SPLUNC1 regulates LPS-induced progression of nasopharyngeal carcinoma and proliferation of myeloid-derived suppressor cells

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Research Article

Keywords: NPC, tumor microenvironment, SPLUNC1, CXCL-2/CXCR-2

Posted Date: July 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1822446/v1
Abstract

Background: Nasopharyngeal carcinoma (NPC) is one of the aggressive malignant tumors with high mortality, and the proliferation of myeloid-derived suppressor cells (MDSCs) could promote the metastasis of NPC through inhibiting the function of T cells. Meanwhile, SPLUNC1 was known to inhibit the malignant behavior of NPC cells, while the detailed function of SPLUNC1 in LPS-modified immune microenvironment of NPC remains unclear.

Methods: To assess the impact of SPLUNC1 in immune microenvironment during the progression of NPC, NPC cells were exposed to LPS and then co-cultured with MDSCs for 48 h. RT-qPCR and western blot were performed to evaluate the mRNA and protein levels, respectively. The levels of cytokines were tested by ELISA. Meanwhile, the expression of CD33+ was tested by flow cytometry.

Results: The expressions of CXCL-2 and CXCR-2 in NPC cells were higher, compared with those in NP69 cells. In contrast, SPLUNC1 level in NPC cells was much lower than that in NP69 cells. SPLUNC1 level was negatively correlated with CXCL-2 and CXCR-2. Overexpression of SPLUNC1 reversed LPS-induced inflammatory responses and proliferation in NPC cells. In addition, SPLUNC1 upregulation could reverse LPS-induced proliferation of MDSCs in tumor microenvironment. Meanwhile, SPLUNC1 overexpression could regulate CXCL-2/CXCR-2 axis.

Conclusion: SPLUNC1 regulates LPS-induced progression of nasopharyngeal carcinoma and proliferation of MDSCs. Thus, our study might provide a theoretical basis for discovering new strategies against NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is confirmed to be the frequent craniofacial malignancy with biological characteristics [1]. It has been often diagnosed at the advanced stage, and this made the high mortality among the patients with NPC [2, 3]. Nowadays, the major treatments of NPC contain radiotherapy, chemotherapy, surgery and other comprehensive clinical treatments, while the outcomes remain not ideal [4]. In addition, tumor metastasis is known to cause the poor prognosis of patients with NPC [5, 6]. Thus, it is essential to discover the novel strategies for developing the therapy against NPC.

Short palate lung and nasal epithelial clone 1 (SPLUNC1) is a multi-functional protein which is involved in innate defense [7]. In addition, it is reported to be highly abundant in human body [8]. SPLUNC1 contains can specifically bind to LPS on the cell wall of Gram-negative bacteria [9]. In addition, it has been reported that SPLUNC1 was highly expressed in normal nasopharyngeal tissues, and it could inhibit the tumorigenesis of NPC [10]. On the other hand, Immune regulation in cancer is a research hotspot in recent years. Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells with suppressor function. MDSC massively expands and activates in NPC, inhibits the function of killer T cells, and promotes tumor invasion and metastasis [11]. Meanwhile, SPLUNC1 exerted the protective effect against LPS-induced acute lung injury, and SPLUNC1 was found to inhibit the recruitment of MDSCs to the spleen [12].
However, the detailed function of SPLUNC1 in LPS-induced MDSCs during the progression of NPC remains unexplored.

Alternative signaling through CXCR2 and its ligands CXCL2/CXCL8 is a critical part of glioblastoma angiogenesis [13]. CXCR2 overexpression can promote EMT in NPC cells by activating ERK/GSK-3β/Snail signaling [14]. CXCL2/MIF-CXCR2 signaling promotes the recruitment of myeloid-derived suppressor cells and correlates with bladder cancer prognosis [15]. Increased neutrophil infiltration in Splnc1 KO mice was accompanied by elevated levels of chemokines [16, 17]. Therefore, we speculate that CXCL-20/CXCR-2 pathway might be involved in the differentiation of MDSCs. Accordingly, it could be hypothesized that SPLUNC1 regulates MDSC differentiation via CXCL-2/CXCR-2 in the LPS-induced immune microenvironment of NPC.

