EBV-EBNA1 constructs an immunosuppressive microenvironment for nasopharyngeal carcinoma by promoting the chemoattraction of Treg cells

Shaofen Huo,1 Yunfan Luo,1 Rui Deng,1 Xiong Liu,1 Jie Wang,1 Lu Wang,2 Bao Zhang,3 Fan Wang,1 Juan Lu,1 Xiangping Li 1

ABSTRACT

Background Nasopharyngeal carcinoma (NPC) is primarily caused by the Epstein-Barr virus (EBV) infection in NPC endemic areas. EBNA1 is an EBV-encoded nuclear antigen, which plays a critical role in the maintenance and replication of EBV genome. However, the mechanisms of EBNA1-promoted NPC immune escape are unknown. Regulatory T (Treg) cells are among the key regulators in restraining antitumor responses. However, the mechanisms of accumulation of Treg cells in NPC have not been defined. This study attempted to identify the detailed mechanisms of EBNA1 functions as a tumor accelerator to promote NPC immune escape by enhancing chemoattraction of Treg cells.

Methods mRNA profiles were determined by next-generation sequencing in NPC cells. In vitro and in vivo assays were performed to analyze the role of EBNA1 in regulation of recruitment of Treg cells. Colocation and coimmunoprecipitation analyses were used to identify the SMAD3/c-JUN complex. Chromatin immunoprecipitation assay and dual luciferase reporter assays were designed to demonstrate c-JUN binding to miR-200a promoter and miR-200a targeting to CXCL12 3'Untranslated Regions. The hepatocellular carcinoma models were designed to demonstrate universality of the CXCL12-CXCR4-Treg axis in promoting immune evasion of various tumors.

Result A novel molecular mechanism was identified that involves EBV-EBNA1-stimulated chemotactic migration of Treg cells toward NPC microenvironment by upregulation of the transforming growth factor-β1 (TGFβ1)-SMAD3-Pi3K-AKT-c-JUN- miR-200a-CXCL12-CXCR4 axis in the NPC microenvironment. CXCL12 efficiently attracts CXCR4-positive Treg cells to remodel an immunosuppressive microenvironment.

Conclusions EBV-EBNA1 promotes chemotactic migration of Treg cells via the TGFβ1-SMAD3-Pi3K-AKT-c-JUN-miR-200a-CXCL12-CXCR4 axis in the NPC microenvironment. These results suggest that EBV-EBNA1 may serve as a potential therapeutic target to reshape the NPC microenvironment.

INTRODUCTION

Statistical data indicate that 129,079 new nasopharyngeal carcinoma (NPC) cases were reported in 2018 worldwide, and approximately, 71% of new cases were in East and Southeast Asia.1 In the NPC-endemic areas, Epstein-Barr virus (EBV) infection is believed to be necessary for NPC development and is considered the most important aetiological factor in pathogenesis.2 Many studies demonstrated that EBV employs a wide range of immune evasion strategies to interfere with the innate and adaptive immune responses during latent infection.3 A better understanding of the immune evasion mechanisms of EBV will improve the development of novel EBV-directed therapies in NPC. However, the exact mechanisms of the process remain unknown.

EBNA1 is an EBV-encoded nuclear antigen, which plays a critical role in the maintenance and replication of EBV genome and postmitotic EBV genome segregation and is the only EBV protein expressed in all types of EBV-infected cells.4 Numerous studies have focused on the proliferation, invasion and metastasis induced by EBNA15-7 and the significance of serum EBNA1 antibody for disease diagnosis and prognosis.8-10 However, the role of EBV-EBNA1 in NPC immune escape is unknown.
Regulatory T (Treg) cells express CD4+CD25−CD127low/− cell surface markers and FOXP3+ intracellular marker and are regarded as a predictor of unfavorable prognosis in multiple cancers. Our previous report demonstrated a significant positive correlation between the density of EBV-DNA in the plasma and Treg cells in the peripheral blood of NPC patients. We also found that EBV-EBNA1-positive NPC cells converted naïve T cells into Treg cells by upregulation of transforming growth factor-β1 (TGFβ1) in vitro. However, the role and immanent mechanism of the effect of EBV-EBNA1 on the chemotactic migration of Treg cells have not been investigated. Therefore, we attempted to identify the detailed mechanisms of EBV-EBNA1-promoted chemotraction of Treg cells, which promotes NPC immune escape.

