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TNF-α-elicited miR-29b potentiates resistance to apoptosis in peripheral blood monocytes from patients with rheumatoid arthritis

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Abstract

CD14-positive monocytes from patients with rheumatoid arthritis (RA) are more resistant to apoptosis, which promotes their persistence at the inflammatory site and thereby contributes crucially to immunopathology. We sought to elucidate one mechanism underlying this unique pathogenesis: resistance to apoptosis and the potential involvement of miR-29b in this process. CD14-positive peripheral blood monocytes (PBMs) from RA patients were observed to be resistant to spontaneous apoptosis compared to PBMs from healthy volunteers. Intriguingly, expression of miR-29b was significantly upregulated in PBMs from RA patients than those from healthy volunteers, and this upregulation was correlated with RA disease activity. Functionally, forced expression of the exogenous miR-29b in CD14-positive Ctrl PBMs conferred resistance to spontaneous apoptosis and Fas-induced death, thereafter enhancing the production of major proinflammatory cytokines in there cells. Following identification of the potential miR-29b target transcripts using bioinformatic algorithms, we showed that miR-29b could directly bind to the 3′-UTR of the high-mobility group box-containing protein 1 (HBP1) and inhibited its transcription in PBMs. Importantly, stable expression of the exogenous HBP1 in differentiated THP-1 monocytes effectively abolished miR-29b-elicited resistance to Fas-induced apoptosis. Finally, among patients with RA and good clinical responses to immunotherapy, expression levels of miR-29b were significantly compromised in those treated with infliximab (a TNF-α inhibitor) but not in those treated with tocilizumab (a humanized mAb against the IL-6 receptor), pointing to a potential association between miR-29b activation and TNF-α induction. The available data collectively suggest that TNF-α-elicited miR-29b potentiates resistance to apoptosis in PBMs from RA patients via inhibition of HBP1 signaling, and testing patients for miR-29b/HBP1 expression ratios may provide more accurate prognostic information and could influence the recommended course of immunotherapy.

Keywords Rheumatoid arthritis (RA) · Peripheral blood monocytes (PBMs) · miRNA · Apoptosis · TNF-α

Introduction

Rheumatoid arthritis (RA), a chronic, progressive, systemic autoimmune disease, is characterized at the molecular level by abnormal pro-inflammatory response and resistance to

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apoptosis in many cell types including T-cells, RA-fibroblast-like synoviocyte (RA-FLS), peripheral blood monocytes (PBMs). Hyperproduction of distinct pro-inflammatory cytokines (e.g. TNF-α, IL-1ß, IFN-γ, IL-6, IL-8, IL-12, IL-17, IL-18 and IL-32), in concert with abnormal activation of growth factor pathways (e.g. FGF2 and VEGF), potentiates an aberrant survival of RA-FLS [1]. Likewise, while increased autophagy in $CD4^+$ T-cells from RA patients leads to T cell hyperactivation and apoptosis resistance [2], disturbed transmembrane TNF (tmTNF) reverse signaling is able to interfere with apoptosis of RA PBMs [3]. Other molecular events that cause an aberrant survival consist of persistent activation of JAK/STATs, SAPK/MAPK and PI3K/Akt/mTOR pathways [1]. Apparently, multiple mechanisms are certain to coexist to confer apoptosis resistance in RA, and a better knowledge of the molecular basis determining such impaired homeostasis should contribute significantly to both understanding and treatment of the disease.

Based on expression levels of CD14 and CD16, PBMs have been subdivided into three groups: classical (CD14++CD16−), intermediate (CD14++CD16+) and nonclassical (CD14+CD16++) monocyte subsets. CD14+CD16++PBMs are a rather small cell population in peripheral blood and only represents ~ 10% of PBMs [4]. Intriguingly, in patients with chronic inflammatory diseases (e.g. RA), there is an increased subset of monocytes with a nonclassical phenotype [5]. To this end, accumulated data evidence that nonclassical PBMs are also significantly increased in synovial fluid from patients with RA and have an activated phenotype [6]. The nonclassical PBMs have been shown to possess the unique capability to promote Th1 as well as Th17 responses of autologous peripheral CD4 memory T cells [7], and are a major source of TNF [8]. Thus, the nonclassical PBMs, though constituting a small percentage of PBMs, definitely play important roles during the pathogenesis of RA. During the disease progression of RA, recruited nonclassical PBMs exhibit activated phenotypes, which are characterized by high resistance to apoptosis and robust production of proinflammatory cytokines such as IL-1, TNF-α and IL-6 [9]. Uncontrolled active PBMs with persistent inflammation subsequently cause damage to articular cartilage, underlying bone and tendinous cords. These unique subtypes of PBMs thus function as key players in RA [10, 11]. However, little information is available regarding the regulation of apoptotic function in nonclassical PBMs and the role for this process in RA progression. This information is critical, as persistence of monocytes at the inflammatory site is the prerequisite for enhanced systemic inflammatory response, and thereby contributes essentially to immunopathology [10]. Thus, the regulation of apoptotic function in PBMs could represent a desirable target for promoting PBMs removal, preventing persistent inflammation and enhancing immunotherapy effectiveness.

MicroRNAs (miRNAs), a class of small, well-conserved, non-coding RNAs that regulate a vast array of cellular processes by targeting the three prime untranslated region (3′-UTR) of target genes, are emerging as the largest contributors to the diversity of cellular functions under both physiological and pathological conditions [12]. Compelling evidence supports that miRNAs serve as influential mediators of monocyte-driven inflammation. For example, miR-9 is up-regulated in human polymorphonuclear neutrophils after TLR4 activation by LPS stimulation and miR-9 regulates the inflammatory response by directly targeting the NFKB1 signaling [13]. miRNA-31 over-expression ameliorates synovial cells apoptosis induced by RA [14]. Likewise, miR-199a-3p, miR-708-5p and miRNA-124a have all been shown to target key signaling pathways and thereby regulate fundamentally the pathogenesis of RA in synovial cells [15–17]. While miRNAs have been studied extensively in synovial cells, there is scant information vis-à-vis the functional roles of these miRNAs in PBMs thereof.

As miR-29b has been reported to be significantly upregulated in inflammation in general and in PBMs and fibroblast-like synoviocytes in particular [18], we sought to investigate whether there is a direct role for this miRNA in the resistance to PBMs apoptosis. In this study, we provide the evidence that miR-29b functions as an essential regulator of monocyte/macrophage apoptosis during the progression of RA. Our combined analysis should pave the way for a better understanding of the relationship between posttranscriptional molecular alterations and systemic induction of apoptotic resistance in RA.

