The LMP1/Lgals1-NF-κB-IRF1-PDL1 axis promotes immune escape in nasopharyngeal carcinoma

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Abstract

Background: Recurrence and metastasis of nasopharyngeal carcinoma (NPC) is closely related to immune escape. It is known that LMP1 promotes the immune escape of NPCs by controlling the expression of PD-L1. However, the underlying molecular mechanisms remain to be fully characterized.

Methods: We demonstrated LMP1 activates downstream pathways in vitro, and confirmed LMP1/Lgals1 interaction and inhibition of PD-L1 expression by Lgals1 inhibitor OTX008 in vitro by Western blot and flow cytometry. Then we analyzed the clinical correlation among LMP1, Lgals1, IRF-1 and PD-L1. Finally, we confirmed that OTX008 enhances the immunogenicity of nasopharyngeal carcinoma cells by T-cell killing assay, ELISA and flow cytometry.

Results: We demonstrated that LMP1 interacts with Lgals1 to regulate IRF1 via NF-κB signaling pathway, promoting the expression of PD-L1 in NPCs. LMP1 and Lgals1 are both found to be tightly linked to recurrence and metastasis of NPC based on clinical data. Targeting Lgals1 by using OTX008, a specific Lgals1 inhibitor, is able to restore the immunogenic environment in NPCs. Our findings reveal a regulatory axis for PD-L1 expression in NPC cells, and targeting one component in this axis, Lgals1, is effective in boosting the immunogenicity of NPCs, providing a therapeutic avenue for treating NPC.

Conclusion: In summary, we showed here that LMP1 can interact with Lgals1, and this complex can influence PD-L1 expression by regulating IRF-1 through NF-κB signal pathway in NPC cells, and inhibition of Lgals1 improves immunogenicity of NPC cells.

1. Background

Nasopharyngeal carcinoma (NPC) is the most common malignant tumors of head and neck, and is particularly prevalent in southern China and regions of Southeast Asia with an annual incidence of 15-50 cases per 100,000 persons[1, 2]. Although the 5-year survival rate after radiotherapy and chemotherapy had exceeded 80%, distant metastasis and local recurrence still plague the clinical treatment of NPC[3, 4]. Once recurrence or metastasis occurs, the 5-year survival rate was less than 10%[5, 6]. Our previous studies have shown that programmed cell death 1 ligand 1 (PD-L1) is highly expressed in 75.5% of tumor cells in fresh specimens of nasopharyngeal carcinoma (n = 102) by immunohistochemical assay, suggesting that PD-L1 immunotherapy may represent a promising way in curing nasopharyngeal carcinoma[7]. However, a large percent of patients have limited response to immunotherapy due to immunosuppression imposed by NPC cells[8, 9]. Therefore, illustrating the molecular mechanisms associated with immunosuppression is urgently needed, and targeting immune-suppressive pathways will improve the efficacy of immunotherapy and disease outcome.

PD-L1, a transmembrane glycoprotein, binds to programmed cell death 1 (PD-1) protein on T lymphocytes and suppresses its activation[10, 11]. A variety of tumor cells express PD-L1 and utilize the PD-L1/PD-1 checkpoint for immune evasion[12].
Epstein-Barr virus (EBV) is strongly associated with the etiology of nasopharyngeal carcinoma\textsuperscript{[4, 13]}. Latent membrane protein 1 (LMP1), a principal oncogene of EBV, contributes to the initiation, invasion, and metastasis of NPC\textsuperscript{[14]}. It has been reported that LMP1 promotes NPC cells escaping from immunosurveillance, especially cytotoxic T lymphocyte responses that kill virus-infected cells\textsuperscript{[15]}. Meanwhile, tumors secrete various types of growth factors and cytokines to shape an immunosuppressive environment. Lgals1, also known as Galectin-1, is a carbohydrate-binding protein, and highly expressed and secreted into the surrounding milieu by solid tumors including lung, melanoma, breast, and NPC\textsuperscript{[16, 17]}. Here, we found that LMP1 interacts with Lgals1 to promote the transcription of NFkB as well as its phosphorylation, leading to the upregulation of IRF1 and PD-L1 in NPC cells. Furthermore, inhibition of Lgals1 boosts the immunogenicity of NPC cells.

