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Hypoxia enhances IL-10-producing B cell generation through upregulating high-mobility group B1 on tumor cell-released autophagosomes

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ABSTRACT

As common features of human solid tumors, hypoxia and nutrient starvation play multifaceted roles in cancer progress. However, the mechanisms are far from clear. Our previous work has indicated that tumor cell-released autophagosomes (TRAPs) are sufficient to suppress anti-tumor immune response in mouse by inducing IL-10-producing B cells through high-mobility group B1 (HMGB1). Here, we hypothesized that hypoxia or starvation might exert immunosuppressive effect through upregulating HMGB1 on TRAPs. We found that HMGB1 on TRAPs from human hepatocellular carcinoma cell line HepG2 played a significant role in IL-10-producing B cell induction. HMGB1 in tumor cells was upregulated under hypoxia and starvation, but only hypoxia significantly enhanced the level of HMGB1 present on the surfaces of TRAPs. Moreover, hypoxic TRAPs induced more IL-10-producing B cells with suppressive activities on $CD4^+$ and $CD8^+$ T cells. The finding indicates the role of TRAPs as a messenger of hypoxic response to enhance immunosuppression in tumor microenvironment.

1. Introduction

Hypoxia and nutrient starvation are common features of solid tumors, which play critical roles in tumor progress, invasion, metastasis, and therapeutic resistance. Various pathways underlying these processes have been revealed. For instance, Starvation or hypoxia-induced autophagy can help tumor cell survival and promote cancer growth in some types of advanced cancers [1–3]. Hypoxia has been reported to induce the expression of arrays of genes associated with cell survival, proliferation, metabolism, apoptosis, stemness, angiogenesis, and epithelial-to-mesenchymal transition (EMT) in hypoxia-inducible factor 1 (HIF-1)-dependent or HIF-1 independent manners [4–7].

Damage-associated molecular pattern molecules (DAMPs) such as high-mobility group B1 (HMGB1), heat shock proteins, S100 are released in response to stress and cell death. In the center of solid tumors, hypoxia and starvation, as the most pervasive microenvironmental stresses, are sufficient to trigger DAMPs release [8]. The roles of these DAMPs in cancers are controversial [9]. On the one hand, DAMPs may exert protective functions by alerting the immune system to the presence of dying tumor cells [10]. On the other hand, accumulating evidence show that multiple DAMPs also exert immunosuppressive effects that ultimately promote the development or progression of tumors [11,12].

Autophagy is an evolutionarily conserved intracellular process with formation of double-membrane vesicles termed autophagosomes [13,14]. In degradative autophagy pathway, misfolded proteins or damaged organelles are sequestered in autophagosomes, and are ultimately fused with lysosomes for degradation and recycling [15]. In contrast to degradative autophagy, autophagosomes of secretory autophagy pathway could bypass lysosome fusion and be released to extracellular milieu carrying a wide range of cytoplasmic substrates, such as IL-1β and autophagic vacuoles [16,17]. In physiological scenarios, autophagy proceeds at low basal levels. While in tumor cells under stress conditions such as starvation and hypoxia, autophagy can be dramatically induced and influence malignant transformation and cancer progression. Studies over the past decade indicate that although regarded as “double-edged sword” that can either inhibit or promote growth of tumor, it is generally accepted that autophagy prevents

Abbreviations: DAMPs, damage-associated molecular pattern molecules; EMT, epithelial-to-mesenchymal transition; HIF-1, hypoxia-inducible factor 1; HMGB1, high-mobility group B1; MDSC, myeloid-derived suppressor cells; TRAPs, tumor cell-released autophagosomes

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cancer development in pre-malignant lesions, but promotes advanced cancer growth [18,19]. Though Numerous studies have focused on the intracellular recycling function of autophagy in cancer, little is known about the role of tumor cell-released autophagosomes (TRAPs).

Our previous studies have indicated the effects of TRAPs on the immunological functions of B cells [20], neutrophils [21] and macrophages [22]. TRAPs from murine tumor cells can induce IL-10 production of B cells which potently suppress T cell proliferation and antitumor response. And HMGB1, one member of DAMPs, was found to play a significant role in the IL-10-producing B cells induction [20]. Since the expression of DAMPs in tumor cells can be dramatically enhanced by hypoxia and starvation in center of tumors, whether hypoxia or starvation can influence HMGB1 present on TRAPs and subsequently modulate the immunosuppressive effect of TRAPs needs to be demonstrated.