Materials and methods

Patients and healthy volunteers

Patients with RA who had not received any immunotherapy (n = 20) or who had received Tocilizumab (n = 11, patients received i.v injection of Tocilizumab at a dose range from 4 to 8 mg/kg every 28 days, with a median treatment duration with iv Tocilizumab of 16.2 months) or Infliximab therapy (n = 10, initial dose was 3 mg/kg body weight and subsequent dose escalation could be increased up to 10 mg/kg, with a minimum duration of 6 months) as routine care for uncontrolled arthritis were recruited from Department of Rheumatology and Immunology, Xi'an Institute of Rheumatology, Xi'an No.5 Hospital during from October 2014 to May 2016. RA were diagnosed at least 1 year according to the criteria described in *American College of Rheumatology 1987 revised classification criteria for RA*. Moreover, healthy individuals (n = 15) recruited from staff in our hospital served as normal controls. The clinical parameters of the patients and healthy donors were described in

Supplementary Table 1. Laboratory investigation of the human blood samples include C-reactive protein (CRP), rheumatoid factor (RF) and erythrocyte sedimentation rate (ESR). All participants gave the infirmed consent for donation of their PBMs. The procedures involved in the human study strictly conformed to the *2008 Revised Declaration of Helsinki* and were approved by the Ethics Committees of Xi'an No. 5 Hospital under number XAIRHC-002013-016.

Preparation and treatment of human PBMs

The human nonclassical (CD14+CD16++) PBMs were isolated and purified from peripheral blood samples using EasySep™ Human Monocyte Isolation Kit [19] (STEMCELL, Beijing, China, purity > 94%), as per the manufacturer's instructions. PBMs were cultured in RPMI 1640 containing 100 U/mL of penicillin G, 100 mg/mL of streptomycin, 2 mM of L-glutamine and 10% fetal calf serum (FCS, Thermo Fisher Scientific, Shanghai, China) at 37 °C under a 5% CO_2 humidified atmosphere [10]. To manipulate the expression levels of miR-29b, PBMs were transfected with miR-29b mimics or mimics negative control (Mimics-NC) (Sigma-Aldrich, Shanghai, China) using MISSION Transfection Reagent (Sigma-Aldrich) for 48 h, as per the manufacturer's instruction. The siGLO Red transfection indicator (also known as Dy547) is a fluorescent oligonucleotide duplex that localizes to the nucleus, thus permitting unambiguous visual assessment of uptake into mammalian cells. It is ideal for use in optimization experiments to determine optimal siRNA, crRNA:tracrRNA, or microRNA reagent transfection conditions and for monitoring relative delivery efficiency [20]. So we used Dy547 here as a qualitative indicator of delivery efficiency of miR-29b mimics. To study the potential induction of miR-29b expression by TNF-α, PBMs were treated either with different doses of TNF-α (R&D Systems, Shanghai, China) for 24 h or with 100 ng/mL of TNF-α for different durations, followed by RT-qPCR analysis of miR-29b expression, as described below.

Measurement of cell survival and apoptosis

PBMs were cultured for 22 h to induce spontaneous apoptosis [10]. For anti-Fas antibody-mediated apoptosis, freshly isolated PBMs or PBMs with different transfections were cultured as above, in the presence of 50 ng/mL of agonistic anti-Fas antibody (clone CH11, Merck Millipore, Hong Kong, China) or IgM isotype control [10], for 22 h. Cell viability was then evaluated spectrophotometrically by MTT assay at 490 nm. Apoptosis was measured using an apoptosis ELISA kit (Roche Diagnostics, Shanghai, China) according to the manufacturer's instructions. Final spectrophotometry for apoptosis assay was developed by measuring the absorbance at 405 nm on an ELISA plate reader (Bio-Rad, Beijing, China).

RT-qPCR

Total RNA including miRNA was isolated and purified using the miRNeasy Mini Kit (Qiagen, Shanghai, China), as instructed by the manufacturer. Levels of mature miR-29b were quantified using the TaqMan® MicroRNA Reverse Transcription Kit and TaqMan miRNA assays provided by Applied Biosystems (Thermo Fisher Scientific). To determine the expression levels of other target genes, RNA samples were reverse transcribed using ProtoScript® II Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA), followed by qPCR analysis using an ABI Prism 7300 instrument and SYBR Green reagents (Thermo Fisher Scientific). Relative strengths of expression of different targets were compared using the $2^{-\Delta\Delta}$Ct method, with *18S* RNA or human U6 snRNA serving as internal controls. Primers used for gene expression analysis were as follows: high-mobility group box-containing protein 1 (HBP1, gene accession number NM_001244262.1), 5′-TGAAGGCTG TGATAATGAGGAAGAT-3′ and 5′-CATAGAAAGGGT GGTCCAGCTTA-3′ [21]; *CD14* (gene accession number NM_000591.4), 5′-AAGCACTTCCAGAGCCTGTC-3′ and 5′-TCGTCCAGCTCACAAGGTTC-3′; and *18S* (gene accession number M10098.1), 5′-CTCGCCGCGCTCTAC CTACCTA-3′ and 5′-ATGAGCCATTCGCAGTTTCACTGT A-3′ [22].

Human cytokines ELISA

At the end of cell culture, supernatants were collected and immediately subjected to measurement of human cytokines using the V-PLEX Human Cytokine 30-Plex Kit (Meso Scale Diagnostics, Rockville, MD, USA) following the manufacturer's instructions.

Western blotting

Western blotting was carried out according to our previous work [23]. Membranes were incubated with different primary antibodies including rabbit anti-HBP1 (Abcam, Shanghai, China) and rabbit anti-β-ACTIN (Abcam). β-ACTIN served as loading control.

Luciferase reporter assay

The DNA fragments (~1.1 kb) harboring the putative miR-29b-binding site in human *HBP1* 3′-UTR were subcloned into the pGL4-Luc reporter vector (Promega, Beijing, China), with the aid of the 2.0 Dry-Down PCR cloning kit (Clontech, Shanghai, China). The site-directed mutagenesis of the

miR-29b binding-site in *HBP1*/3′-UTR was achieved using QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). For the reporter assay, 5×10^6 of NIH/3T3 cells were transfected with the wild-type reporter construct of pGL4-Luc-*HBP1*/3′-UTR or mutated reporter construct, along with miR-29b mimics or Mimics-NC, using ViaFect (Promega). 48 h later, cells were harvested and the relative luciferase activity was determined using a Luciferase Reporter Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China).

Generation and treatment of THP-1/HBP1 cells

To stably express the exogenous HBP1, we employed the ViaFect reagent (Promega). Despite the protocol recommended by the manufacturer, we made the following modifications: (i) We used ViaFect reagent for transfection because it has been tested to be successful in transfection of THP-1 cells. (ii) We used 20% autologous human serum (PAA) instead of 10% FBS in culture supplementation. (iii) THP-1 cells (Sigma-Aldrich) were adjusted to 2.5×10^6 cells/transfection cuvette. (iv) The concentration of pCMV3-HBP1 plasmids were adjusted to 0.5 µg/cuvette. Following transfection, culture medium was replaced with fresh medium supplemented with 200 µg/mL of Hygromycin (Thermo Fisher Scientific) every 2 to 3 days until resistant clones appeared. After 3–4 weeks, the positive clones were harvested to examine HBP1 expression using Western blotting. To induce THP-1/HBP1 cells to differentiate into macrophages, we cultured THP-1/HBP1 cells in the RPMI 1640 medium containing 5 ng/mL of phorbol 12-myristate 13-acetate (PMA) for 48 h [24]. Cells were then subjected to miR-29b mimics/Mimics-NC transfection, followed by cell survival and apoptosis assays, as described above.

