Colonization of experimental murine breast tumours by *Escherichia coli* K-12 significantly alters the tumour microenvironment

Stephanie Weibel,1,2,3,5 Jochen Stritzker,2,6 Matthias Eck,4 Werner Goebel,1 and Aladar A. Szalay1,2,3,5,6*

Departments of 1Microbiology and 2Biochemistry, Biocenter, University of Wuerzburg, 97074 Wuerzburg, Germany. 3Rudolf Virchow Center, Research Center for Experimental Biomedicine, and 4Institute of Pathology, University of Wuerzburg, 97080 Wuerzburg, Germany. 5Institute for Molecular Infection Biology, University of Wuerzburg, 97070 Wuerzburg, Germany. 6Genelux Corporation, San Diego Science Center, San Diego, CA 92109, USA.

Summary

The successful application of live bacteria in cancer therapy requires a more detailed understanding of bacterial interaction with the tumour microenvironment. Here, we analysed the effect of *Escherichia coli* K-12 colonization on the tumour microenvironment by immunohistochemistry and fluorescence microscopy in the murine 4T1 breast carcinoma model. We described the colonization of tumour-bearing mice, as well as the spatiotemporal distribution of *E. coli* K-12 in the 4T1 tumour tissue over a period of 14 days. The colonization resulted within 3 days in large avascular necrotic tissue, redistribution of hypoxic areas and an enhanced collagen IV deposition within the colonized tumour tissue, which changed the tumoral perfusion of systemically injected immunoglobulins. In addition, *E. coli* K-12 colonization led to the redistribution of tumour-associated macrophages, forming a granulation tissue around bacterial colonies, and also to an increase in TNFα and matrix metalloproteinase 9 expression. Colonization of 4T1 tumours by *E. coli* K-12 resulted in strong reduction of pulmonary metastatic events. These new insights will contribute to the general understanding of the tumour–microbe cross-talk and to the design of bacterial strains with enhanced anticancer efficiency.

Introduction

During the past several years, bacterial genera such as *Clostridium, Bifidobacterium, Salmonella, Vibrio, Listeria*, and *Escherichia* have been reported to selectively replicate within solid tumours (Pawelek et al., 1997; Kimura et al., 1980; Agrawal et al., 2004; Yu et al., 2004; Zhao et al., 2005). Some of them gained also importance as live vectors in cancer therapy (Pawelek et al., 2003). One of the most promising advantages bacteria have over conventional chemo- and radiotherapy is the ability to specifically target tumours. Among the different bacterial strains used so far in tumour therapy, only a few have resulted in complete rejection of solid tumours (Wei et al., 2007). Therapeutic modalities using bacteria in combination with radio-, chemo- or immunotherapy, or as delivery vehicles for therapeutic molecules, have often resulted in additive antitumour effects (Jain and Forbes, 2001; Ryan et al., 2006). Although bacteria are successfully used as tumour-targeting agents, little is known about the modulation of the tumour microenvironment by the microbes and their products.

The unique metabolic microenvironment of solid tumours may be one reason for the specific bacterial accumulation. Forbes et al. (2003) showed that *Salmonella typhimurium* became trapped in the chaotic vasculature of tumours and replicated preferentially in the necrotic tumour tissue. However, the metabolic properties of the tumour microenvironment are not the only determining factors for bacterial accumulation and localization. Malignant tumours are complex tissues composed of ever-evolving neoplastic cells and non-neoplastic cellular elements, including fibroblasts, endothelial cells, macrophages and lymphocytes, surrounded by an extracellular matrix (ECM) (Mueller and Fusenig, 2004). The majority of these components interact and communicate with each other mainly by soluble growth factors, matrix-degrading enzymes and other bioactive molecules, leading to matrix remodelling, angiogenesis and metastasis (van Kempen...
et al., 2006). Today, it is widely accepted that the tumour microenvironment, which is mainly orchestrated by inflammatory cells, is an indispensable participant in neoplastic formation and tumour progression (Coussens and Werb, 2002). Evidence from clinical and experimental studies indicates that especially macrophages, which are educated by the tumour microenvironment (Pollard, 2004) and represent the major component of inflammatory infiltrates (Bingle et al., 2002), play a crucial role in solid tumour progression (Mantovani et al., 2002; Lewis and Pollard, 2006). Another critical factor in cancer development is the presence of a growing family of metalloendopeptidases known as matrix metalloproteinases (MMPs). These proteases, which are produced by a variety of vertebrate cells, cleave components of the ECM like collagens and thereby play a central role in tissue remodelling, invasion and metastasis (Stamenkovic, 2003). However, it is becoming increasingly clear that MMPs are also implicated in the functional regulation of a host of non-ECM molecules that include growth factors and their receptors, cytokines and chemokines (Stamenkovic, 2003). They therefore contribute profoundly to cancer progression and inflammatory processes.