In this study, we explored the influence of hypoxia and starvation on HMGB1 level present on TRAPs. The results showed that hypoxia upregulated HMGB1 level on TRAPs, which enhanced IL-10 production of B cells with ability to inhibit T cell proliferation. This finding uncovered the role of TRAPs as a messenger of hypoxic response to enhance immunosuppression in tumor microenvironment.

2. Materials and methods

2.1. Cell culture and treatment

Human hepatocellular carcinoma cell lines HepG2 and Huh7 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). HepG2 and Huh7 cells were cultured in DMEM (Wisent) supplemented with 10% heat-inactivated FBS (Wisent) and 1% penicillin/streptomycin (Wisent) and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

For the incubation of cells under hypoxic state, cells were incubated in an airtight chamber (Thermo), which was flushed with a gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 for 24 h at 37 °C. For starvation, cells were digested with 0.25% trypsin and incubated in Earle's Balanced Salt Solution (EBSS, Wisent) in an airtight chamber at 37 °C with 5% CO_2 for 24 h. For hypoxia/starvation condition, cells were treated the same as in starvation state but incubated at 37 °C with 1% O_2, 5% CO_2 and 94% N_2 for 24 h.

2.2. TRAPs isolation and characterization

TRAPs were harvested from human hepatocellular carcinoma cell lines as previously described [19]. Briefly, tumor cell suspension was spun at 450 g for 7 min to remove cells and debris. The supernatant was further centrifuged at 12,000 *g* for 15 min to pellet TRAPs-containing large extracellular vesicles. TRAPs were finally resuspended in PBS and total protein concentration was measured using a BCA Protein Assay Kit (Pierce) following the manufacturer's instructions. TRAPs were then stained with anti-LC3B mAb (Novus Biological, NB600-1384AF488). The purity and LC3 content were respectively verified by flow cytometry (Fig. S1A) and western blot (Fig. S1B). The exosome markers CD63 and TSG101 were also examined by Western blot as previously described [21]. No exosome markers were detected in our samples.

The morphology of TRAPs was examined by transmission electron microscopy. Samples were prepared according to routine techniques. Briefly, the samples were fixed in 100 mmol/L sodium cacodyate, 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.064% picric acid, 0.1% ruthenium red, post-fixed with osmium tetroxide plus potassium ferricyanide and embedded in Epon. Electron microscopic images were obtained from a JEM-1011 (JEOL, Tokyo, Japan) transmission electron microscope at 60 kV (Fig. S1C).

2.3. Western blot analysis

Total protein of cells or TRAPs was extracted using lysis buffer. Protein samples were heated at 100 °C for 10 min before SDS-PAGE. Equal amount of protein samples was separated by 8% or 12% SDS-PAGE gels and transferred onto PVDF membranes. After washed 5 times with TBST, the membranes were blocked with 5% skim milk in TBS for 1 h at room temperature and incubated with corresponding primary antibodies: HIF-1α (1:1000; Abcam), HMGB1 (1:1000; Sigma), LC3B (1:1000; Sigma), and β-actin (1:1000; Proteintech) overnight at 4 °C. The membranes were washed 5 times with TBST and subsequently incubated with their respective secondary antibodies (1:5000; CST) for 1 h at room temperature. Detection of bound antibodies was visualized by chemiluminescence using ECL substrate (Thermo). Quantitative analysis was performed through the software Gel-Pro Analyzer, with the bands of β-actin or LC3 as loading controls.

2.4. TRAP-induced B cell generation

PBMCs were isolated from healthy donor blood through Ficoll-Paque Plus (GE Healthcare), and then B cells were purified from PBMCs using CD19 dynabeads (Invitrogen). Purified B cells were co-cultured with TRAPs (3 μg/mL) for 72 h in complete RPMI-1640. In some experiments, B cells were pretreated with neutralizing monoclonal antibodies against TLR-2, TLR-4, or isotype control (Biolegend).

