IL-17A Increases Multiple Myeloma Cell Viability by Positively Regulating Syk Expression

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Abstract

PURPOSE: Elevated IL-17 produced by T_h17 cells was reported to promote myeloma cell growth and inhibit immune function in multiple myeloma (MM). IL-17A was also reported to promote MM growth through IL-17 receptors and enhance adhesion to bone marrow stromal cells (BMSCs). Spleen tyrosine kinase (Syk) influences MM cell survival and migration. Herein we aimed to investigate whether Syk was involved in the regulative role of IL-17A in the viability of MM cells. *METHODS:* Cell viability was determined using CCK8 assay. The production of cytokine including IL-17A was evaluated with ELISA. Western blotting assay was used to determine protein expression levels of Syk and nuclear factor κB (NF-κB) related molecules. mRNA expression level of RORγt was detected with reverse transcription quantitative polymerase chain reaction. *RESULTS:* IL-17Awas highly expressed in MM patients and was able to induce MM cell viability. Following analysis indicated that the effects of IL-17A were mediated by Syk/ nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Immunoprecipitation also indicated that Syk is involved in IL-17A–induced Act1-TRAF6 complex formation and TRAF6 polyubiquitination in MM cells. *CONCLUSIONS:* Taken together, our study indicated that IL-17A increases MM cell viability through activating NF-κB signal pathway via positively regulating Syk expression. Syk also participates in the formation of IL-17R-proximal signaling complex (lL-17R-Act1-TRAF6), which is essential for IL-17A–mediated NF-kB activation. These investigations highlight that inhibition of Syk may be a potential therapeutic option for neoplastic diseases such as MM.

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Introduction

Multiple myeloma (MM) is a malignant plasma cell (PC) disorder characterized by proliferation of malignant plasma cells in bone marrow [1]. A hallmark of MM is the presence of interplay between PC and bone marrow microenvironment [2]. Microenvironment is a complex system that causes functional damage of host immune system [3]. The host immune system and the bone marrow microenvironment play an essential role in the PC survival, apoptosis, migration/invasion, and drug resistance [4]. Abnormalities of T cell number or function have been observed in patients with MM [5]. CD4 helper T lymphocytes are critical in both antibody-mediated and cell-mediated immune responses [6]. T-helper 17 (TH17) cells, a subset of CD4 T cells, produce IL-17 and IL-22 under the polarization condition of interleukin-1(IL-1), IL-23,IL-6, and IL-21and with or without transforming growth factor-beta (TGF-β) [7]. Highly expressed TGF-β and IL-6 in myeloma patients enhance the

generation of TH17 cells [8]. IL-17A is a prototypic member of the IL-17 family and mediate the expression of various chemokines and cytokines such as granulocyte-colony stimulating factor (GM-CSF), IL-6, TGF-β, and intercellular adhesion molecule-1 in a variety of cell

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types [9]. IL-17A is dramatically upregulated in blood and bone marrow in MM and enhances MM cell proliferation through IL-17 receptor [10]. IL-17A has also been reported to play an important role in bone disease development in myeloma by regulating osteoclast formation and activation [11]. These results revealed the important role of IL-17A in the progression and development of MM.

Spleen tyrosine kinase (Syk), which belongs to the Src family, is a cytosolic nonreceptor protein tyrosine kinase (PTK) involved in various biological functions [12]. Syk is mainly expressed in hematopoietic cells including B cells, monocytes, and macrophages, thereby modulating the immune responses [12]. Syk was served as an essential component in the BCR signaling pathway, Fc receptors, and adhesion receptors [13]. Activation of Syk plays an important role in proinflammatory cytokine-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in airway epithelial cells [14,15]. Studies have shown that MM cells express Syk and p-Syk and interfere with Syk expression, effectively inhibiting the proliferation, migration, and survival of MM cells [16]. Another study found that incubation of MM cells with Syk inhibitors and primary MM cells resulted in increased cell proliferation and decreased migration, as well as dose-dependent inhibition of NF-κB and mitogen-activate d protein kinase (MAPK) signaling pathways [17]. Importantly, targeting Syk might be a promising therapeutic method for MM that is more efficient when combined with MAPK inhibitors [18]. These investigations suggested the promising role of Syk in MM.