Based on these backgrounds, we sought to explore the function of SPLUNC1 in LPS-induced immune microenvironment of NPC. We hope this research would provide new theoretical basis for discovering new methods against NPC.

**Material And Methods**

**Cell culture**

The human NPC cell lines (5-8F) was maintained in our lab, which used in our previous study [10]. Human nasopharyngeal epithelial cells (NP69) was bought from BLUEFBIO (Shanghai, China). All cells were maintained in DMEM (Gibco, USA) containing FBS (10%, HyClone, USA) and streptomycin/penicillin (1%) at 37°C and 5% CO2.

**MDSC differentiation assay**

CD33+ cells were separated from healthy peripheral blood mononuclear cells (PBMCs) using human CD33 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) [18, 17]. Isolated CD33+ cells were co-cultured with NPC cells in 24-well plates using a transwell system (0.4-mm pore, Corning) at a ratio of 1:5 for 48 h [17]. CD33+ cells cultured in medium alone were evaluated as a control. A panel of collected cells was used to analyze MDSC marker expression via flow cytometry (CD33+ and CD11b). The study was conducted with the informed consent of all participants and approved by Hunan Cancer Hospital.

**Cell transfection**

NPC cells were transfected with pcDNA3.1 (oe-NC), pcDNA3.1-SPLUNC1 (oe-SPLUNC1) or pcDNA3.1-CXCL-2 (oe-CXCL-2) for 48 h applying Lipofectamine 2000 (Invitrogen) in line with the protocol of manufacturer.

**RT-qPCR**
TRIzol (TaKaRa, Tokyo, Japan) was applied to extract the total RNA in line with the manufacturer's protocol. PrimeScript Kit (CWBio, Beijing, China) or miRNA cDNA Synthesis Kit (1st Strand, CWBio) was applied to synthesize the cDNA in accordance with the manufacturer's protocol. ABI7500 system was applied in RT-qPCR analysis using SYBR Green. RT-qPCR was done in three times as the following protocol: 94°C for 2 min, followed by 35 cycles (30 s at 94°C and 45 s at 55°C). The primers originated from GenePharma. \(2^{-\Delta \Delta CT}\) was applied for data quantification as previously described [19]. The primers originated from GenePharma. SPLUNC1: F, 5'-GTCCTGCTGAGTTGGTTCA-3' and R 5'-GCAAGAAATAGTTCAAGCTCAGCA-3'. CXCL2: F, 5'-TGTTGACGGCAGGAAATGTA-3' and R 5'-TGCTCTAAGACAGAGGGAAACA-3'. CXCR2: F, 5'-GCTGAGAATATGCAGCTTCA-3' and R 5'-CCTCAAGGTTGGTGTAGTC-3'. GAPDH: F, 5'-ACAGCCTCAAGATCATCAGC-3' and R 5'-GGTCATGAGTCCTTTCCAGAT-3'.

CCK8 assay

NPC cells (5×10^3) were seeded overnight. LPS was bought from Sigma (cat no. 297-473-0, St.Lious, MA, USA). Afterwards, cells were exposed to LPS, LPS + oe-NC (cells were transfected with oe-NC and then treated with 1 μg/mL LPS), LPS + oe-SPLUNC1 (cells were transfected with oe-SPLUNC1 and then treated with 1 μg/mL LPS), LPS + oe-CXCL-2 (cells were transfected with oe-CXCL-2 and then treated with 1 μg/mL LPS), LPS + oe-SPLUNC1 + oe-NC (cells were co-transfected with oe-NC and oe-SPLUNC1, and then treated with 1 μg/mL LPS) or LPS + oe-SPLUNC1 + oe-CXCL-2 (cells were co-transfected with oe-CXCL-2 and oe-SPLUNC1, and then treated with 1 μg/mL LPS) for 48 h. CCK-8 (Dojindo, 10 μl) was added to the cells for 2 h at 37°C. Finally, microplate reader (Bio-Tek) was applied for assessing the absorbance (450 nm).

Enzyme linked immunosorbent assay (ELISA)

The concentrations of IL-1β (cat.no KE00021, Proteintech, USA), IL-6 (cat.no KE00139, Proteintech, USA), TNF-α (cat.no KE00068, Proteintech, USA) in supernatants of NPC cells were detected by ELISA kits.

