR analysis of these cytokines in human monocyte-derived macrophages after challenge with rsTRAIL. Data are mean ± SD (n = 3) of three independent experiments. *, p < 0.05; **, p < 0.01 compared with control.

FIGURE 1: TRAIL induces the expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in macrophages. (A) Serum from TRAIL-stimulated mice was analyzed for IL-1β, IL-6, and TNF-α using ELISA. The expression of these cytokines was detected in peritoneal macrophages from TRAIL-treated mice by q-PCR. Data are shown as mean ± SD (n = 5). (B) q-PCR analysis of IL-1β, IL-6, and TNF-α in mouse peritoneal macrophages after stimulation with rsTRAIL for the indicated times. The mRNA levels were normalized relative to the expression of β-actin. The supernatant from TRAIL-stimulated macrophages was analyzed using ELISA. Data are shown as mean ± SD (n = 5). (C) q-PCR analysis of these cytokines in human monocyte-derived macrophages after challenge with rsTRAIL. Data are mean ± SD (n = 3) of three independent experiments. *, p < 0.05; **, p < 0.01 compared with control.

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TRAIL-induced miR-146a expression negatively regulated the proinflammatory gene expression

Taganov et al. (2006) demonstrated that miR-146a is a negative feedback regulator that targets IRAK1 and TRAF6. We previously demonstrated that miR-146a negatively regulates the proinflammatory cytokines IL-1β and IL-6 by regulating IRAK1 expression in mouse macrophages (Jiang et al., 2012). In addition, we found that miR-146a expression was up-regulated in the TRAIL-treated human breast cancer cell line MDA-MB-231 (Wang et al., 2013a). Therefore, we hypothesized that miR-146a also regulated proinflammatory mediators in macrophages under TRAIL stimulation. As shown in Figure 2A, miR-146a expression increased significantly (threefold) in peritoneal macrophages from the mice injected intravenously with rsTRAIL compared with the untreated controls. Moreover, miR-146a expression increased significantly in a dose- and time-dependent manner in response to rsTRAIL, reaching a peak of a fourfold increase at 6 h and then decreasing to basal level over the next 18 h in response to rsTRAIL, compared with the untreated cells (Figure 2B). This result was repeatable in human monocyte-derived macrophages (Figure 2C). To determine the effects of miR-146a in TRAIL-treated macrophages, we transfected miR-146a inhibitors and mimics into mouse peritoneal macrophages. At 48 h after transfection, the macrophages were stimulated with rsTRAIL for 3 h, and then the expression of the proinflammatory cytokines was detected by quantitative PCR (q-PCR). As shown in Figure 2D, the overexpression of miR-146a by transfection with miR-146a mimics and followed by rsTRAIL treatment down-regulated the levels of IL-1β, IL-6, and TNF-α in the macrophages. However, the miR-146a inhibitor increased the expression of proinflammatory cytokines in TRAIL-treated cells. These findings indicate that TRAIL-induced miR-146a expression is negatively associated with the production of IL-1β, IL-6, and TNF-α in mouse macrophages.

TRAIL-induced miR-146a expression was dependent on NF-κB activation

It has been reported that miR-146a expression in innate immunity is driven predominantly by NF-κB, and there are two NF-κB binding sites in the upstream 550 base pairs of pre miR-146a (Taganov et al., 2006; Cameron et al., 2008; Lukiw et al., 2008). Our previous studies demonstrated that TRAIL induced miR-146a expression in MDA-MB-231 breast cancer cells in an NF-κB–dependent manner (Wang et al., 2013a). We further investigated whether TRAIL-induced miR-146a expression in macrophages was also regulated by NF-κB activation. As shown in Figure 3A, phosphorylation of IκBα (p-IκBα) expression increased in a time-dependent manner with rsTRAIL treatment. This paralleled the miR-146a expression (Figure 2B), suggesting that TRAIL-induced miR-146a expression was positively associated with NF-κB activation.

To determine whether miR-146a expression is dependent on NF-κB activation, we analyzed the miR-146a promoter activity in RAW264.7 cells. The cells were transfected with a luciferase reporter plasmid harboring the miR-146a promoter containing two NF-κB
binding sites and subsequently treated with rsTRAIL for 6 h; this was followed by a dual-luciferase reporter assay. As shown in Figure 3B, treatment with rsTRAIL increased the expression of both miR-146a and the overexpression of the p65 subunit of NF-κB. However, IκBα dominant-negative (DN) plasmid transfection completely inhibited NF-κB activity in the TRAIL-treated cells, which had a constitutive inhibitory effect on NF-κB activity (Tang et al., 2009b). These results indicated that TRAIL up-regulates miR-146a promoter activity in an NF-κB-dependent manner.