2.5. ELISA

IL-10 production in supernatant was determined using an ELISA kit according to the manufacturer's instructions (eBioscience). A Microplate Reader (Thermo) was used to measure absorbance at 450 nm.

2.6. Flow cytometry

Flow cytometry was performed as previously described [20]. Briefly, for the detection of HMGB1 on TRAPs, TRAPs were incubated with anti-HMGB1 mAb (Sigma) and then incubated with fluorescence-labeled secondary antibody. For intracellular staining, PMA (50 ng/mL; Sigma-Aldrich, P8139), ionomycin (500 ng/mL; Sigma-Aldrich, I3909), and monensin (2 mM; Abcam, ab120499) were added to the culture 5 h before flow cytometric analysis. The frequency of IL-10-producing B cells was evaluated with CD19-APC (Sigma) and IL-10-PE (Sigma). For T cell proliferation assay, TRAP-induced B cells were incubated with CFSE (5 mM; Invitrogen)-labeled PBMCs in a 96-well plate pre-coated anti-CD3 mAb (2 μg/mL; BD Pharmingen) and anti-CD28 mAb (2 μg/mL; BD Pharmingen) for 96 h. The proliferation of $CD4^+$ or $CD8^+$ T cells was evaluated with CFSE dilution.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. All data were presented as mean ± s.e.m and analyzed by one-way ANOVA. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. TRAPs from human hepatocellular carcinoma cell lines induce IL-10-producing B cells with T cell suppressive activity via HMGB1

Our previous study has showed that TRAPs from murine tumor cell lines could induce splenic B cells to differentiate into IL-10-producing regulatory B cells with suppressive activity on $CD8^+$ and $CD4^+$ T lymphocytes in an IL-10 dependent manner [20]. To confirm the influence of TRAPs from human tumor cell, purified B cells from healthy donors were incubated with TRAPs from HepG2 for 72 h. Higher