Bacterial infection leads in general to activation of both the innate and adaptive immune responses in the host (Trinchieri and Sher, 2007), which in turn result in extensive tissue remodelling known for occurring in wound healing (Coussens and Werb, 2002). Therefore, we hypothesized that tumour colonization by microbes should also lead to fundamental remodelling of the tumour microenvironment. In this study, we analysed the tumour-targeting properties and the intratumoral distribution of the non-pathogenic, Gram-negative bacterium Escherichia coli K-12 in the syngeneic murine 4T1 breast cancer model. We demonstrated the effect on tumour vasculature, tissue hypoxia and distribution of macrophages by fluorescence and confocal microscopy. The investigation of the macrophage population in colonized tumours revealed a concentration of tumour-associated macrophages (TAMs), TNFα expression and MMP-9 expression around bacterial colonies. These findings exhibit morphological resemblance to granuloma-like structures known from mycobacterial infection foci and may therefore contribute to the restriction of bacterial distribution. In addition, we showed that tumour colonization by E. coli K-12 led to reduced metastasis during the colonization period. Understanding of the effects of bacterial tumour colonization on the tumour microenvironment concerning architecture, cytokines and matrix remodelling enzymes will provide an important contribution to the development of bacterial strains with improved anticancer effects and to the design of bacterium-mediated tumour therapies.

Results

E. coli K-12 preferentially accumulates in 4T1 tumours of immunocompetent and immunocompromised mice

To determine the localization of intravenously injected E. coli K-12 in 4T1 tumour-bearing mice, we monitored bacterial load in tumours, livers and spleens over a period of 14 days. As shown in Fig. 1, E. coli K-12 persisted in 4T1 tumours of immunocompromised, as well as immunocompetent mice, over 14 days and was already undetectable in livers and spleens 3 days post injection (p.i.). One hour after systemic administration, only a few bacteria (~1000 colony-forming units (cfu)) of the initial inoculum of 5 × 10⁶ cfu reached the tumour tissue, followed by an exponential increase of bacterial cfu within the first days post inoculation.
24 h. In contrast, the number of bacteria in livers and spleens continuously declined, starting with \(10^5-10^6\) cfu per gram of organ. Twenty-four hours p.i., the localization of \(E.\ coli\ K-12\) had already revealed a 10 000:1 ratio of bacterial numbers in tumours versus livers or spleens and later reached > \(10^7\). Monitoring of 4T1 tumour-bearing athymic nude and BALB/c mice injected with luciferase-encoding \(E.\ coli\ K-12\) pMW-Pxyl-luxABCDE for 2 weeks confirmed the specific accumulation of light-emitting bacteria within 4T1 tumours in vivo (Fig. 1, Fig. S1).