METHODS

Western blot analysis and immunofluorescence
Western blot and immunofluorescence were performed according to an established method.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assay (ChIP) assay was performed to test whether c-JUN is targeted to the miR-200a promoter region using a Pierce agarose ChIP kit (Thermo Scientific, catalog: 26156) according to the manufacturer’s instructions.

Coimmunoprecipitation assay
Coimmunoprecipitation (Co-IP) assay was performed to test the protein-protein interactions (PPI) between c-JUN and SMAD3 proteins using a Pierce Classic magnetic IP/Co-IP kit (Thermo Scientific, catalog: 88804) according to the manufacturer’s instructions.

Luciferase reporter assay
Luciferase reporter assay was performed to test whether miR-200a directly binds to the CXCL12 3’UTR region and whether c-JUN directly binds to the miR-200a promoter region using a dual luciferase reporter gene assay kit (Beyotime Biotechnology, catalog: RG027) according to the manufacturer’s instructions. All dual luciferase reporter vectors contained specific wild-type target sequences or specific mutant sequences.

Facs
Single-cell suspensions were stained with monoclonal antibodies and analyzed using a BD FACScanto instrument according to an established method. Cells were resuspended in PBS and stained with CD4, CD8, CD25 and CD127 monoclonal antibodies for 15–30 min. CD4 positive, CD25 positive, CD127 negative or low expression and CD8 negative cells were identified as Treg cells. Please refer to online supplemental table 2 for the detailed description of antibodies.

A detailed description of the methods and materials can be found in the online supplemental Information.

RESULTS

EBV-EBNA1 promotes chemotactic migration of Treg cells by activating TGFβ1-SMAD3 signaling
Our previous study showed a significant positive correlation between the density of EBV-DNA in the plasma and Treg cells in the peripheral blood of NPC patients. Moreover, EBV-EBNA1-positive NPC cells were shown to convert naïve T cells into Treg cells by upregulation of TGFβ1 in vitro. Hence, we speculated that TGFβ1 plays certain roles in the production of Treg cells and probably in chemotactic migration of Treg cells in EBV-EBNA1-positive NPC microenvironment. The initial results of in vitro migration assay of Treg cells and western blot analysis indicated that the culture supernatant of EBNA1-positive NPC cells promotes the tropism and migration of Treg cells compared with that in the EBNA1-negative groups; however, SB431542 (a specific inhibitor of type I TGFβ receptor kinase) blocked the expression of TGFβ1 induced by EBNA1 and reversed the migration of Treg cells driven by EBNA1 (figure 1A, B, online supplemental figure 1A). To identify potential molecular mechanisms associated with EBV-EBNA1, TGFβ1 and migration of Treg cells, mRNA expression profile was determined by next-generation sequencing in EBNA1-negative and -positive NPC cells. Gene set enrichment analysis showed that TGFβ signaling is enriched in the EBNA1-positive group in the hallmark gene sets database (figure 1C and online supplemental figure 1B). Hierarchical clustering analysis of TGFβ signaling showed that SMAD3 was upregulated in EBNA1-positive cells (figure 1D). Since SMAD family is an intracellular signal transducer and transcriptional modulator activated by the TGFβ family, the activation level of TGFβ1 signaling was measured based on the status of SMAD3. TGFβ1, SMAD3 and phospho-SMAD3 (p-SMAD3) expression levels were considerably higher in EBNA1-positive cells, which is consistent with the sequencing results (figure 1B and online supplemental figure 1C). Moreover, IHC detection in 105 tumor specimens of NPC patients indicated that p-SMAD3 expression is positively correlated with EBV-EBNA1 and TGFβ1 expression, infiltration of Treg cells, advanced clinical stage, higher risk of recurrence, metastasis and death, and shorter overall survival (OS) in NPC patients (figure 1E–G, online supplemental figure 1D) and online supplemental tables 3 and 4). Patients with EBNA1highp-SMAD3high expression pattern or patients with p-SMAD3highTreghigh pathological feature had the worst prognosis (figure 1H). Collectively, these findings suggest that EBV-EBNA1 promotes chemotactic migration of Treg cells by activating TGFβ1-SMAD3 signaling and that enhanced TGFβ1-SMAD3 axis is an indicator of poor prognosis.