We also sought to determine whether TRAIL-induced NF-κB activation increases miR-146a expression. As shown in Figure 3C, the macrophages with the NF-κB subunit p65 knockdown exhibited lower levels of both mature and primary miR-146a transcripts compared with the controls. These results were confirmed by a chromatin immunoprecipitation (ChiP) assay, which showed that the binding of NF-κB p65 to both sites of the miR-146a promoter was increased in TRAIL-stimulated macrophages (Figure 3D). The promoter architecture of miR-146a and the PCR fragment amplified by ChIP is also shown in Figure 3D. The above data suggest that TRAIL-induced miR-146a expression is dependent on NF-κB activation in macrophages.

Histone acetylation was involved in the regulation of TRAIL-induced miR-146a expression

HDACs are enzymes that remove acetyl groups from specific lysine residues in histones to regulate chromatin architecture and gene expression. It has been reported that HDACs participate in the inflammatory response of macrophages (Elsharkawy et al., 2010; Halili et al., 2010; Chen et al., 2012; Kuo et al., 2012). To determine whether HDACs are involved in the regulation of miR-146a expression in TRAIL-stimulated cells, we treated the macrophages with rsTRAIL and/or the HDAC inhibitor TSA. As shown in Figure 4A, there were 2.5-fold and fourfold increases of miR-146a expression with either TSA alone or the combination of TSA plus TRAIL, respectively, compared with the control, suggesting that histone acetylation was involved in the regulation of miR-146a expression in the macrophages, regardless of the presence or absence of TRAIL.
shown in Figure 4B, NF-κB binding to both the I and II sites in the miR-146a promoter region increased significantly with rsTRAIL, TSA, or a combination of both. These data indicate that TRAIL-induced miR-146a expression was associated with the enhanced binding capacity of NF-κB to the miR-146a promoter by histone acetylation in mouse macrophages.

Because histone acetylation played an important role in the regulation of miR-146a expression in response to TRAIL, we hypothesized that TRAIL might regulate the expression of HDAC family members in macrophages. Therefore the mRNA expression of all HDAC family members was assessed using q-PCR in mouse macrophages treated with rsTRAIL over 24 h. As shown in Figure 4C, the mRNA expression levels of all 11 HDAC members in macrophages from mice showed time-dependent changes after treatment with rsTRAIL. The mRNA expression of class I HDACs

To investigate whether TRAIL affected the acetylation status of histone at the miR-146a promoter in LPS-stimulated macrophages, we used a ChIP assay to measure the amount of acetyl-histone H3 associated with two NF-κB binding sites in the promoters of miR-146a after rsTRAIL stimulation. Macrophages were exposed to TSA after rsTRAIL stimulation, and the levels of histone H3 acetylation were analyzed by ChIP assay. Consistent with the expression of miR-146a, the acetyl-histone H3 at the NF-κB binding sites I and II in the miR-146a promoter significantly increased after rsTRAIL treatment compared with unstimulated young cells (Figure 4B). Moreover, inhibition of HDAC activities by TSA, as expected, resulted in a significant increase in the level of histone H3 acetylation on the miR-146a promoter alone or in combination with rsTRAIL treatment (Figure 4B).

NF-κB binding to the miR-146a promoter was further confirmed by a ChIP assay in macrophages treated with rsTRAIL and TSA. As shown in Figure 4B, NF-κB binding to both the I and II sites in the miR-146a promoter region increased significantly with rsTRAIL, TSA, or a combination of both. These data indicate that TRAIL-induced miR-146a expression was associated with the enhanced binding capacity of NF-κB to the miR-146a promoter by histone acetylation in mouse macrophages.

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HDAC1 (and HDAC2) and class II HDACs (HDAC5 and HDAC7) were the opposite of the miR-146a expression levels, which were transiently repressed within 3 h and gradually increased thereafter, reaching a peak at 12 h, and then returned to baseline at 24 h of stimulation, indicating they were negatively associated with miR-146a transcription. The HDAC4 and HDAC10 expression paralleled the miR-146a expression, and the rest were either insensitive to rsTRAIL or had low expression in macrophages. These data indicated that TRAIL-induced miR-146a expression was differentially regulated by HDACs.