**Spatiotemporal distribution of \(E.\ coli\ K-12\) within the tumour tissue**

To investigate bacterial localization within 4T1 tumour tissues, we injected DsRed-expressing \(E.\ coli\ K-12\) intravenously into tumour-bearing BALB/c mice and investigated the bacterial distribution in histologically prepared and actin-labelled whole tumour cross-sections at the indicated time points. As seen in Fig. 2B, 24 h p.i. the bacteria clustered in small (50–100 \(\mu\)m) patchy regions within the tumour tissue. After 3 days (Fig. 2C), the distribution pattern changed dramatically. Most of the bacteria were located between the necrotic and the proliferating tumour tissue for the remainder of the 14-day colonization period (Fig. 2D). The central necrotic tissue was also colonized by \(E.\ coli\ K-12\), but with lower density. In contrast, in the proliferating rim of the tumour no bacteria were detected. The comparison of 4T1 control tumours with colonized 4T1 tumours of equivalent age revealed that the extensive necrotic tissue destruction occurred only in colonized tumours (Fig. 2A and C, Fig. S2). These findings suggest that bacterial colonization promoted the necrotic tissue extension. However, the proliferating rim in colonized 4T1 tumours revealed intact tumour stroma, unaffected by the presence of bacteria elsewhere in the tumour (Fig. 2E). Interestingly, an accumulation of labelled actin and labelled DNA was observed in the tissue around (200–400 \(\mu\)m) the bacteria. To identify this bacteria-free area, we created the term b-zone (b = border) (Fig. 2F). This zone is further characterized by an accumulation of small nucleated cells that are a hallmark of inflammatory cell infiltration (Fig. 2H). The morphology and size of cells within the proliferating rim corresponded to that of viable tumour and stromal cells (Fig. 2G), whereas the cell population in the tumour centre displayed a necrotic phenotype characterized by karyolysis (Fig. 2I). The observed bacterial distribution pattern was also detected in 4T1 tumours of immunocompromised athymic nude mice (data not shown). Surprisingly, the extensive destruction of tumour tissues by bacterial colonization did not lead to significant tumour growth inhibition in 4T1 tumour-bearing mice within 14 days (data not shown).

\(E.\ coli\ K-12\) colonization generates a large avascular, hypoxic space and extensive type IV collagen deposition within 4T1 tumours

To assess the impact of bacterial colonization on the tumour vasculature, we compared the distribution pattern of CD31-positive endothelial cells in 3-days-colonized 4T1 tumours with that of same stage control tumours of BALB/c mice. Analysis of the tumour vasculature of 4T1 control tumours revealed a highly disorganized vessel network that was, however, distributed almost homogeneously throughout the tumour (Fig. 3A). After 3 days of injection of \(E.\ coli\ K-12\) pMW211, there were no CD31-labelled blood vessels detectable in the b-zone as well as in the colonized necrotic tumour centre (Fig. 3B). As loss of tumour vasculature may influence tumour perfusion with blood-borne particles, we were interested to see whether bacterial colonization had an effect on tissue oxygenation. To localize the distribution of hypoxic tissue (\(p_O_2 < 10 \text{ mmHg})\), we injected the hypoxia-marker pimonidazole hydrochloride into tumour-bearing mice and allowed it to circulate for 24 h. Control tumours revealed a patchy distribution pattern of pimonidazole staining, especially within central tumour regions (Fig. 3C). In colonized tumours, most of the pimonidazole-positive areas were concentrated within the b-zone rather than the necrotic centre, which was not stained due to a lack of pimonidazole reduction by viable cells (Fig. 3D). The lack of functional vasculature, as well as the enrichment of hypoxic areas within the b-zone, further points to a putative diffusion barrier, which leads to decreased perfusion of central tumour regions with blood-borne particles. To test this, we injected unspecific rat IgGs intravenously into 1- and 3-days-colonized tumorous animals. The distribution pattern of the extravasated IgG revealed a vessel-like distribution within the central tumour tissue at day 1 (Fig. 3E) and a diffuse accumulation of IgG adjacent to the b-zone at day 3 (Fig. 3F). The extravasation pattern on day 3 supports the possible existence of a putative diffusion barrier at the colonized tumour area and further suggests an increased vessel permeability next to the b-zone. As high amounts of collagen are known to decrease interstitial fluid transport in tumour tissues (Jain, 1987; Zaharoff et al., 2002), we also investigated the distribution of collagen within such tumours. In 3-days-colonized 4T1 tumours, the labelling of type IV collagen, which is part of the endothelial basement membrane, revealed an increased deposition of collagen IV (fibrosis) in colonized tumour areas (Fig. 3G). Further, we frequently identified bacterial colonies that colocalized with collagen IV fibres (Fig. 3H). These results indicated that the colonization of tumours by \(E.\ coli\ K-12\) led to vascular remodelling. This was accompanied by an enormous deposition of type IV collagen and ultimately resulted in decreased perfusion of the tumour tissue.
Tumour colonization enhances MMP-9 expression and alters MMP-9 distribution

As MMP-9 plays an important role in the remodelling of type IV collagen during cancer progression and inflammatory processes (Opdenakker et al., 2001), we analysed the possible contribution of MMP-9 to the extensive type IV collagen deposition observed in colonized 4T1 tumours. The immunohistochemical analysis of uncolonized control tumours labelled with anti-MMP-9 antibody revealed a patchy distribution (Fig. 4A). In contrast, 3-days-colonized 4T1 tumours showed an increased density of actin labelling and Hoechst staining in the tissue between colonized necrotic (n) and proliferating (p) tumour tissue (3 days p.i.); this region of high fluorescence intensity of actin labelling and Hoechst labelling was named the b-zone (b = border).