Statistical analysis

At least three independent experiments were performed in triplicates for each assay. Data presented as mean ± S.E.M. were subjected to statistical analysis using GraphPad Prism 5 software (GraphPad, San Diego CA, USA). Data normality was determined using normal probability plots and compared using *Student's t*-test or one way analysis of variance (ANOVA) as appropriate. The association between levels of miR-29b and the common parameters for RA disease activity was determined using Pearson's linear regression analysis. A *P*-value of below 0.05 was considered as statistically significant.

Results

Up-regulation of miR-29b expression in $CD14^+$-PBMs from RA patients

$CD14^+$ monocytes from PBMs of the patients with RA have been reported to show elevated resistance to apoptosis [11]. Indeed in our study, a significant increase in the percentage of live PBMs (the PBMs purified mostly include $CD14^+CD16^{++}$ subset in our study) was observed in RA patients, compared to control PBMs (SFig. 1, Fig. 1a). A 22-h cell culture resulted in a dramatic ~ 19.7-fold induction of apoptosis in control PBMs, but only caused a modest ~ 5.3-fold induction of apoptosis in RA PBMs (Fig. 1b), indicating that the latter are less prone to spontaneous apoptosis. Interestingly, miR-29b expression was more robust in RA PBMs than in control PBMs, as determined by RT-qPCR (Fig. 1c). This upregulation of miR-29b expression was further found to correlate with CRP (Fig. 1d), RF (Fig. 1e) and ESR (Fig. 1f) in RA patients. miR-29b activation in PBMs is thus associated with the disease progression of RA.

Forced miR-29b expression potentiates resistance to spontaneous or Fas-induced apoptosis in PBMs

To determine whether this resistance to apoptosis is directly caused by miR-29b elevation, we transfected miR-29b mimics or non-targeting Mimics-NC in normal PBMs from healthy donors. Flow cytometry analysis of cells expressing the fluorescent molecule (Dy547)-conjugated mimics showed that ~ 55.1% (± 4.8) of cells were Dy547-positive (Fig. 2a), validating the successful transfection in PBMs. Subsequent RT-qPCR analysis revealed that transfection with mimics resulted in a ~ 2.5-fold induction of miR-29b expression in normal PBMs, relative to Mock or Mimics-NC-transfected PBMs (Fig. 2b). Ectopic expression of exogenous miR-29b in PBMs significantly promotes cell survival following a 22-h culture, as compared to Mock or Mimics-NC-transfected PBMs (Fig. 2c). Consistently, an anti-apoptotic effect of mimics transfection was also observed in PBMs (average absorbance at 405 nm following Mimics 1.252 versus Mock 3.634 and Mimics-NC 3.813, respectively. Figure 2d). To further elucidate whether this resistance was specific to spontaneous apoptosis or a more general feature of cell death, we treated PBMs with an agonistic anti-Fas antibody to induce apoptosis. While Mock or Mimics-NC-transfected PBMs were equally susceptible to killing induced by the anti-Fas antibody compared to IgM isotype control-treated cells, mimics-transfected PBMs tended to be more resistant (Fig. 2e, f). Therefore, miR-29b may inhibit apoptosis induced by both intrinsic and extrinsic stimuli.

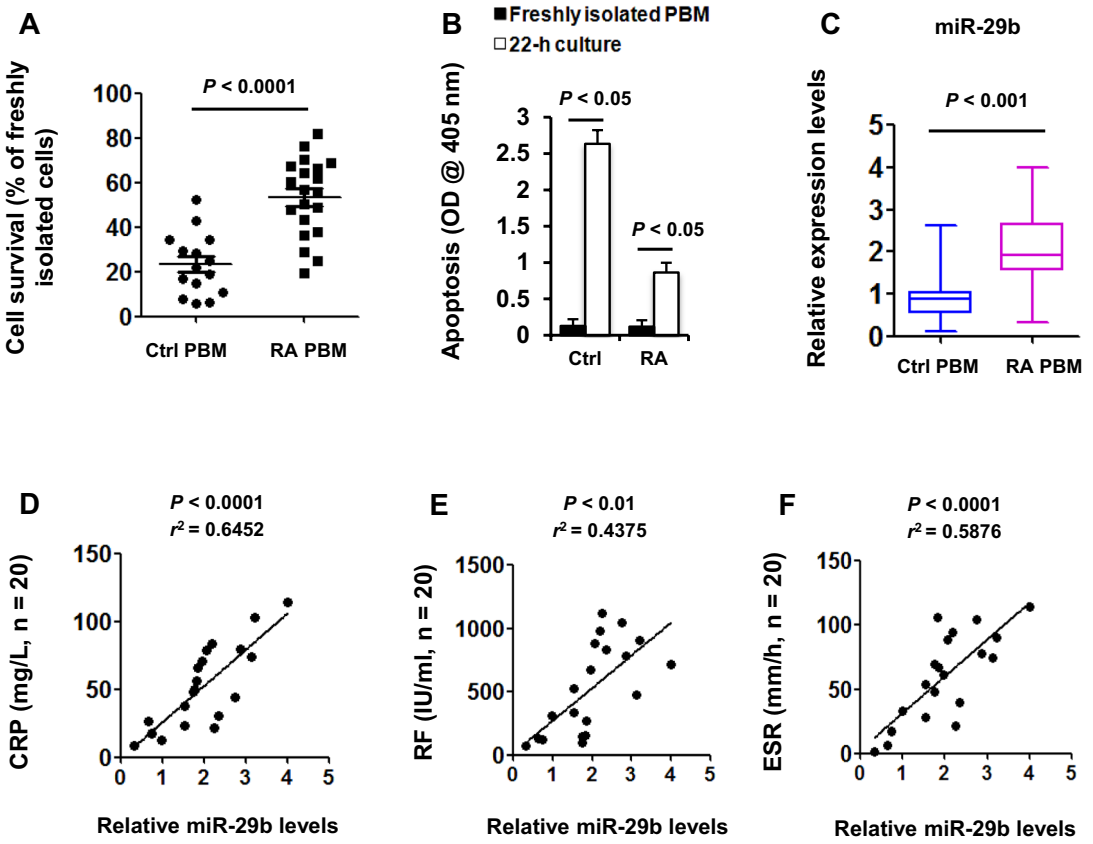

Fig. 1 The human CD14+ monocytes from the peripheral blood (PBMs) of patients with rheumatoid arthritis (RA) show resistance to spontaneous apoptosis and higher expression of miR-29b. The human CD14+ PBMs, isolated using a commercial kit, were cultured for 22 h to induce spontaneous apoptosis, followed by measurement of cell viability (**a**) and apoptosis (**b**) using MTT and ELISA methods, respectively. Relative survival rate was analyzed in which the viable cell numbers (as indicated by MTT assay) in different groups were normalized against the viable cell numbers in freshly isolated PBMs from normal control, which was arbitrarily set at 100%. Results are presented as mean ± S.E.M. **c** Relative expression levels of miR-29b in PBMs from RA patients and normal donors were evaluated using RT-qPCR, and parallel amplification of U6 snRNA served as internal control (n = 3). **d**–**f** The association between levels of miR-29b and the common parameters for RA disease activity, including C-reactive protein (CRP), rheumatoid factor (RF) and erythrocyte sedimentation rate (ESR), was determined using Pearson's linear regression analysis (n = 3)

Promotion of monocyte cytokine production by miR-29b overexpression

Deregulation of proinflammatory response plays a pivotal role in the progression of RA, in both synovial cells [23] and circulating PBMs [10]. In addition to the increased cell survival potential, miR-29b overexpression led to a robust activation in a broad range of chemokines and cytokines, as revealed by the ELISA assays using cell culture supernatants (Fig. 3). miR-29b may thus function as a pluripotent modulator, regulating both cell death and proinflammatory response, in PBMs.