**HDAC1 negatively regulated TRAIL-induced miR-146a expression**

Previous reports demonstrated that the expression of miR-146a, induced by HDAC inhibitors, was inhibited by the overexpression of HDAC1 (Kuo et al., 2012; Gang et al., 2013; Wang et al., 2013b). We determined the effect of HDAC1 on miR-146a expression under rsTRAIL treatment. First, we detected the expression of HDAC1 protein in the mouse peritoneal macrophages stimulated with rsTRAIL for 24 h. In accordance with its mRNA level, HDAC1 protein expression was down-regulated within the first 3 h and gradually increased across 6 to 12 h of rsTRAIL treatment (Figure 5A). This trend was inversely correlated with miR-146a expression in the TRAIL-treated macrophages.

For further assessment of the function of HDAC1, macrophages were transfected with small interfering RNA (siRNA) for HDAC1 (si-HDAC1) or control double-stranded RNA (si-ctrl), and miR-146a expression was analyzed. As shown in Figure 5B, HDAC1 protein expression was significantly reduced after transfection of si-HDAC1, and miR-146a expression was markedly higher in TRAIL-stimulated cells than in control cells at each time point within 24 h. Notably, compared with the controls, knockdown of HDAC1 significantly rescued the expression of miR-146a when cells were stimulated with TRAIL from 6 to 24 h (Figure 5C).

Finally, the extent of HDAC1 and histone H3 acetylation (ace-H3) at the two NF-κB binding sites in the miR-146a promoter were analyzed by ChIP assay within 10 h of stimulation with rsTRAIL. As shown in Figure 5D, HDAC1 binding to NF-κB site I in the miR-146a promoter gradually increased in the first 7 h and then decreased in the next 3 h of rsTRAIL treatment. HDAC1 binding to NF-κB site II in the miR-146a promoter was significantly higher at 7–10 h of rsTRAIL treatment than at all other time points. Moreover, the acetyl-histone H3 at both NF-κB sites in the miR-146a promoter significantly increased and then decreased. This trend was negatively correlated with HDAC1 and paralleled the miR-146a level upon rsTRAIL stimulation (Figure 5D). The data suggest that HDAC1 was a key molecule involved in the regulation of TRAIL-induced miR-146a expression in the mouse peritoneal macrophages.

TAMs display cytotoxic functions against tumor cells under TRAIL stimulation

Because the peritoneal macrophages were sensitive to rsTRAIL, we wondered whether TAMs were also induced by TRAIL to produce proinflammatory cytokines, resulting in promoting or inhibiting effects on tumor cells. The expression of IL-1β, IL-6, and TNF-α was measured in TAMs of TRAIL-treated NCI-H460 allograft tumors (Figure 6A). The NCI-H460 non small cell lung cancer cell line was sensitive to rsTRAIL. The expression of these proinflammatory cytokines in response to TRAIL was uniformly increased in TAMs. Interestingly, the elevated expression of these cytokines was much higher than that in mouse peritoneal macrophages. We then assessed the properties of TRAIL-induced TAMs. To do so, we cocultured NCI-H460 cells with macrophages using a Transwell technique (Figure 6C). THP-1 cell-derived TAMs and NCI-H460 tumor cells were cocultured using Transwells (0.4 μm pore), which permit the exchange of soluble factors but prevent direct cell–cell contact. NCI-H460 cells were treated with rsTRAIL in the absence (control) or the presence of TAMs. Remarkably, the presence of TAMs aggra- vated TRAIL-induced apoptosis (Figure 6D). Accordingly, analysis of TNF expression and secretion by the macrophages showed a significant increase with rsTRAIL treatment (Figure 6E). Given that...
whether the high levels of inflammatory cytokines in TAM were also due to aberrant expression of miR-146a. We therefore tested MiR-146a expression in TAMs with TRAIL treatment. As shown in Figure 6B, miR-146a expression in response to TRAIL was elevated in TAMs. Of note, the level of miR-146a in TAMs was much lower than that in peritoneal macrophages, which might account for the markedly up-regulated expression of inflammatory cytokines in response to TRAIL in TAMs. Our hypothesis was confirmed by the knockdown of miR-146a in TAMs. MiR-146a inhibitors or corresponding control oligonucleotides (negative control) were transiently transfected into THP-1 cell–derived TAMs. NCI-H460 tumor cells were treated with rsTRAIL in the presence or absence of transfected macrophages. As expected, miR-146a inhibitor transfection released the blocking effect of proinflammatory cytokines (such as TNF-α; Figure 6G) and enhanced the antineoplastic ability of TAMs in response to TRAIL (Figure 6F). These data demonstrated that TRAIL stimulation induced TAMs to display an immunosuppressive phenotype and anti-tumor properties, which were negatively regulated by miR-146a.