Fig. 2. Spatiotemporal distribution of E. coli K-12 pMW211 in 4T1 tumours of immunocompetent BALB/c mice. Mice bearing 200–400 mm³ 4T1 tumours were injected with either PBS or 5 ¥ 10⁶ E. coli K-12 pMW211 expressing the DsRed protein.

A–D. Whole tumour cross-sections (100 μm) of control tumours (A) as well as 1- (B), 3- (C) and 14-days-colonized (D) tumours were labelled with Phalloidin-FITC (green); E. coli K-12 colonies (red) within the 4T1 tumour tissue were indicated by arrowhead. A loss in tissue integrity at day 14 p.i. was indicated by asterisks; proliferating (p) and necrotic (n) tumour tissue.

E. The confocal images of a 3-days-colonized 4T1 tumour showed a cut-out of the uncolonized proliferating tumour tissue with intact stroma cells (left) and the colonized tumour region with necrotic tissue identified by karyolysis and decreased actin labelling (right); actin (green) and Hoechst-labelled nuclei (blue).

F. Confocal images of the border region (b-zone) and corresponding Plot profiles of the fluorescence intensity measured in greyscale images showed an increased density of actin labelling and Hoechst staining in the tissue between colonized necrotic (n) and proliferating (p) tumour tissue (3 days p.i.); this region of high fluorescence intensity of actin labelling and Hoechst labelling was named the b-zone (b = border).

G–I. H&E staining of the proliferating 4T1 tumour rim (G), the inflammatory infiltrates within the b-zone (H), the necrotic tissue (I) of a 3-days-colonized 4T1 tumour; the b-zone is indicated by arrowheads.

All images are representative examples. Scale bars represent 5 mm (A–D), 20 μm (E), 100 μm (F), 200 μm (G–I).
showed enhanced MMP-9 labelling, as well as an accumulation of MMP-9 in the b-zone and the colonized tumour regions (Fig. 4B and C). The accumulation within the b-zone coincided with the lack of CD31-positive tumour vasculature, as well as the occurrence of hypoxic tumour areas in colonized 4T1 tumours (see Fig. 3B and D). The enhanced expression of MMP-9 was also confirmed by Western blot analysis, which revealed a threefold increase, compared with control tumours (Fig. 4D). Confocal imaging further revealed the colocalization of fibre-like structures of MMP-9 and type IV collagen within the colonized area, suggesting a direct interaction between MMP-9 and collagen type IV (Fig. 4E–I). In addition, we were able to identify MMP-9 positive cells within the b-zone. We propose that the type IV collagen bound MMP-9 represents predominantly the secreted MMP-9 form, whereas the MMP-9-positive cells within the b-zone represent the...
MMP-9-expressing cells. These results strongly suggest that MMP-9 may play an important role in remodelling and deposition of type IV collagen in colonized 4T1 tumours.

E. coli K-12 colonization alters the distribution of TAMs within 4T1 tumours

Breast tumours are abundantly infiltrated with TAMs (Leek...
Tumour colonization by E. coli K-12 alters the distribution of the TAM population. BALB/c mice bearing 200–400 mm³ 4T1 tumours were injected with either PBS or 5 × 10⁶ E. coli K-12 pMW211 (red). Three days p.i., whole tumour cross-sections (100 μm) of 4T1 control tumours (A) and 3-days-colonized tumours (B–D) were labelled with anti-CD68 antibody to visualize macrophages (green). (C) A higher magnification of the boxed area indicated in (B); b-zone (b), proliferative (p) and necrotic (n) tumour tissue. (D) Confocal image of CD68⁺ macrophages within the colonized tumour area; colocalization of DsRed-expressing E. coli K-12 and CD68⁺ macrophages was indicated by arrowhead. All images are representative examples. Scale bars represent 2 mm (A, B), 200 μm (C), 50 μm (D).

