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Original Articles

Multiple myeloma cell-derived IL-32γ increases the immunosuppressive function of macrophages by promoting indoleamine 2,3-dioxygenase (IDO) expression

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ABSTRACT

The interaction of multiple myeloma (MM) cells with macrophages (MΦs) contributes to the pathophysiology of MM. We previously showed that IL-32 is overexpressed in MM patients. The present study was designed to explore the clinical significance of IL-32 in MM and to further elucidate the mechanisms underlying the IL-32-mediated immune function of MΦs. Our results showed that high IL-32 expression in MM patients was associated with more advanced clinical stage. RNA-sequencing revealed that IL-32γ significantly induced the production of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) in MΦs, and this effect was verified by qRT-PCR, western blotting, and immunofluorescence. Furthermore, MM cells with IL-32-knockdown showed a reduced ability to promote IDO expression. As a binding protein for IL-32, proteinase 3 (PR3) was universally expressed on the surfaces of MΦs, and knockdown of PR3 or inhibition of the STAT3 and NF-κB pathways hindered the IL-32γ-mediated stimulation of IDO expression. Finally, IDO-positive IL-32γ-educated MΦs inhibited $CD4^+$ T cell proliferation and IL-2, IFN-γ, and TNF-α production. Taken together, our results indicate that IL-32γ derived from MM cells promotes the immunosuppressive function of MΦs and is a potential target for MM treatment.

1. Introduction

Multiple myeloma (MM) is an incurable B cell tumor characterized by the accumulation of malignant plasma cells in the bone marrow (BM) [1]. The interaction of malignant plasma cells with the BM microenvironment contributes to their growth, proliferation, invasion, metastasis, and chemoresistance [2]. The MM–BM microenvironment is composed of cells, extracellular matrix, and soluble factors, among which macrophages (MΦs) are an abundant and important component [3,4]. Several studies have shown that the degree of MΦ infiltration can serve as a prognostic marker in newly diagnosed patients with MM [5,6]. Along with promoting angiogenesis through vasculogenic mimicry, MM-associated MΦs (mMΦs) protect MM cells from spontaneous and chemotherapy-induced apoptosis through both contact-mediated and non-contact-mediated mechanisms [7,8]. mMΦs therefore represent a potential target for myeloma treatment [9,10], and it is essential to explore the mechanisms underlying MΦ infiltration and the polarization of normal MΦs to mMΦs. Research has shown that MM cells recruit tumor-supportive MΦs and promote M2-like MΦ polarization through the CXCR4/CXCL12 axis [11]. Our previous study also showed that the chemokines CCL2, CCL3, and CCL14 promoted MΦ infiltration in the MM–BM microenvironment and increased MΦ proliferation [12]. While these studies indicated that cytokines from MM cells affect MΦs, few studies have focused on the MM cell-mediated

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immunosuppressive function of MΦs involving T cells.

Interleukin 32 (IL-32), a proinflammatory cytokine, was identified as natural killer cell transcript 4 (NK4) in activated natural killer cells and T cells [13]. There are more than nine alternatively spliced isoforms of IL-32, each with distinct functions. Among the variants, IL-32β is the most abundant isoform, and IL-32γ appears to be the most potent isoform [14–16]. The definitive receptor of IL-32 has not been identified, although proteinase 3 (PR3) has been revealed to be a specific IL-32-binding protein [17]. Along with being involved in numerous inflammatory and infectious diseases [18,19], IL-32 plays a crucial role in the development of both hematologic malignancies and solid tumors [20,21]. Some reports have shown that IL-32 promotes the progression of various malignancies, such as gastric cancer, lung cancer, and cutaneous T-cell lymphoma [22–24]. However, conflicting reports have shown a tumor suppressive role of IL-32 in colon cancer and melanoma [25,26]. We previously reported that IL-32 is overexpressed in the BM and peripheral blood of MM patients. Moreover, IL-32 induces the production of IL-6 in bone marrow stromal cells (BMSCs) [27]. Recently, another report showed that IL-32 promotes osteoclast differentiation in MM [28]. In this study, we further investigated the association between IL-32 expression and clinical features of newly diagnosed MM patients and explored the effects and underlying mechanisms of IL-32 and MΦs in the MM–BM microenvironment.

2. Materials and methods

2.1. Cell culture

Human MM cell lines RPMI 8226, OPM2, CAG, ARK, MM.1S, ARP-1, H929, and LP-1 were generously provided by Dr. Qing Yi (Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA). All MM cell lines were cultured in RPMI-1640 medium containing 1% L-glutamine and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). To prepare conditioned media (CM), MM cells were seeded at 5×10^5 cells/ml for 24 h. Culture supernatants were collected and stored in aliquots at −80 °C.

PBMCs were isolated from healthy donors after obtaining informed consent. Human MΦs were generated from PBMCs as previously described [4]. Monocytes were cultured in 6-well plates for 1–2 h; non-adherent cells were removed, and adherent monocytes were incubated in medium containing M-CSF (20 ng/ml; R&D Systems, Minneapolis, MN, USA) for 7 days.

$CD4^+$ T lymphocytes were isolated from PBMCs using immunomagnetic bead separation with a $CD4^+$ T cell isolation kit (STEMCELL, Vancouver, Canada). $CD4^+$ T cell purity was evaluated by flow cytometry.

2.2. Immunohistochemistry and immunofluorescence

We detected the expression of IL-32 in formalin-fixed, paraffin-embedded BM biopsy samples using immunohistochemistry. All 23 cases were histopathologically and clinically diagnosed as new, untreated MM at The First Affiliated Hospital, School of Medicine, Zhejiang University from January to June 2017. All patients consented to participation, and the study was approved by the Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Immunohistochemistry was performed as previously reported [29] using an anti-human IL-32 monoclonal antibody (Abcam, Cambridge, UK) and anti-IgG (Proteintech, Rosemont, IL, USA) as an isotype control.

The degree of immunostaining was reviewed by two independent pathologists blinded to all clinical characteristics. Semiquantitative analytical criteria for IL-32 expression levels have been published [29] and involved classifying the percent of positive staining (< 50%, 50–70%, and > 70%) and the strength of staining as follows: absent (−), scarce (±), moderate (+) or strong (++). IL-32 expression was scored as follows: positive: > 70% positivity with scarce (±) to strong (++) staining or 50–70% positivity with moderate (+) to strong (++) staining; weakly positive: 50–70% positivity with scarce (±) staining or < 50% positivity with scarce (±) to strong (++) staining; and negative: < 50% positivity with scarce (±) to absent (−) staining. Positive cases comprised the high expression group, whereas weakly positive and negative cases formed the low expression group.

Immunofluorescence with anti-IDO (Proteintech), anti-CD68, anti-p-STAT3, and anti−NF−κB antibodies (R&D Systems) was performed as previously described [27].

2.3. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Two-tailed Student's *t*-tests were used to determine the significance of differences between experimental groups. Pearson's Chi-square test was used to determine correlations between IL-32 expression and clinicopathological parameters. All tests were considered statistically significant at $P < 0.05$.

Other detailed methods were listed in supplementary materials and methods.

3. Results

3.1. High IL-32 expression in MM correlated with clinical stage

We studied IL-32 expression in BM biopsies from newly diagnosed MM patients by immunohistochemical staining using anti-IgG as an isotype control (Sup.Fig. 1A). IL-32 was detectable in all patients, and approximately 73.9% (17/23) of the samples showed high expression (Fig. 1A). The expression levels of IL-32 and clinicopathological details of the patients are shown in Table 1. IL-32 expression was significantly and positively associated with the International Staging System (ISS) stage ($P = 0.007$) and levels of serum β2-microglobulin ($P = 0.011$), a known prognostic marker in MM [1]. There were no correlations between IL-32 expression and other clinicopathological parameters, including DS stage ($P = 0.156$), albumin ($P = 0.735$), globulin ($P = 0.931$), hemoglobin ($P = 0.526$), creatinine ($P = 0.058$), calcium ($P = 0.296$), lactate dehydrogenase ($P = 0.230$), and plasma cells in BM ($P = 0.535$). Thus, patients with high IL-32 expression had a more advanced ISS stage, indicating that IL-32 may play a role in MM progression.

We next evaluated IL-32 expression in human MM cell lines. Western blotting showed that IL-32 was highly expressed in all tested MM cell lines but was scarcely detected in normal MΦs differentiated from healthy donor PBMCs (Fig. 1B). We stained MM cells for IL-32 and found that it was mainly present in the cytoplasm (Fig. 1C). Quantitative RT-PCR revealed that several isoforms of IL-32 were expressed in MM cells, with IL-32β and IL-32γ being the main isoforms (Fig. 1D). Furthermore, upon treatment of MΦs with different IL-32 isoforms (α, β, and γ), the structural differences among IL-32 isoforms were shown in Supplementary Fig. 1B and IL-32γ was the most active inducer of nuclear factor κB (NF-κB), IκBα, and STAT3 phosphorylation (Fig. 1E). These results are consistent with those of previous studies [16,30]. Thus, we chose IL-32γ for use in subsequent analyses. CCK-8 assay showed that IL-32γ had no direct effect on the proliferation of MM cells (Sup.Fig. 1C–E), suggesting that the role of IL-32γ in MM progression depends on the MM–BM microenvironment.