**DISCUSSION**

In addition to TRAIL’s effective antitumor activity, its immunoregulatory effects are beginning to be studied. Our previous study demonstrated that the TRAIL receptor mediated the release of the inflammatory cytokines IL-8 and TNF-α in an NF-κB–dependent manner and that the TRADD–TRAF2–NIK–IKKα/β signaling cascade was involved in TRAIL receptor–mediated signal transduction in 293T and tumor cell lines (Tang et al., 2009a). In the present study, we demonstrated that TRAIL increased the expression of IL-1β, IL-6, and TNF-α in macrophages of both humans and mice, indicating that TRAIL could trigger innate inflammatory responses.

Cells employ a multilayered molecular mechanism to regulate innate immunity and inflammation. In the present study, we found that the proinflammatory cytokines were transiently induced and subsequently repressed in TRAIL-stimulated macrophages. We therefore deduced that crucial negative regulatory mechanisms were involved in the TRAIL-induced inflammatory response in macrophages. We previously found that there was a TRAIL–NF-κB–miR-146a–CXCR4 axis in MDA-MB-231 cells that suppresses metastasis (Wang et al., 2013a). Thus we hypothesized that a similar TRAIL–NF-κB–miR-146a loop regulates the inflammatory response in cells. This idea was supported by the fact that TRAIL increased miR-146a expression in a time- and dose-dependent manner, and overexpression of miR-146a inhibited the production of IL-1β, IL-6, and TNF-α. Knockdown of the NF-κB p65 subunit suppressed TRAIL-mediated up-regulation of miR-146a. ChIP and luciferase reporter assay further confirmed that TRAIL-mediated elevation of miR-146a expression was NF-κB dependent and correlated with the binding ability of NF-κB p65 to the miR-146a promoter in macrophages.

We also found that pretreatment with the NF-κB signaling inhibitor Bay 11-7082 prevented IL-1β, IL-6, and TNF-α expression in rSTRAIL-induced macrophages (unpublished data), indicating that the NF-κB signaling pathway was involved in the TRAIL-induced inflammatory response. However, there was scarcely any increase of IL-1β and IL-6 mRNA with the TRAIL receptor overexpression in 293T cells (Tang et al., 2009a), suggesting that the precise signaling pathway induced by TRAIL in macrophages might be different from that in 293T cells. Of note, after 24 h of TRAIL stimulation, the activity of NF-κB was still strongly up-regulated (Figure 3A), whereas the expression of miR-146a was reduced to the baseline (Figure 2B). We therefore speculated that there might be a more complicated regulatory mechanism in the process of TRAIL-induced miR-146a expression.