miR-200a suppresses chemotactic migration of Treg cells
miR-200a is a known tumor suppressor in a wide variety of malignant tumors and one of the signature markers for prediction of NPC metastasis, and reduction in the expression of miR-200a at the time of diagnosis is associated with poor prognosis, thus, we hypothesized
that miR-200a may play a role in chemotactic migration of Treg cells. Initially, a direct effect of EBV-EBNA1-TGFβ1-SMAD3 axis on miR-200a expression was tested by qRT-PCR analysis. The expression level of miR-200a was considerably lower in EBNA1-positive NPC cells (figure 2A). Then, NPC cell lines were treated with recombinant human TGFβ1 protein. TGFβ1 induced a rapid and robust reduction in mature miR-200a in all NPC cell lines in a time-dependent manner, even though the basal level of miR-200a in EBNA1-positive cells was

Figure 1  EBV-EBNA1 promotes migration of Treg cells by activating TGFβ1-SMAD3 signaling. (A) SB431542 blocked the migration of Treg cells induced by EBNA1. (B) Upper: TGFβ1, SMAD3 and p-SMAD3 protein expression in EBNA1-positive and -negative NPC cell lines assayed by western blot (WB). Lower: SB431542 blocked the expression of TGFβ1, SMAD3 and p-SMAD3 induced by EBNA1. (C) TGFβ1 signaling is enriched in EBNA1-positive group in the hallmark gene sets database according to GSEA. (D) Differentially expressed genes in EBNA1-negative and -positive cells in TGFβ1 signaling according to hierarchical clustering analysis. (E) Representative EBNA1, TGFβ1 and p-SMAD3 expression in NPC patients in the EBNA1 low and high expression groups according to IHC detection. Scale bars at the bottom right: 500 μm (low magnification) and 75 μm (high magnification). (F) Representative images of tumor-infiltrating Treg cells according to FOXP3 staining. The purple arrows represent FOXP3+ Treg cells in the stroma surrounding the tumor cells. Scale bars at the bottom right: 75 μm. (G) Kaplan-Meier analysis of recurrence risk and overall survival for p-SMAD3 expression groups in 105 NPC patients. (H) Kaplan-Meier analysis of recurrence risk and overall survival for concurrent EBNA1/p-SMAD3 expression groups or concurrent p-SMAD3/Treg pathological feature groups in 105 NPC patients. EBV, Epstein-Barr virus; GSEA, Gene set enrichment analysis; TGFβ1, transforming growth factor-β; Treg, regulatory T; IHC, Immunohistochemistry.
already very low (Figure 2B, online supplemental figure 2A). However, SB431542 treatment rescued this decrease in miR-200a levels (Figure 2C, online supplemental figure 2B). Then, NPC cells were treated with SB431542 at various time points; the results indicated an increase in the level of miR-200a in a time-dependent manner after blockade of autocrine TGFβ1 signaling activity in NPC xenograft groups. Scale bars at the bottom right: 75 μm.
increasing the levels or blocking miR-200a had no effect on TGFβ1 and SMAD3 expression, indicating that miR-200a is a downstream factor in the TGFβ1-SMAD3 axis (figure 2E, online supplemental figure 2D).

Furthermore, the in vitro findings were confirmed in a mouse model, indicating that tumor load and proportion of tumor-infiltrating Treg cells were significantly increased in the 5–8F-EBNA1 group and decreased in...
Collectively, our data indicate that EBV-EBNA1 promotes chemotactic migration of Treg cells by directly targeting the CXCL12 3’UTR and that CXCL12 suppresses mir-200a transcription.