3.2. IL-32γ induces indoleamine 2,3-dioxygenase (IDO) expression in MΦs

Because MΦs are an abundant and important component of the MM microenvironment [4], we sought to determine whether IL-32γ affects the immune functions of MΦs. MΦs were differentiated from PBMCs by M-CSF, and their cell morphology and surface markers were confirmed

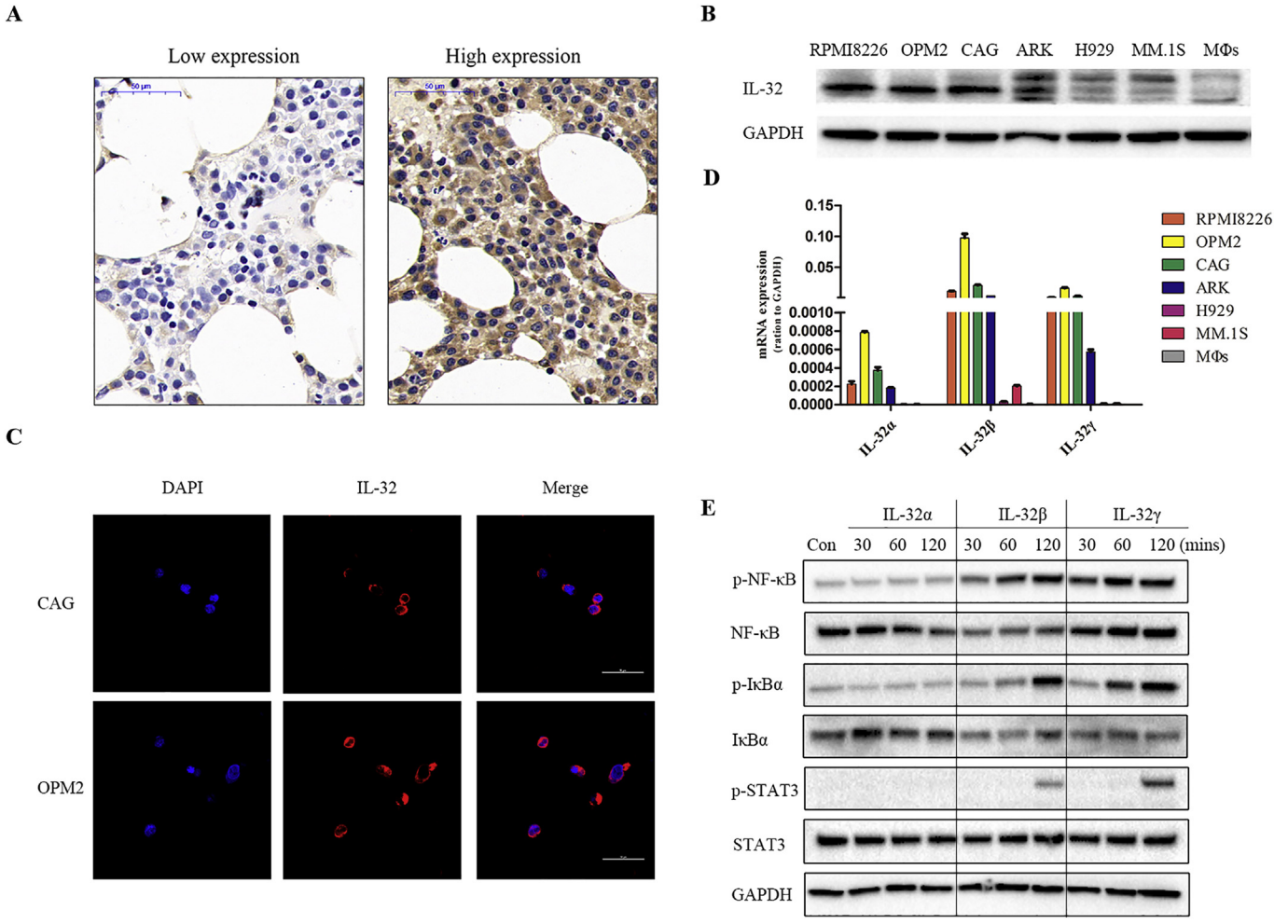

Fig. 1. IL-32 expression in MM patients and MM cells reveals that IL-32γ is the most active subtype. (A) IL-32 expression in BM biopsies of newly diagnosed MM patients was detected by immunohistochemical staining. Scale bar, 50 μm. (B) Protein expression of IL-32 in MM cell lines (n = 6) and monocyte-induced MΦs was measured using western blotting. GAPDH served as the loading control. (C) Immunofluorescence analysis was applied to determine the location of IL-32 in MM cell lines. (D) The mRNA expression of IL-32 isoforms (α, β, and γ) was quantified by qRT-PCR in MM cell lines (n = 6). (E) Monocyte-induced MΦs were stimulated with IL-32α, IL-32β, or IL-32γ (20 ng/ml) for the indicated times, and western blotting was applied to detect the phosphorylation of STAT3, IκBα, and NF-κB.

(Sup.Fig. 2A and B). Then, we cultured MΦs with human recombinant IL-32γ (10 or 40 ng/ml) for 24 h and assessed the global transcriptional profile by RNA-Seq. Compared with untreated cells, IL-32γ-educated MΦs expressed significantly higher levels of immune-related transcription factors, cytokines, and other molecules (Fig. 2A). The most significant change was the dose-dependent increase in the gene expression of *IDO*, which is associated with the immunosuppressive activity of MΦs in some infectious diseases [31,32]. We performed KEGG pathway enrichment analysis to identify canonical signaling pathway activation in the cells. IL-32γ-treated MΦs displayed activation of the JAK-STAT, NF-κB, and MAPK signaling pathways (Fig. 2B), which are highly related to IDO production.

To confirm the results of RNA-Seq, we treated MΦs with IL-32γ at several concentrations (10, 20, or 40 ng/ml) or with the positive control IFN-γ (100 U/ml), which is a known inducer of IDO. Western blotting and qRT-PCR showed that IL-32γ significantly induced IDO production by MΦs in a time- and dose-dependent manner, with untreated MΦs barely expressing IDO (Fig. 2C and D). We also performed immunofluorescence analysis, revealing that IL-32γ-treated MΦs expressed higher levels of IDO than unstimulated MΦs (Fig. 2E). To exclude the possibility that the IL-32γ-induced upregulation of IDO was due to an increase in the proliferation of MΦs, we performed flow cytometry, CCK-8, and CFSE tracking assays. The results revealed that IL-32γ had no effect on the viability or proliferation of MΦs (Sup.Fig. 2C–E). Among the three isoforms (α, β, and γ), IL-32γ was the most active inducer of IDO expression in MΦs (Fig. 2F). Moreover, bortezomib, a common proteasomal inhibitor used to treat MM, inhibited the IL-32γ-induced production of IDO in a dose- and time-dependent manner (Sup.Fig. 3A–C). Taken together, the data showed that IL-32γ significantly promoted IDO expression in MΦs and that this induction could be attenuated by bortezomib.

3.3. MM cells promote IDO production in MΦs through IL-32γ

As confirmed in our previous research and another study, IL-32 is mainly secreted by MM cells [27,28]. Here, we determined whether IDO expression in MΦs is affected by MM cell-secreted IL-32. We collected CM from different MM cell lines and stimulated MΦs for 24 h before evaluating *IDO* expression using qRT-PCR. As shown in Fig. 3A, CM from MM cell lines with high IL-32γ expression (RPMI 8226, OPM2, CAG, and ARK) significantly upregulated *IDO* expression in MΦs, whereas relatively smaller changes in *IDO* levels were observed after stimulation with CM from MM cell lines with low IL-32γ expression (LP-1, ARP-1, and MM.1S). These results showed that IL-32γ expression in MM cell lines was consistent with the ability to induce IDO in MΦs.

To more directly and accurately confirm the effect of MM cell-secreted IL-32, we established OPM2 and CAG cells with knockdown of IL-32 by transfection with *IL32* shRNA. Knockdown efficiency was

Table 1
Correlations between IL-32 expression and clinicopathologic characteristics of patients with multiple myeloma.