Identification of the 3′-UTR of the high-mobility group box-containing protein 1 (HBP1) as the direct target of miR-29b

Using Target scan and miRDB databases, we next screened out 20 genes that may be the potential targets of miR-29b (Fig. 4a). We then focused on HBP1, an apoptosis-related transcriptional factor that participates in multiple cell progressions, including terminal differentiation, senescence induction, and tumor suppression (Fig. 4b), because HBP1 was the only target gene whose expression levels were found

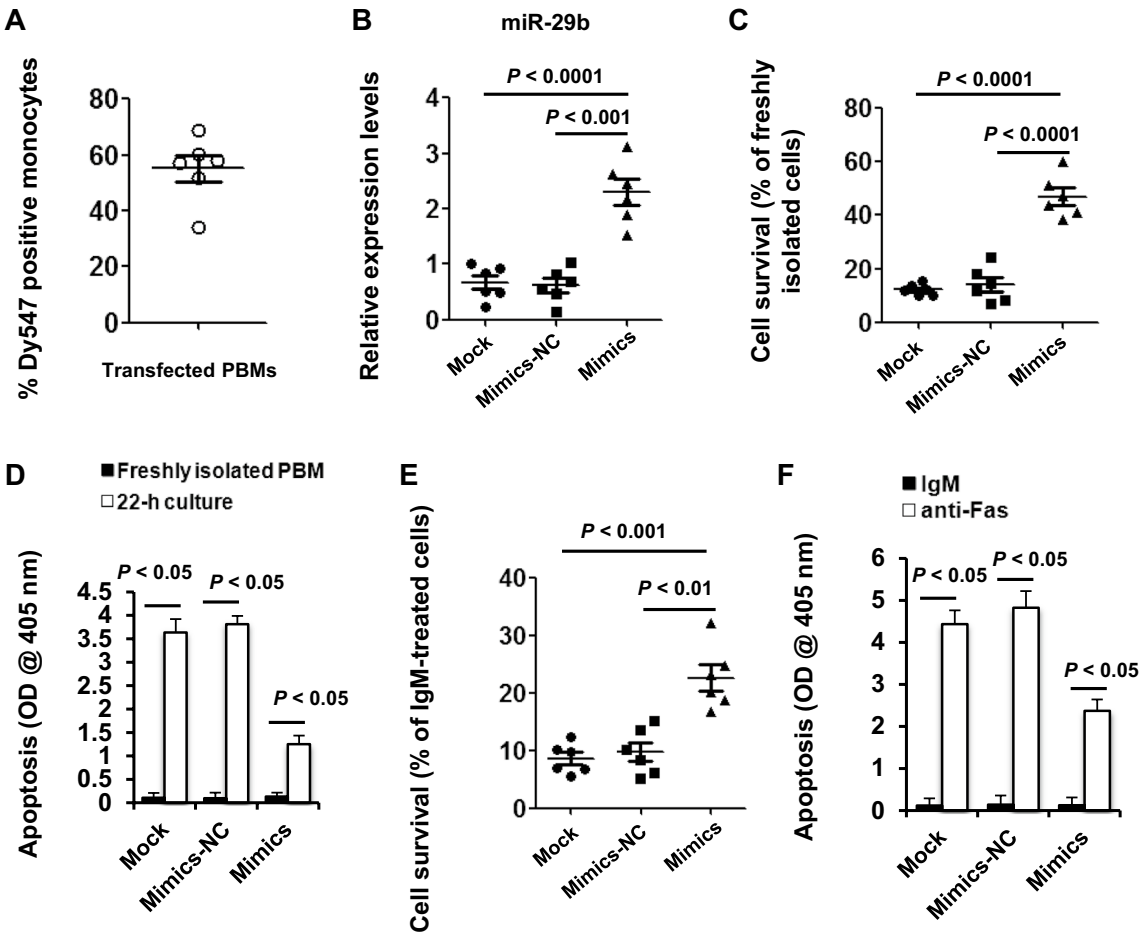

Fig. 2 Overexpression of miR-29b potentiates resistance to spontaneous and Fas-mediated apoptosis in PBMs. **a** The siGLO Red transfection indicator (also known as Dy547) is a fluorescent oligonucleotide duplex that localizes to the nucleus, thus permitting unambiguous visual assessment of uptake into mammalian cells. It is ideal for use in optimization experiments to determine optimal siRNA, crRNA:tracrRNA, or microRNA reagent transfection conditions and for monitoring relative delivery efficiency. Normal donor PBMs were transfected with miR-29b mimics conjugated to a fluorescent molecule Dy547, or with mimics negative control (Mimics-NC). 48 h later, Dy547 positive monocytes were evaluated using flow cytometry (n = 4). **b** 48 h following mimics transfection, PBMs were subjected to RT-qPCR analysis of the relative expression levels of miR-29b (n = 3). **c**, **d** The PBMs with different transfections were cultured for 22 h to induce spontaneous apoptosis, followed by measurement of cell viability and apoptosis as described above (n = 3). **e**–**f** The PBMs with different transfections were cultured in medium containing 50 ng/mL of agonistic anti-Fas antibody for 22 h, followed by measurement of cell viability and apoptosis (n = 3)

to be correlated to the expression levels of miR-29b in RA PBMs (n = 20, $P = 0.0004$, Fig. 4c). Transfection with miR-29b mimics in normal PBMs cells significantly suppressed the expression of HBP1, at both transcriptional (Fig. 4d) and translational (Fig. 4e) levels. To ask whether miR-29b directly targets the 3′-UTR of HBP1, we subcloned the DNA fragments harboring the putative miR-29b-binding site in human *HBP1* 3′-UTR into the pGL4-Luc reporter vector. Cotransfection with the wild-type pGL4-HBP1 3′UTR-Luc reporter plasmid and miR-29b mimics resulted in a ~ 48.7% reduction in the luciferase reporter activity, whereas replacement of wild-type pGL4-HBP1 3′UTR-Luc with mutated pGL4-HBP1 3′UTR-Luc abolished the miR-29b-induced inhibition in the luciferase reporter activity (Fig. 4f, g). HBP1 may thus function as a direct downstream target of miR-29b signaling.