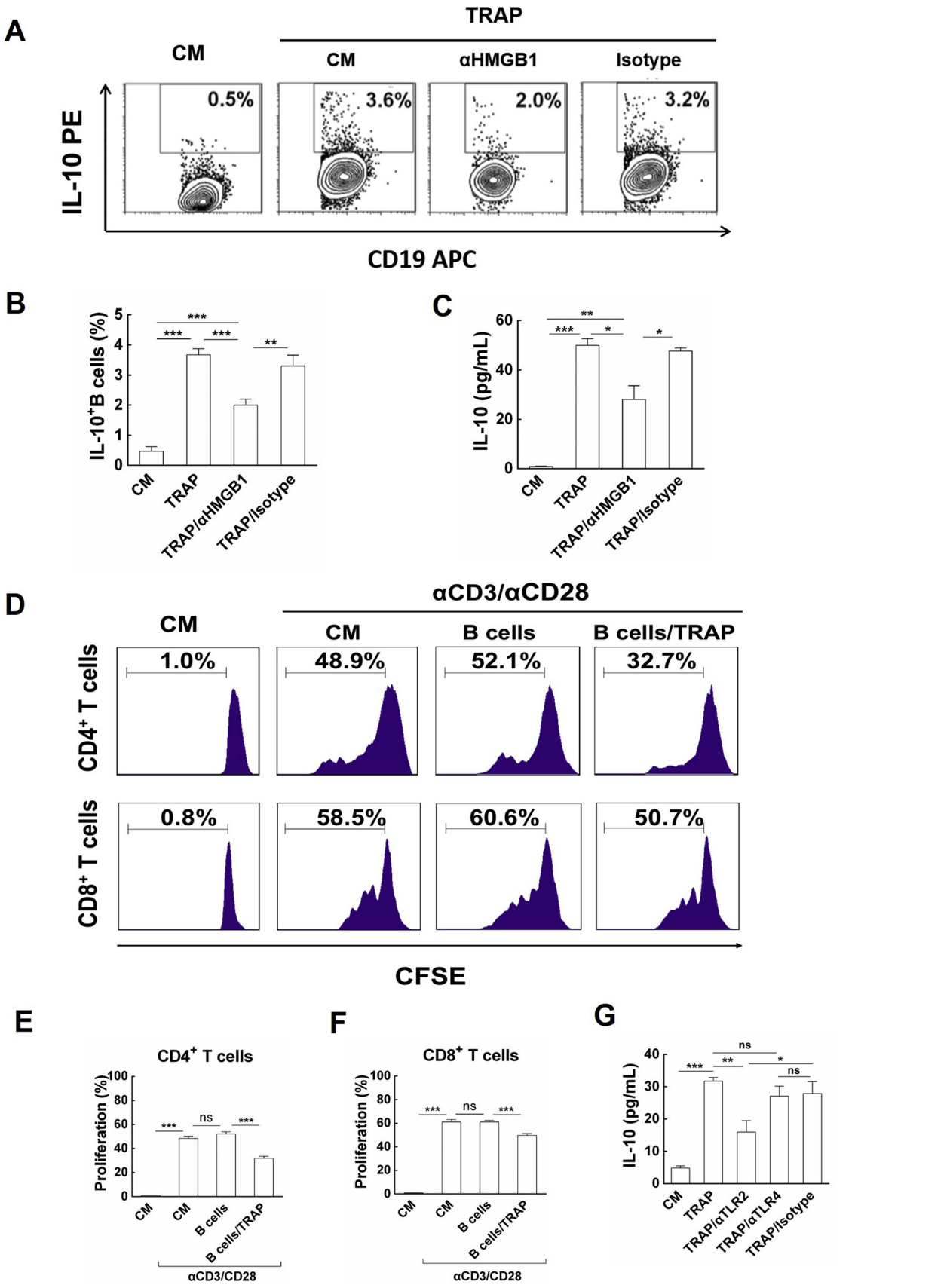

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Fig. 1. TRAPs from HepG2 cells induce IL-10-producing B cells with suppressive activity on T lymphocytes via HMGB1 on TRAPs.
Purified B cells from PBMCs of healthy donors were co-cultured with anti-HMGB1 mAb, or isotype control-treated TRAPs (3 μg/mL) for 72 h in complete RPMI-1640. The frequency of IL-10 producing B cells was evaluated by flow cytometry (A and B). The production of IL-10 by B cells was assessed by ELISA (C). PBMCs from healthy donors were labeled with CFSE and stimulated with plate-bound anti-CD3 and anti-CD28 mAb and then co-cultured with B cells or TRAP-induced B cells for 96 h. T cell proliferation was evaluated by flow cytometry (D, E, and F). Purified human B cells pre-treated with anti-TLR2, anti-TLR4, or isotype were co-cultured TRAPs (3 μg/mL) for 72 h in complete RPMI-1640. IL-10 levels in supernatants were measured by ELISA (G). Data (mean ± s.e.m) represent a typical result from three independent experiments. ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired t-test.

frequency of IL-10$^+$ B cells and elevated IL-10 production were detected in the TRAP-treated B cells when compared with the control B cells (Fig. 1A–C). To verify whether TRAP-induced B cells possessed a regulatory function on T cells, PBMCs of heathy donors were stimulated with activating anti-CD3 and anti-CD28 mAb for 96 h in the presence of TRAP-induced B cells or control B cells. The result showed that the proliferation of CD4$^+$ T and CD8$^+$ T cells was significantly suppressed in the presence of TRAP-induced B cells but not of control B cells (Fig. 1D–F).

HMGB1 on intact TRAPs has been found to be crucial in inducing IL-10 production of B cells in mouse cell model [20]. To explore the role of HMGB1 on human TRAPs, TRAPs from HepG2 cells were pre-treated with anti-HMGB1 antibody. Flow cytometric analysis and ELISA results showed that pre-treatment with anti-HMGB1 antibody attenuated the effect of TRAPs on IL-10 production of B cells (Fig. 1A–C). These results indicated that, consistent with the observations in mouse, HMGB1 from human TRAPs was critical for inducing IL-10 production of B cells. The conclusion was also confirmed in TRAPs from another human hepatocellular carcinoma cell line Huh7 (Fig. S2). We have found that the TRAP-induced IL-10 production depend on TLR2-mediated signal in mouse B cells [20]. To verify if TLR2 on human B cells played a similar role, purified human B cells pre-treated with functional anti-TLR2, anti-TLR4, or isotype control were co-cultured with HepG2-derived TRAPs. The result showed that only TLR2 blockage significantly impaired IL-10 secretion, indicating TLR2 but not TLR4 was involved in TRAP-induced IL-10 production of human B cells. Together, these results demonstrated that human tumor cell-derived TRAPs could induce regulatory B cells with T cell suppressive activity via HMGB1.

