Pumilio RNA binding family member 1 deficiency activates anti-tumor immunity in hepatocellular carcinoma via restraining M2 macrophage polarization

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**Abstract**

Pumilio RNA binding family member 1 (PUM1), which has been implicated in both the progression of colorectal cancer and the regulation of inflammation, has not yet been associated with hepatocellular carcinoma. PUM1 is essential for the transition of tumor-associated macrophages (TAMs) into the M2 polarization state. It does this by inhibiting anti-tumor immunity in hepatocellular carcinoma through a process mediated by TAMs that target CD8+ T cells, as demonstrated in this study using PUM1-knockout mice. By activating the cAMP signaling pathway, we have shown that PUM1 promotes the transformation of TAMs into pro-tumorigenic M2-like phenotypes. In order to emphasize the potential of PUM1 as an objective for immunotherapy centered on TAMs in the treatment of gastric carcinoma, the present investigation revealed the molecular mechanism underlying the pro-tumor role of PUM1 in this cancer.

**Background**

The incidence and mortality of Hepatocellular carcinoma (HCC) rank fifth and third, respectively [1]. The majority of patients are identified at later stages, which generally results in an overall poor prognosis of the carcinoma. Moreover, inflammation, multiple molecular occurrences, and several cellular signaling pathways were all linked to HCC advancement, apart from Hepatitis C virus and Hepatitis B virus infection [2]. Drug resistance, tumor recurrence, and metastasis significantly restrict the effectiveness of HCC treatments despite the availability of numerous treatment options, including targeted therapy and immunotherapeutic medications [3].

The tumor microenvironment (TME) has recently become recognized as having an essential part in the growth of tumors and could be a potential target for cancer therapy [4]. In the TME, tumor-associated macrophages (TAMs) are widely distributed and are regarded as crucial immunosuppressive cellular components [5]. The activation of TAMs is a complicated process that manifests itself in a variety of phenotypes during the progression of tumors [6]. Typically, early-stage tumor-initiating macrophages have an M1 phenotype and engage in anti-tumor actions [7], while the pro-tumorigenic TAMs receive instructions from the tumor cells to develop an immunosuppressive state and encourage the growth of the malignancy by either secreting a variety of anti-inflammatory chemicals or by blocking the cytotoxic activity of CD8+ T cells, thus evading the immune surveillance [8]. TAMs have anti-tumorigenic and pro-tumorigenic plasticity, due to which they are regarded as a potential target for therapeutic intervention against malignancies [9]. Several preclinical investigations have shown that preventing macrophage recruitment or modifying their characteristic phenotype to restricted M2 type might prevent tumor growth and enhance therapeutic response [10–14]. The identification of novel compounds capable of interfering with macrophages may provide novel targets for TAM-centered immunotherapy against cancer, and by possible reversal of the M2 state of TAMs in the tumors may thus be viewed as a potential therapeutic method.

Eukaryotic RNA binding proteins (RBPs) are highly conserved in nature. The PUF family, including the FBF and Pumilio, binds to the sequence regions post-transcriptionally, which are responsible for repressing
gene expression. By binding targets specifically on the mRNAs at the 3'UTR region, the Pumilio-binding elements (PBEs) control cell differentiation and fate determination [15–17]. Recent genetic research on the genes that encode the RNA-binding proteins known as Pumilio-1 (Pum1) and Pumilio-2 (Pum2), members of the mammalian PUF family, have shown the functions of post-transcriptionally controlling growth, reproduction, and development of the eukaryotic brain in addition to malignant tumors progression [18–20]. An earlier study revealed that PUM1 is overexpressed in colon cancer cells that have developed Cetuximab resistance and is associated with TAM [21–22]. However, the mechanism of action of PUM1 in HCC is still largely unknown.

In the current study, we sought to understand better the processes by which PUM1 promotes the development of HCC by demonstrating that PUM1 inhibits anti-tumor immunity in HCC and is necessary for the M2 polarization of TAMs using PUM1-knockout mice. Our findings offer further proof showing the function of PUM1 in the tumor microenvironment in HCC.

