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*J Immunol* published online 26 November 2014
http://www.jimmunol.org/content/early/2014/11/26/jimmunol.1400643

**Supplementary Material**  
[http://www.jimmunol.org/content/suppl/2014/11/26/jimmunol.1400643.DCsupplemental](http://www.jimmunol.org/content/suppl/2014/11/26/jimmunol.1400643.DCsupplemental)

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Extravascular Red Blood Cells and Hemoglobin Promote Tumor Growth and Therapeutic Resistance as Endogenous Danger Signals

Tao Yin,*† Sisi He,*† Xiaoling Liu,*,† Wei Jiang,*,† Tinghong Ye,*,† Ziqiang Lin,*,† Yaxiong Sang,* Chao Su,*† Yang Wan,*† Guobo Shen,*† Xuelei Ma,* Min Yu,† Fuchun Guo,*† Yanyang Liu,* Ling Li,* Qiancheng Hu,*† Yongsheng Wang,*† and Yuquan Wei*†

Hemorrhage is a common clinical manifestation in patients with cancer. Intratumor hemorrhage has been demonstrated to be a poor prognostic factor for cancer patients. In this study, we investigated the role of RBCs and hemoglobin (Hb) in the process of tumor progression and therapeutic response. RBCs and Hb potently promoted tumor cell proliferation and syngenic tumor growth. RBCs and Hb activated the reactive oxygen species–NF-κB pathway in both tumor cells and macrophages. RBCs and Hb also induced chemoresistance mediated, in part, by upregulating ABCB1 gene expression. Tumor growth induced by RBCs was accompanied by an inflammatory signature, increased tumor vasculature, and influx of M2 macrophages. In both the peritoneal cavity and tumor microenvironment, extravascular RBCs rapidly recruited monocyte–macrophages into the lesion sites. In addition, RBCs and Hb increased several nucleotide-binding oligomerization domain–like receptors’ expression and induced IL-1β release. Our results provide novel insights into the protumor function of RBCs and Hb as endogenous danger signals, which can promote tumor cell proliferation, macrophage recruitment, and polarization. Hemorrhage may represent a useful prognostic factor for cancer patients because of its role in tumor promotion and chemoresistance. The Journal of Immunology, 2015, 194: 000–000.
retinoic acid–inducible gene I receptors, C-type lectin receptors, and receptor for advanced glycation end products, play an important role in carcinogenesis and tumor progression (13). Moreover, those studies provide novel targets for cancer therapy. In normal physiological conditions, blood cells are circulating in the vascular systems. We hypothesize that blood exudates are danger signals for inflammation and cancer.

In our present study, we found that extravascular RBCs and hemoglobin (Hb) are endogenous danger signals that trigger inflammatory responses and promote tumor growth and resistance to chemotherapy.

Materials and Methods

Cell isolation and culture

RBCs were isolated from BALB/c and C57BL/6 mice with the density-gradient centrifugation method (Dakewe Biotech). Murine breast cancer cell line 4T1 and melanoma cell line B16 were from American Type Culture Collection. Tumor cells were cultured and propagated in the RPMI 1640 media supplemented with 10% FBS at 37˚C, 5% CO2 atmosphere. Peritoneal macrophages were harvested from BALB/c mice, washed with RPMI 1640 medium, and plated in six-well culture plates. After incubating for 3 h at 37˚C, 5% CO2 atmosphere, nonadherent cells were removed by washing three times, and the adherent macrophages were cultured in six-well plates in RPMI 1640 medium containing 10% FBS.

Western blot

After treatment with RBC lysates (RL) and Hb for 15 min, 30 min, and 24 h, 4T1 tumor cells were harvested and lysed with RIPA buffer containing protease inhibitors. Equal amounts of protein were loaded and resolved by SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane. The membranes were blotted with primary Abs against phospho–NF-κB p65 (Ser189) (Cell Signaling Technology), c-Myc (Epitomics), cyclin D1 (Cell Signaling Technology), and hemoxgenase-1 (HO-1; Abcam), respectively. Macrophages were stimulated with RL and Hb (CUSABIO Life science), and then cell lysates and supernatant were blotted with Abs against phospho–NF-κB p65 (Ser529), IL-1β (Cell Signaling Technology), and caspase-1 (Epitomics), respectively. Then the blots were incubated with HRP-linked secondary Abs. β-actin was the loading control. The blot bands were revealed with the ECL detection system.