3.2. Hypoxia substantially upregulates HMGB1 expression and starvation dramatically enhances autophagy in HepG2 cells

To investigate the effect of hypoxia and starvation on HMGB1 expression and autophagy level in HepG2 cells, we cultured HepG2 cells under normoxia, hypoxia, starvation, or combination of hypoxia and starvation (hypoxia/starvation) for 24 h and collected cells to obtain cell lysate. Western blot analysis showed that HIF-1α, a recognized key mediator in hypoxia [23], was increased in hypoxia and hypoxia/starvation groups compared with in normoxia and starvation groups, indicating the reliability of the hypoxia model (Fig. 2A and B). HMGB1 protein was substantially increased under hypoxia, while slightly increased under starvation. However, LC3-II protein was significantly upregulated under starvation and slightly upregulated under hypoxia. The combination of hypoxia and starvation did not show any additive effect on the level of HMGB1 or autophagy (Fig. 2A and B). These results indicated that hypoxia substantially enhanced the expression of HMGB1, while starvation dramatically enhanced autophagy in hepG2 cells.

3.3. Hypoxia upregulates HMGB1 on TRAPs

To explore the effects of hypoxia and starvation on HMGB1 level in TRAPs, HMGB1 protein in purified TRAPs was examined by Western blot analysis with LC3-II served as internal reference. The results showed that HMGB1 proteins in TRAPs from cells under hypoxia and hypoxia/starvation were increased, and no additive effect was observed under hypoxia/starvation (Fig. 3A and B). These results demonstrated