Variables	IL-32 Immunostaining[a]		P-value
	Low expression(n = 6)	High expression(n = 17)	
Median age, years (range)	60(41–70)	63(47–83)	
Age ≥60	5(83.3%)	11(64.7%)	0.621
Sex (M/F)	3/3	9/8	0.901
Durie-Salmon staging, n (%)			
Stage IIA	–	2(11.8%)	0.156
Stage IIB	–	2(11.8%)	
Stage IIIA	6(100%)	8(47.5%)	
Stage IIIB	–	5(29.4%)	
ISS, n (%)			
Stage I	3(50%)	–	0.007
Stage II	1(16.7%)	2(11.8%)	
Stage III	2(33.3%)	14(82.4%)	
Unknown	–	1(5.9%)	
Median Alb (g/L), (range)	35.9(21.2–45.7)	35.71(21.3–52.8)	
≥35 g/L	4(66.7%)	10(58.8%)	0.735
< 35 g/L	2(33.3%)	7(41.2%)	
Median Glb (g/L), (range)	44.7(24.1–78.4)	56.02(16.3–127)	
≥35 g/L	4(66.7)	11(64.7)	0.931
< 35 g/L	2(33.3)	6(35.3)	
Median β2-microglobulin (mg/L), (range)	9.13(2.68–26.5)	15.52(4.87–58.57)	
≥5.5 mg/L	2(33.3%)	14(82.4%)	0.011
< 5.5 mg/L	4(66.7%)	2(11.8%)	
Unknown	–	1(5.9%)	
Median Hb (g/L), (range)	104.5(81–133)	96.76(56–139)	
≥105 g/L	3(50%)	6(35.3%)	0.526
< 105 g/L	3(50%)	11(64.7%)	
Median Cre (μmol/L), (range)	71.5(45–89)	155.71(51–709)	
≥104 μmol/L	–	7(41.2%)	0.059
< 104 μmol/L	6(100%)	10(58.8%)	
Median Ca^{++} (mmol/L), (range)	2.28(2.18–2.41)	2.27(1.62–2.87)	
≥2.54 mmol/L	–	3(18.8%)	0.296
< 2.54 mmol/L	5(100%)	13(81.3%)	
LDH (U/L), (range)	150.2(69–228)	228.53(94–731)	
Normal	5(83.3%)	14(82.4%)	0.230
High	–	3(17.6%)	
Unknown	1(16.7%)	–	
M protein subtype, n (%)			
IgG kappa	2(33.3%)	3(17.6%)	0.131
IgG lambda	–	7(41.2%)	
IgA	1(16.7%)	–	
IgA kappa	1(16.7%)	–	
IgA lambda	1(16.7%)	2(11.8%)	
Kappa light chain	1(16.7%)	2(11.8%)	
Lambda light chain	–	3(17.6%)	
Plasma cells in bone marrow (%), (range)	16.7(11–22)	17.9(0–58)	0.535

Abbreviations: M: male, F: female, ISS: International Staging System, Alb: albumin, Glb: globulin, Hb: hemoglobin, Cre: creatinine, Ca^{++}: calcium, LDH: lactate dehydrogenase.

[a] IL-32 immunostaining was scored as low expression or high expression, as described in the METHODS.

confirmed at both the mRNA and protein levels (Fig. 3B, Sup.Fig. 4A). IL-32 knockdown did not influence the viability or proliferation of either cell line, as evidenced by CCK-8 assay and flow cytometry (Sup.Fig. 4B and C). We then cultured MΦs with the CM from IL-32-knockdown cells. As shown in Fig. 3C, CM from IL-32-knockdown OPM2 and CAG cells showed a reduced ability compared to that of CM from control MM cells to upregulate IDO. The above experiments showed that while normal MΦs barely expressed IDO, IDO expression could be induced by MM cells. Moreover, patients with high IL-32 expression showed colocalization of IDO expression and $CD68^+$ MΦs (Fig. 3D). These data demonstrated that IL-32 in MM cells contributed to IDO expression in MΦs within the MM–BM microenvironment.

3.4. PR3 is indispensable for IL-32γ-induced IDO expression

PR3, a serine protease, has been reported to be a binding protein for IL-32 [28]. Immunofluorescence staining results showed that PR3 was universally expressed in MΦs (Fig. 4A). Furthermore, the majority of PR3 was detected on the cell surface of MΦs using flow cytometry (Fig. 4B). We then performed IP and GST pulldown experiments, confirming the binding affinity of PR3 for IL-32 (Fig. 4C and D).

To verify the participation of PR3 in IL-32γ-induced IDO expression, we silenced PR3 expression in MΦs using siRNA targeting *PR3*. Western blotting, qRT-PCR, and flow cytometry confirmed PR3 knockdown (Fig. 4E). When MΦs were transfected with *PR3* siRNA, the upregulation of the mRNA and protein expression of IDO induced by IL-32γ decreased significantly, while this was not observed in the control group (Fig. 4F). Moreover, phenylmethanesulfonylfluoride (PMSF), a general serine/cysteine protease inhibitor, markedly downregulated the IL-32γ-induced expression of IDO (Sup.Fig. 3D). The PR3 blocking antibody also attenuated the IL-32γ-induced upregulation of IDO (Sup.Fig. 3E). Taken together, these results demonstrated that the ability of IL-32γ to upregulate IDO production was mediated through a PR3-dependent mechanism.

3.5. Role of STAT3 and NF-κB in IDO regulation

As the KEGG enrichment analysis of the RNA-Seq results showed that IL-32γ significantly activated the JAK-STAT and NF-κB signaling pathways in MΦs (Fig. 2B), western blotting was performed to study the ability of MΦs to respond to IL-32γ. As shown in Fig. 1E, IL-32γ activated the phosphorylation of both STAT3 and NF-κB in MΦs at 30–120 min after stimulation. Furthermore, we treated MΦs with increasing concentrations of IL-32γ (10–40 ng/ml) for 2 h and observed a dose-dependent increase in the phosphorylation of IKKα/β, IκBα, and NF-κB, indicating NF-κB signaling pathway activation (Fig. 5A). Constitutive phosphorylation of STAT3 was also observed in the presence of IL-32γ. Immunofluorescence analysis revealed increased p-STAT3 levels and NF-κB translocation into the nucleus after IL-32γ treatment (Fig. 5B).

To more directly verify the role of STAT3 and NF-κB activation in IL-32γ-induced IDO expression, we blocked these pathways by pretreating MΦs with a STAT3 inhibitor (BP-1-102) or an NF-κB inhibitor (QNZ). These inhibitors indeed decreased STAT3 and NF-κB phosphorylation in MΦs stimulated with IL-32γ (Fig. 5C) and significantly decreased IL-32γ-induced upregulation of IDO mRNA and protein expression (Fig. 5D). Hence, these data indicated that the IL-32γ-driven upregulation of IDO expression was predominantly dependent on the STAT3 and NF-κB pathways.

3.6. IDO produced by IL-32γ-educated MΦs inhibits proliferation and effector function of $CD4^+$ T cells

Several studies have described an immunomodulatory role for IDO in MΦs [31,33]. Therefore, we sought to determine whether IDO produced by IL-32γ-educated MΦs inhibited the proliferation of T cells. $CD4^+$ T cells were isolated from healthy donor PBMCs (purity, > 90%). $CD4^+$ T cells were cultured with the CM from IL-32γ-educated MΦs and stimulated with anti-CD3/CD28 Dynabeads, and proliferation was evaluated by CFSE tracking. The proliferation of $CD4^+$ T cells was significantly diminished when grown in CM from IL-32γ-educated MΦs compared to that in CM from untreated MΦs (Fig. 6A). Moreover, the specific IDO inhibitor 1-MT was applied to elucidate the role of IDO. $CD4^+$ T cell proliferation was almost completely restored by adding 1-