Immunohistochemistry and immunofluorescence

Endothelial cell staining was performed by FITC-conjugated anti-CD31 Abs (BioLegend). Tumor sections were stained with 1:100 dilution of anti-F4/80 Abs (Abcam) and anti-Ki-67 Abs (Merck Millipore), followed by the incubation of secondary Ab, and the immunocomplex was visualized using the streptavidin biotin complex method. Then sections were counterstained with hematoxylin.

MTT assay and apoptosis assay

MTT assay was performed in 96-well plates to determine cell growth rate. A total of 3 × 10^4 cells/well were seeded in 96-well plates. Tumor cells were exposed to 1 × 10^5/ml, 1 × 10^4/ml, 1 × 10^3/ml, 1 × 10^2/ml, and 1 × 10^1/ml RL or 200 μg/ml Hb on the following day. After 24–72 h, the number of tumor cells was calculated. For MTT assay, MTT reagent was added to each well and incubated for another 4 h at 37˚C. Then, the formazan precipitates in the cells were dissolved in 150 μl DMSO after removal of supernatant. Absorbance was determined at 570 nm, and the growth curve was plotted. The 4T1 cell line was prestimulated with RL and Hb for 48 h and then treated with 37.5 nM paclitaxel, 6.67 μM cisplatin, and 5 μM doxorubicin for another 24 h. All of the chemotherapeutical agents were purchased from West China Hospital (Sichuan University). To evaluate the apoptosis, doxorubicin for another 24 h. All of the chemotherapeutical agents were purchased from BD Pharmingen, and CellQuest software (BD Biosciences). CD11b-PerCP–Cy5.5, CD11b-PE, Ly6C–FITC, and CD206–FITC Abs were purchased from BD Pharmingen, and F4/80–PE and F4/80–allophycocyanin Ab were from BioLegend. Intra-cellular reactive oxygen species (ROS) production upon 50 ng/ml LPS, 2 × 10^5 RL, or 200 μg/ml Hb stimulation for 10 min was evaluated by using a dichlorodihydrofluorescein diacetate (CM-H2DCFDA) fluorescence probe (Invitrogen Molecular Probes). A total of 20 μg/ml polymyxin B (Sigma-Aldrich) was used to quench LPS.

RT-PCR

Total RNA was prepared from tumor tissues and the cultured macrophages using an RNA isolation kit (Axygen) and reverse transcribed (TaKaRa) following the manufacturer’s protocols. cDNA was used to amplify ABCB1, arginase1, Tnfα, M1, Fizz1, Mgl1, iNOS, TGF-β, VEGF, IL-10, IL-6, IL-12, IL-1β, TNF-α, ERF, IFN-regulatory factor (IRF) 4, NLRP1a, NLRP1b, NLRP3, and NLRC4. Real-time PCR was performed on a CFX 96 real-time PCR thermocycler (Bio-Rad) using a kit (SYBR Premix Ex Taq; TaKaRa), with 18S RNA as a reference control. Primers used in PCR were shown in Supplemental Table I. Relative gene expression was calculated using the 2^-(ΔΔCt) method with 18S RNA as reference.

Detection of endotoxin in RL preparation

Endotoxin was measured by the tachypleus amebocyte lysate assay according to the manufacturer’s protocols (Chinese Horseshoe Crab Reagent Manufacturer). The amount of endotoxin was <0.25 endotoxin units/1 × 10^5 RL.

In vivo tumor model

BALB/c mice and C57BL/6 mice (6–8 wk old) were purchased from Beijing HFK Bioscience. TL4R−/− and MyD88−/− knockout mice were purchased from The Jackson Laboratory. All animals were maintained under pathogen-free conditions in the animal facility with individual ventilation. All animal experiments were performed according to protocols approved by Sichuan University’s Institutional Animal Care and Use Committee. A total of 1 × 10^3 4T1 cells was coinjected s.c. with 1 × 10^6 and 1 × 10^5 RBCs and an equal number of RBCs lysates into BALB/c mice. A total of 1 × 10^5 B16 cells was coadministered s.c. with 1 × 10^6 RBCs and RL into C57BL/6 mice, TL4R−/−, and MyD88−/− mice. To evaluate the protumor effect of Hb in vivo, 1 × 10^4 4T1 cells were coinjected s.c. with 15, 12, and 5 mg Hb into BALB/c mice, respectively. Tumor growth was monitored every 3 d by measuring large (a) and short (b) length with a sliding caliper. Tumor volume was calculated as a × b^2/2 × 0.52. At the end of the experiments, mice were sacrificed, and the tumors were excised and weighed. Tumor tissues were used further for flow cytometry, immunohistological stain, or RT-PCR.