**Material and methods**

**Mice and Tumor Model**

On a C57BL/6J background, wild-type (WT) and PUM1 knockout (PUM1-/-) mice were developed and acquired from Service Bio-Co., Ltd (Wuhan, China). The Experimental Animal Ethics Committee of Service Bio Co., Ltd (Wuhan, China) authorized the animal research and oversaw their execution according to guidelines. Female WT and PUM1-/- mice (6–8 weeks old) received subcutaneous injections of MHCC97H cells into the right flanks for subcutaneous tumor models. Tumor tissues were removed after the experiment was completed to do further examination. For the current study, we used bone marrow-derived macrophages (BMDMs). The C57BL/6J mice lost macrophages after being intraperitoneally injected with 200 mL of clodronate liposomes (YEASEN, Shanghai, China) 48 hours before the inoculations. Then, a combination of MHCC97H cells and macrophages obtained from WT or PUM1/mice was administered subcutaneously into the macrophage-depleted C57BL/6J mice. After measuring the diameter of the tumor, mice were executed according to protocol, and lymphocytes that entered the tumor were examined, as reported in prior work [23].

**Cell Lines Used and Preparation of Tumor-Conditioned Medium**

The Hep3B, HepG2, HCCLM3, Huh7, and MHCC-97H were the HCC cell lines under study. Moreover, the U937 and THP-1 were the two human monocyte cell lines used in the current work. All cell lines were taken and maintained following proper guidelines from the American Type Culture Collection (Maryland, USA). By treating both cell lines, human U937 cells and THP-1, to PMA (phorbol-12-myristate-13-acetate; 200 nmol/L, Sigma Aldrich, Taufkirchen, Germany) for 48 hours, the cells were transformed into mature macrophages. A complete growth medium was used to culture cancer cells, following its subsequent replacement by a serum-free medium when cells reached about 80% confluency. A fresh batch of
conditioned medium (CM) was obtained after 24 hours, centrifuged for 5 minutes at 500g, filtered through 0.22 µm filters, and then stored at 80°C.

Extraction and Stimulation of Mouse Primary Cells

As previously described, cells obtained from the bone marrow of female WT and PUM1-/- mice were cultured for 7 days in Iscove’s modified Dulbecco’s medium (Gibco, Grand Island, NY, USA) with FBS (concentration 10%) and M-CSF (50 ng/mL; PeproTech, NJ, USA) to obtain BMDMs [23]. BMDMs were cultured for 48 hours with IL4 / IL13 (20 ng/mL, PeproTech) to induce M2 macrophage differentiation. To obtain TAMs, a half volume of tumor CM was mixed with the culture medium being used, and the mixture was then allowed to incubate for a period of 48 hours.

In vitro phagocytosis assays

For this assay, several phenotypes of macrophages were added into 96-well low-attachment plates, and these plates were then co-cultured with Carboxyfluorescein succinimidyl ester (CFSE)-labeled MHCC-97H cells. All cells were collected after two hours, stained with F4/80 antibody, a murine macrophage surface marker, for 30 minutes on ice, and then subjected to flow cytometric analysis. The proportion of F4/ 80+ cells with fluorescence from CFSE was used to calculate the phagocytosis efficiency.

CD8 + T cell suppression assay

To achieve successful isolation of CD8 + T cells of the mouse from C57BL/ 6J mice, a CD8 + T cell isolation kit specific for the mouse was used that was manufactured by Stem Cell Technologies (Vancouver, Canada). After being stimulated with aCD3/ aCD28 and IL-2 (PeproTech) and co-cultured with various phenotypes of macrophages for a time period of 72 hours, CD8 + T cells supplemented with the eBioscience™ Cell Stimulation Cocktail (Invitrogen, Carlsbad, CA, USA) were incubated for 4 hours. The cells were then collected and subjected to flow cytometry analysis to determine whether they proliferated and expressed functional markers.

Statistical analysis

Statistical analysis was done by analysis of variance (ANOVA) or Student’s t-tests among the experimental groups. Whereas a $p < 0.05$ was statistically significant in all experiments. Statistical Package for the Social Sciences (SPSS) v17.0 (IBM, USA) and GraphPad Software (La Jolla, CA, USA) were used for all statistical analyses.