2. Methods

2.1 Patient samples

In this study, informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University. Fresh tissue samples were obtained from 10 patients and were subjected to immunohistochemistry, blood samples were obtained from 40 patient and were subjected to RNA extraction.

2.2 Cell lines and cell culture

Human nasopharyngeal epithelial cells NP69 and NPC cell lines (TW03, and HK1) were used. NP69 was maintained in serum free medium (SFM, Gibco, USA). TW03 and HK1 were maintained in RPMI-1640 Medium (Gibco, USA). TW03 and HK1 were maintained in medium with 10% FBS (Gibco, USA), and all cells were maintained in medium with 1% penicillin/streptomycin (Gibco, USA) in a humidified environment containing 5% CO\textsubscript{2} at 37\degree C.

2.3 Cell transfection

NP69, TW03 and HK1 cells were transfected with pLVX-LMP1 and control plasmid to construct LMP1-overexpression stable cell lines.

2.4 Dual-luciferase reporter assays

The activities of 45 classical signaling pathways were detected by the Cignal Finder Signal Transduction 45-pathway Reporter Array kit (Qiagen, Germany), following the manufacturer’s instructions. NP69 EV and NP69 LMP1OE cells transduced by those plasmids were seeded in the 96-well plate for 48 h. The cells were lysed and the luciferase activities of firefly and renilla were examined with Dual-Luciferase Reporter Assay kit (Promega, USA) following the manufacturer’s instructions. All experiments were performed three times.
2.5 Western blot

Cells were lysed in RIPA lysis buffer containing protease inhibitors. An equal amount of total protein from each sample (20 µg) was resolved on 10-15% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were blocked with 5% BSA for 2 h at room temperature and then incubated with primary antibodies for the detection of LMP1, Lgals1, NF-κB (p65), p-NF-κB (p-p65), IRF-1, PD-L1 GAPDH and β-actin (CST, USA) overnight at 4°C, followed by the appropriate secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The dilution ratio of all primary antibodies was 1:1000. Protein expression was detected using an enhanced chemiluminescent HRP substrate (CST, USA) and imaged with a Chemiluminescence Imaging System (Bio-Rad Laboratories, USA).

2.6 Collection of data from the University of California, Santa Cruz (UCSC), Xena Functional Genomics Browser

The expression of Lgals1 and clinicopathological information for 499 head and neck squamous carcinoma (HNSC) patients in the Cancer Genome Atlas were downloaded from Xena (https://xena.ucsc.edu/) to analyze the relationship between Lgals1 expression and HNSC prognosis.

2.7 RNA Extraction and qRT-PCR

Total RNAs were extracted from blood tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s protocols. Approximately 2 µg of RNA was reverse-transcribed to DNA templates for quantitative RT–PCR (qRT–PCR) using the ReverseAid First Strand cDNA Synthesis kit (Thermo Scientific, USA).

To analyse the gene expression levels quantitatively, qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) using the 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal program was as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. GAPDH was used to normalise the expression of the target genes. The dissociation curve program was used to confirm the specificity of the target amplification product. The primers used in the qRT-PCR analyses were listed in Supplemental Table 1.

2.8 Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from 10 patients, and the slides (4 µM) were deparaffinized and hydrated. Tumor sections were immunostained subsequently for LMP1, Lgals1, IRF-1 and PD-L1. MaxVisionTM kit was applied to each slide (MaixinBiol, China) according to the manufacturer’s instructions. Color was developed by DAB kit (MaixinBiol, China) and slides were counterstained with hematoxylin. The immunostained slides were observed under microscope and scored by two independent pathologists using Image J.