Statistical analysis

Statistical significance was analyzed by Student t test when one group was compared with the control group and one-way ANOVA when compared among more than three groups. Results were expressed as the means ± SD. The p values <0.05 were considered as statistically significant.

Results

RBCs and Hb promote tumor growth

To investigate the role of RBCs in tumor growth, we coinoculated murine 4T1 breast cancer cells with RBCs or RL s.c. into the flanks of BALB/C mice. In early stages, tumor progression in both RBCs and RL groups was dramatically faster than 4T1 cells alone (Fig. 1A), though there was no obvious dose-dependent effect. On day 14 postinoculation (early to middle stages of 4T1 tumor development), tumors in the 10^3 RL/4T1 coinoculation group were significantly larger than that in control group, with tumor weights of 252 ± 16.1 and 85.7 ± 3.8 mg, respectively (Fig. 1B). To verify the generality of our finding in different experimental models,
we injected B16-F10 melanoma cells s.c. into the flanks of C57BL/6 mice. As expected, s.c. injection of B16 melanoma cells and RL resulted in rapid establishment and growth in C57 mice compared with the control group (Fig. 1C). Notably, we did not observe the tumor promotion effect of RBC and RL at the lower doses (data not shown). Hb is the main component of RBC. To investigate whether Hb promoted tumor growth, we inoculated 4T1 tumor cells with various doses of Hb s.c. Hb also robustly promoted tumor growth (Fig. 1D). These results indicate an in vivo tumor-promoting activity of RBC and Hb on tumor growth.

**RBCs and Hb influence tumor cells proliferation**

To investigate whether RBCs promote tumor cell proliferation in vitro, 4T1 breast tumor cells maintained under normal growth conditions were exposed to 1 × 10^5/10^9/mL RL for 72 h, and then their proliferation rate was determined. RL treatment caused a marked increase in the proliferation of 4T1 breast tumor cells, as revealed by cell counting (Fig. 2A) and MTT assay (Fig. 2B). However, the proliferation promotion effect of RBCs was not dose dependent. A total of 1 × 10^5 4T1 cells was coadministered s.c. with various doses of RBCs or RL (1 × 10^6 and 1 × 10^7) into the right flank of BALB/c mice. Tumor volume was measured at 14 d after inoculation (n = 8–13). (B) 4T1 tumors from day 14 were excised, imaged macroscopically, and weighed. (C) Impact of coadministration of large number of RBCs and RL (1 × 10^6) on B16 melanoma progression. On day 15, tumor size was measured (n = 7 to 8). (D) Effect of coinjection of Hb on the growth of 4T1 breast cancer. Total of 1 × 10^5 4T1 cells was coadministered s.c. with various doses of Hb (15, 12, and 5 mg) into the right flank of BALB/c mice. Tumor volume was measured at 14 d after inoculation (n = 6 to 7). *p < 0.05. **p < 0.01. Ctrl, control.

**RBCs and Hb activate ROS-NF-κB pathway and mediate chemoresistance**

Next, we determined whether RBCs mediated its effects on tumor cells by activating intracellular signaling pathways. ROS participates in several physical and pathological processes through their involvement in signal transduction. To investigate the role of ROS in the tumor promotion effect of RBCs and Hb, we detected ROS production upon RL or Hb stimulation by using CM-H2DCFDA fluorescence probe. Upon RBC and Hb stimulation, the level of intracellular ROS was markedly increased (Fig. 3A, 3B). The polynyxin B was not able to impair RL-induced ROS production, which excluded LPS, a potential contaminant of RL and ROS inducer (Fig. 3A). NF-κB, a redox-sensitive transcription factor and a sensor for oxidative stress, can be activated by ROS directly or indirectly (15). We next investigated whether accumulation of ROS by RL or Hb resulted in NF-κB activation. We found that stimulation of tumor cells by RL and Hb led to upregulated phospho-NF-κB p65 (Ser^536) (Fig. 3C). These results strongly suggest that RL and Hb can activate the ROS-NF-κB pathway.

