

An obligatory anaerobic *Salmonella typhimurium* strain redirects M2 macrophages to the M1 phenotype

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Abstract. A genetically engineered *Salmonella typhimurium* strain that may be applied in the medically useful therapeutic strategy of using bacterial agents to target breast cancer in a tumor-bearing nude mouse model has been previously reported. Furthermore, immune cell accumulation in breast tumor types has been observed, particularly distributed in regions surrounding the bacteria. M2 macrophages are associated with breast cancer aggressiveness, whereas M1 macrophages are prone to devouring bacteria and killing cancer cells. Therefore, this engineered tumor-targeting salmonella strain was used in an attempt to reverse the phenotype of M2 macrophages into the M1 phenotype. Subsequent to the co-culture of M2 macrophages with the bacteria for a short time, >50% of the M2 macrophages were invaded by bacteria. These M2 macrophages exhibited a decreased expression of mannose receptor (an M2 phenotypic marker) and increased expression of human leukocyte antigen-antigen D related (an M1 phenotypic marker). The results of the present study indicated that differentiated M2 macrophages may be redirected into the M1 phenotype following exposure to the engineered bacteria stimulus. This effect may be a potential mechanism by which bacteria retard tumor growth. Thus, this engineered bacterium may be a useful candidate for targeting and redirecting M2 macrophages into the M1 phenotype.

Introduction

The potential use of bacteria for cancer treatment has been extensively investigated in previous years. Bacteria, including *Bifidobacterium* (1,2), *Clostridium* (3) and *Salmonella* have been demonstrated to preferentially target and replicate in the hypoxic and necrotic regions of a tumor, resulting in tumor repression (4-7). In a previous study, a synthetic biology approach was used to generate the novel *Salmonella typhimurium* strain YB1 (YB1) (8). This bacterium specifically colonizes and proliferates in the hypoxic/necrotic areas of the tumor, but avoids normal organs and retards tumor growth (8). Furthermore, a previous study reported that numerous macrophages accumulate in breast tumors and are associated with a poor prognosis (9).

Macrophages are heterogeneous cells that respond differently to various stimulating signals and display numerous different phenotypes (5). The M1 and M2 macrophage phenotypes represent the two extremes of a broad range of macrophage functional states. Fully polarized M1 (or classically activated) macrophages are stimulated by microbial agents or pro-inflammatory factors, including lipopolysaccharide (LPS), whereas M2 (or alternatively activated) macrophages respond to anti-inflammatory molecules, including interleukin-4 (IL-4) (10,11). Macrophages located in the stroma of breast cancer tissues [known as tumor-associated macrophages (TAMs)] are primarily M2 macrophages activated by IL-4-producing cluster of differentiation (CD)4+ T cells (12). TAMs are the most notable migratory hematopoietic cell type in the tumor microenvironment and promote the invasiveness of breast cancer cells (13).

Clinically, a large amount of macrophage infiltration in tumor sections from patients with breast cancer has been observed using CD68 immunohistochemical staining. TAMs are associated with breast cancer aggressiveness and promote cancer metastasis, whereas M1 macrophages are prone to killing cancer cells and devouring bacteria (14). Furthermore, studies have revealed that TAMs (which are primarily M2 macrophages activated by IL-4) exhibit a CD206^{high}/human leukocyte antigen-antigen D related (HLA-DR)^{low} phenotype that is associated with immune suppression (15-17). Therefore, CD206 and HLA-DR may be used as markers for M1 and

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M2 macrophage phenotype analysis (15). In the present study, the newly engineered tumor-targeting YB1 strain was used in order to attempt to redirect M2 macrophages into the M1 phenotype. More than half of the M2 macrophages devoured the bacteria after 2 h of co-culture. These M2 macrophages exhibited a decreased CD206 expression and an increased HLA-DR expression. Therefore, the IL-4-activated M2 macrophages switched from the CD206^{high}/HLA-DR^{low} phenotype to the CD206^{low}/HLA-DR^{high} phenotype subsequent to co-culture with the engineered YB1 strain. The present study indicates that differentiated M2 macrophages may be redirected into an M1 phenotype following exposure to different stimuli. This finding may reflect a potential mechanism by which bacteria retard tumor growth. Therefore, these engineered bacteria may be used as a vector to target tumors.