2.9 Co-immunoprecipitation assay
For the anti-LMP1 immunoprecipitation, LMP1 was immunoprecipitated using an anti-LMP1 antibody (1:100; CST, USA), and the co-immunoprecipitated proteins were then detected using an anti-Lgals1 antibody (1:2500; CST, USA). For the anti-Lgals1 immunoprecipitation, Lgals1 was immunoprecipitated using an anti-Lgals1 antibody (1:250; CST, USA), and the co-immunoprecipitated proteins were then detected using an anti-LMP1 antibody (1:1000; CST, USA).

2.10 Flow Cytometry

PE-conjugated anti-human PD-L1, human IgG isotype control antibodies, PE-conjugated anti-human CD69, APC-conjugated anti-human CD3 and PE-conjugated anti-human CD8 antibodies were purchased from Biolegend. Cells were assayed using a flow cytometer, and data were processed using the accompanying software (CytExpert, Beckman Coulter, Beijing, China).

2.11 Immunofluorescence

Cells were plated onto coverslips and fixed with cold 4% paraformaldehyde on ice for 15 min. The cells were then penetrated by treatment with 0.5% Triton X-100 for 15 min. The cells were blocked with 3% BSA and incubated with primary antibodies against corresponding target proteins at 4°C overnight. Then, secondary antibodies were added, and the cells were incubated at 37°C for 1h. The sections were counterstained with DAPI and observed using a confocal microscope (Leica SP8).

2.12 Sorting of T cells by magnetic beads and T-cell killing assays

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation from healthy donors. Then, to isolate the CD8+ T-cells from PBMCs, monocytes were depleted by plastic adherence and any cell clamps were removed using a 70 µm pre-separation filter (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8+ T-cells were positively isolated from the peripheral blood lymphocytes using magnetic beads-conjugated anti-CD8 mAbs kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Subsequently, the cells were stimulated with 5 µg/ml of anti-CD3 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and 5 µg/ml of anti-CD28 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of 100 IU/ml recombinant human interleukin-2. To maintain T lymphocyte expansion, half-volume medium exchange was performed every 3 days with fresh medium and recombinant human IL-2.

TW03 cells were seeded at 1 × 10^5/well in a 96-well U-bottom plate, co-cultured with CD8+ T-cells at different effector-to-target (E/T) ratio for 18 h. The cell-free supernatant was collected, and cytotoxicity was assessed using an LDH activity kit (Sigma, MAK066).

2.13 ELISA

The concentration of perforin, granulysin (GZMB), IFN-γ, IL-2 and Lgals1 in cell culture supernatants were measured using the ELISA Kit (R&D systems, USA) according to the manufacturer’s instructions.
2.14 Statistical Analysis

All data were listed as mean with SD and analyzed by SPSS 20.0 (IBM, USA) and Graphpad Prism 7.0 (GraphPad Software Inc., USA). Student’s t-test was adopted to confirm statistical significance. A P value < 0.05 was indicated statistical significance.

3. Results

3.1 LMP1 promotes the expression of PD-L1 by regulating IRF-1

It has been shown that Epstein-Barr virus (EBV) encoded latent membrane protein LMP1 in nasopharyngeal carcinoma (NPC) induces PD-L1 expression to promote immune escape of tumor cells. Our previous studies have shown that the expression level of PD-L1 in 102 fresh specimens of nasopharyngeal carcinoma by immunohistochemical assay and found that the proportion of tumor cells with high PD-L1 expression (50%) was 75.5%, suggesting that PD-L1 immunotherapy may have a promising application in nasopharyngeal carcinoma[7].

To further investigate the regulatory role of LMP1 on PD-L1, we used the Gene Reporter Assays (Qiagen) to examine the changes of 44 important signaling pathways after overexpression of LMP1 (NP69 LMP1OE) compared to control (NP69 EV) in nasopharyngeal epithelial cells NP69. The results showed that the most significant changes of key effector transcription factor activities were observed in NF-κB, IRF-1 and E2F (Figure 1A). Several previous articles have reported that NF-κB signaling pathway regulates PD-L1 expression[18, 19], while PD-L1 regulation by transcription factor IRF-1 was first confirmed in lung cancer cells in February 2018[20].