To investigate the regulatory associations between CXCL12 and chemotactic migration of Treg cells, the in vitro findings were verified in an in vivo migration assay; the results indicated that overexpression of CXCL12 enhanced migration of Treg cells and decreased the CD8/Treg ratio; blockade of the CXCL12 axis by AMD3100 (a selective antagonist of CXCL12-mediated chemotaxis) suppressed the migration of Treg cells and increased the CD8/Treg ratio (figure 3H). Additionally, CXCL12 expression was positively correlated with EBV-EBNA1 expression, infiltration density of Treg cells, advanced clinical stage, high risk of recurrence, metastasis and death in NPC patients (figures 1E and 3L, online supplemental figure 3F and online supplemental tables 3 and 4). NPC patients with CXCL12^{high}Treg^{high} pathological feature had the worst prognosis (figure 3J).

Collectively, our data indicate that EBV-EBNA1 promotes chemotactic migration of Treg cells by upregulating CXCL12 expression, and CXCL12 acts as a target gene of mir-200a and as an inhibitor of mir-200a transcription.

c-Jun directly binds to mir-200a promoter and induces a c-JUN-mir-200a-CXCL12-P13K-AKT-c-JUN feedback loop

We hypothesized that CXCL12 suppresses mir-200a transcription via certain transcriptional factors, which interact with the mir-200a promoter region. Bioinformatics analysis (UCSO, PROMO, JASPAR and Genomatix) was used to predict possible transcription factors for CXCL12.
that bind upstream of the 3 kbp miR-200a promoter region. Six putative c-JUN binding sites were identified and named site1, site2, site3, site4, site5 and site6 (online supplemental figure 4A). To evaluate whether CXCL12 regulates miR-200a expression via cJUN and the signals of the PI3K/AKT pathway upstream of cJUN, NPC cells were treated with recombinant human CXCL12 protein or CXCL12-specific siRNA, respectively. Overexpression of CXCL12 increased p-PI3K, p-AKT, cJUN and p-cJUN expression and did not change the total PI3K and AKT levels; however, blockade of CXCL12 had the opposite effect, suggesting that CXCL12 is an upstream positive regulator of the PI3K-AKT-cJUN signals (figure 4A). Moreover, cJUN-specific siRNAs considerably increased miR-200a transcription and rescued the suppressive effect of CXCL12 on miR-200a transcription, suggesting that cJUN is an upstream regulator of miR-200a (figure 4B).

Subsequent ChIP-qPCR assay demonstrated that cJUN protein was recruited to site1 and site6 (figure 4C). Then, miR-200a promoter dual luciferase reporter assay confirmed the exact association between cJUN and these two sites (online supplemental figure 4B). WT and MUT6 promoter-dependent luciferase activities were significantly reduced by cotransfection with the cJUN overexpression plasmid, and MUT1 and MUT1 +6 promoter-dependent luciferase activities did not change, implying that cJUN suppressed miR-200a promoter-dependent transcription by directly binding at site1 (figure 4D).

Interestingly, overexpression of cJUN upregulated CXCL12 protein and blockade of c-JUN reduced CXCL12 expression, suggesting that CXCL12 is positively regulated by cJUN and serves as a downstream factor of cJUN (figure 4E). Moreover, IHC assay in the xenograft model confirmed that CXCL12 expression is positively associated with cJUN. The cJUN-CXCL12 axis was dramatically upregulated in the 5–8F-EBNA1 group and downregulated in the 5–8F-miR-200a group compared with that in the 5–8F-NC group (figures 3C and 4F). In NPC patients, cJUN and p-cJUN levels were positively correlated with the expression of EBNA1 and CXCL12, infiltration of Treg cells, advanced clinical stage, high risk of recurrence and metastasis, and short OS (figures 1E, 3I, 4G and H, (online supplemental figure 4C) and online supplemental tables 3 and 4). In addition, patients with the EBNA1highp-cJUNhigh expression pattern suffered from the highest recurrence rate and had a tendency for shorter survival even if it was not statistically significant (figure 4H). Moreover, patients with p-cJUNhighTreghigh pathological feature had the worst prognosis (figure 4H).

Thus, our study demonstrates the exact molecular mechanism of EBV-EBNA1-dependent regulation of chemotactic migration of Treg cells mediated by the cJUN-miR-200a-CXCL12-PI3K-AKT-cJUN feedback loop.