Materials and methods

Patient samples and macrophage immunohistochemistry staining. All tumor samples from breast-infiltrating ductal carcinomas were obtained from female patients (mean age, 45 years; age range, 35-55 years) at the Guangdong Women and Children's Hospital (Guangdong, China). The samples were used with written informed consent and ethical approval was obtained from the Internal Review and the Ethics Boards of Guangdong Women and Children's Hospital (Guangdong, China).

The samples were fixed in 10% formalin for >2 h at room temperature, paraffin-embedded (3 min at 56°C) and sectioned into 5 μ M-thick slices. The macrophages were visualized by immunohistochemistry staining using an anti-CD68 antibody (cat. no. M0814; dilution, 1:200; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), and sections were treated using this antibody overnight at 4°C. For details, please refer to reference (18).

Bacterial culture. The bacterial YB1 strain was cultured in lysogeny broth medium overnight (12 to 16 h) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with chloramphenicol and 2,3-diaminopropionic acid (Sigma-Aldrich; Merck KGaA) at 37°C (8).

Isolation and activation of human monocyte-derived macrophages. Institutional ethical approval was obtained from the Internal Review and the Ethics Boards of Guangdong Women and Children's Hospital, Guangdong, China prior to conducting the study. Human mononuclear cells were isolated from 100 ml peripheral blood of healthy donors by Ficoll density gradient centrifugation (20°C at 250 x g for 20 min), as previously described (18). The resulting monocyte-derived macrophages were activated by the addition of IL-4 (45 ng/ml) to the culture medium for 3 days (19), and LPS (20 ng/ml) was added as a control.

Bacteria and macrophage co-culture. Isolated macrophages were activated by IL-4. The bacterial YB1 strain, which carried the green fluorescent protein (GFP)-tagged plasmid, was added to the macrophage culture for a final bacterial concentration of 5x10⁶/ml. The cultures were incubated at 37°C under hypoxic conditions as previously described (8) for 2 h.

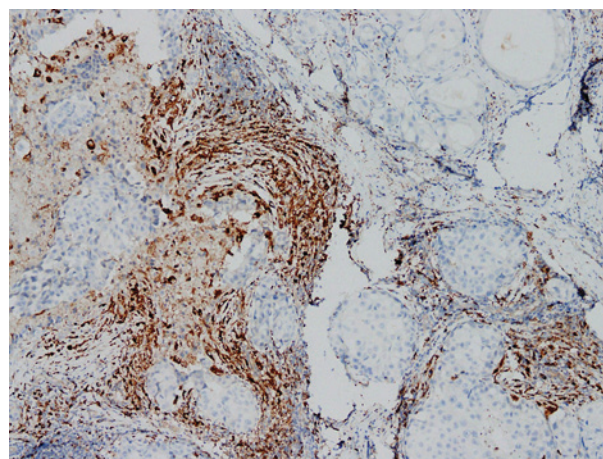


Figure 1. Immunohistochemical staining of breast cancer tissue revealing the mannose receptor-positive macrophages present (magnification, x100).

Then, the bacteria were washed away and the macrophages were harvested for further analysis.

Flow cytometry. Following the co-culture, the macrophages were fixed in paraformaldehyde (4%) at 4°C for 0.5-2 h. The macrophage phagocytic rate of YB1 (GFP) was detected using flow cytometry. CD206 or HLA-DR expression levels in the macrophages were also determined using flow cytometry. Briefly, the macrophages (10⁵-10⁶/ml) were collected, fixed in 4% paraformaldehyde at 4°C for 0.5-2 h, blocked with 5% bovine serum albumin for 20 min at room temperature and stained using a phycoerythrin-conjugated CD206 (dilution, 1:20; cat no. 321105; BioLegend, Inc., San Diego, CA, USA) or allophycocyanin-conjugated HLA-DR (dilution, 1:20; cat no. 307609; BioLegend, Inc.) antibody for 1 h at 4°C. The corresponding isotype control was included in each test. Then, CD206 or HLA-DR expression was analyzed using flow cytometry. FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA) was used to analyze the data.