HBP1 overexpression counteracts miR-29b-induced resistance to apoptosis

To unequivocally elucidate the role of the miR-29b-HBP1 axis in the regulation of PBMs apoptosis, we generated the THP-1 cells that stably expressed the exogenous HBP1 (designated as THP-1/HBP1) (Fig. 5a). To be noted, as shown by additional experiment (SFig. 2), stable expression of HBP1 in THP-1 cells had no any effects on their polarization. THP-1/HBP1 cells were then treated with 5 ng/mL of PMA to induce differentiation to macrophages

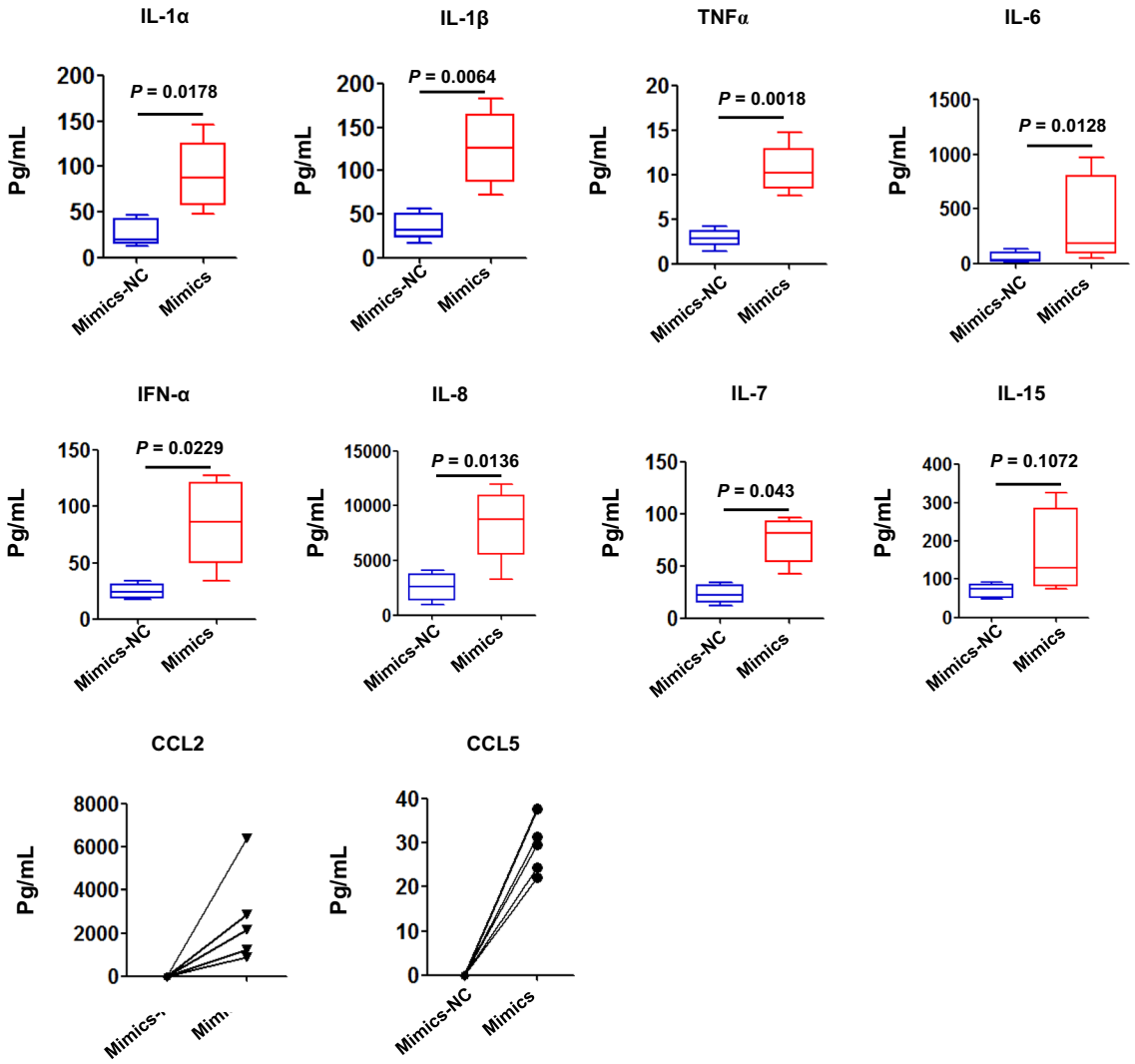

Fig. 3 miR-29b overexpression causes a robust production of proinflammatory cytokines in PBMs. PBMs with different transfections were cultured for another 10 h, and supernatants were then collected and immediately subjected to measurement of human cytokines using the V-PLEX Human Cytokine 30-Plex Kit (n = 6)

(Fig. 5b). To further confirm the differentiation process, we analyzed the expression of CD14 using RT-PCR and FACS, because CD14 has been shown to be an essential surface marker of differentiated macrophages [24]. In particular, CD14 was expressed abundantly during THP-1/HBP1 differentiation induced by PMA, relative to negative expression of CD14 in untreated THP-1/HBP1 cells (Fig. 5c, d). Intriguingly, the THP-1/HBP1 cells transfected with miR-29b mimics had higher cell viability (Fig. 5e) and lower apoptosis upon anti-Fas stimulation than Mimics-NC-transfected cells (Fig. 5f). By contrast, HBP1 overexpression effectively abolished miR-29b mimics-induced resistance to apoptosis in THP-1/HBP1 cells (Fig. 5e, f). Together, we reason that augmentation of HBP1 expression is critical for blocking the miR-29b-repressed PBMs apoptosis.

Translation of miR-29b molecular effects to clinical application

Over the duration of the study, we unexpectedly observed that among patients with RA and good clinical responses to immunotherapy, miR-29b expression noticeably decreased in those treated with infliximab (a TNF-α inhibitor), but not in those treated with tocilizumab (a humanized mAb against the IL-6 receptor) (Fig. 6a). These data point to a potential association between miR-29b activation and TNF-α induction. To validate this, we treated the primary cultured normal PBMs with TNF-α. Apparently, miR-29b expression was induced both dose- and time-dependently along the incubation with TNF-α, with highest values being observed in PBMs treated with higher concentrations of TNF-α (Fig. 6b) or during the late phases after treatment with 100 ng/ml of