The NF-κB signaling pathway is important in regulating cell proliferation and survival and may mediate chemoresistance and radioresistance (16). Having shown that RL and Hb activated the NF-κB pathway, we investigated a role for RBCs and Hb in chemoresistance, a common and significant clinical phenomenon during anticancer treatment. 4T1 tumor cells exposed previously to RL or Hb exhibited resistance to multiple chemotherapeutical agents, paclitaxel, cisplatin, and doxorubicin, at different levels (Fig. 3D–G). A previous report suggested NF-κB activation was associated with major multidrug transporter ABCB1/P-glycoprotein (P-gp) expression (17). To directly assess the relevance of a multidrug transporter in cancer-induced chemoresistance, we analyzed the expression of ABCB1 with RT-PCR. In this study, a significant increase of ABCB1 expression in 4T1 tumor cells was observed following RL and Hb stimulation in vitro (Fig. 3H). To further confirm this phenomenon in vivo, we examined the ABCB1 expression in the tumor tissues. As expected, RL markedly increased the expression of ABCB1 in the coinoculation group (Fig. 3I). Those results suggest that RBCs mediate multidrug resistance during chemotherapy. These observations appear particularly important, and thus chemotherapy in the vicinity of tumor bleeding may be an ill-timed medication.

**RBCs mediate inflammatory responses and angiogenesis**

We next determined whether RBCs had a role in mediating inflammatory and proangiogenic responses in the tumor microenvironment. Considering large doses of RL and Hb promote tumor growth, the doses we used for subsequent experiments represent considerable hemorrhage within tumors. On day 14 after inoculation, tumors were dissected, and tumor tissues were analyzed by

**FIGURE 1.** RBCs and Hb promote tumor growth in vivo. (A) Effect of coinjection of RBCs on the growth of 4T1 breast cancer. Total of 1 × 10^5 4T1 cells was coadministered s.c. with various doses of RBCs or RL (1 × 10^6 and 1 × 10^7) into the right flank of BALB/c mice. Tumor volume was measured at 14 d after inoculation (n = 8–13). (B) 4T1 tumors from day 14 were excised, imaged macroscopically, and weighed. (C) Impact of coadministration of large number of RBCs and RL (1 × 10^6) on B16 melanoma progression. On day 15, tumor size was measured (n = 7 to 8). (D) Effect of coinjection of Hb on the growth of 4T1 breast cancer. Total of 1 × 10^5 4T1 cells was coadministered s.c. with various doses of Hb (15, 12, and 5 mg) into the right flank of BALB/c mice. Tumor volume was measured at 14 d after inoculation (n = 6 to 7). *p < 0.05. **p < 0.01. Ctrl, control.
RT-PCR for the expression of proinflammatory cytokines. TNF-α, iNOS, IL-6, IL-12, and IL-1β were elevated in the tumor microenvironment in the RL group (Fig. 4A). Notably, among those cytokines, TNF-α was most obvious, at 25 times higher levels (Fig. 4A). As expected, proangiogenic factors VEGF and IL-8 were also upregulated at least three times (Fig. 4A). In addition, we assessed the expression of important immunosuppressive cytokines. Though IL-10 was decreased, we observed the markedly increased expression of TGF-β (Fig. 4A). Thus, RL coinoculation exhibits increased inflammation and immunosuppressive factors. We further detected the source of those cytokines. Both macrophages and tumor cells in the tumor microenvironment could produce inflammatory cytokines, although the cytokine profiles were different (Supplemental Fig. 1).

Considering the increased proinflammatory, proangiogenic, and immunosuppressive factors, we postulated that RBCs might provoke

**FIGURE 2.** RBCs promote tumor cell proliferation. (A and B) Proliferation and viability of 4T1 cells exposed to RL for 72 h were determined by cell counting and MTT assays. (C) 4T1 cells were exposed to murine Hb for 72 h, and the proliferation rate was determined by MTT assays. (D) Western blot analysis for cyclin D1, c-Myc, and HO-1 in 4T1 tumor cells exposed to RL and Hb. (E) Analysis of the proliferative state of tumor cells from day 14 after coinoculation using Ki-67 staining (original magnification ×200). (F) Quantification of Ki-67 index in (E). *p < 0.05, **p < 0.01. Ctrl, control.