Particularly, a recent research showed that, in human keratinocytes, IL-17R–proximal signaling complex is pivotal for IL-17A–stimulated NF-κB activation. Syk mediates the complex formation and tumor necrosis factor receptor–associated factor 6 (TRAF6) polyubiquitination [19]. Another study suggested that IL-17A triggers gene expression and protein secretion through the Syk-dependent NF-κB mechanism [20]. Based on these foundations, we aimed to investigate whether Syk was involved in the regulative role of IL-17A in the viability of MM cells.

Materials and Methods

Cell Culture and Transient Transfection

Human MM cell lines NCI-H929 and U226 were obtained from Cell Library, China Academy of Science, and Bioleaf Biotech (Shanghai, China). Cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY). Human bone marrow stromal cell line HS-5 (Cell Library, China Academy of Science) was cultured in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Three siRNA sequences targeting Syk were designed and transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Western Blot Analysis and Antibodies

The proteins were loaded to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by transferring onto polyvinylidene difluoride (PVDF) filter membranes (Millipore, Bedford, MA). The membranes were incubated with the primary antibodies against IKK (1:1000), IκBα (1:1000), TRAF6 (1:1000), Syk (1:1000), GAPDH (1:1000), p-IKK (1:1000), p-IκBα (1:1000), and horseradish peroxidase–conjugated secondary antibody for 2 hours at room temperature. All antibodies used were purchased from Cell Signaling Technology Danvers. The membranes were scanned by the ECL

detection system. The band density was quantified using the Image J analysis system (Wayne Rasband, National Institutes of Health, Bethesda, MD). The experiments were carried out on three separate occasions.

Cell Viability Assay

MM cells at the density of $1×10^5$ were seeded into a 96-well plate (Corning Inc., Corning, NY) and incubated for 48 hours with or without IL-7A treatment. The cells were treated with 10 μl Cell Counting Kit-8 (CCK8) reagents (Dojindo, Kumamoto, Japan) for an additional 1 hour at 37°C in the dark. The absorbance was measured in a microplate reader (Bio-Rad, Hercules, CA) at a test wavelength of 450 nm.

Co-Immunoprecipitation Assay

The cells were collected and lysed with lysis buffer for 0.5-3 hours at 4°C. The supernatants were precleared with 30 μl protein A/G at 4°C for 1 hour. Then, 30 μl of supernatants served as input, and the rest of the supernatants were allocated into two parts and incubated with the Syk antibody and IgG at 4°C overnight. The samples were incubated with 30 μl protein A/G at 4°C for 2 hours. Finally, the precipitates were collected and washed three times and boiled for 10 minutes with SDS sample buffer followed by Western blot.

Quantitative PCR

Total RNA was extracted with RNeasy kit according to the manufacturer's instructions (QIAGEN). Reverse transcription of RNA to cDNA was performed with the RETRO script kit (Ambion) according to the manufacturer's protocol. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out using the Applied Biosystems 7500 apparatus with the SYBR Green PCR master mix (Applied Biosystems) according to manufacturer's instructions. The specific primers for human RORγt mRNA were as follows 5′-GCT GGTTAG GAT GTG CCG-3′ and reverse primer 5′-GGATGC TTT GGC GAT GA-3′.

Statistical Analysis

Data are presented as means ± SD. Each experiment was repeated at least three times using the Statistical Package for the Social Science SPSS 20.0 software (SPSS Inc., An IBM Company, Chicago, IL). The probability of .05 or less was considered statistically significant.