In addition, the concentrations of Arg-1 (cat. no ab230930, Abcam, USA), iNOS (cat. no ab253217, Abcam, USA) and PD-L1 (cat. no ab214565, Abcam, USA) in NPC cell supernatants were detected by ELISA kits (Proteintech, USA) according to the manufacturer's protocol.

The levels of CXCL2 (cat. no MBS455320) and CXCR2 (cat. no MBS2516149) in supernatants of NPC cells were detected by ELISA kits in line with the manufacturer’s protocol.

Western blotting

RIPA (Beyotime) was applied to extract the total protein. BCA kit (Beyotime) was applied for protein quantification. SDS-PAGE (10%) was applied to separate the protein (40 μg per lane). Subsequently, proteins were removed to PVDF membranes (Invitrogen). Primary antibodies were applied to incubate the membranes overnight: anti-SPLUNC1 (proteintech; 10413-1-AP, 1:200, USA), anti-CXCL-2 (Abcam;
ab275879, 1:1000), anti-CXCR-2 (proteintech; 20634-1-AP, 1:1000) and anti-β-actin (proteintech; 66009-1-lg, 1:5000) after 5% skim milk was applied to block the membranes for 1 h. After that, secondary antibodies (HRP-conjugated, proteintech; SA00001-1, 1:5000) were applied to incubate the membranes for 1 h. ECL kit (Thermo Fisher Scientific) was applied to analyze the protein bands. β-actin was applied for data quantification.

Statistical analysis

Three independent experiments were applied in each group, and mean ± standard deviation (SD) was applied to express the data. Meanwhile, spearman correlation analysis was performed to test the correlation between SPLUNC1 and CXCL-2/CXCR-2. One-way analysis of variance (ANOVA) followed by Tukey’s test (more than 2 groups, Graphpad Prism 7) or Student’s t-test (only 2 groups) was applied to analyze the changes. P<0.05 suggested a significant difference.

Results

SPLUNC1 was downregulated in NPC

To investigate the role of SPLUNC1, CXCL-2 and CXCR-2 in NPC, RT-qPCR and western blot were performed. As indicated in Figure 1A and 1B, the levels of CXCL-2 and CXCR-2 in NPC cells were significantly higher, compared with those in NP69 cells. In contrast, the expression of SPLUNC1 in NPC cells was much lower than that in NP69 cells (Figure 1A and 1B). In addition, the expression of SPLUNC1 was negatively correlated with CXCL-2 and CXCR-2 (Figure 1C and 1D). To sum up, SPLUNC1 was downregulated in NPC.

Overexpression of SPLUNC1 reversed LPS-induced proliferation and inflammatory responses in NPC

To investigate the function of SPLUNC1 in NPC, NPC cells were transfected with pcDNA3.1-SPLUNC1. As shown in Figure 2A, the expression of SPLUNC1 in NPC cells was notably upregulated by pcDNA3.1-SPLUNC1. LPS greatly increased the proliferation of NPC cells, while this phenomenon was partially reversed by SPLUNC1 upregulation (Figure 2B). Consistently, the levels of pro-inflammatory cytokines in NPC cell supernatants were notably upregulated by LPS, which were significantly restored by pcDNA3.1-SPLUNC1 (Figure 2C). Furthermore, LPS significantly decreased the level of SPLUNC1 and upregulated the expressions of CXCL-2 and CXCR-2 in NPC cells, while the effect of LPS on these proteins was greatly inhibited in the presence of pcDNA3.1-SPLUNC1 (Figure 2D and 2E). Taken together, overexpression of SPLUNC1 reversed LPS-induced proliferation and inflammatory responses in NPC.

Overexpression of SPLUNC1 alleviated LPS-induced proliferation of MDSCs in tumor microenvironment of NPC

In order to investigate the function of SPLUNC1 in tumor microenvironment of NPC, CD33+ cells were isolated from PBMCs, and then co-cultured with NPC cells. As shown in Figure 3A, the distribution of
MDSCs was significantly increased when co-cultured with NPCs, and this phenomenon was further aggravated by LPS. However, the effect of LPS was greatly restored by overexpression of SPLUNC1 (Figure 3A). In addition, the concentrations of PD-L1, Arg-1, iNOS, CXCL-2 and CXCR-2 in supernatants of MDSCs were significantly increased when co-cultured with NPC cells, and this phenomenon was further enhanced by LPS (Figure 3B). Furthermore, SPLUNC1 upregulation partially abolished the effect of LPS (Figure 3B). All these results suggested that overexpression of SPLUNC1 alleviated LPS-induced proliferation of MDSCs in tumor microenvironment of NPC.