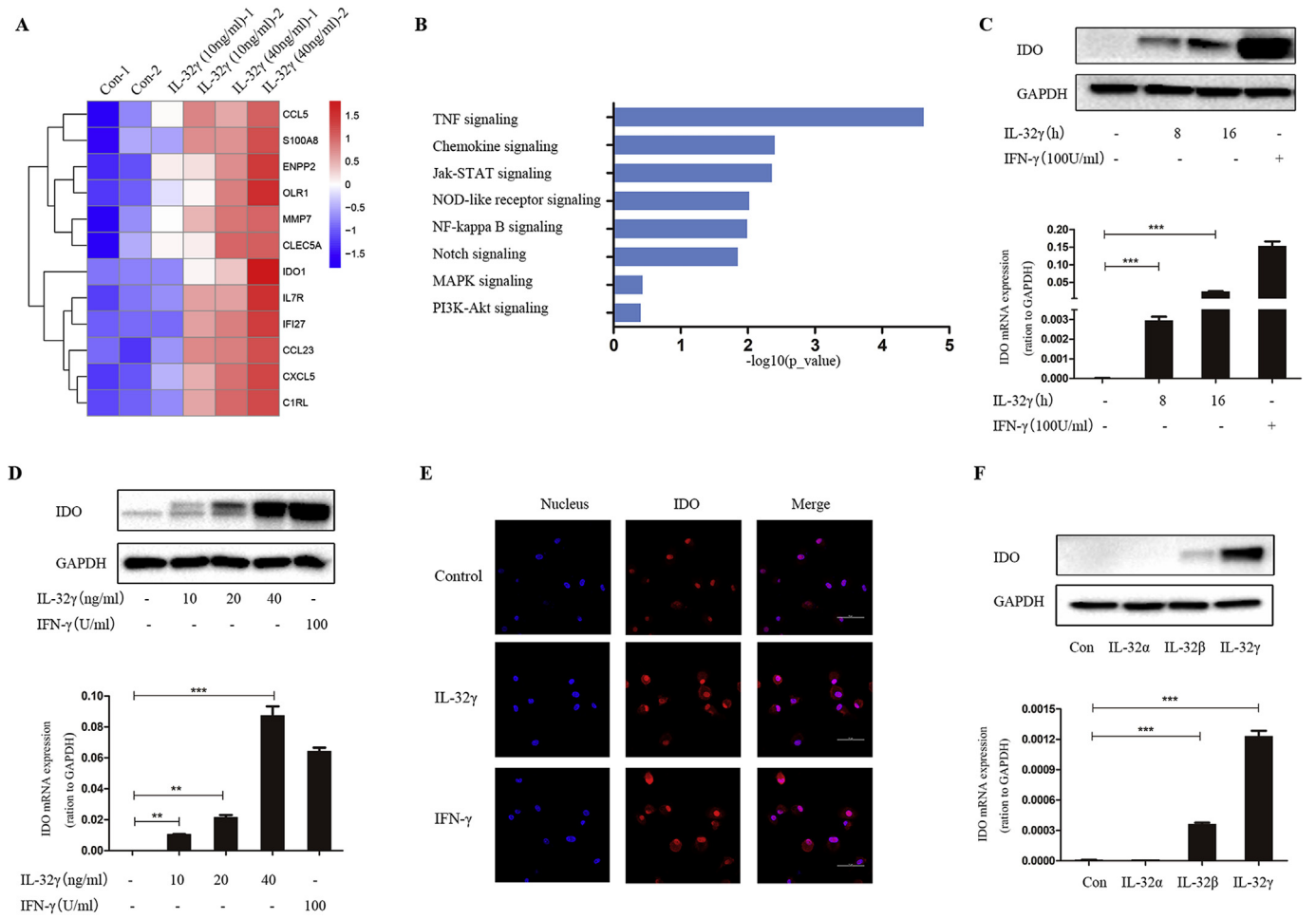

Fig. 2. IL-32γ stimulates the production of IDO by MΦs. (A) MΦs were stimulated with IL-32γ (10 or 40 ng/ml) for 24 h. RNA (biological samples from two donors) was extracted for RNA-sequencing, and the $\log_2$ fold change in a selection of immune-related genes is shown as a heat map. (B) KEGG enrichment of obvious canonical pathways, as indicated, in IL-32γ-treated MΦs. MΦs were treated with IL-32γ (20 ng/ml) for the indicted times (C) or with several concentrations of IL-32γ (10, 20, or 40 ng/ml) for 24 h (D), along with IFN-γ (100 U/ml) as a positive control. Western blotting and qRT-PCR were applied to detect IDO expression. Similar results were observed in three independent experiments. (E) Immunofluorescence analysis was performed to assess the production of IDO in MΦs stimulated with IL-32γ (20 ng/ml) or IFN-γ (100 U/ml) for 24 h. Scale bar, 50 μm. (F) MΦs were treated with different IL-32 isoforms (α, β, and γ, 20 ng/ml) for 24 h, followed by detection of IDO expression.

MT to CM from IL-32γ-educated MΦs (Fig. 6A and B). These results indicated a potential inhibitory effect of IDO produced by IL-32γ-educated MΦs on $CD4^+$ T cell proliferation.

To explore whether the IDO produced by IL-32γ-educated MΦs influences cytokine production by $CD4^+$ T cells, we performed intracellular cytokine staining. Proinflammatory cytokines generated by $CD4^+$ T cells, including IL-2, IFN-γ, and TNF-α, play a crucial role in the immune system. We found that the CM from IL-32γ-educated MΦs significantly suppressed the production of these three cytokines (Fig. 6C). This finding was replicated using $CD4^+$ T cells from six independent healthy volunteers (Fig. 6D). Moreover, we compared the effects of CM from IL-32γ-stimulated PR3-knockdown MΦs and IL-32γ-stimulated control MΦs. Flow cytometry revealed that CM from IL-32γ-stimulated PR3-knockdown MΦs increased the production of IL-2 ($P = 0.066$), IFN-γ ($P = 0.058$), and TNF-α ($P = 0.034$) as compared with that in the control group (Sup.Fig. 5). However, MΦs from different donors may be needed to confirm the statistical significance of this result. To exclude the possibility that T cell suppression was due to nutritional depletion in CM, we assessed the viability and apoptosis of $CD4^+$ T cells and confirmed that $CD4^+$ T cells exhibited good viability during cultivation (Sup.Fig. 2F). These data demonstrated that CM collected from IDO-positive IL-32γ-educated MΦs impaired $CD4^+$ T cell function, as demonstrated by decreased IL-2, IFN-γ, and TNF-α production in response to activation.

4. Discussion

We report herein that high IL-32 expression in MM patients was associated with more advanced clinical stage. IL-32γ in MM cells induced IDO production in MΦs through PR3, which was dependent on the activation of the STAT3 and NF-κB pathways. IDO-positive IL-32γ-educated MΦs suppressed the proliferation of $CD4^+$ T cells and their IL-2, IFN-γ, and TNF-α production in response to polyclonal stimulation.

IL-32 is a proinflammatory cytokine involved in various inflammatory diseases and cancers [18]. Previous studies have demonstrated that different isoforms of IL-32 elicit distinct effects on malignant tumors [34–36]. Our group first reported that IL-32 expression was higher in BM and peripheral blood from patients with MM than in that from healthy controls and that IL-32 was mainly secreted by MM cells [27]. A recent study employing database analysis revealed that high IL-32 expression is associated with reduced survival of MM patients [28]. In the present study, we demonstrated that high IL-32 expression was positively correlated with ISS stage and levels of serum β2-microglobulin, a known prognostic marker of MM. Because a specific IL-32γ antibody is not commercially available, we tested the total expression of IL-32 in BM biopsies using immunohistochemical staining.