Subsequently, we examined the changes of NF-κB, IRF-1 and PD-L1 after overexpression of LMP1 in nasopharyngeal epithelial cells NP69 and nasopharyngeal carcinoma cells TW03(Figure 1B). The results showed that in NP69 and TW03 overexpression of LMP1 activated NF-κB signaling pathway and contributed to the elevation of IRF-1 and PD-L1 protein levels.

We recruited the fresh tissue of 40 patients, after which mRNA was extracted to analyze the expression correlation between LMP1, Lgals1, IRF-1 and PD-L1 (Supplemental Table 2). The results showed that the expression levels of LMP1, Lgals1, IRF-1 and PD-L1 showed a positive correlation between each other(Figure 1C).

3.2 LMP1/Lgals1 influence PD-L1 expression by regulating IRF-1 in NPC cells

To further clarify the regulatory mechanism of LMP1 on PD-L1 expression, we are concerned that some investigators have screened potential interacting proteins of LMP1 by bimolecular fluorescence complementation experiments, among which Lgals1 has attracted our attention.

We verified the interaction between LMP1 and Lgals1 by immunoprecipitation (Co-IP) in an NP69 LMP1 stable overexpression cell line (NP69 LMP1OE). The results showed that the interaction between LMP1
and Lgals1 did exist (Figure 2A-B).

To demonstrate if Lgals1 involved in the regulation of PD-L1 by LMP1, we performed experiments using the Lgals1 inhibitor OTX008. First we performed experiments with inhibitor concentration and time gradient in TW03. The results showed that 10 μM OTX008 treatment for 24h resulted in a very significant inhibition of Lgals1 (Figure 2C). Subsequently, we examined the changes of PD-L1 after the addition of OTX008 under LMP1 overexpression. Unexpectedly, OTX008 could significantly inhibit the phosphorylation levels of NF-κB, the expression of IRF-1 and PD-L1 under LMP1 overexpression in a concentration-dependent effects (Figure 2D). We also treated the cells with different concentrations of BAY 11-7085 (which is an inhibitor of NF-κB activation), and showed the same results as OTX008 treatment, suggesting that Lgals1 influence PD-L1 expression by regulating IRF-1 through NF-κB signaling pathway. Meanwhile, we detected the expression of PD-L1 on the surface of TW03 cells by flow cytometry and found that PD-L1 expression was reduced after OTX008 treatment in the presence of overexpressed LMP1 (Figure 2E), indicated that Lgals1 inhibitor OTX008 can attenuate the expression of PD-L1 on the cell surface.

In addition, we also validated the knockdown of Lgals1 by siRNA. The results showed that knockdown of Lgals1 in LMP1 overexpressing TW03 resulted in significant downregulation of both RNA levels and protein levels of IRF-1 and PD-L1 (Figure 2F-G).

3.3 Lgals1 may play an important function in nasopharyngeal carcinoma

To elucidate the role of Lgals1 in nasopharyngeal carcinoma, we first analyzed the gene expression profile of nasopharyngeal carcinoma from GEO database (GSE61218), and found that Lgals1 expression levels were significantly higher in nasopharyngeal cancer tissues compared with normal nasopharyngeal tissues (Figure 3A). In addition, we also examined the expression of LMP1, IRF-1 and PD-L1 in nasopharyngeal cancer tissues compared with normal nasopharyngeal tissues, and the results were consistent with Lgals1 (Figure 3A). The above results imply that Lgals1 may play a cancer-promoting role in nasopharyngeal carcinoma. Meanwhile, we examined the expression levels of Lgals1, LMP1, and PD-L1 in clinical samples. The results showed that the transcript levels of Lgals1, LMP1 and PD-L1 were significantly higher in nasopharyngeal cancer tissue samples (17 cases) than in normal nasopharyngeal epithelial tissue samples (15 cases) (Figure 3B).

Subsequently, We analyzed the expression correlation between Lgals1, IRF-1 and PD-L1 using the TCGA database data. The results showed that the expression levels of Lgals1, IRF-1 and PD-L1 showed a positive correlation between each other (Figure 3C).