**Overexpression of CXCL-2/CXCR-2 aggravated LPS-induced inflammatory responses in NPC cells**

To investigate the relation between SPLUNC1 and CXCL-2 in NPC, NPC cells were transfected with pcDNA3.1-CXCL-2, and then western blot was used. The data revealed that pcDNA3.1-CXCL-2 significantly upregulated the level of SPLUNC1 in NPC cells (Figure 4A). In addition, CXCL-2 upregulation notably increased the proliferation of NPC cells (Figure 4B). Consistently, the concentrations of IL-6, IL-1β and TNF-α in NPC cell supernatants were greatly increased by pcDNA3.1-CXCL-2 (Figure 4C). The mRNA and protein levels of CXCL-2 and CXCR-2 in NPC cells were significantly upregulated by pcDNA3.1-CXCL-2 (Figure 4D and 4E). Taken together, overexpression of CXCL-2/CXCR-2 aggravated LPS-induced inflammatory responses in NPC cells.

**Overexpression of SPLUNC1 might reverse LPS-induced proliferation and inflammatory responses in NPC cells through mediation of CXCL-2/CXCR-2 axis**

To further investigate the mechanism by which SPLUNC1 regulates LPS-induced proliferation and inflammatory responses in NPC cells, CCK8 assay was performed. As indicated in Figure 5A, the proliferation of LPS-treated NPC cells was significantly inhibited by overexpression of SPLUNC1, which was partially rescued in the presence of pcDNA3.1-CXCL-2. Additionally, the concentrations of IL-1β, IL-6 and TNF-α in supernatants of LPS-treated NPC cells were notably inhibited by overexpression of SPLUNC1, while these phenomena were markedly abolished by CXCL-2 upregulation (Figure 5B). Meanwhile, pcDNA3.1-SPLUNC1 significantly increased the level of SPLUNC1 and inhibited the expressions of CXCL-2 and CXCR-2 in LPS-treated NPC cells, while the effect of pcDNA3.1-SPLUNC1 was attenuated by CXCL-2 upregulation (Figure 5C and 5D). To sum up, overexpression of SPLUNC1 might reverse LPS-induced proliferation and inflammatory responses in NPC cells through mediation of CXCL-2/CXCR-2 axis.

**Overexpression of SPLUNC1 might alleviate LPS-induced MDSC proliferation in tumor microenvironment of NPC cells through mediation of CXCL-2/CXCR-2 axis**

Finally, to further confirm the relation between SPLUNC1 and CXCL-2/CXCR-2 axis in tumor microenvironment of NPC cells, flow cytometry was performed. As shown in Figure 6A, the distribution of MDSCs in CD33+ cells co-cultured with LPS-treated NPC cells was significantly inhibited by SPLUNC1 overexpression, while this phenomenon was partially abolished by CXCL-2 overexpression. In addition, the concentrations of Arg-1, PD-L1, iNOS, CXCL-2 and CXCR-2 in CD33+ cells co-cultured with LPS-treated...
NPC cells were notably inhibited by overexpression of SPLUNC1, which were rescued in the presence of pcDNA3.1-CXCL-2 (Figure 6B). In summary, overexpression of SPLUNC1 might alleviate LPS-induced MDSC proliferation in tumor microenvironment of NPC cells through mediation of CXCL-2/CXCR-2 axis.

**Discussion**

It has been reported that SPLUNC1 was involved in NPC tumorigenesis, and it was confirmed to play key roles in innate immune [20, 21]. In addition, it has been indicated that SPLUNC1 could inhibit the inflammatory responses caused by LPS [22, 23]. In this research, we found SPLUNC1 was downregulated in NPC, and its overexpression could restore LPS-induced proliferation and inflammatory responses in NPC cells. Thus, our data were consistent to these previous reports, suggesting that SPLUNC1 could act as an inhibitor in NPC cell growth.