Results

PUM1 deficient mice reduced the MHCC-97H tumor growth and polarized TAMs to M2-like phenotype

Subcutaneous injection of MHCC-97H cells in WT and PUM1 knockout mice was simultaneously done to explore the involvement of PUM1 in the tumor microenvironment. PCR testing verified that the mice had
the expected genotype, and the PUM1 was in considerably low concentration in the primary BMDMs of PUM1-/- mice (Fig.S1A). We observed that the development of MHCC-97H tumors in PUM1-/- mice were noticeably slower than that of the WT group (Fig. 1A and B). The immune cell infiltration in the MHCC-97H tumors was investigated afterward. Tumors from PUM1-/- mice demonstrated reduced TAMs infiltration upon comparison to the tumors of WT mice. Despite this, there was no discernible variation in the frequency of all hematopoietic cells such as CD3e, CD11b, dendritic cells (DC), CD4 + T, and CD8 + T cells (Fig. 1C and D). More investigation showed that PUM1 deficiency suppressed CD206 + TAM cells (Fig. 1E) but increased the ratio of M1 to M2 macrophages and the frequency of IFN-γ + CD8 + T cells upon comparison to MHCC-97H-WT animal (Fig. 1F and G), suggesting that PUM1-/- mice had an attenuated M2-like immunosuppressive phenotype of TAMs. Although the difference was non-significant, we showed that the PUM1-/- mice had a lower ratio of monocytes in the blood (Fig.S1B). Additionally, PUM1-/- mice displayed lower blood serum levels of IL-10 (Fig.S1C), suggesting that the controlled phenotype change of TAMs to an M2-like state may help to prevent cancer formation in PUM1-/- animals.

**PUM1 deficient macrophages are less polarized toward M2-like phenotypes**

The effects of PUM1 deficiency on macrophages were then verified in vitro. Firstly, BMDMs were isolated and invigorated with IL-4 and IL-13 from PUM1-/- mice (PUM1-/-/BMDMs) or WT mice (WT-BMDMs). PUM1-/- BMDMs had lower levels of ARG-1 and IL10, two typical M2 markers, compared to WT-BMDMs. In line with the current finding, the fraction of macrophages (CD206 + M2) was lower in PUM1-/-/BMDMs (Fig. 2A and B). These findings suggest that PUM1 is necessary for polarization events in macrophages. For a better representation of the influence of PUM1 in the TME, WT-BMDMs were cultivated with CM isolated from the MHCC-97H cell culture (MHCC-97H-CM), which caused an upregulation of PUM1 in TAMs (Fig. 2C). The use of CM that was obtained from multiple human tumor cell cultures produced results that were comparable (Fig. 2D). In addition, we examined the differences in the state (phenotype) of BMDMs derived from wild-type and PUM1-/- animal. In comparison to their WT mouse counterparts, PUM1-/- BMDMs cultivated with MHCC-97H-CM showed enhanced gene expression, which was related to M1 type of macrophages (Fig. 2E) and reduced the expression of those genes involved in M2 type macrophages (Fig. 2F). Additional evidence suggested that PUM1-/- macrophages had less M2 polarization which was shown by flow cytometric analysis, and resulted in enhanced expression of CD86 and a decreased expression of CD206 (Fig. 2G and H).

**Deficiency of PUM1 increases phagocytosis and decreases tumor-promoting activity**

Next, we evaluated how PUM1 could impact macrophages’ ability to phagocytose tumor cells. WT- or PUM1-/- BMDMs were co-cultured with CFSE-labeled MHCC-97H cells, respectively. Upon comparison to the WT mice, M2 macrophage from PUM1-/- mice showed enhanced phagocytosis of MHCC-97H cells (Fig. 3A). Similar outcomes were also seen in BMDMs treated with MHCC-97H-CM.
MHCC-97HCM, PUM1-/- BMDMs demonstrated increased macrophage phagocytosis than the WT-BMDMs. (Fig. 3B). Next, we explored how PUM1 affected tumor cell proliferation and migration with the assistance of macrophages. The CM boosted the capacity of MHCC-97H cell proliferation from WT macrophages. However, it was inhibited by CM from PUM1-/- macrophages upon comparison to WT-BMDMs. (Fig. 3C and D).