miR-146a regulated the expression of IL-1β, TNF-α, and IL-6 in TRAIL-stimulated peritoneal macrophages, we were interested in whether the high levels of inflammatory cytokines in TAM were also...
The currently accepted view is that miRNA expression is dominated at the transcriptional level and regulated by epigenetic mechanisms (O’Donnell et al., 2005; Zhao et al., 2005; Zhang et al., 2012). Many studies have demonstrated that HDACs are involved in controlling the transcription of NF-κB-dependent genes in immune and inflammatory responses (Calao et al., 2008; Esharkawy et al., 2010; Shakespear et al., 2011; Zhou et al., 2013). Our previous study showed that the HDAC inhibitor TSA up-regulates miR-146a expression by increasing the binding of NF-κB to the mir-146a promoter in osteoarthritis fibroblast-like synoviocytes (Wang et al., 2013b). Thus, we speculated that HDAC might participate in miR-146a transcriptional activation in a promoter-dependent manner in TRAIL-stimulated cells. We observed that inhibition of histone deacetylation by TSA up-regulated miR-146a expression with or without TRAIL administration in macrophages (Figure 4A). To determine whether the induction of miR-146a by TSA was due to the regulation of histone acetylation in the miR-146a promoter, we conducted a ChIP assay and found that TSA up-regulated the NF-κB–binding activity and histone H3 acetylation levels at both NF-κB binding sites in the miR-146a promoter, suggesting that histone acetylation regulates miR-146a expression by altering the promoter activity and subsequently regulating NF-κB binding to the promoter (Figure 4B). There is evidence that histone acetylation/deacetylation indirectly regulates NF-κB–dependent genes by altering the activity of the upstream IKK (Yin et al., 2001; Takada et al., 2006), but it remains unclear whether TSA also indirectly regulates miR-146a transcription in macrophages, which is an interesting direction for further research. Furthermore, as shown in Figure 5D, the extent of HDAC1 and ace-H3 at the two NF-κB binding sites in the miR-146a promoter was regulated by rSTRAIL, which provides direct evidence of a linear TRAIL-HDAC/TSA-miR-146a pathway. The additive effects of TRAIL and TSA, as shown in Figure 4B, are interesting, and the complicated regulatory network may account for these. TRAIL-stimulated miR-146a expression may also be negatively controlled by a feedback loop. In this case, TRAIL treatment will strengthen the feedback and show subdued promoting effect on the acetylation of histone H3 at the two NF-κB binding sites in the miR-146a promoter. Thus combined treatments of TRAIL and TSA show additive effects. Within 24 h of TRAIL stimulation, the mRNA trends of the HDAC subtypes varied, indicating that HDACs regulated multiple genes and had diverse functions in the TRAIL-mediated immune response of macrophages (Figure 4C). HDAC subtypes (HDAC1, 2, 4, 5, 7, 11) that had both negative correlation with miR-146a and relatively high expression levels were involved in the regulation of the transcription of miR-146a or proinflammatory cytokines. HDAC1, a class I HDAC, has been reported to associate with the immune response and to inhibit proinflammatory genes such as IL-1β, IL-6, TNF-α, IL-8, Ccl2, and Cxcl10 (Zhong et al., 2002; Esharkawy et al., 2010; Janzer et al., 2012). Wang et al. (2013b) noted that an increase in miR-146a expression by the HDAC inhibitors was prevented by the overexpression of HDAC1, 4, and 6, suggesting that they contribute to inhibition of miR-146a expression. The present study provides the first direct evidence that HDAC1 associates with the negative regulation of miR-146a expression at the transcriptional level in TRAIL-treated macrophages. Further research is necessary to evaluate the roles of other HDACs in TRAIL-induced miR-146a transcription.

TRAIL is best known for its ability to induce apoptosis in cancer cells without severe toxicity to most normal cells, which has led to several clinical trials to evaluate its antitumor potential. Experimental evidence shows that this antineoplastic agent may regulate the tumor microenvironment (Diao et al., 2013). We were therefore most concerned with the characteristics of TAMs in response to TRAIL. Inflammation is a key component of the tumor microenvironment. It is already clear that the production of proinflammatory cytokines (e.g., IL-1β, TNF-α, IL-6) in TAMs is insufficient because of defective NF-κB activation in response to the IFNγ or TLR4 signals, and TAMs promote tumor cell growth under this circumstance (Allavena et al., 2008). However, recent studies have demonstrated that, under certain conditions or stimulations, TAMs display cytotoxic functions against tumor cells (Duluc et al., 2009; Beatty et al., 2011; Rolny et al., 2011). We therefore wondered whether TRAIL induced TAMs to produce proinflammatory cytokines and display cytotoxic functions against tumor cells. In line with peritoneal macrophages, biosynthesis of IL-1β, IL-6, and TNF-α in TAMs was significantly promoted by rSTRAIL administration. At least partly due to the lower sensitivity of miR-146a expression in response to TRAIL, the changes in the proinflammatory cytokines were much greater.

We found that TAMs showed toxicity toward tumor cells in the presence of TRAIL in vitro, and this effect of macrophages was negatively controlled by miR-146a. By assessing the TNF-α secretion of TRAIL-treated TAMs, we inferred that the tumor-suppressing effect of TRAIL-induced TAMs was probably related to the proinflammatory phenotype. The relationship between inflammation and cancer has long been discussed and is extremely complicated. Take TNF-α, for example, which was most strikingly sensitive to TRAIL treatment. Until now, the effect of TNF-α on oncogenesis has been obscure. It is thought to have double-edged roles in cancer development and metastases (Yang et al., 2006; Woo et al., 2013). Li and his colleagues demonstrated that low levels of endogenous TNF-α act as a tumor promoter (Li et al., 2009), and high doses have antitumor activity. So, the specific impact of TRAIL-induced and TAM-secreted proinflammatory cytokines on tumor cells is of great importance. Although the detailed mechanism of TRAIL-treated macrophages’ induction of tumor-killing activity remains to be explored, the data presented here illustrate for the first time that TRAIL can re-educate TAMs to adopt proinflammatory phenotype and to display cytotoxic functions against tumor cells. We demonstrated that TNF-α acts in conjunction with other TAM-derived factors, and the overall balance of tumor-inhibiting and tumor-promoting factors will produce the net effect of tumor suppression. Macrophage colony-stimulating factor (M-CSF or CSF1) is a prominent cytokine known to regulate myeloid development, macrophage differentiation, and proliferation. CSF1-mediated signaling has been shown to be critical for sustaining macrophage numbers in tumors and also for promoting tumor proliferation, angiogenesis, invasion, and metastasis (Escamilla et al., 2015). Thus far, few reports examining whether TRAIL or miR-146a regulates CSF-1 are available, and it remains unclear whether TRAIL treatment can influence tumor metastasis. An interesting direction for further research would be to evaluate an association between TRAIL treatment and CSF-1 secretion in TAMs.