We also performed immunohistochemical assays for LMP1, Lgals1, IRF-1 and PD-L1 on tissue sections from patients with nasopharyngeal carcinoma. The results showed that the expressions of Lgals1, IRF-1 and PD-L1 were lower in the low LMP1 expression group. Conversely, the expression of Lgals1, IRF-1 and PD-L1 were also higher in the high LMP1 expression group, indicating a significant positive expression correlation between them (Figure 3D).
We analyzed the relationship between Lgals1 or PD-L1 and the prognosis of patients using the TCGA database data. We extended our analysis to patients with squamous carcinoma of the head and neck. The data of 499 head and neck squamous carcinoma patients in the TCGA database were analyzed, and the results showed that the overall survival rate of patients with low Lgals1 or PD-L1 expression was significantly higher than that of patients with high Lgals1 or PD-L1 expression (Figure 3E).

3.4 OTX008 inhibits the immune escape of NPC cells

To further confirm that Lgals1 can inhibit the immune escape of NPC cells by suppressing PD-L1 expression, we performed the T-cell killing assays. We sorted CD8+ T cells by magnetic beads (Figure 4A), and performed induction and differentiation of the T cells, which were then co-cultured with NPC cells TW03 under different treatments with different ratios of effector cells (E) and target cells (T). The results showed that T cells were more capable of killing OTX008-treated LMP1 overexpressing TW03 cells than the control group (Figure 4B-C).

To clarify the reasons for the above changes in T-cell killing assays, we detected indicators reflecting T cell killing ability and T cell activity in the supernatant of co-cultured cells, such as perforin, granulysin (GZMB), IFN-γ and IL-2 by ELISA kits, to detect whether the killing ability of T cells was changed. The results showed that in the supernatants of T cells co-cultured with OTX008-treated LMP1 overexpressing TW03, all of the above indicators significantly elevated compared to the control group (Figure 4D). These results indicated that the killing ability and activity of T cells co-cultured with OTX008-treated LMP1 overexpressing TW03 were enhanced significantly compared with the control group. We also examined the secretion of Lgals1 in the cell supernatant and found that there was no significant change in the secretion of Lgals1 after OTX008 treatment (Figure 4D).

Meanwhile, we examined changes in T cell surface CD69, one of the earliest markers upregulated after T cell activation, by flow cytometry after co-culture. The results showed that the expression of CD69 on the surface of T cells co-cultured with OTX008-treated LMP1 overexpressing TW03 cells was significantly higher compared to the control group (Figure 4E), indicating that T cell activity was higher after co-culture with OTX008-treated LMP1 overexpressing TW03 cells compared to the control group.

In summary, we propose a mechanistic model for the regulation of PD-L1 expression after LMP1 binding to Lgals1 (Figure 5). LMP1 can interact with Lgals1, and this complex can influence PD-L1 expression by regulating IRF-1 through NF-κB signal pathway in NPC cells. OTX008, a targeted inhibitor of Lgals1, can regulate the immune escape process of nasopharyngeal carcinoma cells. Our findings suggest that Lgals1 may serve as an important target for inhibiting immune escape in nasopharyngeal carcinoma, to lay solid foundations for translational medicine research.

4. Discussion

Recurrence and metastasis of nasopharyngeal carcinoma is closely related to immune escape[21]. Previous study have shown that LMP1 can promote the immune escape of nasopharyngeal carcinoma
cells by controlling expression of PD-L1[15, 22, 23]. In this study, we further investigated the mechanism behind the regulation, and try to find key members involved in this process, and it may will be as a therapeutic target against immune escape.

At first, we screened the transcription factor which LMP1 can induce, to find the transcription factor regulates PD-L1, and further proved LMP1 can elevated the expression of IRF-1 in nasopharyngeal carcinoma cells. We also found the transcription factor activity of E2F was elevated by LMP1. The E2F family plays a crucial role in the control of cell cycle, and can mediate both cell proliferation and TP53/p53-dependent apoptosis[24-26]. How E2F involved in the regulation of PD-L1 by LMP1 needs to be further investigated.