**Tgfβ1 suppresses miR-200a by enhancing the formation of the Smad3/c-Jun complex**

To evaluate whether TGFβ1 regulates miR200a expression through the cJUN-dependent pathway, NPC cells were treated with recombinant human TGFβ1 protein or SB431542 in the presence or in the absence of cJUN expression. TGFβ1 promoted the expression of cJUN, SMAD3 and p-SMAD3 and suppressed miR200a expression and SB431542 reversed these effects (figure 5A,B). However, cJUN overexpression by transient transfection neutralized the stimulatory effect of SB431542 on miR200a but did not neutralize the suppressive effect of SB431542 on TGFβ1, SMAD3 or p-SMAD3 (figure 5A,B). Moreover, blockade of cJUN partially neutralized the suppressive effect of TGFβ1 on miR200a but did not neutralize the stimulatory effect of TGFβ1 on SMAD3 or p-SMAD3 (figure 5A,B). These results suggest that cJUN is a downstream factor of the TGFβ1-SMAD3 axis and TGFβ1 suppresses miR-200a by stimulating the SMAD3-cJUN pathway.

To analyze the details of the interactions of TGFβ1, SMAD3 and cJUN, possible PPI were predicted by bioinformatics analysis (STRING, Genomatix, BIOGRID and BioPlex); the results indicated that SMAD3 and cJUN may form a protein–protein complex at the AP-1/SMAD site. Thus, we hypothesized that TGFβ1, which is activated by EBV-EBNA1, can modulate the transcription and subsequent profound immunosuppression via the SMAD3/cJUN complex in the microenvironment of EBV-infected NPC cells. Quadruplet-color confocal microscopy colocalization analysis demonstrated that TGFβ1 protein is located in the cytoplasm, and SMAD3 and cJUN proteins are located in the cytoplasm and nucleus in EBV-EBNA1-positive NPC cells (figure 5C).

Subsequent co-IP analysis showed that SMAD3 and cJUN interact weakly in the absence of EBV-EBNA1 and exogenous TGFβ1 stimulus (5–8F). However, the interaction between SMAD3 and cJUN was enhanced after stimulation with exogenous recombinant human TGFβ1 protein (5–8F-TGFβ1) or exogenous recombinant active EBV-EBNA1 protein (5–8F-EBNA1) (figure 5D). Interestingly, blockade of the TGFβ1 pathway by SB431542 neutralized the stimulatory effect of EBV-EBNA1 and significantly weakened the interaction between SMAD3 and cJUN (5–8F-EBNA1-SB) (figure 5D). These results suggest that SMAD3/cJUN interaction is governed by the upstream EBNA1-TGFβ1 axis in EBV-EBNA1-infected NPC cells. In the clinical cohort, NPC patients with high TGFβ1 or high p-SMAD3 expression had higher cJUN and p-cJUN expression (figures 1E, 4G and 5E). Patients with the p-SMAD3highp-cJUNhigh expression pattern suffered from the highest recurrence rate and the shortest OS (figure 5F).

Overall, the results indicate that TGFβ1 suppresses miR-200a by enhancing the formation of the SMAD3/cJUN complex, and EBV-EBNA1 acts as a comprehensive regulator of cellular gene expression by governing the SMAD3/cJUN interaction in a TGFβ1-dependent manner.

**Tgfβ1 induces CXCL12 production by suppressing miR-200a**

To verify whether TGFβ1 influences CXCL12 expression in a miR-200a-dependent manner, NPC cells were treated with recombinant human TGFβ1 protein for various periods of time; the results indicate that production of...
CXCL12 protein was elevated in a time-dependent manner (Figure 6A). However, SB431542 blocked the induction effect of TGFβ1 on CXCL12 expression when NPC cells were pretreated with SB431542 in the presence or in the absence of TGFβ1 (Figure 6B). These results suggest that TGFβ1 positively regulates CXCL12 expression. Moreover,