In summary, we demonstrated that TRAIL-stimulated macrophages released the proinflammatory cytokines IL-1β, IL-6, and TNF-α and induced systemic mild inflammation. The innate immune responses were negatively controlled by miR-146a and finely regulated by HDAC. HDAC1, a primary class I HDAC expressed in macrophages, was directly involved in the repression of miR-146a transcription, especially in the late stage of TRAIL stimulation. These findings provide a primary mechanistic insight into the inflammatory response.
response and immunoregulation in TRAIL-mediated macrophages and offer valuable background for the further study of the physiological and pathological functions of TRAIL. Most importantly, our study sheds light on the regulation of TAMs by TRAIL in tumor tissues and suggests that macrophage re-education is an important component of TRAIL's antitumor activity.

**MATERIALS AND METHODS**

**Animal experiments**

BALB/c and C57/BL6 male mice obtained from the Institute of Zoological Sciences, Chinese Academy of Medical Sciences, in Beijing, were used. The animals were kept away from potential endemic viral pathogens, which could influence the inflammatory response. Five-to six-week-old C57/BL6 male mice were randomized into two groups (n = 6). rsTRAIL (20 mg/kg per day every other day) was administered by intravenous injection (i.v.). Phosphate-buffered saline (PBS) was administered as a control. One week later, the peritoneal macrophages and blood serum were collected before the mice were killed. All animal procedures were performed in accordance with the Committee on the Use and Care of Animals, Chinese Academy of Medical Sciences.

**Cell culture and treatment**

Peritoneal macrophages were isolated from BALB/c male mice. Mice were injected intraperitoneally with 2.5 ml of 3% thioglycollate (Difco, Detroit, MI). Three days later, the peritoneal exudate cells were isolated by washing the peritoneal cavity with ice-cold PBS. These cells were incubated for 2 h, and the adherent cells were used as peritoneal macrophages.

The primary human monocytes were isolated from fresh blood of healthy volunteers by the Ficoll density gradient technique (Tianjin TBD Biotech Development Center, China). Then the CD14+ monocytes were purified through fluorescent cell sorting and cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT) and 104 U/ml recombinant human SCF-1 (R&D Systems, Minneapolis, MN) for 7 d. The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki and were approved by the local ethics committee.

The human embryonic kidney fibroblast 293T, human leukemic monocyte line THP-1, and murine leukemic macrophage line RAW264.7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human 293T cells were maintained in high-glucose DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT) and 104 U/ml recombinant human SCF-1 (R&D Systems, Minneapolis, MN) for 7 d.

The primary human monocytes were isolated from fresh blood of healthy volunteers by the Ficoll density gradient technique (Tianjin TBD Biotech Development Center, China). Then the CD14+ monocytes were purified through fluorescent cell sorting and cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT) and 104 U/ml recombinant human SCF-1 (R&D Systems, Minneapolis, MN) for 7 d.

**Measurement of miRNA and mRNA expression**

Total RNA was extracted from each cell sample using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. First-strand cDNA was synthesized with 2 μg of total RNA as template and M-MLV reverse transcriptase (Promega, Madison, WI). The Taqman miRNA assay (Applied Biosystems, Foster City, CA) was used to quantitatively detect the expression of miR-146a following the manufacturer’s instructions. Quantification of miRNAs or mRNA by real-time q-PCR was performed in an ABI 7300HT thermocycler at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold values were converted to relative gene expression levels using the 2−ΔΔCT method (Livak and Schmittgen, 2001). The data were normalized to that of internal control GAPDH or U6. The primer sequences for q-PCR are shown in Table 1.

**Cytokine detection**

The expressions of IL-1β, IL-6, and TNF-α in mice plasmas or culture supernatants of cells treated with TRAIL were determined by using corresponding enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA) according to the manufacturers’ instructions.