Results

Elevated Levels of Th17 Cells and IL-17A Cytokine in MM Patients

We firstly determined the frequency of Th17 cells in bone marrow tissues and peripheral blood in MM and control groups. The orphan nuclear receptor RORγt is the specific transcription factor of Th17 cells and is necessary for the generation of TH17 cells [21]. Results from qRT-PCR showed that the mRNA expression levels of RORγt in both bone marrow tissues and peripheral blood from MM patients were significantly higher than that from normal controls($P < .05$; Figure 1A). We further analyzed the protein level of proinflammatory cytokine IL-17A secreted by Th17 cells in bone marrow tissues and peripheral blood by ELISA, respectively. Remarkable increase in the level of IL-17A was found in bone marrow tissues from MM patients compared with those from healthy controls (25.990 vs. 22.880 pg/ml; $P < .05$; Figure 1B). Consistently, significant upregulation of serum IL-17A from MM group compared with control group was also observed (131.056 vs. 9.900 pg/ml; $P < .05$; Figure 1C).
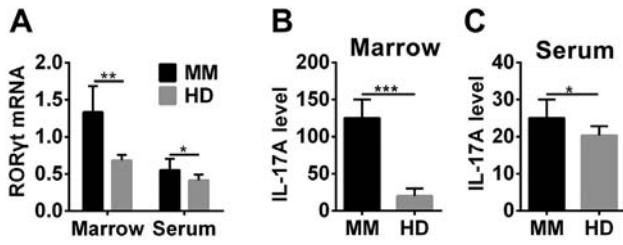
Figure 1. Elevated levels of Th17 cells and IL-17A cytokine in MM patients.**(A)** The RORγt mRNA expression level was detected in bone marrow tissues and peripheral blood from 10 pairs of MM patients and healthy donors by using RT-qPCR. **(B)** The IL-17A production from bone marrow was detected in 10 pairs of MM tissues and normal tissues by using ELISA assay. **(C)** The expression of serum IL-17A was detected in 10 pairs of MM patients and healthy donors by using ELISA assay. "*" denotes $P < .05$, "**" denotes $P < .01$, and "***" denotes $P < .001$ Data are expressed as the mean ± SD.
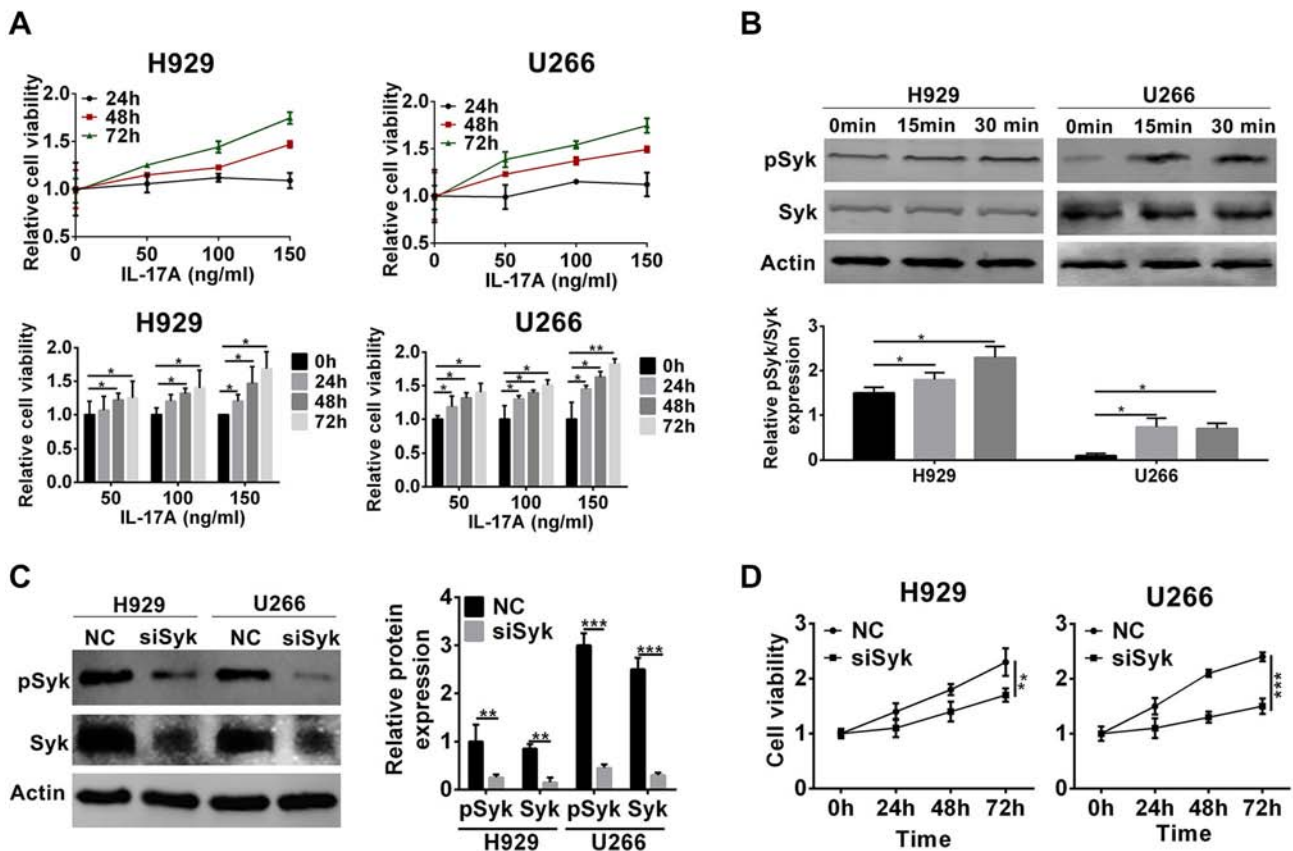
IL-17A Increases Viability of MM Cells Through Regulating Syk Expression