**PUM1 deficiency limits the TAM-mediated inhibition of CD8 + T cells**

TAMs have a suppressive effect on T-cell proliferation and the anti-tumor activity that accompanies it. Thus, we examined the immune modulatory effects of PUM1 in phagocytes by co-culturing M2 or CM-treated macrophages with CD8 + T cells. Co-culturing CD8 + T cells with M2 macrophages derived from PUM1-/- mice resulted in an increase in the ratio of activated CD8 + T cells to the total number of CD8 + T cells, as was proven by our research. This hypothesis was backed even further by the increase in CD8 + T cells that were positive for granzyme B, IFN-γ, or TNF alpha in comparison to the WT controls (Fig. 4A-C). CD8 + T cells with tumor CM-treated macrophages generated from PUM1-/- mice yielded similar findings. Following the co-culture technique with CM-treated PUM1-/- phagocytes, an apparent elevation of classic cytotoxic cytokines in CD8 + T cells were seen (Fig. 4D-F). These findings highlight PUM1’s critical involvement in generating immunosuppressive TAM.

**Activation of cAMP signaling by PUM1 induces macrophage M2 polarization**

After that, we assessed the amount of cAMP produced and found that it increased in WT-BMDMs treated with IL4 / IL13 or MHCC-97H-CM but reduced in PUM1-/- BMDMs (Fig. 5A and B). In PUM1-/- macrophages, forskolin also significantly increased the quantity of IL-10 and ARG-1 (Fig. 5C–F). This data indicates PUM1 stimulating cAMP signaling to cause macrophage M2 polarization.

**PUM1-/- BMDM inhibits MHCC-97H tumor growth and enhances antitumor immunity**

Further, we wanted to evaluate whether PUM1-/- BMDMs will reconfigure TME and demonstrate anti-tumor effects *in vivo*. To examine this, MHCC-97H cells and PUM1-/- or WT BMDMs were combined before being implanted into mice with pre-depletion macrophages (C57BL/6J). PUM1-/- macrophage-containing MHCC-97H xenografts showed slower tumor development than those combined with WT macrophages (Fig. 6A and B). After this, the impact of PUM1-/- macrophages on the turnover of TAM and the infiltration of different immune cell groups, specifically CD8 + T cells in TME, was examined. The frequency of CD45 + cells (which are the hematopoietic cells) did not change, according to an analysis of immune cells infiltrating tumors. In the PUM1-/- macrophage co-cultured MHCC-97H group, TAMs tended to decline, although this trend did not achieve statistical significance (Fig. 6C and D). Contrarily, the
number of CD86 + M1 type of macrophages rose, whereas the infiltration of CD206 + M2 type of macrophages significantly decreased (Fig. 6E and F). These findings imply that PUM1 transformed TAM into "anti-tumor" M1-like phenotypes from M2-like phenotypes which are known to cause tumor progression. The phenotypic change of TAMs was accompanied by tumors containing PUM1-/- macrophages displaying noticeably increased MHC class II on DC (Fig. 6G) and some of IFN-g + CD4 + T cells (Fig. 6H), showing increased activation of DC and responses was Th1-dominant. Additionally, the PUM1-/- macrophage group had enlarged granzyme B + CD8 + T cells (Fig. 6I) and IFN-γ + CD8 + T cells (Fig. 6J), with a decreased number of PD-1 + CD8 + T cells as shown in Fig. 6K. The results showed that PUM1-/- BMDM modified the immunosuppressive TME to improve anti-tumor immunity in the TME.

**Discussion**

A significant portion of the TME comprises TAMs, which exhibit substantial plasticity in both function and phenotype in response to a wide variety of stimuli. Established tumors with TAMs are typically biased in the direction of the pro-tumorigenic M2 characters, encouraging tumor growth with reducing immunological anti-tumor response. Their presence tends to result in a poor prognosis in most malignancies [24]. TAMs have thus been regarded as a crucial aim for immunotherapy against cancers. In this study, we should identify an essential regulatory function of PUM1 regarding TAMs in the development of HCC, where PUM1 is critical for inducing an immunosuppressive phenotype in macrophages and blocking an anti-tumor immune response in TME. As a result, we conclude that PUM1 is a promising target for TAMs that aim to treat HCC using anti-tumor immunotherapy.