**Western blot analysis**

Proteins extracted from cells were separated by SDS–PAGE (5% acrylamide for spacer gel and 12% acrylamide for separation gel) and transferred to polyvinylidene difluoride membrane (Amer sham Biosciences, Piscataway, NJ). The membrane was incubated with specific antibodies and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Zhongshan Goldenbridge Biotechnology, Beijing, China). The primary antibodies included mouse anti-HDAC1 monoclonal antibody (Millipore, Billerica, MA), rabbit anti-ixBα and p-ixBα pAbs (Cell Signaling Technology, Beverly, MA). All primary antibodies were used at concentrations of 1/500–1/1500. Specific protein bands were visualized using an ECL chemiluminescence system (Amer sham Biosciences, Buckinghamshire, UK) followed by exposure to X-ray film.

**Plasmid, siRNA, and transfection**

The pGL3-miR-146a promoter reporter plasmid (miR-146a promoter-luc) was a pGL-basic vector containing a 560-base pair upstream sequence of the predicted transcriptional start site of the pri-miR-146a (primary transcript of miR-146a; Taganov et al., 2006). The NF-κB p65 submit overexpression vector was a kind gift from Jae Won Rhee (Rhee et al., 2007). The kappa B (ixB) dominant-negative (ixB DN, ixB (S32A/S36A)) plasmid was used to inhibit NF-κB activity (Tang et al., 2009b). MiR-146a mimics (double-stranded RNA oligonucleotides) and inhibitors (single-stranded chemically modified oligonucleotides) (GenePharma, Shanghai, China) were used to overexpress and inhibit miR-146a expression in cells. Negative control mimics or inhibitors (GenePharma, Shanghai, China) were transfected to act as matched controls. HDAC1 siRNA (sc-35422, Santa Cruz Biotechnology, Santa Cruz, CA) and NF-κB p65 siRNA (sc-37007, Santa Cruz Biotechnology) were used to inhibit the activity of HDAC1 and NF-κB p65 subunit, respectively. Macrophages described in the Cell culture and treatment section were transfected with RNAs at a final concentration of 10 nM using INTERFERin (Polyplus-Transfection SA, Illkirch, France) according to the manufacturer’s instructions.
Chromatin immunoprecipitation (ChIP) assay
The peritoneal macrophages (1–2 × 10^7 cells per dish) were cultured with or without 1 μg/ml TRAIL for the indicated time. Then the cells were cross-linked by formaldehyde (Sigma-Aldrich, St. Louis, MO) and collected for extraction of chromatin. The ChIP assay was performed using the EZ ChIP kit (Millipore, Billerica, MA) according to the protocol. The fragmented chromatin DNA (200–500 base pairs in length) was immunoprecipitated with ChIP anti–NF-κB P65 subunit (Abcam, Cambridge, MA), anti-acetylated histone H3 (Millipore), or anti-HDAC1 (Millipore) antibody, respectively; and the corresponding IgG antibody was used for unspecific binding. q-PCR quantitation of precipitated chromatin fragments was performed for the final test. The primers were designed to amplify two reported NF-κB binding sites (I and II) in miR-146a promoter, respectively (Table 1). The negative control primers, which have been reported by Staab et al. (2007), were used to represent specificity of ChIP reactions. The DNA purified from the sonicated nuclear lysate was used as an input control. Expression of a target DNA sequence was normalized to the input control DNA and represented as fold enrichment compared with the nontreated control (set as onefold).

Isolation of TAMs from tumor tissue
Five- to six-week-old BALB/c nude mice were housed under specific pathogen-free conditions. Animal experiments proceeded in accordance with the guidelines of the National Institutes of Health. NCI H460 cells (5 × 10^7) in 50 μl of PBS were injected subcutaneously into the right flanks of 12 nude mice. Twenty days later, the animals were randomized into two groups: rSTRAIL (200 μg per mouse every other day) was administered by i.v. injection, and PBS was administered as a control. Two weeks later, the animals were killed, and the tumor tissues were surgically excised.