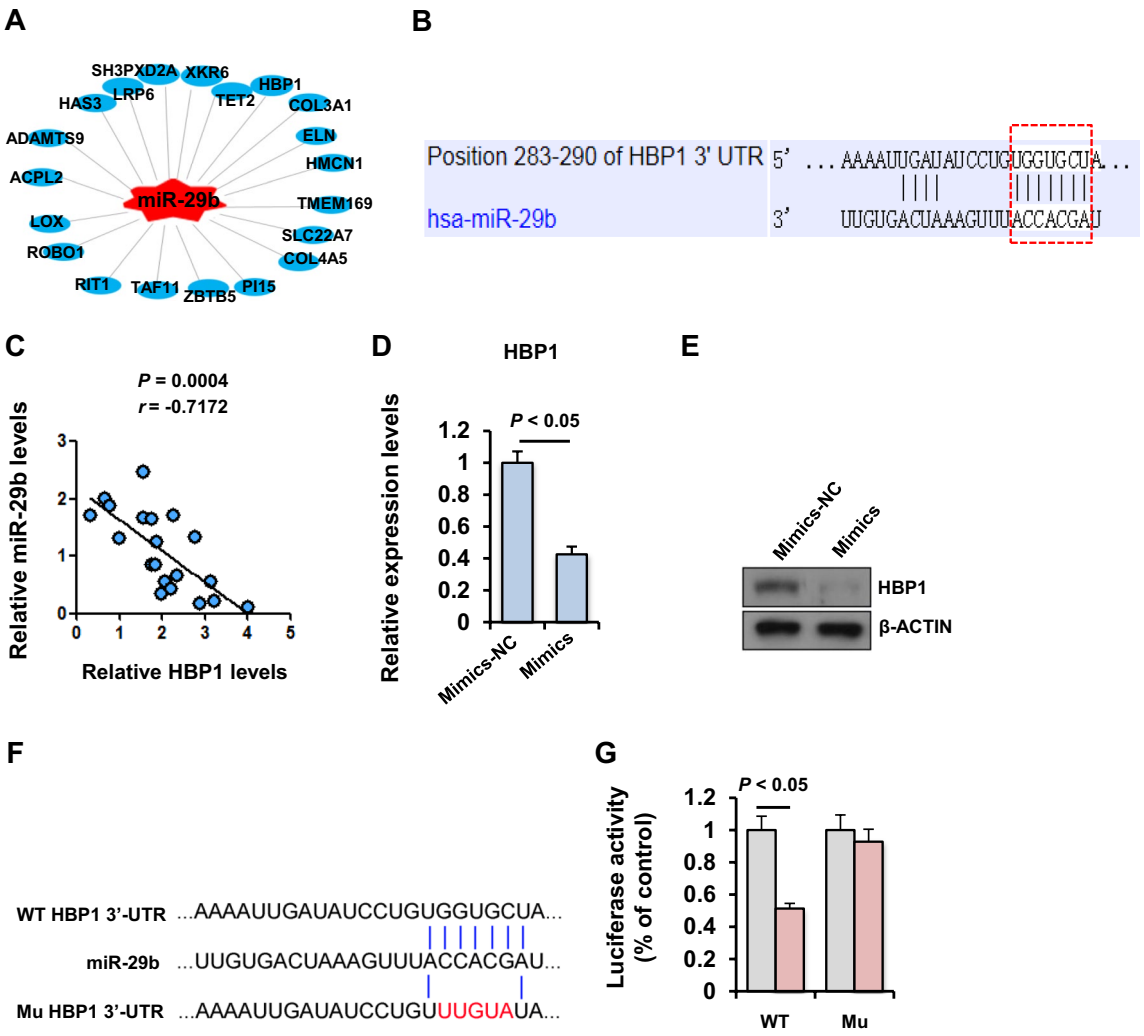

Fig. 4 High-mobility group box-containing protein 1 (HBP1) functions as the direct target of miR-29b. **a** Prediction of putative target genes of miR-29b by online programs. **b** Predicted miR-29b binding sites in the 3′-UTR of *HBP1*. **c** The association between levels of miR-29b and *HBP1* mRNA in PBMs from RA patients (n = 20) was determined using Pearson's linear regression analysis. Normal donor PBMs were transfected with miR-29b mimics or Mimics-NC. 48 h later, HBP1 expression levels were assessed in the transfected PBMs using RT-qPCR (**d**) and Western blotting (**e**) (n = 3). **f** Predicted mutated miR-29b binding sites in the 3′-UTR of *HBP1*. **g** Luciferase reporter assay with co-transfection of wild-type or mutant *HBP1*-3′-UTR and miR-29b mimics in NIH/3T3 cells

TNF-α (Fig. 6c). Therefore, TNF-α functions as a potent stimulant for the miR-29b induction in PBMs.

Discussion

Recent high-throughput studies have identified a bunch of miRNAs that are expressed aberrantly in PBMs from patients with autoimmune diseases [25, 26]. The function and underlying mechanisms of these miRNAs, however, remain largely unknown. In this regard, miR-29b was selected and subjected to further investigation regarding its regulatory role in apoptotic PBMs based on three criteria: (i) miR-29b is essentially expressed in macrophages and its expression levels changes along the polarization of macrophages [27], indicating that changes in the expression levels of this unique miRNA may serve as circulating signatures in macrophage gene expression that occur in different exogenous activating conditions. (ii) Misexpression of miR-29b has been observed in different immune disorders such as aging [28], atherosclerosis [29] and Alzheimer's disease [30], independent groups. (iii) Functional evidence of miR-29b as a fundamental regulator of apoptosis has been presented by multiple publications [31–33]. We observed that miR-29b expression was significantly upregulated in RA PBMs compared to normal PBMs, and a stepwise increase in miR-29b expression was even more prominent in PBMs with high clinical stage than those in PBMs with low or moderate