Western blotting. Cells were lysed using radioimmunoprecipitation assay lysis buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) and protease inhibitors (Sigma-Aldrich; Merck KGaA). Cells were lysed with lysis buffer for 15-20 min at 4°C. HLA-DR expression was determined according to a previously described protocol (18). The proteins (20 μ l per lane) were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk for 2 h at room temperature and then incubated with antibodies against HLA-DR (1:5,000; rabbit monoclonal IgG antibody; cat no. ab92511; Abcam, Cambridge, UK) overnight at 4°C. Subsequent to washing using 0.1% TBST (50 mM tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) three times (10 min each), the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Chicago, IL, USA) for 1 h at room temperature. Then, the membranes were washed again using 0.1% TBST three times (10 min each). The HRP signal was visualized using enhanced chemiluminescence (ChemiDoc MP Imaging System;

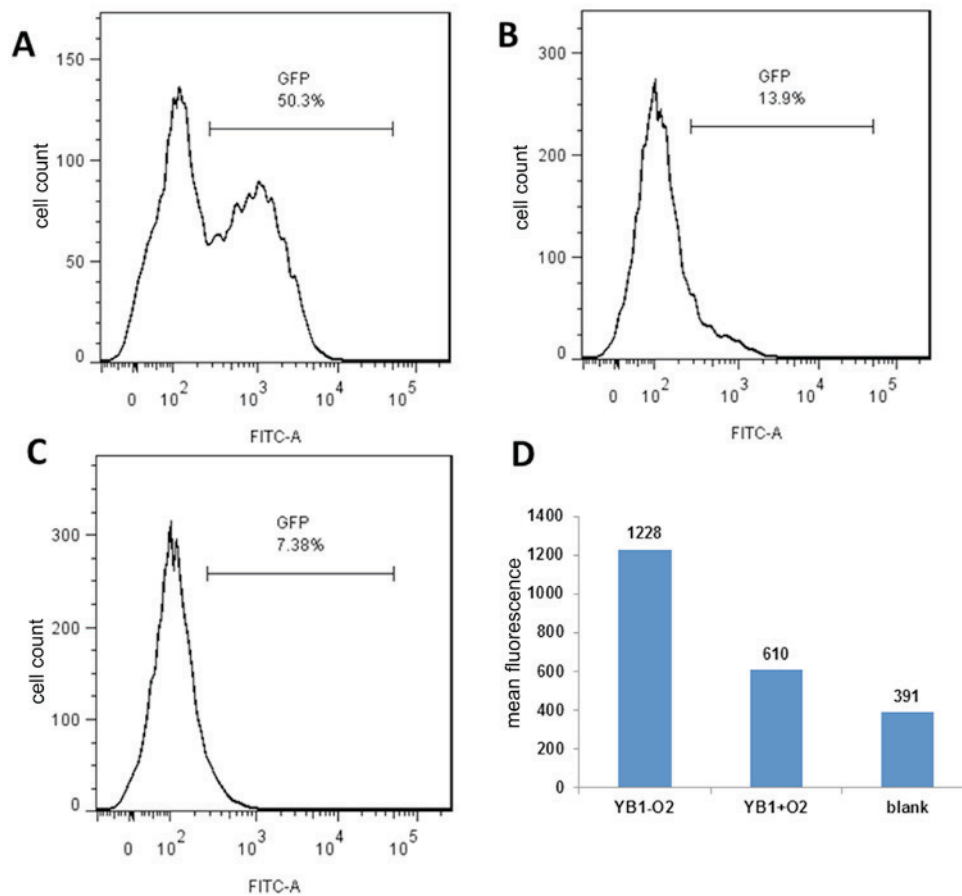


Figure 2. YB1 strain invasion rate between hypoxic and normal conditions, determined by flow cytometry analysis. YB1 invasion rate under (A) hypoxic and (B) normal conditions, with (C) demonstrating the results of the blank control. (D) Mean fluorescence between hypoxic and normal conditions. YB1-O₂: under hypoxic conditions; YB1+O₂: under normal conditions. GFP, green fluorescent protein; FITC-A, fluorescein isothiocyanate; YB1, *Salmonella typhimurium* strain YB1.

Bio-Rad Laboratories, Inc., Hercules, CA, USA), and analyzed using Image Lab (version 5.2.1; Bio-Rad Laboratories, Inc.).