We further confirmed the presence of the LMP1/Lgals1 complex, and demonstrated that LMP1/Lgals1 can regulate IRF-1 through the NF-κB signaling pathway. We also confirmed the involvement of Lgals1 in the immune escape of nasopharyngeal carcinoma cells by T-cell killing assay, ELISA and flow cytometry. Lgals1, also known as Galectin-1, is a member of the galactoside-binding protein family, which has immunosuppressive function and it is abundantly expressed and inhibits maternal rejection of the fetus[17, 27]. Some investigators have outlined the role of the Galectin family in head and neck tumors, including proliferation, invasion, apoptosis, angiogenesis, and immune escape[28-30]. The currently reported association of Galectin-1 with immune escape focuses on its apoptotic effects on activated T cells, they found tumor-secreted Galectin-1 mediates immune evasion by preventing T cell migration into the tumor[31]. In our study, we focused on the changes of Lgals1 on PD-L1 expression in tumor cells. We also examined the secretion of Lgals1 in cell supernatants in Figure 4D, and found no significant changes, suggesting that the inhibition of immune escape we obtained by OTX008 is still mainly due to changes in PD-L1 expression by inhibiting Lgals1 in tumor cells. It is possible that Lgals1 has effects on both tumor cells and T cells, which provides more solid evidence for its potential as a drug target.

We had attempted to construct Lgals1 knockout cell lines by using the Crispr-Cas9 system. Unfortunately, we only successfully screened Lgals1 knockout monoclonal in TW03, and no changes of PD-L1 expression level were observed in these Lgals1 knockout monoclonal cell lines (Data not shown). We speculate that there may be other Lgals family members to compensate for the function of Lgals1 during the screening of stable knockout cell lines, and there may be functional redundancy. However, when we performed transient stimulation with the Lgals1-targeted inhibitor OTX008, this functional compensation came too late to be implemented, hence the phenomenon we observed.

We also tried to find the binding sites of LMP1 and Lgals1 to lay a theoretical foundation for finding inhibitors that alter their interaction. However, we finally failed to identify the binding site. We will further investigate it in the future, with the aim of finding the inhibitors of breaking their interactions, thus affecting the regulation of PD-L1 by the LMP1/Lgals1 complex more specifically.

Our findings suggest that Lgals1 serve as an important target for inhibiting immune escape in nasopharyngeal carcinoma, which may provide a good basis for future clinical translation.
5. Conclusion

In summary, we showed here that LMP1 can interact with Lgals1, and this complex can influence PD-L1 expression by regulating IRF-1 through NF-κB signal pathway in NPC cells, and inhibition of Lgals1 improves immunogenicity of NPC cells.

Abbreviations

E2F, E2F transcription factor; EBV, epstein-barr virus; ELISA, enzyme-linked immuno sorbent assay); EV, empty vector; FFPE, formalin-fixed paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRF-1, interferon regulatory factor-1; Lgals1, lectin, galactoside-binding, soluble, 1; LMP1, latent membrane protein 1; NF-κB, nuclear factor Of kappa light polypeptide gene enhancer in B-cells 3; NPC, nasopharyngeal carcinoma; OE, overexpression; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; TCGA, the cancer genome atlas.

Declarations

Declarations of interest: none

Ethical Approval and Consent to participate

This study received approval from Xiamen university ethics committees (XMULAC20170063).

Consent for publication

This manuscript has been read and approved by all the authors for publication and has not been submitted and is not under consideration for publication elsewhere.

Availability of supporting data

Supplemental Table 1. Primers in This Study.

Supplemental Table 2. General information and clinical characteristics of patients.

Competing interests

Authors declare no competing financial interest.

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Authors' contributions

Qin Lin, Qicong Luo, Yixing Chen and Zhiqin Li were involved in the experimental design; Qin Lin, Yixing Chen and Fang Xie wrote the manuscript; Yixing Chen and Fang Xie conducted the experiments; Dayu Lin, Jianfang Zhuo and Jianhao Chen helped to conduct the experiments and statistical analysis. All authors read and approved the final manuscript.