Tumor tissues were chopped into small pieces and then incubated for 1 h at 37°C in 5 ml of HBSS (10% FBS) containing 125 U/ml of collagenase I (Sigma, St. Louis, MO), 125 U/ml of collagenase IV (Sigma, St. Louis, MO), 60 U/ml of Dnase I (Boehringer Mannheim, Indianapolis, IN), and 60 U/ml of hyaluronidase (Sigma, St. Louis, MO). Supernatants were harvested and then depleted of red blood cells with ACK Lysis Buffer (Leagene, Beijing, China). Cell suspensions were passed through a fine screen mesh and then seeded in 100-mm dishes (3.5 × 10^7 cells in a final volume of 10 ml incomplete RPMI 1640 medium). After 1 h of incubation, nonadherent cells were washed off. More than 90% of the adherent cells were macrophages.

Cell survival assays
NCI H460 tumor cells (1.5 × 10^5) were seeded into a 24-well plate. After 48 h of treatment, the medium was removed, and 0.2 ml of crystal violet staining solution was added to each well. The plate was returned to the incubator for 30 min for the uptake of the violet dye crystals. Thereafter the staining solution was removed, and the cells were rapidly washed with ddH2O. Tumor cell images were captured; this was followed by the addition of 0.5 ml 50% acetic acid solution to extract the dye from the cells. Finally, the plate was transferred to a microplate reader equipped with a 590-nm filter to measure the absorbance of the extracted dye. Cells without any treatment were used as the negative control. Cell viability was converted as the percentage of the negative control.

### Table 1: Primers used for q-PCR analysis.

|        | Forward                     | Reverse                     |
|--------|-----------------------------|-----------------------------|
| **Real-time q-PCR** |                             |                             |
| IL-1β  | CCGTGTGGTTTCTCCTGTTGCT     | GCCTAATGTCCCCCTTGTGAACCA    |
| IL-6   | CAGAAGGGTGGCTAAGGACCA      | AGCGACCTAGTTTGGCGAGTAG      |
| TNF-α  | CCTGTGTCCTCCTCTTTG         | TCAGTGATGTAAGGACGCTG       |
| HDAC1  | TCTGCTGCCTGCGACTCTAC-3'    | GTAGGAGCAGTCTATTGGAGATCT    |
| HDAC2  | TGGAGGAGGCTACACACACTC      | TTTGAAACACAGGTGACTGT       |
| HDAC3  | CTCCCCCTTCCTCTAAACT        | TTTGATGGAAGGACAGACT       |
| HDAC4  | CAGACAGCAAGGCCCCTCTCTA     | AGACCTGTGGAACCTTGG         |
| HDAC5  | TGAGAGGCGAGGCCCTTCACT      | CTTCCAAGTGCCACTCCACAC      |
| HDAC6  | ACCGGATGACCCTGGCAACT       | TCCAGGGCACATTGACAGTA       |
| HDAC7  | TTTCAAGCCAGGCCCCCCAGTG     | AAGACAGGACAGGACCTCAG       |
| HDAC8  | TGGAGATGCCAGAGGACACC       | TTTGGGACCTTCACAAAGGA       |
| HDAC9  | TGGAGCACGCGAGGGCAAGAAGA    | TTGGGGACCTTCACAAAGGA       |
| HDAC10 | CGGACCACCTTACTGGAACA       | TTGGGGACCTTCACAAAGGA       |
| HDAC11 | GGAATTGAGGTGGGGCAAGCAG     | GCCCAGCGTTGTACACACCA       |
| β-Actin| GACCTGACAGACTCTCTG         | AGACACGACACTGTGTTG         |
| **ChIP-PCR** |                             |                             |
| NF-κB p65 I | CTTGAAAAGGCCAACACAGGCTCGTG | GCGGGCAGCTCGATGGGCT        |
| NF-κB p65 II | AGCGAGTAAGCCTCGCAGCCTCCC  | CATTCCGACCCAGCGAGCTA       |
| Negative control | ATGGTTGCCACTGGGGATCT     | TGCCAA AGCCTAGGGGAAGA    |
THP1–macrophage differentiation and Transwell coculture assay

THP1 cells were differentiated in Transwell inserts (BD Biosciences, San Diego, CA). To differentiate THP1 cells into tumor-associated macrophages, we treated THP1 cells with 200 ng/ml of 12-O-tetradecanoylphorbol-13-acetate for 6 h. Subsequently, the culture medium was replaced by NCi H460 tumor cell–conditioned medium. After differentiation for 24 h, the inserts were washed in RPMI three times before being placed in wells with preplated NCi H460 tumor cells. Experiments using rSTRAIL treatment were performed for 48 h by adding the reagent to the wells, so that both cell populations were exposed to the same conditions.

Statistical analysis

All the experiments were performed at least three times. The data are shown as mean ± SD. Significance was determined with Student’s t test. p Values were also used to measure the statistical significance.

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