CXCL-2 and CXCR-2 were confirmed to be the key mediators in inflammatory responses [24]. In addition, CXCL-2 and CXCR-2 were reported to be involved in the tumor microenvironment of multiple cancers [25, 26]. In this study, we found CXCL-2/CXCR-2 upregulation could reverse SPLUNC1-mediated inhibition of inflammatory responses during the progression of NPC. In addition, CXCL-2 overexpression could restore the effect of SPLUNC1 overexpression on the distribution of MDSCs. Thus, it could be suggested that SPLUNC1 overexpression could reverse LPS-modified tumor microenvironment of NPC through regulation of CXCL-2/CXCR-2 axis. Meanwhile, Ou et al found that SPLUNC1 reduces the inflammatory response of nasopharyngeal carcinoma cells infected with the EB virus by inhibiting the TLR9/NF-κB pathway [27], and our study was similar to this previous study. TLR9/NF-κB pathway has been confirmed to induce the inflammatory responses caused by LPS [28, 29]. Thus, the similar function between CXCL-2/CXCR-2 and TLR9/NF-κB pathway might contribute to the similarity between our research and Qu et al.

iNOS were known to be the key mediator in inflammation and tumor microenvironment [30, 31]. In addition, iNOS was confirmed to be the downstream protein of TLR9/NF-κB pathway [32]. On the other hand, Arg-1 could play a vital role in inflammatory responses and immune tolerance [33, 26]. PD-L1 was the key target for immune therapy in cancer [34–37]. Hence, our study revealed that SPLUNC1 overexpression could reverse LPS-modified tumor microenvironment in NPC through regulation of PD-L1, Arg-1 and iNOS.

Indeed, some shortcomings exist in this work: 1) the downstream targets of SPLUNC1 in NPC remain unexplored; 2) the mechanism by which SPLUNC1 regulates PD-L1 remains largely unknown. Therefore, more investigations are essential in coming future.

To sum up, SPLUNC1 regulates LPS-modified immune microenvironment in NPC through regulation of myeloid-derived suppressor cells. Thus, this study might shed novel insights on discovering new targets against NPC.

**Declarations**
Author contributions

Hui Wang put forward to the hypothesis and revised the manuscript. Pan Chen wrote this manuscript. Ling Tang, Ling Peng and Huai Liu performed the part of experiment. Data analysis in the study was conducted by Tengfei Xiao, Wangning Gu and Hongmin Yang. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 82173201), the Natural Science Foundation of Hunan Province (No. 2021JJ30422), and Hunan Cancer Hospital Climb Plan (2020NSFC-A001). The authors received no financial support for the research, authorship, or publication of this article.

Conflict of interests

These authors declared no competing interests in this research.

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Figures
Figure 1

**SPLUNC1 was downregulated in NPC.** (A) The mRNA levels of SPLUNC1, CXCL-2 and CXCR-2 in NPC and NP69 cells were detected by RT-qPCR. (B) The protein levels of SPLUNC1, CXCL-2 and CXCR-2 in NPC and NP69 cells were detected by western blot. (C) The correlation between SPLUNC1 and CXCL-2 was analyzed by spearman correlation analysis. (D) The correlation between SPLUNC1 and CXCR-2 was analyzed by spearman correlation analysis. *P<0.05 compared to NP69.

Figure 2

**Overexpression of SPLUNC1 reversed LPS-induced proliferation and inflammatory responses in NPC.** (A) NPC cells were transfected with oe-NC or oe-SPLUNC1. The protein level of SPLUNC1 in NPC cells was tested by western blot. NPC cells were treated with LPS, LPS + oe-NC (cells were transfected with oe-NC and then treated with 1 μg/mL LPS) or LPS + oe-SPLUNC1 (cells were transfected with oe-SPLUNC1 and...
then treated with 1 μg/mL LPS). (B) The proliferation of NPC cells was tested by CCK8 assay. (C) The concentrations of IL-1β, IL-6 and TNF-α in supernatants of NPC cells were assessed by ELISA. (D) The mRNA expressions of SPLUNC1, CXCL-2 and CXCR-2 in NPC cells were tested by RT-qPCR. (E) The protein expressions of SPLUNC1, CXCL-2 and CXCR-2 in NPC cells were tested by western blot. *P<0.05 compared to control or oe-NC. #P<0.05 compared to LPS.