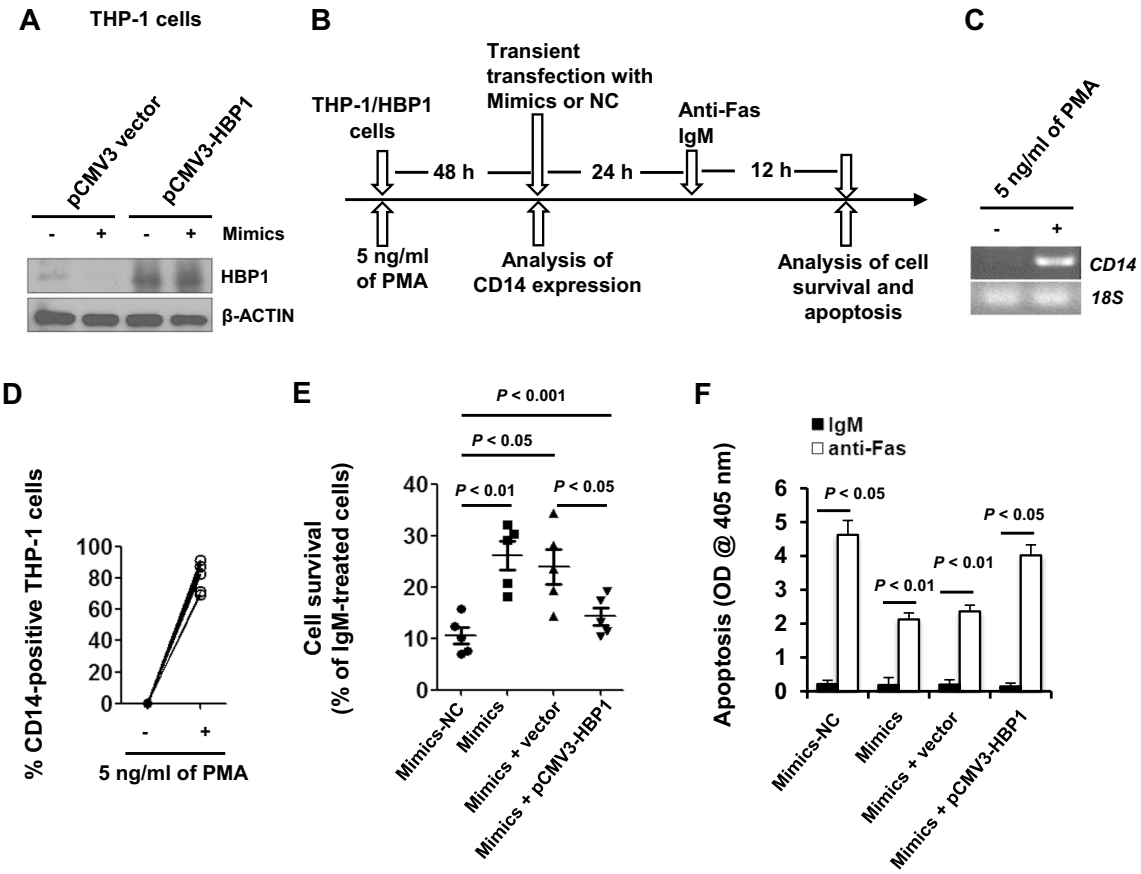

Fig. 5 Overexpression of HBP1 attenuates miR-29b-induced resistance to Fas-mediated apoptosis. **a** Establishment of THP-1 cells stably expressing the exogenous HBP1 was validated using Western blotting (n = 4). **b** Protocols used for induction of THP-1 cells differentiation into macrophages by treatment with 5 ng/mL of phorbol 12-myristate 13-acetate (PMA) for 48 h. Successful induction of THP-1 cells differentiation into macrophages was validated by assessing CD14 expression using RT-PCR analysis (**c**) and flow cytometry (**d**) (n = 3). **e**–**f** The THP-1/HBP1 cells with different transfections were cultured in medium containing 50 ng/mL of agonistic anti-Fas antibody for 22 h, followed by measurement of cell viability and apoptosis (n = 3), as described above

clinical stage, suggesting a close association between miR-29b levels and RA progression (Fig. 1).

The miR-29 family of miR (miR-29a, b-1, b-2 and c) is a key player in T-cell differentiation and effector function, with deficiency causing thymic involution and a more inflammatory T-cell profile [34]. To be specific, IFN-γ itself enhances miR-29b expression in T cells and miR-29b directly regulates T-bet and IFN-γ, thus establishing a novel regulatory feedback loop [35]. Likewise, miR-29b modulates innate and antigen-specific immune responses in mouse models of autoimmunity [36]. miR-29b thus serves as a potent immune modulator. In the context of other cellular function, deregulation of miR-29b expression has been associated with the apoptotic cell death process in many cell types. For example, miR-29b expression is elevated in lipopolysaccharide (LPS)-treated human umbilical vein endothelial cells (HUVECs) and overexpression of miR-29b aggravates cell inflammatory injury via enhancement of cell apoptosis [37]. Likewise, miR-29b suppresses cellular proliferation and promotes apoptosis of pulmonary artery smooth muscle cells (PASMCs), thereby contributing much to the progression of pulmonary arterial hypertension (PAH) [38]. In contrast to these findings, our gain-of-function approaches demonstrate that ectopic expression of miR-29b promotes resistance to apoptosis in PBMs (Fig. 2). Taken together, miR-29b can function as either a promoter or a suppressor of monocyte apoptosis, which largely depends on cell context, cell types and differentiation status of cells. Moreover, miR-29b overexpression enhances survival of monocytes under resting-state and stress-state (induced by the agonistic anti-Fas antibody) conditions (Fig. 2), suggesting that the anti-apoptotic effect of miR-29b may be applicable to both intrinsic and extrinsic pathways. As a consequence of this resistance to apoptosis, miR-29b overexpression resulted in a robust production of the proinflammatory cytokines in PBMs (Fig. 3). Considering that these proinflammatory cytokines, in particular TNFα, IL-1 and IL-6, help to recruit additional leukocytes to the inflamed joint, cause acute phase reactions