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References


Figure 1

LMP1 promotes the expression of PD-L1 by regulating IRF-1.

(A) Screening of downstream signaling pathways for LMP1 activation in NP69 empty vector (EV) and NP69 LMP1-overexpression (OE) stable cell lines, the transcription factor activities of NF-κB, IRF-1 and E2F were stimulated by LMP1. (B) The protein expression level of NF-κB, p-NF-κB, IRF-1 and PD-L1 were elevated by LMP1 in NP69 and TW03 (detected by western blot). (C) The RNA expression level of LMP1, Lgals1, IRF-1 and PD-L1 showed a positive correlation (detected by qRT-PCR).
**Figure 2**

LMP1/Lgals1 influence PD-L1 expression by regulating IRF-1 in NPC cells.

(A) Interaction of LMP1 and Lgals1 detected using Anti-LMP1 by a co-immunoprecipitation assay in NP69 empty vector(EV) and NP69 LMP1-overexpression(OE) stable cell lines. (B) Interaction of Lgals1 and LMP1 detected using Anti-Lgals1 in NP69 LMP1-overexpression(OE) stable cell lines. (C) The protein expression levels of Lgals1 were reduced treated with Lgals1 inhibitor OTX008 (10 uM and 20 uM) in TW03. (D) The protein expression level of Lgals1, P-NF-κB, NF-κB, IRF-1 and PD-L1 (detected by western blot) under different concentrations of OTX008 and BAY 11-7085 in LMP1 overexpression(OE) TW03. (E) The protein expression level of PD-L1 on the surface of LMP1 overexpression(OE) TW03 were decreased
by treating with Lgals1 inhibitor OTX008 (detected by flow cytometry). (F) The RNA expression level of IRF-1 and PD-L1 were down-regulated under knockdown of Lgals1 in LMP1 overexpression (OE) TW03 (detected by qRT-PCR). (G) The protein expression level of IRF-1 and PD-L1 were down-regulated under knockdown of Lgals1 in LMP1 overexpression (OE) TW03 (detected by western blot).

Figure 3

Lgals1 may play an important function in squamous carcinoma of the head and neck.
(A) The expression level of LMP1, Lgals1, IRF-1 and PD-L1 in nasopharyngeal carcinoma tissues (Tumor) were significantly higher than normal nasopharynx tissues (Normal) (from GEO database GSE61218). (B) The significant expression differences of Lgals1, LMP1 and PD-L1 (detected by qRT-PCR) between nasopharyngeal carcinoma tissues and normal nasopharynx tissues in our clinical samples. (C) The expression levels of Lgals1, IRF-1 and PD-L1 (from Head-Neck Squamous Cell Carcinoma (HNSC) patients in TCGA database) showed a positive correlation. (D) The expression levels of LMP1, Lgals1, IRF-1 and PD-L1 in tissue sections of patients with nasopharyngeal carcinoma by immunohistochemistry. (E) Compared with those of low Lgals1 or PD-L1, Lgals1 or PD-L1 high-expressing HNSC patients (from TCGA database) had significantly reduced overall survival.
**Figure 4**

**OTX008 causes enhanced killing ability of T cells and inhibits the immune escape of NPC cells.**

(A) Sorting of T cells by magnetic beads. (B) T-cell killing assay. The ratios of effector cells (E) and target cells (T) were 1:8, 1:16 and 1:32. (C) Statistics of cytotoxicity of T cell killing assay, the cytotoxicities were elevated by OTX008 treatment. (D) The concentration of IFN-γ, IL-2, granulysin(GZMB), perforin and Lgals1 in the supernatant of co-culture cells (detected by ELISA assays). (E) The protein expression level of CD69 on the surface of T cells were elevated by co-culturing with LMP1 overexpression(OE) TW03 which treated with Lgals1 inhibitor OTX008.

**Figure 5**

**Model of the role of LMP1 binding to Lgals1 in regulating PD-L1 expression**

**Supplementary Files**

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