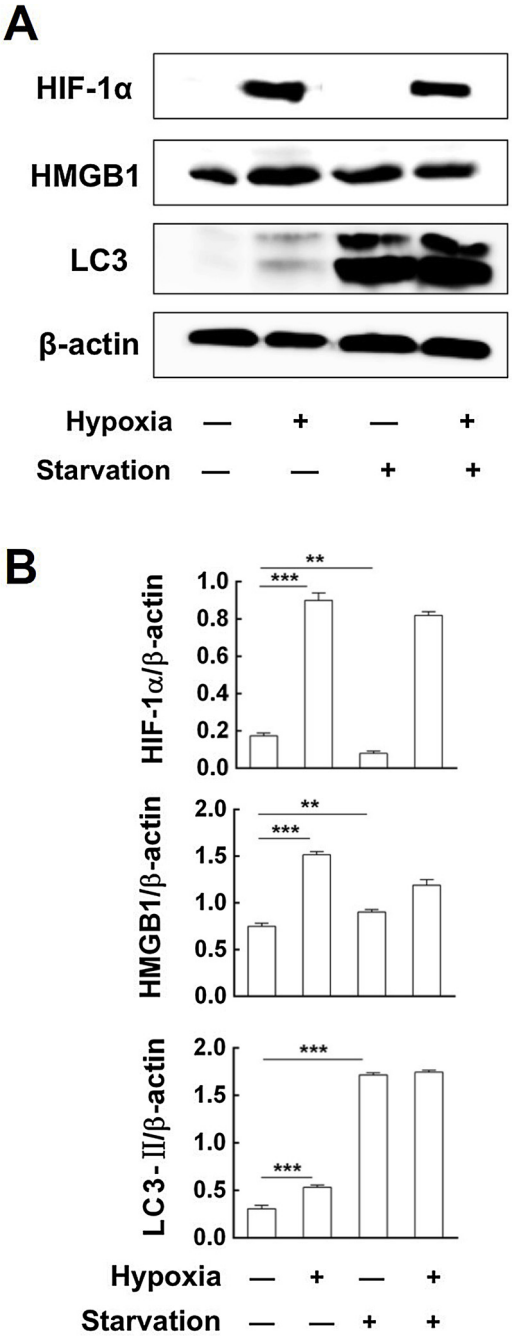

Fig. 2. Hypoxia substantially upregulates HMGB1 expression and starvation dramatically enhances autophagy level in HepG2 cells.
HepG2 cells pre-exposed to normoxia (20% O_2), hypoxia (1% O_2), starvation (EBSS) or hypoxia/starvation (1% O_2 and in EBSS) for 24 h. Cells were collected and lysed. Expression of HIF-1α, HMGB1 and LC3 under the indicated conditions were analyzed by western blot (A). Quantitative analysis of western blot with β-actin served as control (B). Data (mean ± s.e.m) are representative of three independent experiments. ns, $p > 0.05$, **$p < 0.01$, ***$p < 0.001$ by unpaired t-test.

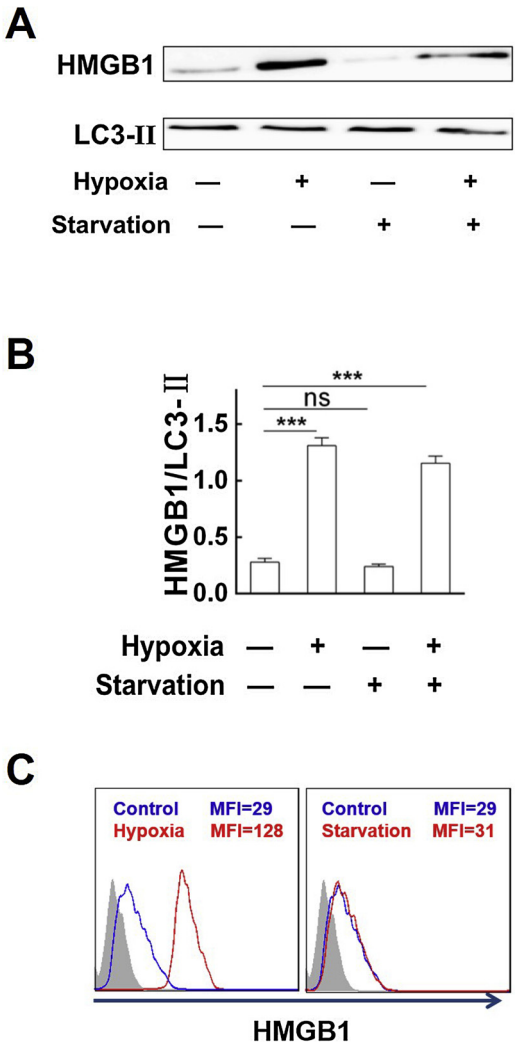

Fig. 3. Hypoxia enhances HMGB1 level on TRAPs.
HMGB1 in TRAPs under the indicated conditions was analyzed by western blot (A). Quantitative analysis of western blot with LC3-II served as control (B). HMGB1 on the surface of TRAPs under the indicated conditions was determined by flow cytometry (C). Data (mean ± s.e.m) represent a typical result from three independent experiments. ns, $p > 0.05$, ***$p < 0.001$ by unpaired t-test.

that HMGB1 protein in TRAPs was significantly upregulated under hypoxia but not starvation.

Our previous study revealed that membrane-bound HMGB1 on TRAPs played a crucial role in IL-10-producing B cell induction. To detect if HMGB1 on the surface of TRAPs was upregulated under hypoxia or starvation, flow cytometry was applied to examine HMGB1 on TRAPs from cells under different situations. The result showed that under hypoxia the median fluorescent intensity (MFI) of HMGB1 rose from 29 to 128, but no significant change was observed under starvation (Fig. 3C). This result indicated that the membrane-bound HMGB1 on TRAPs was significantly increased under hypoxia, but not under starvation.

3.4. TRAPs from cells under hypoxia induce more IL-10-producing B cells with suppressive activity on T cells

Since TRAPs from cells under hypoxia carry more membrane-bound HMGB1 which plays an important role in IL-10-producing B cells induction, we hypothesized that TRAPs from cells under hypoxia could induce more IL-10-producing B cells. TRAPs from cells under different conditions were co-cultured with purified B cells from healthy donor blood for 72 h. IL-10-producing B cells and IL-10 production in supernatant were detected. Consistent with our previous studies, TRAPs significantly induced generation of IL-10-producing B cells. Moreover, TRAPs from cells under hypoxia had an enhanced capacity to induce IL-10-producing B cells than those from cells under normoxia or starvation (Fig. 4A–C). Furthermore, these hypoxic TRAP-induced B cells inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells more potently than those induced by TRAPs from cells under normoxia or starvation (Fig. 4D and E). Together, these results demonstrated that TRAPs from cells under hypoxia could induce more IL-10-producing B cells with capacity to suppress T cell proliferation.