**FIGURE 3.** RBCs activate ROS–NF-κB pathway and mediate chemoresistance. (A and B) LPS, RL, and Hb were added into the culture media, and CM-H₂DCFDA was used to measure ROS generation at 10 min. Polymyxin B was used to quench LPS. (C) Immunoblotting for phospho–NF-κB p65 (Ser536), following RL and Hb exposure, was performed at indicated times. 4T1 tumor cells pretreated with RL and Hb exposed to 37.5 nM paclitaxel (D and E), 6.67 μM cisplatin (F), and 5 μM doxorubicin (G). Flow cytometry was performed to detect viable cells. Cell viability was considered as Annexin V<sup>−</sup> propidium iodide (PI) cells. (H) RT-PCR analysis of ABCB1/P-gp expression in 4T1 tumor cells after stimulated with RL for 24 h. (I) Influence of RL on ABCB1/P-gp expression in tumor microenvironment. Tumors were dissected 14 d after inoculation. ABCB1/P-gp level was analyzed by RT-PCR. *p < 0.05, **p < 0.01. Ctrl, control.
angio genesis in the tumor microenvironment. To analyze whether RBCs has any impact on tumor angiogenesis, we analyzed vasculature in tumor samples after 14 d inoculation. Tumors with RL coinoculation revealed a markedly higher vascular density than that in controls (Fig. 4B, 4C). To exclude the possibility that the difference between the two groups resulted from different tumor volumes, we compared inflammatory factors and tumor vasculature at the same tumor volume. In this study, we first inoculated 4T1 cells and then RL and 4T1 cells 4 d later. Two weeks after control tumor cell inoculation (hereafter referred to as Ctrl D14), namely, 10 d after RL and 4T1 coinoculation (RL D10), tumors in both group reached the same volume (Supplemental Fig. 2A). Tumor tissues were then evaluated for inflammation and vasculature. There are also elevated inflammatory factors and higher tumor vasculature in the RL D10 group than in the Ctrl D14 group (Supplemental Fig. 2B–D). Collectively, RBCs mediate inflammatory response and foster the development of vasculature in the tumor microenvironment.

RBCs recruit macrophages to promote tumor growth

Tumor-infiltrating leukocytes are key players in the development of cancer. Among tumor-associated leukocytes, macrophages are generally regarded as accomplices in the tumor microenvironment that steel tumor progression (18). To investigate whether macrophages participate in the tumor promotion of RBCs, we examined macrophages in the tumor sections. At 14 d after establishment, immunohistochemical analysis showed a significant increase in the infiltration of F4/80+ macrophages in the RL group as compared with control (Fig. 5A). Moreover, M2-associated genes arginase1, Fizz1, Ym1, and Mgl1 were upregulated in the RL group (Fig. 5B). By flow cytometry, enumeration of immune cell phenotypes demonstrated increased CD11b+F4/80+CD206+ M2 macrophages in the tumor microenvironment in the presence of RL (Fig. 5C, 5D). In addition, we found the M2 macrophage polarization factor IRF4 was increased in the RL coinoculation group (Fig. 5E). We further compared macrophages in the tumor microenvironment at the same volume. We still found more M2-type macrophages in the RL D10 group than Ctrl D14 group, suggesting that increased M2 macrophages were not the result of enlarged tumor volume (Supplemental Fig. 2E–G).

We therefore reasoned that RBCs might trigger macrophage recruitment by producing factors attracting macrophages to the tumor microenvironment and analyzed the expression of several cytokines and chemokines in the tumor tissues. RT-PCR analysis of miRNAs provided evidence that S100A8, S100A9, S100A10, CCL2, CSF1, CSF2, CXCL12, CCL17, and placental growth factor were upregulated in the RL group (Fig. 5F). To investigate the direct effect of RBCs on macrophage polarization, we treated peritoneal macrophages from normal mice with RL for 24 h. RT-PCR showed that, though RL mildly increased the expression of Ym1 and Mgl1, RLC did not significantly upregulate other M2 macrophage-associated genes like arginase1 and Fizz1 (Fig. 5G) and slightly induced VEGF and IL-10 expression (Fig. 5H). Notably, RL upregulated the expression of IL-6, IL-12, IL-1β, TNF-α, and iNOS (Fig. 5I). Collectively, though RBCs did not directly polarize macrophages into the M2 type, RBC could recruit and polarize macrophages into M2 macrophages in the tumor microenvironment.

To test whether other immunosuppressive cells participated in the tumor promotion effect of RBCs, we evaluated myeloid-derived suppressor cells and regulatory T cells in the tumor microenvironment. We found that there were no significant changes of those two cell types compared with control (data not shown). Those data suggested myeloid-derived suppressor cells and regulatory T cells were not the main factors in the tumor progression induced by RL.