Our findings suggest that PUM1 was necessary for both the usual M2 polarization of macrophages and the induction of an immunosuppressive phenotype in macrophages in the tumor microenvironment (TME). Furthermore, PUM1 inhibition or knockdown in macrophages increased the number of the pro-inflammatory subtype of macrophages. This work explored PUM1’s role in remodeling TAMs and the subsequent suppression of immunity in HCC. When compared to MHCC-97H-WT mice, our research discovered that PUM1-/- animals had reduced levels of CD206 + TAM and TAM, with higher levels of IFN-γ + CD8 + T. These findings suggest that PUM1 had an immunosuppressive influence on the microenvironment of the developing tumor in addition to inhibiting the development of cancer. Additionally, our *in vitro* data showed that PUM1 encouraged TAMs to polarize into a pro-tumoral state. PUM1-/- macrophage inhibited tumor growth and improved immunity against cancer *in vivo*, as demonstrated by a sharp decline in the M2-like TAMs during tumor infiltration and an increase in anti-tumor lymphoid cell populations such as CD4+ effector T cells, mature DC, and CD8+ T cells. The immunological microenvironment of a carcinoma influences how various immunotherapeutic approaches will work [25]. Reprogramming immune cells and boosting anti-tumor immunity while overcoming treatment resistance caused by the microenvironment may be possible with the use of immunosuppressive TAMs and other similar agents. [26–29]. PUM1 might be a promising target for improving immunotherapies.
Various extracellular stimuli stimulate the second messenger cAMP through GPCRs [30–36]. Furthermore, cAMP and IL-4 work together synergistically to increase the expression of STAT6, and they may play the role of co-factors in reprogramming the macrophages. We further verified in the current study that cAMP contributed to the differentiation of TAMs in the TME via PUM1. Additionally, in PUM1-deficient macrophages, inducing cAMP synthesis restored M2-like behaviors. Our findings suggest that deficiency of PUM1 in TAMs prevents polarization of macrophages toward pro-tumor phenotypes with M2-like characteristics by reducing cAMP signaling.

**Conclusions**

Our findings demonstrate the significant regulatory function of PUM1 in TAMs. It enhances the immunosuppressive phenotype of macrophages, blocks the response of anti-tumor immunity, and also speeds up the growth of tumor in HCC. Because of this, PUM1 is a prospective pharmacological target for anti-tumor immunotherapy in HCC with a focus on TAM, and it may help in breaking the resistance against immune checkpoint blockade (ICB) therapies.

**Declarations**

**Conflict of Interest**

The study had no possible conflicts of interest, according to the authors.

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**Author contributions**

Liu Ouyang and Chen-ming Ni designed the research. All of the authors carried out the experiments, performed data analysis, wrote the manuscript. All of the authors revised the manuscript. All of the authors have read and approved the final manuscript.

**References**


Figures

**Figure 1**

PUM1 deficiency in mice inhibits the formation of MHCC-97H tumors and initiates the transformation of TAMs to M2-like character. $2 \times 10^6$ (n=3) MHCC-97H cells were subcutaneously implanted into 8 weeks old female WT (n=3; triplicate) and PUM1 knockout (PUM1-/-) mice (n=3). The experimental mice were executed after 21 days. (A) Growth curve obtained for each tumor. (B) Weights were taken of each tumor.
Flow cytometry examination of the various immune subsets present in the tumor. Data showing infiltration of (C) CD11b+, DC, CD3e, CD4+ T cells, and CD8+ T cells and (D) TAMs (CD11b+ F4/80+) were examined by flow cytometry via fluorescently labeled antibodies. (E) M2-like TAMs (CD11b+ F4/80+ CD206+), (F) M1 (CD11b+ F4/80+ CD86+)/M2 ratio, and (G) IFN-γ+ CD8+ T cells were also checked using flow cytometer. The obtained data have been reported as the mean accompanied by the standard deviation (SD) (n=3). Here, the p-values (* p < 0.05) were calculated via a two-tailed Student’s t-test. #non-significant result upon comparison to the control (WT) group.
Deficiency of PUM1 in the macrophages resistant to M2 polarization (A) BMDM taken from WT and PUM1 -/- mice were subjected to either treatment with or not treated with IL4 / IL13 (6 hours). We performed qPCR of ARG-1 and IL-10 to measure their expression. (B) For 48 hours, we performed the culturing of BMDMs with IL4 / IL13. Flow cytometry made it feasible to determine the percentage as well as the mean fluorescence intensity of F4/80+ CD206+ M2 phagocytes. (C) The expression of PUM1 in BMDMs cultivated with CM from MHCC-97H cells for a duration of 48 hours (Western blot shown in left panel) and 6 hours (qPCR shown in the right panel). (D) Western blot (48 hours) and qPCR (6 hours) of the expression of PUM1 in U937 macrophages in CM from HCC cells. WT and PUM1/BMDMs were co-cultured with MHCC-97H-CM for 6 hours; qPCR was used to detect the expression of genes associated with (E) inflammation or (F) macrophage with immunosuppressive polarization state, with β-actin as a control. Flow cytometry (48 hours) was used to evaluate the expression of (G) CD86 and (H) CD206. All of our generated data are represented as mean±SD (n=3). As previously mentioned, we used a two-tailed Student’s t-test to calculate statistical significance (* p < 0.05), upon comparison to the WT group.