4. Discussion

As one of the hallmarks of human solid tumor, hypoxia is associated with malignant transformation and cancer progression, and may represent a predictor of poor clinical outcomes [6,7]. Numerous studies have revealed multifaceted roles of hypoxia, and the rapidly increasing understanding of tumor hypoxia has helped greatly in screening novel anticancer agents [24]. In this study, we examined HMGB1 level on TRAPs from tumor cells under hypoxia. The results suggested a novel explanation for the association between hypoxia and tumor progression: in the environment of hypoxia, immunosuppression could be enhanced by the increase of HMGB1 present on TRAPs.

DAMPs represent a large range of chemically unrelated mediator entities that are released following stress or cell death. As tumors grow, cancer cells are inevitably exposed to multiple kinds of stress such as hypoxia and starvation, and it has become evident that DAMPs can be dramatically induced and released in a wide range of tumors. The results of this study indicated that membrane-bound DAMPs on TRAPs could also be induced and released to the extracellular milieu in the tumor microenvironment of hypoxia. Paradoxical dual effects of HMGB1 in tumors were reported under different circumstances. During chemotherapy and radiotherapy, HMGB1 can enhance DC-mediated antigen presentation and elicit anti-tumor responses [10]. However, HMGB1 is also known to facilitate tumor progression by directly affecting tumor cell growth [25] and inducing immunosuppressive cell formation [11]. Su and colleagues demonstrated that HMGB1 secreted by cancer cells facilitate monocytic myeloid-derived suppressor cells (MDSC) differentiation from bone marrow progenitors and contribute to conversion of monocytes into MDSC-like cells [12]. Our previous study demonstrated that membrane-bound HMGB1 on intact TRAPs is critical for inducing IL-10-producing B cells which could exert inhibitory activities on T cells and ultimately impair antitumor immune response [20]. The results of this study indicated that under hypoxia, human tumor cells could release TRAPs with more membrane-bound HMGB1, which enhances the induction of IL-10-producing B cells and results in more potent inhibitory activity on T cells. These data together with previous studies suggested that HMGB1 might be a potential tumor immunotherapy target.

In the previous study we found that TRAPs from murine cells induced IL-10 producing B cell differentiation via the activation of TLR2-MyD88-NF-κB signal pathway. In this study, TLR-2 blockage on human B cells partially impaired IL-10 production, indicating that other receptors are involved in the human TRAP-induced B cell differentiation. Pre-treatment of mouse-derived TRAPs with a functional anti-HMGB1 antibody almost completely abolished IL-10 production, suggesting that HMBG1 is the major ligand on TRAPs to stimulate TLR-2-mediated IL-10 production in mouse B cells. However, the current data showed that IL-10 production in human B cells was partially attenuated by HMGB1 blockage (Figs. 1A–C, S2A, B and C), suggesting that other molecules on human TRAPs also play roles in this IL-10-producing B cell induction.

Mounting reports have demonstrated that starvation can enhance autophagy and DAMPs expression. In our system, we confirmed that under starvation, autophagy was dramatically induced and HMGB1 was