Next, the effect of IL-17A on viability of MM cell lines H929 and U266 was examined *in vitro*. As shown in Figure 2A, exogenous IL-17

significantly promoted viability of MM cell lines H929 and U266, as assessed by CCK8 assay in a time- and dose-dependent manner ($P < .05$). To assess whether Syk was involved in IL-17A–induced enhancement in the viability of MM cells, the protein expression of Sykin response to IL-17A (150 ng/ml) stimuli was detected at different time points. Western blotting analysis showed that IL-17A treatment upregulated Syk expression in H929 and U266 cell lines in a time-dependent manner ($P < .05$; Figure 2B). To further explore the underlying mechanism, Syk was knocked down by specific small interfering RNA (siSyk) and transfected into H929 and U266 cell lines treated with exogenous IL-17 (150 ng/ml). The silencing efficiency of siRNA was confirmed (Figure 2C). Importantly, the increased MM cell viability upon IL-17A treatment was prevented by Syk silencing in a time-dependent manner ($P < .05$; Figure 2C). Collectively, Syk was involved in IL-17A–induced enhancement in the viability of MM cells.

IL-17A Activates NF-κB Signaling Pathway via Positively Regulating Syk in MM Cells

Since NF-κB plays an important role in regulating MM cell viability, we speculated that IL-17A/Syk–induced cell proliferation might be associated with NF-κB signaling. As shown in Figure 3, *A* and *B*, IL-17A stimuli resulted in the phosphorylation of IKK and