Figure 4  c-JUN directly binds to miR-200a promoter and generates a c-JUN-miR-200a-CXCL12- PI3K-AKT-c-JUN feedback loop. (A) Overexpression of CXCL12 increased PI3K-AKT-c-JUN signaling expression; however, CXCL12-specific siRNA decreased the PI3K-AKT-c-JUN signaling pathway expression. (B) The c-JUN-specific siRNAs increased miR-200a level after blockade of c-JUN and rescued the suppressive effect of CXCL12 overexpression on miR-200a transcription. (C) ChIP assay demonstrated c-JUN binding to the miR-200a promoter region at site1 and site6. (D) miR-200a promoter dual luciferase reporter assay verified that c-JUN directly binds to miR-200a promoter. (E) c-JUN positively regulates CXCL12 expression. (F) IHC detection of c-JUN and p-c-JUN expression in various NPC xenograft groups from figure 3F1. Scale bars at the bottom right: 75 μm. (G) Representative c-JUN and p-c-JUN expression in NPC patients in EBNA1 low and high expression groups according to IHC. Scale bars at the bottom right: 500 μm (low magnification) and 75 μm (high magnification). (H) Kaplan-Meier analysis of recurrence risk and overall survival for c-JUN and phospho-c-JUN expression groups, and the recurrence risk and overall survival for the concurrent EBNA1/p-c-JUN expression groups and the concurrent p-c-JUN expression and infiltration of Treg cells groups in 105 NPC patients. ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; NPC, nasopharyngeal carcinoma; Treg, regulatory T.
various concentrations of miR-200a mimics partially neutralized the stimulatory effect of TGFβ1 on CXCL12 expression in a dose-dependent manner, suggesting that TGFβ1 regulates CXCL12 expression in a miR-200a-dependent manner (figure 6C). Interestingly, the effect of TGFβ1 or SB431542 on CXCL12 mRNA level was not significant in agreement with the finding that miR-200a inhibits CXCL12 by a post-transcriptional mechanism (online supplemental figure 5A). To detect the stimulatory effect of CXCL12 on the migration of Treg cells induced by the TGFβ1-miR-200a axis, the culture supernatant was collected after the treatment of the cells with miR-200a mimics or recombinant human TGFβ1 protein for 72 hours; exogenous and endogenous TGFβ1 proteins were depleted by incubation with TGFβ1-neutralizing antibody for 2 hours. Various concentrations of miR-200a mimics partially neutralized the stimulatory effect of TGFβ1 on CXCL12 expression and ultimately reduced the migration of Treg cells in a dose-dependent manner (figure 6C,D and online supplemental figure 5B). In NPC patients, CXCL12 level was positively correlated with TGFβ1 and p-SMAD3 expression (figures 1E, 3I and 6E). Patients with p-SMAD3high-CXCL12high expression pattern suffered from the worst prognosis (figure 6F). Thus, these results suggest that the stimulatory effect of TGFβ1 on CXCL12 expression is predominantly mediated by the suppression of miR-200a, and chemotactic migration of Treg cells is controlled by the TGFβ1-SMAD3-miR-200a-CXCL12 pathway in NPC.

CXCL12 mediates chemoattraction of Treg cells by upregulating the CXCR4 receptor of Treg cells and the CXCL12-CXCR4-Treg regulatory pathway is universal in various malignant solid tumors

To determine the mechanism involved in CXCL12 axis-induced recruitment of Treg cells, involvement of CXCR4 receptor was investigated. The ligand-binding receptor function was blocked with AMD3100; the data indicate that the migration of Treg cells and CXCR4 expression in Treg cells were considerably suppressed by AMD3100 in vitro (figure 7A and online supplemental figure 6A) and in vivo (figure 3H1–H3) despite the stimulatory effects of CXCL12. Furthermore, similar phenomenon was detected in other animal models. The expression of CXCR4 in Treg cells was significantly increased in the 5–8F-EBNA1 group and