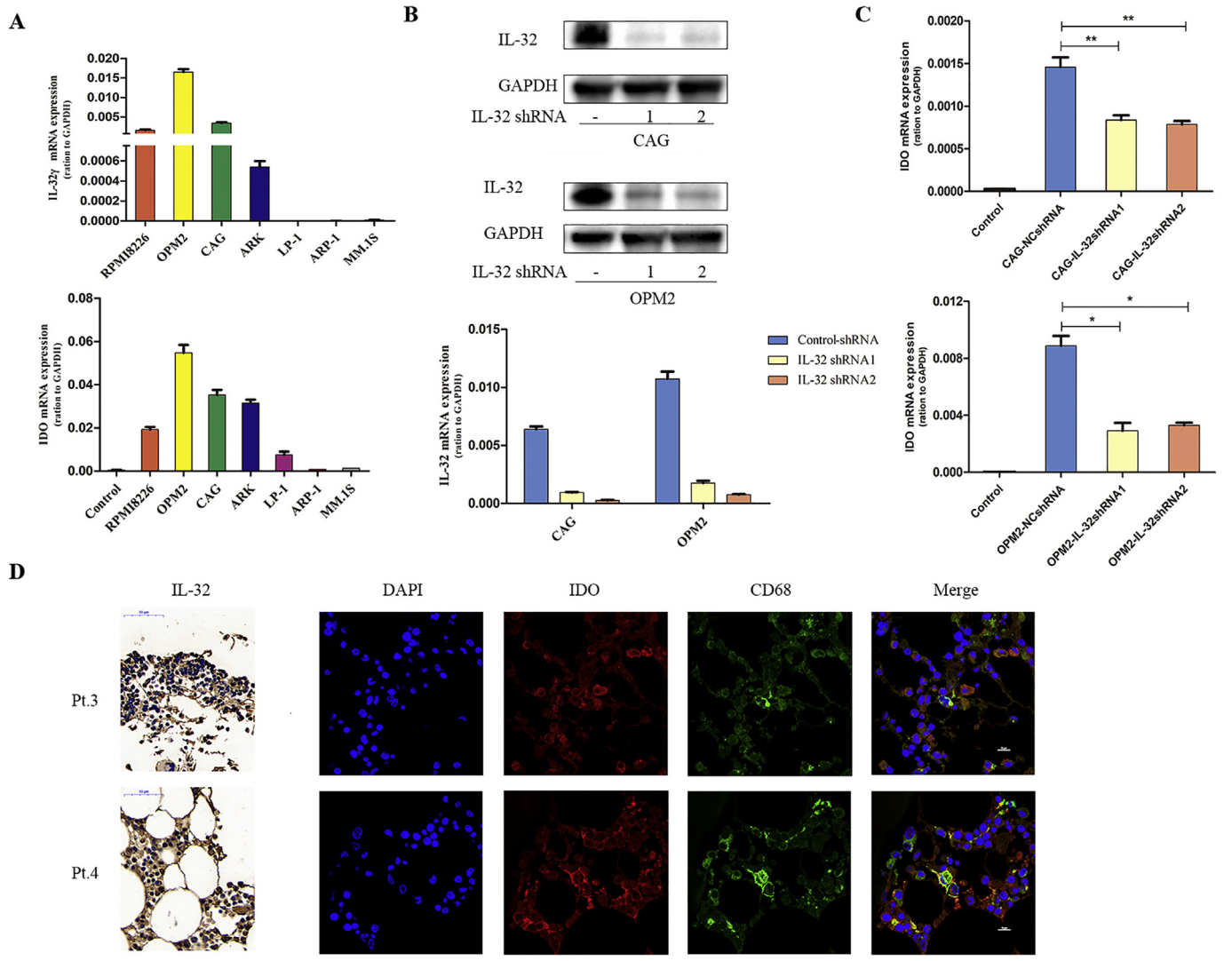

Fig. 3. MM cell-derived IL-32γ promotes IDO expression in MΦs. (A) IL-32γ expression in MM cell lines and IDO expression in MΦs educated by CM from the corresponding MM cells were quantified by qRT-PCR. (B) OPM2 and CAG cells were transfected with control shRNA or *IL32* shRNA, and the expression of IL-32 was examined by western blotting (cell lysate) and qRT-PCR. (C) MΦs were stimulated with CM from IL-32-knockdown OPM2 and CAG cells for 24 h, and qRT-PCR was performed to measure *IDO* expression. Data are presented as the mean ± SD. *P < 0.05. (D) Representative immunofluorescence images of IDO (red), CD68 (green), and DAPI (blue) in BM biopsies from MM patients with high IL-32 expression (Scale bar, 50 μm) showing colocalization of IDO and $CD68^+$ MΦs. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The associations between IL-32 expression and chemotherapy response, progression-free survival, and overall survival should be investigated in the future. We found that IL-32γ was the main subtype in MM cells and was the most active isoform, consistent with previous research findings [30]. Thus, we chose to explore the functions of IL-32γ in MM.

MΦs are important components of the MM–BM microenvironment and play a crucial role in the pathophysiology of MM [37,38]. Current evidence suggests that MΦs exhibit high plasticity and are often educated to differentiate into several functional states by tumor-derived cytokines, including IL-10 and TGF-β [39,40]. In this study, we found that several MM cell lines could induce the expression of the immunosuppressive molecule IDO in MΦs, and this ability was consistent with IL-32 expression in MM cells. Importantly, the capacity of MM cells to induce IDO production was reduced upon IL-32 knockdown. Interestingly, $CD68^+$ MΦs in BM biopsies of MM patients with high IL-32 expression showed strong IDO expression; however, the association between IL-32 expression and IDO expression should be further explored in more samples. This finding indicated that MM cells facilitate MΦ transformation into a more immunosuppressive phenotype, and this process may partly depend on IL-32. Our data showed that IL-32γ, the major isoform in MM cells, significantly induced IDO mRNA and protein expression in MΦs. IDO mediates immunosuppression by catalyzing tryptophan conversion into kynurenine, which can result in tryptophan deprivation for T cells [41]. An immunomodulatory effect of IDO has also been found in MΦs [32]. Our data strongly indicated that IDO-positive IL-32γ-educated MΦs suppressed the proliferation of $CD4^+$ T cells and their cytokine production. IL-32 induces the production of IL-6 by BMSCs [27] and promotes osteoclast differentiation in MM [28]. Our study involving MΦs provides a better understanding of the immune-related functions of IL-32 in the MM–BM microenvironment.

IL-32 plays an important role in tumor progression through intracellular and extracellular biological functions [20]. Knockdown of intracellular IL-32 in MM cells did not influence the viability or proliferation of MM cells, suggesting that IL-32 acts as an extracellular cytokine. While the IL-32 receptor that mediates extracellular signal transduction has not yet been identified, PR3 has been reported to be a binding protein of IL-32 according to surface plasmon resonance [17]. The binding between PR3 and IL-32 was independent of its enzymatic activity. However, PR3 enhances the activities of IL-32 by limited

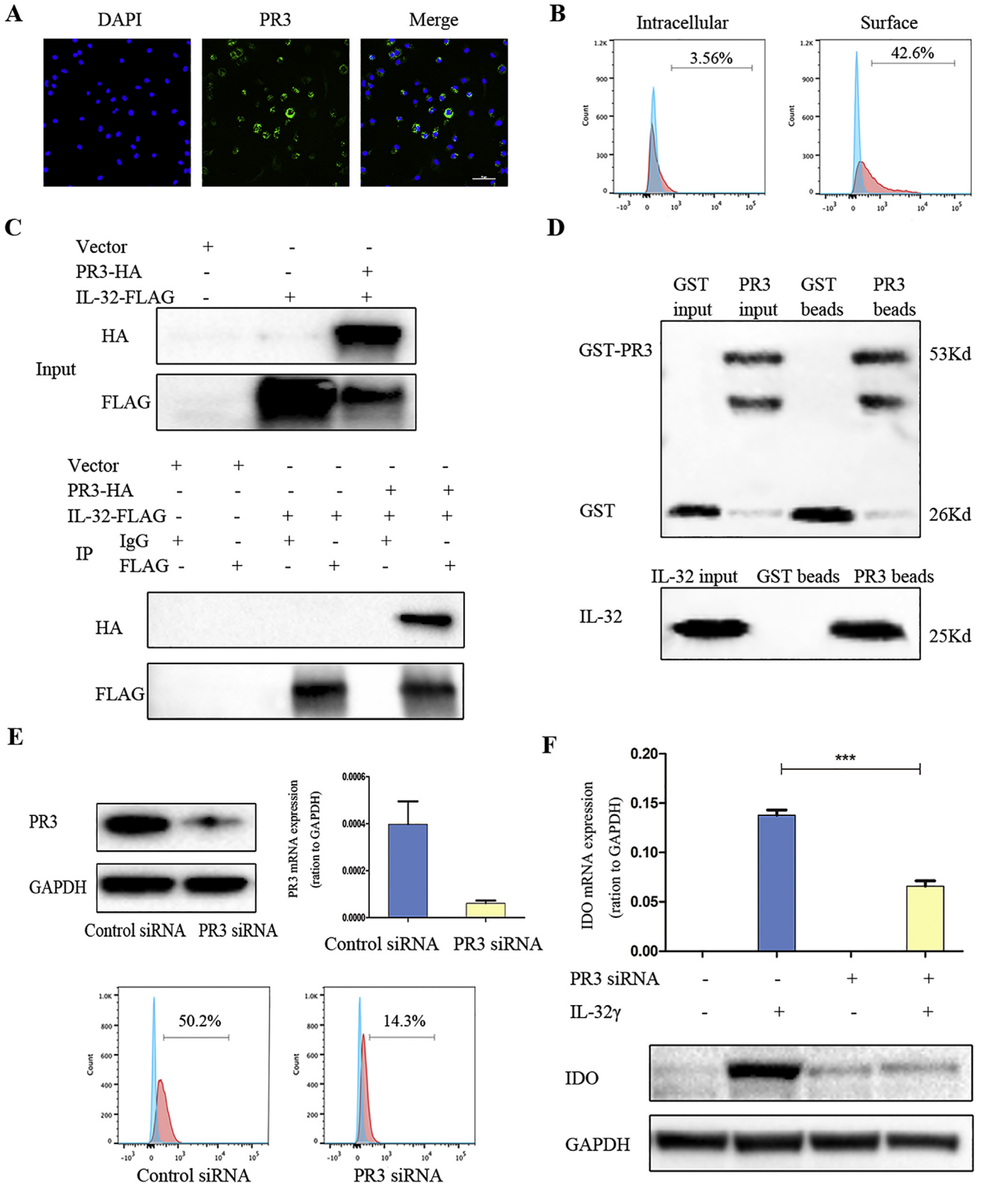

Fig. 4. PR3 is indispensable for IL-32γ-induced IDO expression. (A) The expression of PR3 in monocyte-induced MΦs was detected by immunofluorescence analysis. Scale bar, 50 μm. (B) Flow cytometry was performed to identify the intracellular and surface expression of PR3 in MΦs. The binding affinity of PR3 for IL-32 was examined by GST pulldown (C) and IP experiments (D). (E) MΦs were transfected with control siRNA or *PR3* siRNA, and PR3 expression was detected by western blotting, qRT-PCR, and flow cytometry. (F) PR3-knockdown MΦs were stimulated with or without 20 ng/ml IL-32γ for 24 h. Western blotting and qRT-PCR were performed to measure the expression of IDO. Similar results were obtained in three independent experiments. PR3 knockdown resulted in a reduced ability of IL-32γ to induce IDO expression. ***P < 0.001.