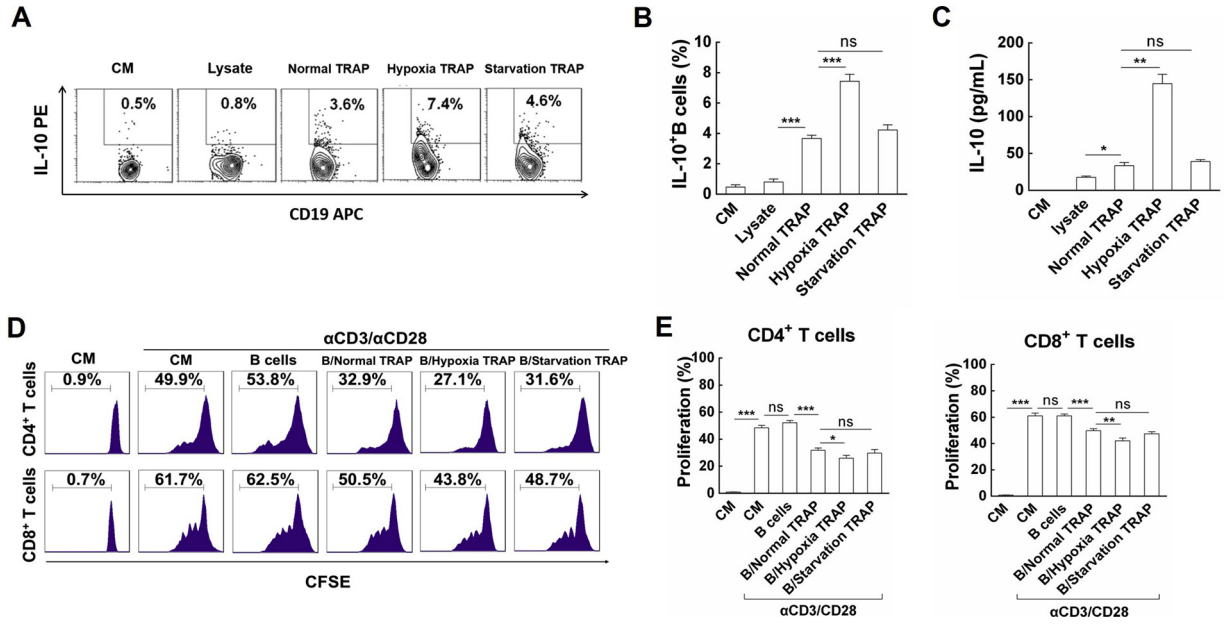

Fig. 4. TRAPs from cells under hypoxia induce more IL-10-producing B cells with T cell suppression activity.
Purified B cells were co-cultured with TRAPs from cells cultured in normoxia, hypoxia or starvation for 72 h in complete RPMI-1640. The frequency of TRAP-induced B cells was evaluated by flow cytometry (A and B). The production of IL-10 by B cells was assessed at 72 h by ELISA (C). CFSE-labeled PBMCs from healthy donors were stimulated with plate-bound anti-CD3 and anti-CD28 mAb and then co-cultured with B cells or TRAP-induced B cells for 96 h. T cell proliferation was evaluated by flow cytometry (D and E). Data (mean ± s.e.m) are representative of three independent experiments. ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired t-test.

significantly upregulated. However, the membrane-bound HMGB1 on TRAPs was not increased. Even so, starvation might still enhance immunosuppression in tumor tissues, because more TRAPs could be released due to the upregulated autophagy level.

In addition to inducing IL-10-producing B cells, our recent researches have shown that TRAPs are capable to regulate other types of immune cells and exert their immunosuppressive functions through various mechanisms. TRAPs can be rapidly and effectively phagocytized by neutrophils and promote neutrophil apoptosis which could inhibit proliferation and activation of T cells in a cell contact and ROS-dependent manner [22]. Besides, TRAPs could convert macrophages into an immunosuppressive M2-like phenotype characterized by the expression of PD-L1 and IL-10. And these TRAPs-induced M2-like macrophages are capable to suppress T cell function through PD-L1-PD-1 signaling. Furthermore, in vivo studies have demonstrated that all the above TRAPs-induced immune cells (IL-10-producing B cells, M2-like macrophages and apoptotic neutrophils) play significant roles in impairing anti-tumor T cell response and in turn enhancing tumor progression. The current study found that under hypoxia, IL-10 production of B cells could be enhanced by the increase of HMGB1 present on TRAPs. Future studies focused on neutrophils and macrophages are worthy to be carried out to explore how hypoxia and starvation influence their functions through TRAPs.

In summary, we found that HMGB1 on TRAPs was significantly upregulated under hypoxia, which enhanced the generation of IL-10-producing B cells with suppressive activity on T cells, indicating that hypoxia may contribute to the suppression of antitumor immunity by upregulating DAMPs on TRAPs.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.09.005.

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