**Figure 3**

**Overexpression of SPLUNC1 alleviated LPS-induced proliferation of MDSCs in tumor microenvironment of NPC.** Cells were divided into five groups: CD33+, CD33+ + 5-8F (5-8F cells were co-cultured with CD33+ cells), CD33+ + 5-8F + LPS (5-8F cells were exposed to 1 μg/mL LPS for 48 h, and then co-cultured with CD33+ cells), CD33+ + 5-8F + LPS + oe-NC (5-8F cells were transfected with oe-NC and then treated with 1 μg LPS for 48 h. Then, 5-8F cells were co-cultured with CD33+ cells), and CD33+ + 5-8F + LPS + oe-SPLUNC1 (5-8F cells were transfected with oe-SPLUNC1 and then treated with 1 μg LPS for 48 h. Then, 5-8F cells were co-cultured with CD33+ cells). (A) The distribution of MDSCs was tested by flow cytometry. (B) The concentrations of PD-L1, Arg-1, CXCL-2, CXCR-2 and iNOS in supernatants of NPC cells were tested by ELISA. *P<0.05 compared to CD33+. #P<0.05 compared to CD33+ + 5-8F. &P<0.05 compared to CD33+ + 5-8F + LPS.
Figure 4

Overexpression of CXCL-2/CXCR-2 aggravated LPS-induced inflammatory responses in NPC cells. (A) NPC cells were transfected with oe-NC or oe-CXCL-2. The protein level of SPLUNC1 in NPC cells was tested by western blot. *P<0.05 compared to oe-NC. NPC cells were treated with LPS + oe-NC or LPS + oe-CXCL-2 (cells were transfected with oe-CXCL-2 and then treated with 1 μg/mL LPS). (B) The proliferation of NPC cells was assessed by CCK8 assay. (C) The concentrations of IL-1β, IL-6 and TNF-α in supernatants of NPC cells were assessed by ELISA. (D, E) The expressions of SPLUNC1, CXCL-2 and CXCR-2 in NPC cells were tested by RT-qPCR and western blot. *P<0.05 compared to oe-NC + LPS.

Figure 5

Overexpression of SPLUNC1 might reverse LPS-induced proliferation and inflammatory responses in NPC cells through mediation of CXCL-2/CXCR-2 axis. NPC cells were treated with LPS + oe-NC, LPS + oe-SPLUNC1, LPS + oe-SPLUNC1 + oe-NC (cells were co-transfected with oe-SPLUNC1 and oe-NC, and then treated with 1 μg/mL LPS) or LPS + oe-SPLUNC1 + oe-CXCL-2 (cells were co-transfected with oe-
SPLUNC1 and oe-CXCL-2, and then treated with 1 μg/mL LPS). (A) The proliferation of NPC cells was evaluated by CCK8 assay. (B) The concentrations of IL-1β, IL-6 and TNF-α in supernatants of NPC cells were assessed by ELISA. (C, D) The expressions of SPLUNC1, CXCL-2 and CXCR-2 in NPC cells were tested by RT-qPCR and western blot. *P<0.05 compared to oe-NC + LPS. #P<0.05 compared to LPS + oe-SPLUNC1.

Figure 6

Overexpression of SPLUNC1 might alleviate LPS-induced MDSC proliferation in tumor microenvironment of NPC cells through mediation of CXCL-2/CXCR-2 axis. Cells were divided into five groups: CD33+ 5-8F, CD33+ + 5-8F + LPS, CD33+ + 5-8F + LPS + oe-NC and CD33+ + 5-8F + LPS + oe-SPLUNC1. (A) The distribution of MDSCs was tested by flow cytometry. (B) The concentrations of PD-L1, Arg-1, CXCL-2, CXCR-2 and iNOS in supernatants of NPC cells were tested by ELISA. *P<0.05 compared to CD33+ + 5-8F + LPS + oe-NC. #P<0.05 compared to CD33+ + 5-8F + LPS + oe-SPLUNC1.