Figure 3

Deficiency of PUM1 enhanced the phagocytic property of macrophages and lowered its tumor-progressing properties. 1×10⁵ BMDMs were taken (WT and PUM1-/- mice) and invigorated with IL-4 / IL-13 or MHCC-97H in CM for a time period of 48 hours, followed by co-culturing them. 2×10⁵ CFSE-fluorescently labeled MHCC-97H was used for additional 2 hours. Data of phagocytosis is represented as
(A) M2- macrophages or (B) CM- macrophages and determined using the flow cytometry technique. (C) and (D) Showed the action of PUM1 on the proliferation and migration of tumor cells mediated by macrophages. BMDMs (PUM1-/- and WT) were exposed for 48 hours with IL-4 / IL-13 or MHCC-97H-CM, and macrophage-specified CM was harvested. MHCC-97H cells was used to cultured either (C) M2- CM or (D) TAM- CM. Two-tailed Student's t-test was employed to determine p-values. *p < 0.05, upon comparison to the WT group.

Figure 4

Deficiency of PUM1 restricts the suppression of TAM-mediated CD8+ T cell. PUM1-deficient macrophages recovered co-cultured CD8+ T cell activities. The number of macrophages seeded was 1×10^5 and taken from either wild-type and PUM1-/- animals and invigorated with (A-C) IL-4/IL-13 or (D)-(F) MHCC-97H-CM given for a duration of 48 hours, and then CD8+ T cell (number of cells = 5-10×10^5) were co-cultured for 72 hours. The method of flow cytometry was utilized to analyze the three most prevalent cytotoxic...
cytokines found in CD8+ T cells, the IFN-γ, TNF-α, and Granzyme B (GzmB). The obtained data have been reported as the mean accompanied by the SD (n=3). The two-tailed Student’s t-test was used to analyze the data's statistical significance (*p < 0.05), and comparison was made with the WT group.

Figure 5

Activation of the cAMP signaling pathway by PUM1 is responsible for the M2 polarization of macrophages. (A&B) IL4 / IL13 (shown in the left panel) or MHCC-97H-CM (shown in the right panel) stimulated WT and PUM1-/-BMDMs for a duration of 2 hours, and cAMP levels were checked. (C-F) Forskolin pretreated was done on WT and PUM1/ macrophages, and next treatment with IL4 / IL13 was done for a duration of 6 hours. IL-10 and ARG-1 expression was determined. Data generated in the above assays are represented as mean ± SD (n=3). The statistically significant findings (p-value) were determined with the use of an analysis of variance (ANOVA). * p < 0.05.
Figure 6

**PUM1/ BMDM inhibits MHCC-97H tumor growth and enhances anti-tumor immunity.**

Subcutaneous injections of MHCC-97H cells (2×10^6 cells) were given to C57BL/6J mice that had their macrophage levels previously decreased and were added with WT or PUM1-/- phagocytes (5×10^5 cells). (A) The volume of the tumor and (B) weight of the tumor are presented at indicated days. (C)-(K) Analysis
of immune subsets in the TME by flow cytometry. (C) CD45+ cell infiltration amid living cells. The observed infiltration data of (D) TAMs (CD11b+F4/80+), (E) CD86+TAMs, (F) CD206+ TAMs, (G) MHC on DC, (H) IFN-γ+CD4+T cells, (I) IFN-γ+CD8+T cells, (J) GzmB + CD8+T cells, and (K) PD1+ CD8+T cells were evaluated. The preceding data is provided as a mean along with an SD (n=3). The p-values were computed using a two-tailed version of the student's t-test. *p < 0.05, # non-significant result, upon comparison with the WT-BMDMs group.

**Supplementary Files**

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