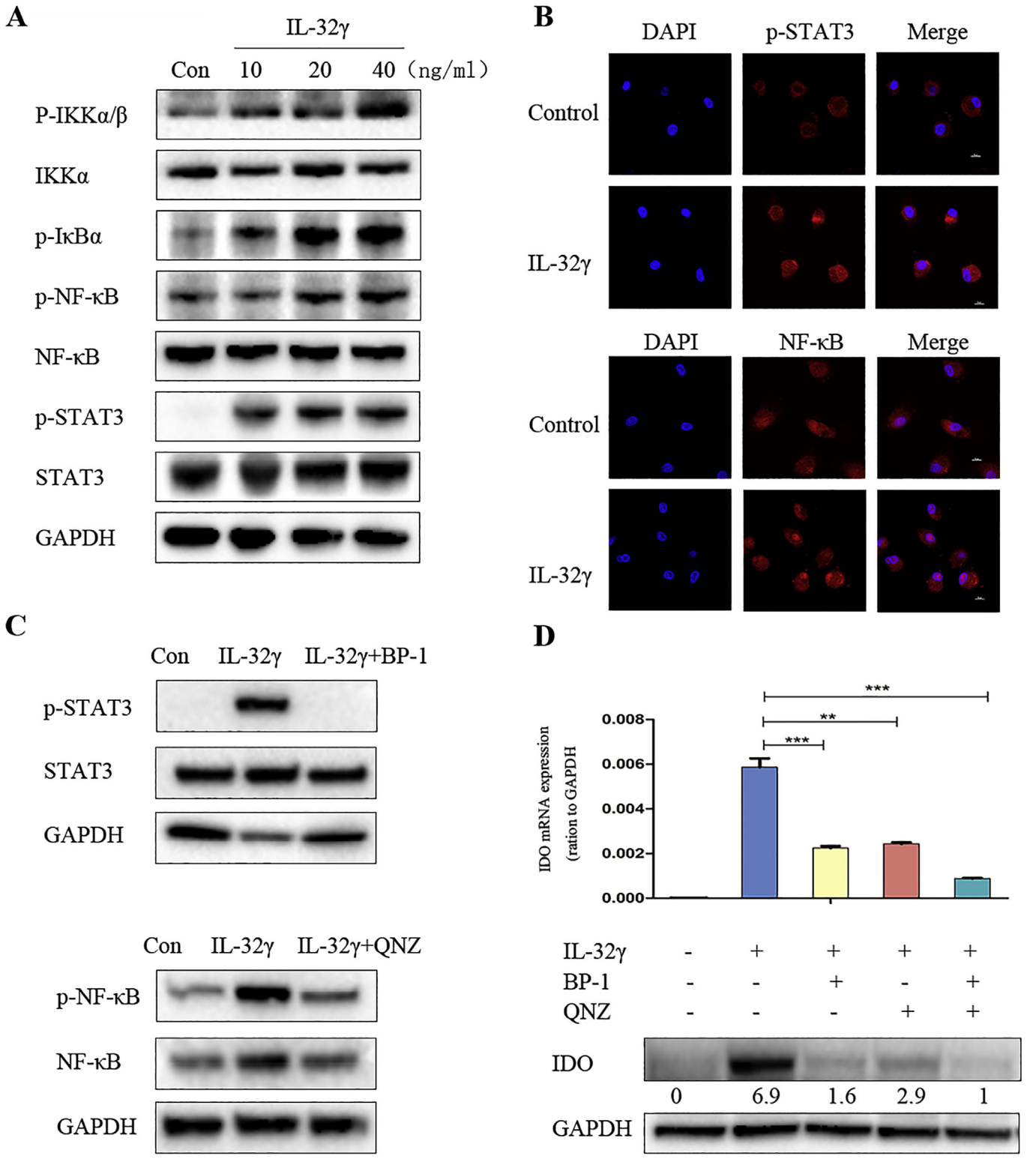

Fig. 5. STAT3 and NF-κB are key signaling pathways in IL-32γ-induced IDO expression in MΦs. (A) Phosphorylation of STAT3, IKKα/β, IκBα, and NF-κB in MΦs stimulated or not with IL-32γ (10–40 ng/ml) for 2 h was examined by western blotting. (B) MΦs were treated with or without 20 ng/ml IL-32γ for 2 h, and immunofluorescence analysis and confocal microscopy were performed to detect the intensity and localization of p-STAT3 and NF-κB. Scale bar, 50 μm. (C) After pretreatment with or without the STAT3 inhibitor BP-1-102 (10 μM) or the NF-κB inhibitor QNZ (10 μM) for 2 h, MΦs were stimulated or not with IL-32γ (20 ng/ml) for 2 h. Western blotting was applied to define the effect of the inhibitors. (D) After pretreatment with the inhibitors, MΦs were stimulated with IL-32γ (20 ng/ml) for 24 h, and IDO expression was subsequently examined by western blotting and qRT-PCR. The quantified density is shown below the bands. Similar results were obtained in three independent experiments. **P < 0.01.

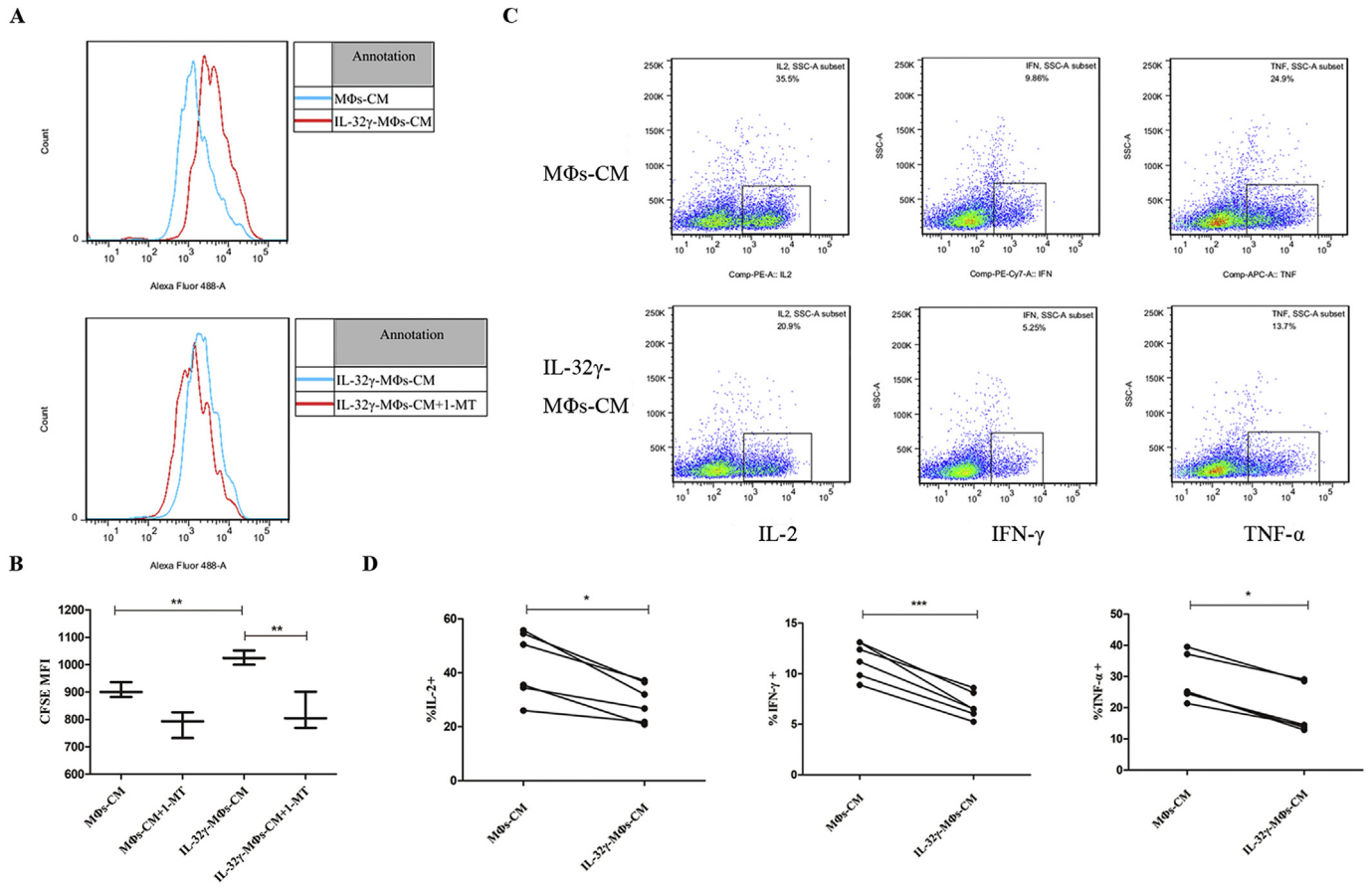

Fig. 6. IL-32γ-educated MΦs inhibit the proliferation and effector function of CD4$^+$ T cells. (A) CD4$^+$ T cells isolated from PBMCs were labeled with CFSE, plated in CM from IL-32γ-educated MΦs, and stimulated with anti-CD3/CD28 antibodies in the absence or presence of 1-MT (100 μM) for 3–5 days. The CFSE intensity of CD4$^+$ cells was measured by flow cytometry. (B) Cell division and proliferation are indicated by the reduction in CFSE intensity, shown as the median fluorescence intensity (MFI). **P < 0.01. (C) For intracellular IL-2, IFN-γ, and TNF-α staining assays, the cells were incubated with Leukocyte Activation Cocktail for 4 h and subsequently stained with the appropriate antibodies for flow cytometry analysis. (D) Cumulative data for intracellular IL-2, IFN-γ, and TNF-α staining in CD4$^+$ T cells from six independent donors are shown. Values are presented as the mean ± S.D. *P < 0.05, ***P < 0.001.