RBCs and Hb act as endogenous danger signals

In the normal state, RBCs are in the circulation system. We postulated that extravascular RBCs could be danger signals to organisms, and those danger signals could initiate immune and repair mechanisms. RBCs induced the influx of macrophages in the tumor microenvironment when inoculated with 4T1 tumor cells. To further determine directly whether extravascular RBCs recruit macrophages, we administered RBCs into the peritoneal cavity of normal BALB/c mice and measured the frequency of F4/80+ monocyte–macrophages. A significant and rapid increase in the percentages of F4/80+ monocyte–macrophages was observed after RBCs administration (Fig. 6A, 6C). Consistent with that in the peritoneal cavity, enhanced monocyte–macrophages response was also noted following intratumoral injection of RBCs in tumor tissues (Fig. 6B, 6D). However, this phenomenon does not last for a long time. Twelve hours later, the number of monocyte–macrophages began to decrease. Thus, we demonstrated that, in the normal peritoneal cavity and tumor microenvironment, extravascular RBCs could
rapidly recruit monocyte–macrophages into bleeding sites, though the fine mechanism needed further investigation.

PAMPs and DAMPs can be recognized by immune cells through a group of pattern recognition receptors (19). Next, we investigated the pattern recognition receptors that mediated the protumor effect of RBCs. We focused on TLR4, which was a known receptor for hemin (20). We inoculated s.c. B16 melanoma cells and RL in the flanks of TLR4^2/2 and MyD88^2/2 mice (MyD88 signals downstream of many TLRs and IL-1 family receptors). RL still promoted markedly the melanoma growth in TLR4^2/2 and MyD88^2/2 mice (Supplementary Fig. 3). Thus, the protumor effect of RL was not TLR4 dependent. RBCs induced gene expression of many proinflammatory mediators in macrophages. Among those factors, IL-1β secretion is often induced by inflammasome activation. We examined the release of IL-1β into the conditioned medium of peritoneal macrophages. Active IL-1β was increased after 20 h of RL and Hb stimulation, as determined by Western blotting (Fig. 6E). Interestingly, we found that RL and Hb could increase pro–IL-1β expression independent of LPS. We next assessed the activation of NF-κB in response to RL stimulation. Consistent with the results in the tumor cells, RL also activated NF-κB pathway in peritoneal macrophages (data not shown), which might increase the expression of pro–IL-1β. To test whether RL and Hb activate caspase-1, we measured activated caspase-1 in peritoneal macrophages exposed to RL and Hb. Immunoblot analysis showed that both RL and Hb promoted cleavage of caspase-1 (Fig. 6F), indicating that RL- and Hb-induced release of IL-1β is mediated by activated caspase-1. Furthermore, the mRNA levels of NLRP1a, NLRP1b, NLRP3, and NLRC4 in peritoneal macrophages were examined after exposure to RL and Hb for 24 h. We observed a notable increase in NLRP1b and NLRP4 levels and a slight increase in the level of NLRP1a and NLRP3 (Fig. 6G). In addition, RL also markedly elevated NLRP1b, NLRP3, and NLRC4 levels in tumor tissue (Fig. 6H). Collectively, we provide several lines of evidence that extravascular RBCs and Hb act as endogenous signals to initiate inflammation and immune response.

Discussion

In this study, we demonstrate that extravascular RBCs and Hb effectively promote tumor growth. The effects of RBCs on the tumor progression are pleiotropic. RBCs can stimulate tumor cell proliferation directly and remodel the tumor stroma, characterized by a hostile proinflammatory microenvironment, enriched M2 macrophages, and increased tumor vasculature. RBCs are endogenous danger signals that can promote inflammatory responses and monocyte–macrophages recruitment. Oxidative stress induced by RBCs and Hb results in elevated intracellular ROS levels. Consistent with this role for RBC and Hb, we found that RBCs and Hb regulated NF-κB, an important transcriptional factor required for cell proliferation and survival.