Figure 5  TGFβ1 suppresses miR-200a by enhancing the formation of the SMAD3/c-JUN complex. (A) (B) WB and qRT-PCR detection verified that c-JUN is a downstream factor of the TGFβ1-SMAD3 axis; TGFβ1 suppressed miR-200a by activating the SMAD3-c-JUN pathway. (C) Quadruple-color confocal microscopy colocalization of TGFβ1, SMAD3 and c-JUN. (D) Coimmunoprecipitation (Co-IP) analysis indicated that SMAD3 and c-JUN can form a complex to mediate TGFβ1-induced transcription and that EBNA1 determines the SMAD3/c-JUN interaction in a TGFβ1-dependent manner. (E) NPC patients with high TGFβ1 or high p-SMAD3 expression had higher c-JUN and p-c-JUN expression compared to those in patients with low TGFβ1 or low p-SMAD3 expression. (F) Kaplan-Meier analysis of recurrence risk and overall survival for concurrent p-SMAD3/p-c-JUN expression groups in 105 NPC patients. ANOVA, analysis of variance; NPC, nasopharyngeal carcinoma; TGFβ1, transforming growth factor-β1; WB, western blot.
decreased in the 5–8F-miR-200a group compared with that in the control (5–8F-NC), which explains the mechanism of EBV-EBNA1 antigen-promoted migration of Treg cells and miR-200a-suppressed migration of Treg cells (figures 2F1–F3). Blockade of the TGFβ1-SMAD3 axis by SB431542 inhibited CXCL12 expression in vitro (figure 6B) or migration of Treg cells in vivo (figures 2G1–G3) and reduced CXCR4 expression in infiltrating Treg cells (figures 2G1–G3). However, in these three animal models, the CD4/CD8 ratio and CXCR4 expression in CD8+ T cells were similar in all groups, implying that the CXCL12-CXCR4 ligand/receptor pair is unlikely to be responsible for regulation of the functional migration of CD8+ T cells in the NPC microenvironment (figures 2F1–F3, G1–G3 and 3H1–H3). Thus, the data indicate that the CXCL12-CXCR4 ligand/receptor pair is the main direct regulator of chemotactic migration of Treg cells, and the EBV-EBNA1-TGFβ1-SMAD3-miR-200a-CXCL12 axis mediates the migration of Treg cells by regulating the CXCR4 receptor of Treg cells.

To provide additional support for the role of CXCL12 in stimulation of the migration of Treg cells and to assess whether the CXCL12-CXCR4 pathway is universal in various tumor contexts, a humanized immune reconstruction nude mouse model of human hepatocellular carcinoma was produced by intraperitoneal injection of HCCLM3 cells into immunocompromised nude mice; additionally, a well-established immunocompetent mouse model of hepatocellular carcinoma was produced by intraperitoneal injection of Hepa1-6 mouse hepatoma cells into the C57BL/6 syngeneic hosts (figure 7B1-B3, 7C1-C3). In HCCLM3 and Hepa1-6 animal models, the tumor load, proportion of tumor-infiltrating Treg cells and expression of CXCR4 in Treg cells were significantly increased in the CXCL12 treatment groups and decreased in the AMD3100 treatment groups compared with those in the control groups (figure 7B1-B3, 7C1-C3, (online supplemental figure 6B,C). Moreover, the CD8/Treg ratio was decreased in the CXCL12 treatment groups and increased in the AMD3100 treatment groups (figure 7B2-3 and 7C2-3). Unexpectedly, the CD4/CD8 ratio was reversed due to a decrease in the proportion of CD4+ T cells and an increase in CD8+ T cells in each group in the immunocompetent C57BL/6 mouse model (figure 7C2-C3); this effect was not detected in the immunocompromised nude mouse models, which did not have fully functional T cell lineages (figures 2F2–F3, 2G2–G3, 3H2–H3 and 7B2–B3), implying that tumor caused local immune senescence in a real immunocompetent syngeneic host, which may be another synergistic mechanism for tumor immune escape. Overall, our results in NPC and hepatoma models demonstrate the universal role of the CXCL12-CXCR4 axis in chemotactic migration of Treg cells in various malignant solid tumors.

Thus, EBV-EBNA1 promotes chemoattraction of Treg cells by upregulation of the TGFβ1-SMAD3-PI3K-AKT-c-JUN-CXCL12-CXCR4 axis and downregulation of miR-200a (figure 7D).