Figure 2. IL-17A increases viability of MM cells through regulating Syk expression.(A) IL17A promoted the viability of MM cells. H292 cells and U266 cells were incubated with IL-17A at concentrations of 50, 100, and 150 ng/ml for 24, 48, and 72 hours, respectively. Cell viability was evaluated by CCK8 assay. (B) The protein expression of p-Syk and total Syk is detected in H292 cells and U266 cells treated with 150 ng/ml of IL-17A for 15 and 30 minutes using Western blot. (C) The protein expression of p-Syk and total Syk is detected in H292 cells and U266 cells, which were transfected with negative control small interfering RNA (NC) or siSyk and then treated with IL-17A at concentration of 150 ng/ml. (D) The cell viability was detected in H292 cells and U266 cells, which were transfected with siSyk or NC for 24 hours and then treated with IL-17A at concentration of 150 ng/ml for 24, 48, and 72 hours. Cell viability was evaluated by CCK8 assay. "*" denotes $P < .05$, "**" denotes $P < .01$, and "***" denotes $P < .001$ Data are expressed as the mean ± SD.
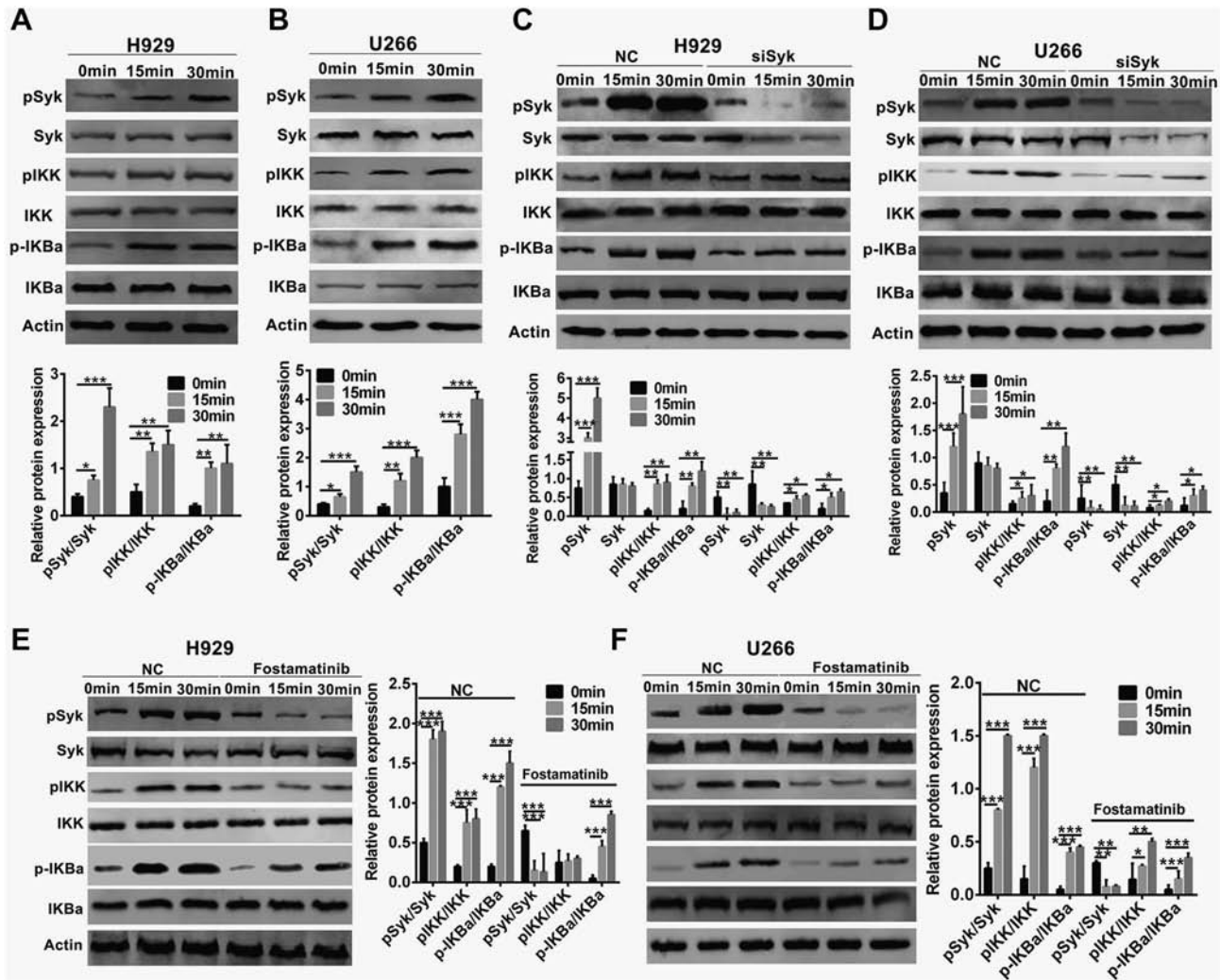
Figure 3. IL-17A activates NF-κB signaling pathway via positively regulating Syk in MM cells.(A, B) The protein expression of p-Syk, Syk, p-IKK, IKK, p-IκB, and IκB was detected in H292 cells and U266 cells when treated with IL-17A at concentration of 150 ng/ml for 15 and 30 minutes by using Western blot. (C, D) The protein expression levels of p-Syk and total Syk were detected in H292 cells and U266 cells transfected with the siSyk or negative control for 48 hours and then treated with IL-17A at 150 ng/ml for 15 and 30 minutes. (E, F) The protein expression levels of p-Syk and total Syk were detected in H292 cells and U266 cells pretreated with Syk inhibitor for 30 minutes and then treated with IL-17A at 150 ng/ml for 15 and 30 minutes. "*" denotes $P < .05$, "**" denotes $P < .01$, and "***" denotes $P < .001$. Data are expressed as the mean ± SD.