**FIGURE 5.** RBCs recruit and polarize macrophages in the tumor microenvironment. (A) 4T1 tumor cells were inoculated with RL into BALB/c mice. Fourteen days later, tumor sections were prepared. Macrophages in tumors were determined by immunohistochemical analysis of tumor tissue with anti-F4/80 Abs. Original magnification ×400. (B) RT-PCR analysis of M2-specific gene signature in tumor tissues. M2-specific genes were higher in the RL group. (C) Tumors were minced, and single-cell suspensions were analyzed by flow cytometry. Analysis of F4/80^+CD206^+ M2 macrophages in the tumor microenvironment. Plots were gated on CD11b^+ immune cells. (D) Quantification of CD11b^+F4/80^+CD206^+ M2 macrophages in tumor tissue at day 14 after inoculation. Recruitment of M2 macrophages in the RL group was increased. (E) RT-PCR analysis of M2 macrophage polarization factor IRF4 in the tumor tissues. IRF4 was greatly increased by RL. (F) RT-PCR analysis of chemokines associated with macrophage recruitment in tumor tissues. (G) Macrophages were isolated from peritoneal cavity and treated with RL for 24 h, and the M2-specific genes were analyzed with RT-PCR assay. (H) RT-PCR analysis of VEGF, IL-10, and TGF-β in RL-treated macrophages. (I) RT-PCR analysis of proinflammatory genes, IL-6, IL-12, IL-1β, TNF-α, and iNOS in RL-treated macrophages. *p < 0.05, **p < 0.01. Ctrl, control.
NF-κB can be activated by ROS and is a highly pleiotropic transcription factor, which induces expression of many gene products including cyclinD1 and c-myc. We found that the known cell growth–promoting factors c-myc and cyclin-D1 were markedly increased upon RL and Hb stimulation. HO-1 degrades heme into carbon monoxide, biliverdin, and ferrous iron and protects against oxidative injury. After exposure to RL and Hb, HO-1 is upregulated in tumor cells, which regulates cell proliferation and facilitates angiogenesis (14). Thus, we demonstrate that RBCs aberrantly activate the NF-κB pathway and function as a regulator of tumor cell proliferation, which has not previously been described. In this study, we should mention that lower doses of RL and Hb did not obviously promote tumor growth, suggesting that only extensive intratumor hemorrhages accelerate tumor progression.

Previous studies showed that NF-κB can mediate chemoresistance and radioresistance (22). It is tempting to speculate that RBCs can mediate the resistance of tumor cells to chemotherapeutical agents. In vitro, both RL and Hb induce resistance to paclitaxel, cisplatin, and doxorubicin. Our results are consistent with other reports. Heme accumulation was detrimental to cells (23). Interestingly, tumor cells can use BCRP to reduce heme or porphyrin accumulation (24). NF-κB activation was associated with major multidrug transporter ABCB1/P-gp expression (17). In the current study, we provide evidence that, in murine breast cancer cells, ABCB1 was upregulated under heme stress. RBCs also induce ABCB1 gene expression in the tumor microenvironment. Conceivably, it is not a good chance for chemotherapy in the treatment of cancer when the tumor is in the vicinity of bleeding.

Tumor-associated macrophages were M2-like type, which are characterized by high expression of markers of alternative activation, such as Arg1, Ym1, Fizz1, and Mgl1 (25). Our data suggest that, during tumorigenesis, RBCs may facilitate the recruitment of macrophages into the tumor microenvironment, as assessed by flow cytometry and immunofluorescent staining of tumor sections. RBCs variably upregulated the production of many chemokines, including S100A8, S100A9, S100A10, CCL2, CSF1, CSF2, CCL17, CXCL12/SDF-1, and placental growth factor. Those chemokines are all associated with macrophage recruitment. Usually, RBCs are in the circulation system in the normal state. Therefore, it is conceivable that extravascular RBCs could be danger signals. We then directly injected RBCs exogenously into the abdominal cavity and tumors and found that RBCs could recruit monocyte–macrophages into the lesion site immediately after the injection of RBCs.

Additionally, more CD11b+ F4/80+ CD206+ macrophages, which indicate an M2 type, were detected in the tumor microenvironment,
accompanied by the upregulated M2-specific gene signature. However, the RBC per se is not able to skew macrophage polarization into M2 macrophages. Conversely, ex vivo macrophages stimulated with RBC lysates produce high levels of proinflammatory cytokines, including IL-6, IL-12, IL-1β, and TNF-α, and enhance the expression of iNOS. The maintenance of M2 polarization state might be due to microenvironmental cues such as continuous exposure to the inflammatory and immunosuppressive factors induced by RBCs in tissue. Immunosuppression and proangiogenic activity are the main mediators for tumor progression induced by M2 macrophages (25). We evaluated the immunosuppressive and angiogenic factors in the tumor tissues. RBCs induced a 16-fold upregulation of TGF-β, IL-6, IL-8, and VEGF are also increased in the presence of RBCs. The elevated cytokines and the presence of M2 macrophages are associated with potent proangiogenic capability. As expected, coinoculation of RL results in a remarkably higher degree of vascularization in tumors. Extravascular RBCs are effective stimuli for angiogenesis and exert potent proangiogenic activity in the tumor progression.