Results

Macrophage infiltration in breast tumor tissues. The engineered YB1 strain may specifically target and survive in a solid tumor. To confirm macrophage infiltration in the breast tumor, clinical breast cancer samples were collected and CD68 immunohistochemistry staining was used to demonstrate the macrophage distribution. As presented in Fig. 1, a large number of macrophages (CD68-positive) infiltrated the breast tumor, particularly in the tumor-associated stromal border, a result consistent with previous reports (12). This result implies that bacteria may be able to target macrophages localized in the breast tumor.

YB1 invaded M2 macrophages under hypoxic conditions. The engineered YB1 strain only survives under hypoxic conditions (8). However, whether YB1 may invade M2 macrophages under hypoxic conditions remains unknown. Thus, an anaerobic microenvironment was stimulated *in vitro* and co-cultured M2 macrophages with the YB1 strain with the GFP-tagged plasmid for 2 h. Flow cytometry analysis was used to determine the YB1 invasion rate. As presented in Fig. 2A, YB1 had a high invasion rate under hypoxic conditions; after 2 h, >50%

of the macrophages were invaded. In contrast, the invasion rate was very low under normal conditions (21% oxygen) (Fig. 2B) as YB1 may not survive. Fig. 2C presented the results of the blank control. It was additionally identified that the mean fluorescence was substantially higher under hypoxic conditions than under normal conditions, indicating that >1 bacterium invaded each macrophage (Fig. 2D).

M2 macrophages exhibited increased HLA-DR and decreased CD206 expression. Subsequent to co-culturing M2 macrophages with YB1, the macrophages were collected and flow cytometry was used to determine the HLA-DR expression levels (Fig. 3A). LPS activated the macrophages to express higher levels of HLA-DR compared with those in the IL-4-activated macrophages. Notably, the YB1 alone strain induced the highest HLA-DR expression in macrophages, suggesting the high efficiency of YB1 in activating the M1 macrophage phenotype. When the macrophages were activated in advance with IL-4 and then co-cultured with YB1, an increase in HLA-DR expression was observed compared with LPS or IL-4 alone activated macrophages. Western blotting confirmed these results (Fig. 3B). Next, the M2 macrophage phenotype marker CD206 was examined (Fig. 3C) and it was identified that IL-4-activated macrophages expressed higher CD206 levels compared with LPS-activated macrophages. The YB1 strain alone reduced the CD206 expression levels compared with