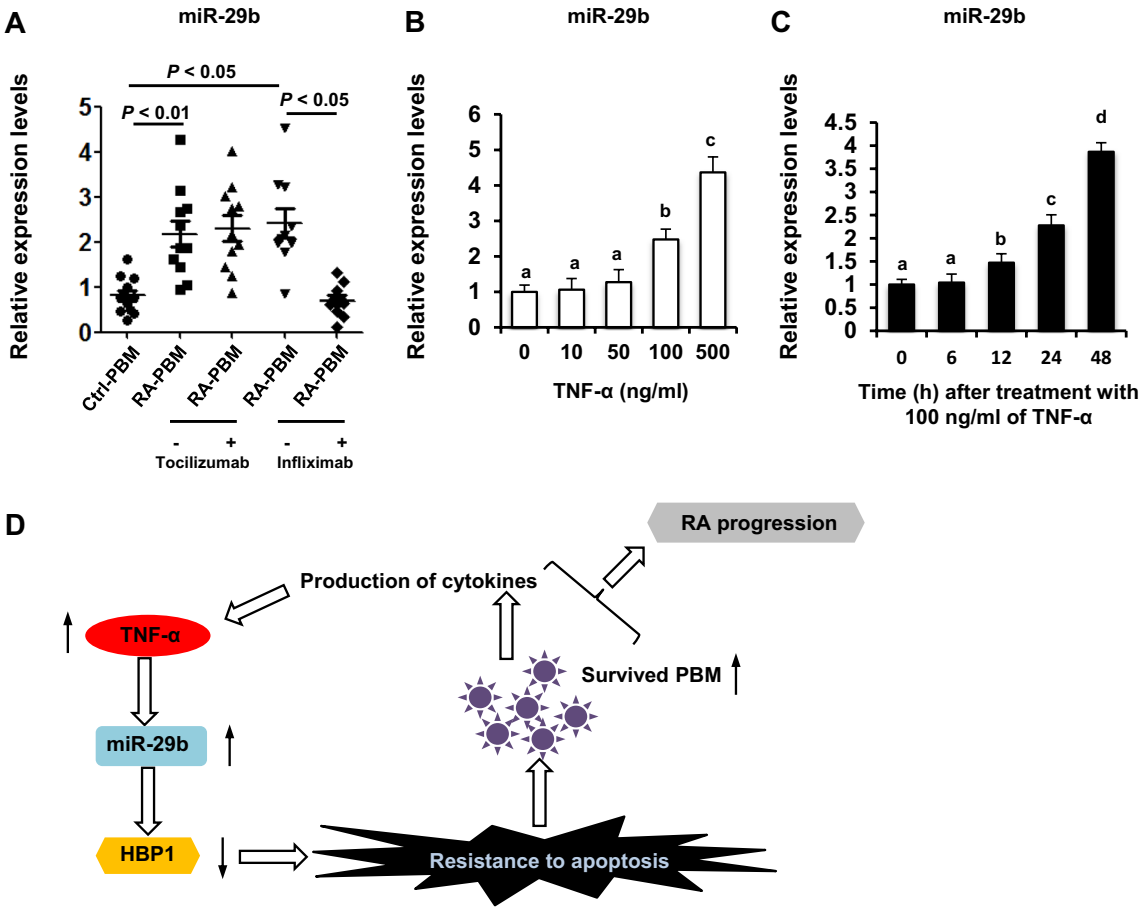

Fig. 6 Modulation of miR-29b expression by TNF-α signaling in PBMs. **a** Relative expression levels of miR-29b before and after the indicated immunotherapy were evaluated using RT-PCR in isolated PBMs (n = 3). **b** PBMs from RA patients were treated with different doses of TNF-α for 24 h, followed by RT-qPCR analysis of miR-29b expression (n = 3). Different superscript letters denote significant difference ($P < 0.05$). **c** PBMs from RA patients were treated with 100 ng/mL of TNF-α for different durations, followed by RT-qPCR analysis of miR-29b expression (n = 3). Different superscript letters denote significant difference ($P < 0.05$). **d** Proposed working model for the current study

and induce cartilage damage [39], increased miR-29b thus bears a significant biological effect along the progression of RA. Base on the aforementioned findings, we reason that miR-29b may function as a critical converging point linking proinflammatory response to apoptosis in RA. In favor of this hypothesis, miR-29b has been shown to be functionally involved in Toll-like receptor control of glucocorticoid-induced apoptosis in human plasmacytoid dendritic cells [40]. To this end, it could potentially be exploited further for therapeutic intervention for RA.

By profiling PBMs in the setting of ectopic miR-29b expression, we have identified HBP1 as the potential downstream target, and we further confirmed the direct binding of miR-29b onto the 3′-UTR of HBP1 using a luciferase reporter assay (Fig. 4). Importantly, stable expression of HBP1 can counterbalance the promoting effects of ectopic miR-29b expression on resistance to apoptosis in differentiated THP-1 cells (Fig. 5). Therefore, the potentiation of resistance to apoptosis in PBMs by miR-29b is mediated, at least in part, through negative regulation of HBP1 signaling. HBP1 is a multifunctional transcriptional factor which participates in multiple cell progressions, including senescence induction, apoptosis regulation, differentiation termination, and tumor suppression, in a variety of tissues and cell types [41, 42]. Regarding the function/regulation of HBP1, two fundamental aspects are worthy to note: (i) HBP1 regulates fundamentally the activation of cell apoptosis. For instance, HBP1 promotes cell death by enhancing the expression levels of p21, an essential mediator of p53-dependent apoptosis, through reinforcing p53 stability via inhibition of Mdm2-mediated ubiquitination of p53. HBP1 is thus believed to be a potent tumor suppressor. In untransformed cells (i.e. granulosa cells), granulosa-specific ablation of Hbp1 causes reduced apoptotic signals in mouse granulosa cells and promotes follicle growth and oocyte production, thus conferring a critical control of ovarian reserve [43]. Consistently

in our study, overexpression of HBP1 restored the sensitivity to the agonistic anti-Fas-elicited apoptosis in differentiated THP-1 cells (Fig. 5). These findings collectively suggest that the pro-apoptotic effect of HBP1 is relatively conserved in different systems. It will be of future interest to deconvolute whether a miR-29b/HBP1/p21 axis is also at play in RA PBMs. (ii) HBP1 expression is tightly regulated by epigenetic mechanisms. Multiple miRNAs including miR-21, miR-19a and miR-155 have been reported to directly target the 3′-UTR of HBP1 in both transformed and normal cells [44–46]. Our current study thus extends these knowledges by identifying miR-29b as a direct upstream regulator of HBP1 signaling in PBMs. Nevertheless, interpretation of the results obtained on THP-1 cells should be cautious based on the follwoing facts: (i) THP-1 cells were originally derived from the peripheral blood of a childhood case of acute monocytic leukemia (M5 subtype). So it can be considered as an immature monocyte-like cell line [47]. (ii) Primary monocytes are different from THP-1 cells in many aspects. For example, THP-1 cells express lower levels of CD14 and thus are less responsive to LPS. Similarly, human cationic antimicrobial protein (CAP37) and heparin both demonstrate weak biological effects in THP-1 cells than in primary monocytes [48]. In this context, it will be more appropriate to consider THP-1 cells as an apparently ideal monocyte proxy for translational research.

The other significant outcome of the current study is the regulation of miR-29b by TNF-α. The overproduction of TNF-α at the inflammatory sites (synovial cells or PBMs) is one of the key inflammatory cascades that drive inflammation and subsequent joint destruction [49]. Moreover, TNF-α bears a wide variety of biological activities: recruitment of inflammatory cells, modulation of cell death and regulation of immune response. In this context, treatment with TNF-α-inhibitors (including first-generation agents Etanercept, Infliximab, and Adalimumab, and second-generation agents Certolizumab and Golimumab) has proven to be highly effective in RA therapy [50]. However, due to the pleiotropic functions played by this unique pro-inflammatory cytokine, TNF-α blockade, during the long-term treatment, frequently causes severe side-effects including serious infections [51], occurrence of psoriasis [52] and increased risks of hematologic and solid malignancies [50]. Because we have shown that miR-29b expression was regulated by TNF-α signaling, and because epigenetic modification mediated by miRNAs can be easily affected by metabolic signals and inflammatory response [10], testing patients for circulating TNF-α/miR-29b expression ratios may thus provide more accurate prognostic information and could influence the recommended course of anti-TNF-α treatment. Moreover, utilities of combined miR-29b-HBP1 manipulation agents may help to combat the unwanted side-effects caused by TNF-α-inhibitors, thus representing a plausible therapeutic strategy to optimize the anti-TNF therapy. To be noted, we have observed that TNF-α can regulate the expression levels of many other miRNAs in PBMs (data not shown). It will be of future interest to deconvolute whether TNF-α modulate PBMs apoptosis through regulation of other distinct miRNAs.

As mentioned earlier, RA renders resistance to apoptosis in many cell types including T-cells, RA-FLS and PBMs. Interestingly, miR-29b has been shown to regulate T-bet expression and IFN-γ production by establishing a novel regulatory feedback loop with these two key factors in T cells [35]. Moreover, our ongoing study demonstrates that miR-29b expression is significantly upregulated in synovial tissues from RA compared to that in synovial tissues from OA (data not shown). Therefore, It will also be of interest to investigate if a TNF-α/miR-29b/HBP1 axis is also at play in T and synovial cells.

In summary, we show that miR-29b is significantly upregulated in RA PBMs. miR-29b serves both as a target and a component of the TNF-α signaling in the regulation of inflammatory responses. The TNF-α-elicited miR-29b promotes resistance to both intrinsic and extrinsic apoptosis in PBMs, and this pro-apoptotic function is mainly mediated through negative regulation of HBP1 signaling (Fig. 6d). The striking observation obtained in the current communication stresses the need for future intensive endeavors aimed at epigenetic modification by miRNAs in monocyte/macrophage survival.

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