IκBα ($P < .05$). We further determined whether Syk was involved in IL-17A–induced NF-κB activation in MM cells. The results showed that silencing of Syk dramatically decreased IL-17A–triggered phosphorylation of IKK and IκBα (Figure 3, C and D). Besides, we also found that fostamatinib, an inhibitor of Syk, also significantly decreased IL-17A–triggered NF-κB activation (Figure 3, E and F). These results directly suggested that IL-17A could regulate NF-κB signaling via Syk in MM cells.
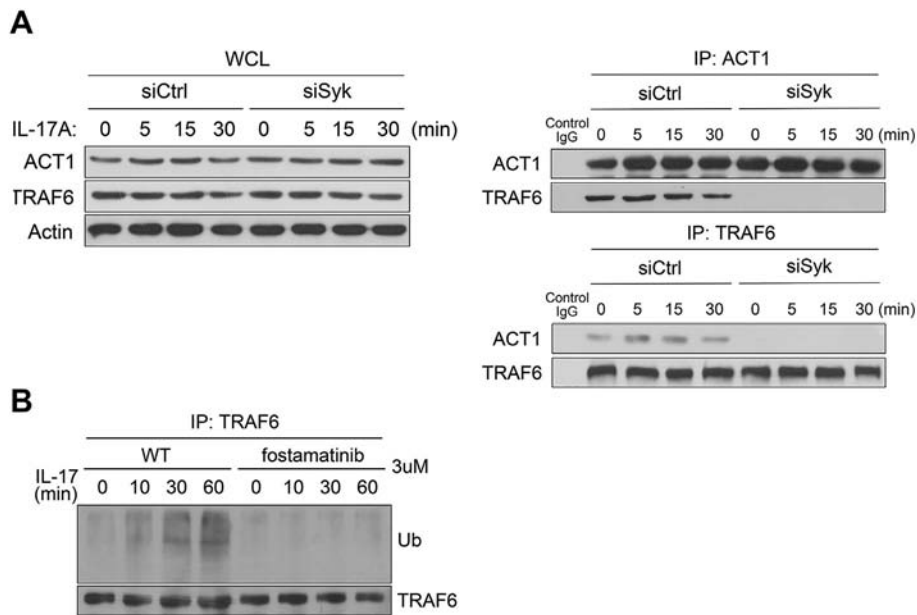
Involvement of Syk in IL-17A–Induced Act1-TRAF6 Complex Formation and TRAF6 Polyubiquitination in MM Cells

Since IL-17 receptor (IL-17R)–proximal signaling complex (IL-17R-Act1-TRAF6) is essential for IL-17–mediated NF-κB activation, we speculated that Syk was involved in IL-17–mediated NF-κB activation through IL-17R-Act1-TRAF6 complex formation and TRAF6 polyubiquitination in MM cells. Results of co-immunoprecipitation with Act1 or TRAF6 antibody showed that

Syk knockdown significantly blocked the binding between TRAF6 and Act1, suggesting that Syk is involved in the proximal signalosome complex formation in MM cells (Figure 4A). We further sought to examine the function of Syk in TRAF6 ubiquitination in MM cells. We found that IL-17 treatment was able to time-dependently increase the TRAF6 ubiquitination, while fostamatinib abolished the effect of IL-17 on TRAF6 ubiquitination (Figure 4B). Taken together, IL-17A–induced Act1-TRAF6 binding and TRAF6 polyubiquitination were mediated by Syk in MM cells.

Discussion

MM is a malignant plasma cell proliferative disease, accounting for about 10% of hematologic malignancies [22]. Abnormal immune cells and cytokines are important components of myeloma microenvironment and play an important role in the development of tumors [23]. Th17 cells are a newly discovered T cell subpopulation that secretes IL-17 and is involved in the development of various tumors and

Figure 4. Involvement of Syk in IL-17A–induced Act1-TRAF6 complex formation and TRAF6 polyubiquitination in MM cells.(A) H929 cells were transfected with siSyk or NC for 72 hours followed by treatment of IL-17A (150 ng/ml) at 10, 30, and 60 minutes, and total cell lysates were immunoprecipitated with IgG, anti-Act1, and anti-TRAF6. (B) H929 cells were incubated with fostamatinib at 3 μM for 30 minutes followed by treatment of IL-17A (150 ng/ml) at 10, 30, and 60 minutes. The cell lysates were immunoprecipitated with TRAF6 antibody and analyzed K63 polyubiquitination.

autoimmune diseases [24]. Th17 cells are considered as therapeutic targets for serious tumors and autoimmune diseases [25].