Inflammation has been long thought to promote tumor promotion and progression (26). Anti-inflammation agents, including nonsteroidal anti-inflammatory drugs, reduce cancer incidence according to clinical observations (27, 28). In this study, we found that the levels of proinflammatory chemokines IL-6, IL-12, IL-1β, and TNF-α were higher in malignant tissues in the presence of RBCs. We even found a 25-fold rise in TNF-α expression. Among these cytokines, the protumorigenic effect of TNF-α and IL-6 is well established, which can influence all stages of tumor development, including initiation, progression, and metastasis (29). TNF-α has a known role in inflammation, angiogenesis, tumor progression, and tissue remodeling (28). Endogenous TNF-α can act as a tumor promoter (30). The tumor-promoting properties of TNF-α may be associated with its ability to activate both AP-1 and NF-κB signaling pathways, which stimulate cell proliferation and survival (26). Blocking TNF-α reduces colorectal carcinogenesis associated with chronic colitis in mice (31). We also revealed that those elevated inflammatory factors were associated with both tumor cells and macrophages. Thus, as danger signal molecules, extravascular RBCs initiate a robust sterile inflammation and foster the cancer progression.

Heme has been demonstrated to be a TLR4 activator (32). A recent study found that extracellular heme triggers acute chest syndrome, and this lethal type of acute lung injury was mediated by TLR4 syndrome, and this lethal type of acute lung injury was mediated by TLR4. We next focused on the NLR inflammasome. The inflammasome is a proteolysis complex that cleaves and activates pro–IL-1 and pro–IL-18 to generate IL-1 and IL-18 (33). Several endogenous danger signals, such as ATP (34) and uric acid (10), can activate inflammasome and induce IL-1β secretion. Based on our findings, we proposed that RBCs and Hb could be associated with inflammasome activation, whereas the mechanism should be further investigated. The activation of inflammasome is mediated by NLR proteins that respond to exogenous and endogenous stimuli. We observed caspase-1 activation and IL-1β maturation in response to RL and Hb. RL could upregulate NLRP1a, NLRP1b, NALP3, and NLR-C4 expression in macrophages. RBCs and Hb can directly generate active IL-1β with no need for LPS. This may result from the NF-κB activation to generate pro–IL-1β. The microenvironmental IL-1β is required for angiogenesis and invasiveness of different tumor cells in vivo (35). A recent paper demonstrated that IL-1β induced secretory of IL-17 by CD4+ T cells and then blunted the anti-cancer efficacy of the chemotherapy (36).

In summary, our study provides novel insights into protumor functions of extravascular RBCs and Hb as endogenous danger signals. RBCs modulate several aspects of the malignant phenotype: tumor proliferation, inflammation, angiogenesis, macrophage recruitment and polarization, and chemoresistance. Extensive intratumor hemorrhage may represent a pool prognostic factor for cancer patients.

Disclosures

The authors have no financial conflicts of interest.

References


Table S1. Primer sequences are listed below.

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Supplementary Figure 1. Tumor cells and macrophages produce inflammatory cytokines in tumor microenvironment remodeled by RBC. Flow cytometry analysis of inflammatory factors in the 4T1 tumor tissues 14 days after co-inoculation with RL. CD11b+F4/80+ macrophages (A) and CD45- tumor cells (B) were intracellularly stained with antibodies against IL-1β, VEGF, iNOS, IL-6, TNFα and IL-12.
Supplementary Figure 2. RBC lysates provoke inflammation, angiogenesis, M2 macrophages influx in tumor microenvironment. A, $1 \times 10^9$ RL plus $1 \times 10^5$ 4T1 and $1 \times 10^5$ 4T1 tumor cells alone were first inoculated at day 0, and then $1 \times 10^9$ RL and $1 \times 10^5$ 4T1 cells four days later. Two weeks after control tumor cells inoculation (Ctrl D14), namely, 10 days after RL and 4T1 co-inoculation (RL D10), tumors in both group reached the same volume. Tumors in the RL D14 group had a larger volume compared with that in Ctrl D14 and RL D10 group. B, RT-PCR analysis of inflammatory genes in the tumor tissues in Ctrl D14 and RL D10 groups. C, 4T1 tumor sections in Ctrl D14 and RL D10 groups were stained by CD31-FITC antibody. D, Quantification of microvessel densities in tumor sections between Ctrl D14 and RL D10 groups. E, Analysis of F4/80+CD206+ M2 macrophages in the tumor microenvironment in Ctrl D14 and RL D10 groups. Plots were gated on CD11b+
Supplementary Figure 3. RBC lysates promote tumor growth in TLR4-null and MyD-88-null mice. $1 \times 10^5$ B16 melanoma cells were co-administered subcutaneously with $1 \times 10^9$ RL into the right flank of TLR4-/- (A) and MyD88-/- (B) mice. Tumor volume was measured at indicated times (n=6-7).