**DISCUSSION**

EBV-positive NPC microenvironment is heavily infiltrated with lymphoid stroma. Latent EBV-infected NPC cells use several specific mechanisms to form and shape the tumor microenvironment to their own benefit, especially immune escape.26 Treg cells are one of the key regulators in restraining antitumor immune responses,27 and are considered to be associated with immune escape of...
various tumors, including human ovarian carcinoma,\(^{16}\) melanoma\(^{28}\) and hepatocellular carcinoma.\(^{17}\) Our previous report showed that the plasma EBV-DNA density has significant positive association with the number of Treg cells in the peripheral blood of NPC patients.\(^{13}\) EBNA1 is an EBV-encoded nuclear antigen which plays
a critical role in EBV genome maintenance, replication and postmitotic segregation and is the only EBV protein expressed in all types of EBV-infected cells.3 We have previously demonstrated that EBV-EBNA1 promotes the production of Treg cells in a TGFβ1-dependent manner.11

However, the role and specific mechanism of EBV-EBNA1 in chemotraction of Treg cells remains poorly understood. The present study is the first to demonstrate that EBV-EBNA1 regulates chemotactic migration of Treg cells via the TGFβ1-PI3K-AKT-c-Jun-miR-200a-CXCL12-CXCR4 feedback loop to create an extremely immunosuppressive microenvironment in NPC. Our results link latent EBV infection and construction of immunosuppressive microenvironment in NPC. Mrizak et al previously demonstrated that EBV-EBNA1 promotes the latent infection will provide an opportunity to develop novel EBV-directed immunotherapies to reshape the tumor microenvironment.

Mechanistic analysis revealed that EBNA1 overexpression in NPC cells upregulates TGFβ1 expression and activates the downstream SMAD3/c-Jun protein–protein complex, which binds to the promoter region of miR-200a and represses miR-200a transcription. However, another study showed that miR-200a increases c-Jun expression by stabilizing its mRNA in proliferating cells.29 This contradiction suggests a feedback loop between miR-200a and c-Jun. In our study, CXCL12 was confirmed for the first time as a direct target of miR-200a. Importantly, in addition to the repressive effect of miR-200a on CXCL12 expression, CXCL12 overexpression can suppress miR-200a transcription through the PI3K/AKT/c-Jun pathway. These results confirm direct interaction of miR-200a, c-Jun and CXCL12, which form a c-Jun-miR-200a-CXCL12-PI3K-AKT-cJun feedback loop to regulate chemotactic migration of Treg cells.

Previous studies have reported that EBV-encoded latent genes can induce immune escape through several mechanisms, including downregulation of HLA expression to interfere with the antigen-presenting machinery, upregulation of the coinhibitory receptor expression to interrupt T cell activation, production of immunosuppressive cytokines and prevention of innate immune responses.30 Several recent publications focused on the modulation of the tumor microenvironment induced by EBV latent genes, especially by the well-characterized LMP1 oncogene.31 However, the role of EBNA1 in immune modification associated with escape from immune attack is incompletely understood. Our study demonstrates that EBNA1 overexpression in NPC cells induces the secretion of CXCL12 chemokine and efficiently attracts CXCR4-positive Treg cells to remodel an immunosuppressive microenvironment. This result provides a link between EBV infection and chemokine-mediated immunosuppressive microenvironment in NPC. Mrizak et al obtained similar results, which indicated that NPC-derived exosomes contain CCL20 chemokine that converts conventional CD4+ T cells into suppressive Treg cells, and recruitment of Treg cells into the tumor increases their suppressive functions.32 Interactions between EBNA1 and recruitment of Treg cells represent a newly defined mechanism of EBV-induced immune evasion in NPC.

In conclusion, our study demonstrates a previously unrecognized mechanism of EBNA1-induced chemotraction of Treg cells mediated by the TGFβ1-PI3K-AKT-c-Jun-miR-200a-CXCL12-CXCR4 axis in the NPC microenvironment. Given the critical roles of Treg cells in immune escape, a more comprehensive understanding of the crosstalk between Treg cells and EBV latent infection will provide an opportunity to develop novel EBV-directed immunotherapies to reshape the tumor microenvironment.

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Contributors This study was designed by SH and JL and supervised by XL. Experiments were primary conducted by SH. Patient enrolment and follow-up were done by RD. Specimen preparation was done by XL, YL, JW, LW, BZ and FW prepared NPC cells for NGS detection and conducted qRT-PCR screening according to NGS results. The administrative support was obtained from XL. The manuscript was written by SH and approved by all authors.

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ORCID iD Xiangping Li http://orcid.org/0000-0002-0238-3557

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