cleavage [17]. Another study further revealed that IL-32γ increases PR3 activity and subsequently triggers protease-activated receptor 2 (PAR2) signaling. The IL-32γ-PAR2 axis utilizes the TRIF and Ras-Raf-1 pathways to exert its biological activities [42]. Our research strongly confirmed the binding affinity of PR3 for IL-32 using GST pulldown and IP experiments. Importantly, the capacity of IL-32 to induce IDO expression was significantly reduced upon PR3 knockdown in MΦs. Although another study has indicated that IL-32 promotes IDO expression by immune cells in HIV-infected lymphatic tissue [43], the relevant mechanisms remain poorly understood. Our data verified that PR3 is an important mediator of the pro-IDO effect of IL-32γ. Signal transduction-mediated IDO expression involves the p38 mitogen-activated protein kinase (p38MAPK), JAK/STAT, and NF-κB pathways [44]. We found that IL-32γ significantly activated STAT3 and NF-κB in a dose- and time-dependent manner, while no obvious changes in the phosphorylation of p38MAPK were observed. Moreover, inhibitors of STAT3 and NF-κB decreased the ability of IL-32γ to promote IDO expression. Although IL-32γ increased STAT1 phosphorylation, a STAT1 inhibitor did not downregulate IL-32γ-induced IDO expression (data not shown). Thus, we suggest that the IL-32γ-induced upregulation of IDO expression is predominantly dependent on the STAT3 and NF-κB pathways in MΦs.

In conclusion, our study showed that MM cell-derived IL-32γ induced IDO production in MΦs through PR3 and the downstream STAT3 and NF-κB pathways, resulting in the suppression of the proliferation and effector function of CD4$^+$ T cells (Fig. 7). High IL-32 expression in MM may contribute to an immunosuppressive microenvironment by upregulating IDO production in MΦs, promoting MM progression.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Haimeng Yan: Conceptualization, Formal analysis, Writing - original draft. **Mengmeng Dong:** Data curation, Writing - review & editing. **Xinling Liu:** Software, Writing - review & editing, Software. **Qiang Shen:** Investigation, Resources. **Donghua He:** Data curation, Funding acquisition, Methodology. **Xi Huang:** Conceptualization. **Enfan Zhang:** Project administration. **Qingxiao Chen:** Methodology. **Xing Guo:** Visualization. **Jing Chen:** Conceptualization. **Gaofeng Zheng:** Project administration, Funding acquisition. **Gang Wang:** Resources. **Jingsong He:** Methodology, Supervision. **Qing Yi:** Validation. **Zhen Cai:** Funding acquisition, Project administration, Validation.

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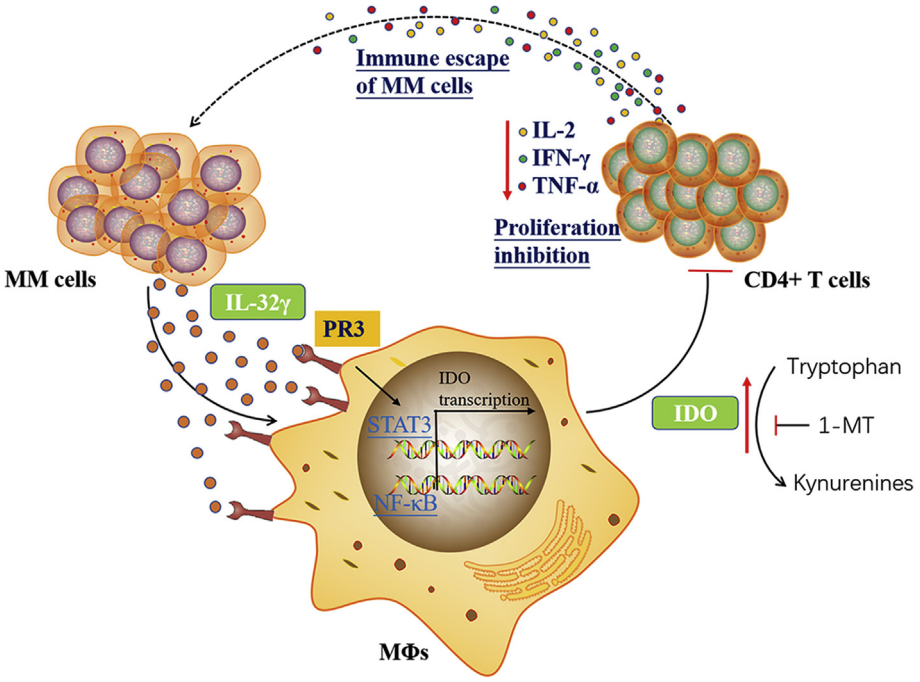

Fig. 7. Summary of main findings presented in the article. MM cell-derived IL-32γ induced IDO expression in MΦs through PR3 and downstream STAT3 and NF-κB signaling, leading to the suppression of the proliferation and effector function of $CD4^+$ T cells.

editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.012.

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References

[1] M.S. Raab, K. Podar, I. Breitkreutz, P.G. Richardson, K.C. Anderson, Multiple myeloma, Lancet 374 (2009) 324–339.
[2] M. Moschetta, Y. Kawano, K. Podar, Targeting the bone marrow microenvironment, Immunol. Rev. 263 (2015) 160–172.
[3] G. Shay, L. Hazlehurst, C.C. Lynch, Dissecting the multiple myeloma-bone microenvironment reveals new therapeutic opportunities, J. Mol. Med. 94 (2016) 21–35.
[4] Y. Zheng, Z. Cai, S. Wang, X. Zhang, J. Qian, S. Hong, H. Li, M. Wang, J. Yang, Q. Yi, Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis, Blood 114 (2009) 3625–3628.
[5] S. Panchabhai, K. Kelemen, G. Ahmann, S. Sebastian, J. Mantei, R. Fonseca, Tumor-associated macrophages and extracellular matrix metalloproteinase inducer in prognosis of multiple myeloma, Leukemia 30 (2016) 951–954.
[6] M.N. Andersen, N.F. Andersen, S. Rodgaard-Hansen, M. Hokland, N. Abildgaard, H.J. Moller, The novel biomarker of alternative macrophage activation, soluble mannose receptor (sMR/sCD206): implications in multiple myeloma, Leuk. Res. 39 (2015) 971–975.
[7] Y. Zheng, J. Yang, J. Qian, P. Qiu, S. Hanabuchi, Y. Lu, Z. Wang, Z. Liu, H. Li, J. He, P. Lin, D. Weber, R.E. Davis, L. Kwak, Z. Cai, Q. Yi, PSGL-1/selectin and ICAM-1/CD18 interactions are involved in macrophage-induced drug resistance in myeloma, Leukemia 27 (2013) 702–710.
[8] J. Chen, D. He, Q. Chen, X. Guo, L. Yang, X. Lin, Y. Li, W. Wu, Y. Yang, J. He, E. Zhang, Q. Yi, Z. Cai, BAFF is involved in macrophage-induced bortezomib resistance in myeloma, Cell Death Dis. 8 (2017) e3161.
[9] A. Gutiérrez-González, M. Martínez-Moreno, R. Samaniego, N. Arellano-Sánchez, L. Salinas-Muñoz, M. Relloso, A. Valeri, J. Martínez-López, Á.L. Corbí, A. Hidalgo, Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma, Blood 128 (2016) 2241–2252.
[10] Q. Wang, Y. Lu, R. Li, Y. Jiang, Y. Zheng, J. Qian, E. Bi, C. Zheng, J. Hou, S. Wang, Therapeutic effects of CSF1R-blocking antibodies in multiple myeloma, Leukemia 32 (2018) 176–183.
[11] K. Beider, H. Bitner, M. Leiba, O. Gutwein, M. Koren-Michowitz, O. Ostrovsky, M. Abraham, H. Wald, E. Galun, A. Peled, A. Nagler, Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype, Oncotarget 5 (2014) 11283–11296.
[12] Y. Li, Y. Zheng, T. Li, Q. Wang, J. Qian, Y. Lu, M. Zhang, E. Bi, M. Yang, R. Frederic, Chemokines CCL2, 3, 14 stimulate macrophage bone marrow homing, proliferation, and polarization in multiple myeloma, Oncotarget 6 (2015) 24218–24229.
[13] C.A. Dahl, R.P. Schall, H.L. He, J.S. Cairns, Identification of a novel gene expressed in activated natural killer cells and T cells, J. Immunol. 148 (1992) 597–603.
[14] J.W. Kang, Y.S. Park, D.H. Lee, M.S. Kim, Y. Bak, S.Y. Ham, S.H. Park, H. Kim, J.H. Ahn, J.T. Hong, D.Y. Yoon, Interaction network mapping among IL-32 isoforms, Biochimie 101 (2014) 248–251.
[15] C. Goda, T. Kanaji, S. Kanaji, G. Tanaka, K. Arima, S. Ohno, K. Izuhara, Involvement of IL-32 in activation-induced cell death in T cells, Int. Immunol. 18 (2006) 233–240.
[16] J.D. Choi, S.Y. Bae, J.W. Hong, T. Azam, C.A. Dinarello, E. Her, W.S. Choi, B.K. Kim, C.K. Lee, D.Y. Yoon, S.J. Kim, S.H. Kim, Identification of the most active interleukin-32 isoform, Immunology 126 (2009) 535–542.
[17] D. Novick, M. Rubinstein, T. Azam, A. Rabinkov, C.A. Dinarello, S.H. Kim, Proteinase 3 is an IL-32 binding protein, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 3316–3321.
[18] J.T. Hong, D.J. Son, C.K. Lee, D.Y. Yoon, D.H. Lee, M.H. Park, Interleukin 32, inflammation and cancer, Pharmacol. Ther. 174 (2017) 127–137.
[19] F. Calabrese, S. Baraldo, E. Bazzan, F. Lunardi, F. Rea, P. Maestrelli, G. Turato, K. Lokar-Oliani, A. Papi, R. Zuin, P. Sfriso, E. Balestro, C.A. Dinarello, M. Saetta, IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 178 (2008) 894–901.
[20] Y.J.E. Sloot, J.W. Smit, L.A.B. Joosten, R.T. Netea-Maier, Insights into the role of IL-32 in cancer, Semin. Immunol. (2018), https://doi.org/10.1016/j.smim.2018.03.004.
[21] A.M. Marcondes, A.J. Mhyre, D.L. Stirewalt, S.H. Kim, C.A. Dinarello, H.J. Deeg, Dysregulation of IL-32 in myelodysplastic syndrome and chronic myelomonocytic leukemia modulates apoptosis and impairs NK function, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 2865–2870.
[22] C.Y. Tsai, C.S. Wang, M.M. Tsai, H.C. Chi, W.L. Cheng, Y.H. Tseng, C.Y. Chen, C.D. Lin, J.I. Wu, L.H. Wang, K.H. Lin, Interleukin-32 increases human gastric cancer cell invasion associated with tumor progression and metastasis, Clin. Canc. Res. 20 (2014) 2276–2288.
[23] Q. Zeng, S. Li, Y. Zhou, W. Ou, X. Cai, L. Zhang, W. Huang, L. Huang, Q. Wang, Interleukin-32 contributes to invasion and metastasis of primary lung adenocarcinoma via NF-kappaB induced matrix metalloproteinases 2 and 9 expression, Cytokine 65 (2014) 24–32.
[24] H. Suga, M. Sugaya, T. Miyagaki, M. Kawaguchi, H. Fujita, Y. Asano, Y. Tada, T. Kadono, S. Sato, The role of IL-32 in cutaneous T-cell lymphoma, J. Invest. Dermatol. 134 (2014) 1428–1435.