In view of the important role of immune cells in MM microenvironment, it is necessary to study the abnormal expression and mechanism of Th17 cells and IL-17A in MM. We firstly found that the frequency of Th17 cells and level of IL-17A were elevated in MM. Further study revealed that IL-17A could promote MM cell viability. The underlying mechanism was subsequently explored. It is found that TRAF6 is highly expressed in myeloma cells and known to regulate the downstream NF-κB signaling pathways and participates in the proliferation and apoptosis of myeloma cells [26]. NF-κB is a pivotal regulator of the immune system and is involved in a variety of neoplastic diseases, which is known to be involved in IL-17A stimulation [27]. Consistently, in our study, we found that IL-17A activated the NF-κB signaling pathway and enhanced TRAF6 ubiquitination. It raises the possibility that the promotive effects of IL-17A on MM cells may be associated with NF-κB signaling pathway.

Syk, as a nonreceptor tyrosine kinase, is widely expressed in hematopoietic cells; plays a central role in B cell or T cell proliferation, differentiation, and survival; and is involved in innate immune and acquired immune responses [17]. Syk inhibitors are also used to treat a variety of autoimmune diseases (such as rheumatoid arthritis, asthma, allergic rhinitis) and have entered clinical trials [29]. Recent study identified Syk as a new therapeutic target because Syk inhibitors effectively inhibit MM cell proliferation through NF-κB signaling pathway [28]. In this study, IL-17A could induce Syk phosphorylation, and the increased MM cell viability upon IL-17A treatment was prevented by Syk silencing in a time-dependent manner, demonstrating that Syk was involved in IL-17A–induced enhancement in the viability of MM cells. IL-17A could also induce the phosphorylation of Syk, IKK, and IκBα. Besides, IL-17A stimulated the interaction between Syk and TRAF6. In addition, silencing of Syk

attenuated IL-17A–induced NF-κB activity. These results indicated that Syk is an upstream molecular of NF-κB signaling pathway and acts on IL-17R-Act1-TRAF6 complex.

TRAF6 is a tumor necrosis factor (TNF) superfamily and Toll-like / interleukin-1 receptor (Toll / IL-1) superfamily co-linker molecule that is important for intracellular signaling pathways [30]. Consistent with our results, previous studies have shown that Syk can be recruited to TRAF6 / Act1 or TRAF6 / TAK1 complexes in IL-17R signaling, and Syk may be a common regulator of TRAF6-dependent signaling pathways, with specific mechanisms of action depending on IL-17A stimulation [14]. However, the involvement of Syk in IL-17R signaling has not yet been fully understood. These results indicated that Syk plays an important role in positively regulating IL-17A–mediated TRAF6 signaling and cellular function. In this study, although we clearly observed that IL-17A enhances the interaction between Syk and TRAF6, whether Syk specifically modulates the molecular mechanism underlying the ubiquitination of the K63 locus of TRAF6 requires further validation. Detecting the ubiquitination alterations in the K63 locus of TRAF6 and ubiquitination after interfering with Syk gene expression is necessary. Moreover, we suspect that Syk may be recruited to the TRAF6 signal complex, but the specific mechanisms in MM need further study.

In conclusion, we showed that Syk is an important molecule in the IL-17A–medicated NF-κB signaling pathway. The results demonstrated that IL-17A increases Syk expression, which regulates the ubiquitination of TRAF6, affects the downstream NF-κB signaling pathway, and consequently results in the enhanced MM cell viability. This study provides a better understanding of IL-17A–mediated signaling pathways but also provide potential therapeutic target for MM.

Acknowledgement

Conflict of Interest

The authors declare that there is no conflict of interest.

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