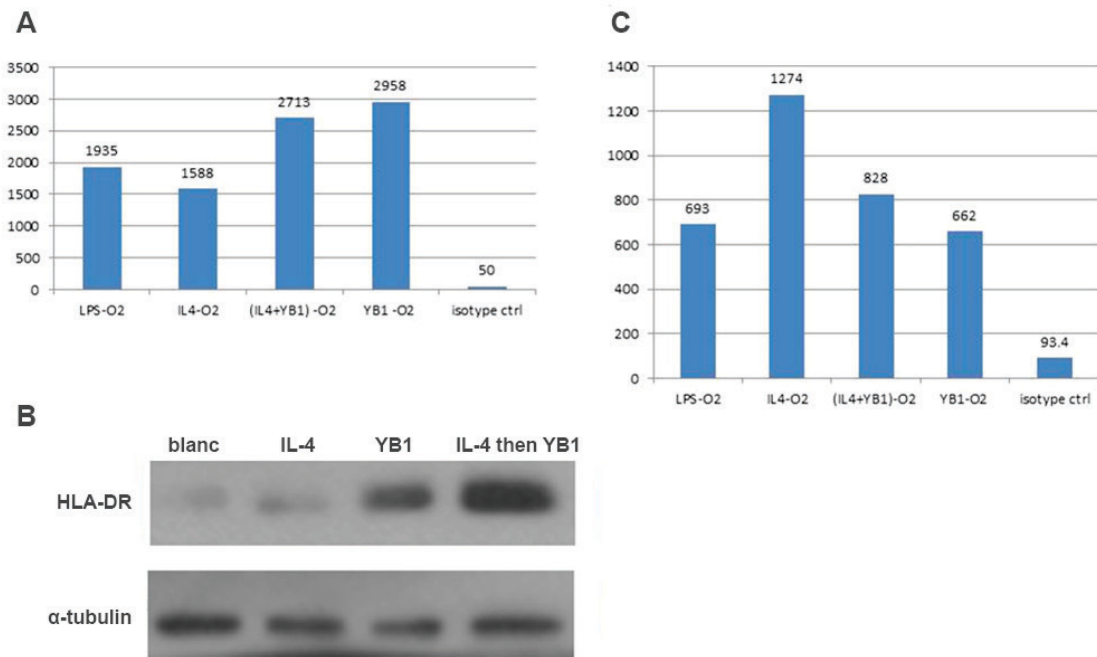


Figure 3. M2 macrophages exhibited increased HLA-DR and decreased CD206 expression levels. (A) HLA-DR expression levels in M2 macrophages activated using LPS, IL-4, YB1, a combination of YB1 and IL-4 or an isotype. (B) HLA-DR expression confirmed using western blotting. (C) CD206 expression levels in M2 macrophages activated using LPS, IL-4, YB1, a combination of YB1 and IL-4 or an isotype. HLA-DR, human leukocyte antigen-antigen D related; CD206, mannose receptor; LPS, lipopolysaccharide; IL, interleukin; YB1, *Salmonella typhimurium* strain YB1.

every other group. Notably, the YB1 strain reduced CD206 expression in the IL-4-activated M2 macrophages.

Discussion

Although a broad range of macrophage subsets have been identified, the two major macrophage populations are the M1 (classically activated) macrophages and M2 (alternatively activated) macrophages (20). These two different types of macrophages have different effects on tumor progression. As early as 1980, studies have demonstrated that bacterial LPS activates macrophages (21-23) to specifically kill tumor cells including breast cancer cells but has no effect on normal cells (24,25). This effect on macrophages requires LPS for maintenance (26). Additionally, macrophages themselves possess a phagocytic ability. When a tumor occurs, macrophages migrate to the tumor location, as directed by the action of chemokines, and devour the tumor cells (27). Macrophages may kill tumor cells, though it remains unknown why this killing effect halts tumor growth and distant metastasis (28). Beyond the tumor immune escape mechanism (29), studies have identified that macrophages are induced by tumor cells in the tumor microenvironment and develop tumor-promoting properties (M2 type, otherwise known as TAMs) (30,31). Statistical data analyses have revealed that the proportion of TAMs in solid tumor tissues may be as high as 80% (29). Likewise, numerous macrophages have been detected in clinical breast cancer samples and, furthermore, macrophage infiltration and breast cancer metastasis are associated (32-34). However, although M1 and M2 macrophages serve different functions in tumor progression, there is no absolute boundary between the two types of macrophages. In the tumor microenvironment, factors including the MHC expression level in

tumor cells and the oxygen pressure in the microenvironment affect the macrophage phenotype (29). Therefore, the phenotype of differentiated macrophages may change. This finding indicates a novel target of tumor treatment: Macrophages in the tumor microenvironment. If M2 macrophages may be directed to become the M1 type, one of the drivers of tumor progression would be eliminated, and result in the gain of one more helper to kill tumor cells.

Deng *et al* (35) has demonstrated that suppression of heme oxygenase-1 in TAMs in a breast cancer mouse model alternatively activates the switching of the M2 macrophage type to the classically activated M1 macrophage type. However, the 'weapons' used to specifically target macrophages in breast cancer are still lacking.

In the present study, a novel engineered *Salmonella* strain (YB1) was reported to induce increased HLA-DR expression and decreased CD206 expression in differentiated M2 macrophages. These M2 macrophages changed from the CD206^{high}/HLA-DR^{low} phenotype to the CD206^{low}/HLA-DR^{high} phenotype, indicating an M2 to M1-type switch. This result suggests a potential use for the engineered tumor-targeting bacteria YB1 in redirecting M2 type macrophages into the M1 type and thus suppressing tumor growth.

Overall, the results suggest that the engineered bacterial YB1 strain may be a good candidate for targeting and redirecting M2 macrophages into the M1 type. In addition to its tumor targeting ability, these bacteria may survive and proliferate in the tumor microenvironment; therefore, the effects would be long-lasting, and the activation of the M1 type would be sustained. Furthermore, for safety, this engineered *Salmonella* YB1 strain is controllable and may be eliminated by antibiotics. Finally, the genetic background of YB1 is clear and may be engineered to carry further 'weapons' in order to kill cancer cells.

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