[25] J.H. Oh, M.C. Cho, J.H. Kim, S.Y. Lee, H.J. Kim, E.S. Park, J.O. Ban, J.W. Kang, D.H. Lee, J.H. Shim, S.B. Han, D.C. Moon, Y.H. Park, D.Y. Yu, J.M. Kim, S.H. Kim, D.Y. Yoon, J.T. Hong, IL-32 gamma inhibits cancer cell growth through inactivation of NF-kappaB and STAT3 signals, Oncogene 30 (2011) 3345–3359.
[26] H.M. Yun, J.H. Oh, J.H. Shim, J.O. Ban, K.R. Park, J.H. Kim, D.H. Lee, J.W. Kang, Y.H. Park, D. Yu, Y. Kim, S.B. Han, D.Y. Yoon, J.T. Hong, Antitumor activity of IL-32 beta through the activation of lymphocytes, and the inactivation of NF-kappaB and STAT3 signals, Cell Death Dis. 4 (2013) e640.
[27] X. Lin, L. Yang, G. Wang, F. Zi, H. Yan, X. Guo, J. Chen, Q. Chen, X. Huang, Y. Li, Interleukin-32α promotes the proliferation of multiple myeloma cells by inducing production of IL-6 in bone marrow stromal cells, Oncotarget 8 (2017) 92841–92854.
[28] M. Zahoor, M. Westhrin, K.R. Aass, S.H. Moen, K. Misund, K.M. Psonka-Antonczyk, M. Giliberto, G. Buene, A. Sundan, A. Waage, Hypoxia promotes IL-32 expression in myeloma cells, and high expression is associated with poor survival and bone loss, Blood Adv 1 (2017) 2656.
[29] C. Sorrentino, E. Di Carlo, Expression of IL-32 in human lung cancer is related to the histotype and metastatic phenotype, Am. J. Respir. Crit. Care Med. 180 (2009) 769–779.
[30] A. Osman, F. Bhuyan, M. Hashimoto, H. Nasser, T. Maekawa, S. Suzu, M-CSF inhibits anti-HIV-1 activity of IL-32, but they enhance M2-like phenotypes of macrophages, J. Immunol. 192 (2014) 5083–5089.
[31] Y. Xue, H. Xiao, S. Guo, B. Xu, Y. Liao, Y. Wu, G. Zhang, Indoleamine 2,3-dioxygenase expression regulates the survival and proliferation of *Fusobacterium nucleatum* in THP-1-derived macrophages, Cell Death Dis. 9 (2018) 355.
[32] W.L. Liu, Y.H. Lin, H. Xiao, S. Xing, H. Chen, P.D. Chi, G. Zhang, Epstein-Barr virus infection induces indoleamine 2,3-dioxygenase expression in human monocyte-derived macrophages through p38/mitogen-activated protein kinase and NF-kappaB pathways: impairment in T cell functions, J. Virol. 88 (2014) 6660–6671.
[33] T.S. Johnson, D.H. Munn, Host indoleamine 2,3-dioxygenase: contribution to systemic acquired tumor tolerance, Immunol. Invest. 41 (2012) 765.
[34] Y.H. Kang, M.Y. Park, D.Y. Yoon, S.R. Han, C.I. Lee, N.Y. Ji, P.K. Myung, H.G. Lee, J.W. Kim, Y.I. Yeom, Y.J. Jang, D.K. Ahn, J.W. Kim, E.Y. Song, Dysregulation of overexpressed IL-32 alpha in hepatocellular carcinoma suppresses cell growth and induces apoptosis through inactivation of NF-kappaB and Bcl-2, Cancer Lett. 318 (2012) 226–233.
[35] J. Oh, M. Cho, J. Kim, S. Lee, H. Kim, E. Park, J. Ban, J. Kang, D. Lee, J. Shim, IL-32γ inhibits cancer cell growth through inactivation of NF-κB and STAT3 signals, Oncogene 30 (2011) 3345–3359.
[36] Y.S. Park, J.-W. Kang, D.H. Lee, M.S. Kim, Y. Bak, Y. Yang, H.G. Lee, J. Hong, D.-Y. Yoon, Interleukin-32α downregulates the activity of the B-cell CLL/lymphoma 6 protein by inhibiting protein kinase Cε-dependent SUMO-2 modification, Oncotarget 5 (2014) 8765.
[37] J. Kim, R.A. Denu, B.A. Dollar, L.E. Escalante, J.P. Kuether, N.S. Callander, F. Asimakopoulos, P. Hematti, Macrophages and mesenchymal stromal cells support survival and proliferation of multiple myeloma cells, Br. J. Haematol. 158 (2012) 336–346.
[38] S. Berardi, R. Ria, A. Reale, A. De Luisi, I. Catacchio, M. Moschetta, A. Vacca, Multiple myeloma macrophages: pivotal players in the tumor microenvironment, J Oncol 2013 (2013) 183602.
[39] A. Mantovani, F. Marchesi, A. Malesci, L. Laghi, P. Allavena, Tumour-associated macrophages as treatment targets in oncology, Nat. Rev. Clin. Oncol. 14 (2017) 399–416.
[40] N.B. Hao, M.H. Lü, Y.H. Fan, Y.L. Cao, Z.R. Zhang, S.M. Yang, Macrophages in tumor microenvironments and the progression of tumors, Clin. Dev. Immunol. 2012 (2014) 948098.
[41] G.C. Prendergast, C. Smith, S. Thomas, L. Mandik-Nayak, L. Laury-Kleintop, R. Metz, A.J. Muller, Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer, Cancer Immunol. Immunother. 63 (2014) 721–735.
[42] M. Nakayama, Y. Niki, T. Kawasaki, Y. Takeda, H. Ikegami, Y. Toyama, T. Miyamoto, IL-32-PAR2 axis is an innate immunity sensor providing alternative signaling for LPS-TRIF axis, Sci. Rep. 3 (2013) 2960.
[43] A.J. Smith, C.M. Toledo, S.W. Wietgrefe, L. Duan, T.W. Schacker, C.S. Reilly, A.T. Haase, The immunosuppressive role of IL-32 in lymphatic tissue during HIV-1 infection, J. Immunol. 186 (2011) 6576–6584.
[44] N. Arumuggam, N.A. Bhowmick, H.P. Rupasinghe, A review: phytochemicals targeting JAK/STAT signaling and Ido expression in cancer, Phytother Res. 29 (2015) 805–817.