et al., 1996), which were located mainly in hypoxic tumour tissues and are involved in matrix remodelling (Murdoch et al., 2004). As colonized 4T1 tumours revealed large hypoxic areas and intensified matrix remodelling, we were interested in finding out whether bacterial tumour colonization affects the distribution of TAMs. In control 4T1 breast tumours, we identified by immunohistochemistry a large amount of CD68⁺ macrophages clustered in small subpopulations (Fig. 5A). The colonization by E. coli K-12 led to redistribution of CD68⁺ macrophages, which accumulated in the b-zone and in significant numbers in the avascular colonized area (Fig. 5B and C). As shown in Fig. 5D, CD68⁺ macrophages were also located in close proximity to bacterial colonies and some of these macrophages contained phagocytosed DsRed-expressing bacteria.

**Induction of TNFα expression in 4T1 tumours by E. coli K-12**

Bacteria and bacterial products exhibit immunomodulatory properties that lead to the expression of pro-inflammatory cytokines by cells of the myeloid lineage (e.g. macrophages). The extensive necrosis in the colonized tumour area, as well as the high number of TAMs in the b-zone and in colonized tumour tissues, points to a possible involvement of TNFα, which is known to induce necrotic tissue destruction (Carswell et al., 1975). Histological analysis of 3-days-colonized tumours revealed an increased density of TNFα-expressing cells in the b-zone and in colonized areas (Fig. 6D–G). Similarly, immunostaining for CD68⁺ macrophages also displayed a dense accumulation within the b-zone and in colonized areas. These findings strongly indicate that macrophages may be the source of TNFα expression (Fig. 6A–C). Exposure of 4T1 tumour cells and the murine macrophage cell line J774A.1 to either 10 μg ml⁻¹ lipopolysaccharide (LPS), heat-inactivated or viable E. coli K-12 revealed TNFα secretion in J774A.1 cells but not in 4T1 cells (Fig. 6H). In parallel, the bacterium-induced expression of TNFα in vivo was confirmed by Western blot analysis of 3-days-colonized 4T1 tumours and control tumours (Fig. 6I). These results suggest that bacterium-mediated induction of TNFα expression in macrophages may be one reason for the extensive tissue destruction upon tumour colonization. Moreover, the concentration of TAMs, MMP-9 and TNFα within the b-zone indicate a link between macrophages and these pro-inflammatory molecules in response to bacterial tumour colonization.

**Mice with colonized tumours showed reduced events of pulmonary metastasis**

The accumulation of TAMs (Leek et al., 1996) and increased expression of MMP-9 (Deryugina and Quigley, 2006) is often correlated with enhanced metastasis. Therefore, we investigated the effect of E. coli K-12 colonization in vivo on the metastatic spread of 4T1 cells, which are known to metastasize to the lung via the haematogenous route (Heppner et al., 2000). To track the metastatic spread of the 4T1 cells, we used 4T1-Luc cells, which stably express firefly luciferase (Kelly et al., 2006). After 2 weeks of tumour growth, mice were injected with either PBS or E. coli K-12 pMW211. After 14 days, the lungs were excised and imaged and pulmonary metastases were counted. We defined a metastatic event as any detectable luciferase signal above background in the lungs of tumorous mice. Using this method, metastases were detected within lungs in 87.5% of tumorous 4T1 control mice (n = 8) (Fig. 7). In contrast, only 28.6% of the colonized mice showed pulmonary metastases (n = 7; P < 0.05 in Fisher’s exact test). The results suggested that the colonization of solid 4T1 tumours by E. coli K-12 led to prolonged metastasis-free time within the 2-week colonization period.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd, Cellular Microbiology, 10, 1235–1248
Fig. 6. Colonization of 4T1 tumours by *E. coli* K-12 induces TNFα expression in the b-zone.

A–G and I. BALB/c mice bearing 200–400 mm³ 4T1 tumours were injected with either PBS or 5 × 10⁶ *E. coli* K-12 pMW211.

A–F. (A–C) Confocal images and corresponding Plot profiles of the b-zone of a 3-days-colonized 4T1 tumour labelled with anti-CD68 antibody; (D–F) confocal images and corresponding Plot profiles of the b-zone of 3-days-colonized 4T1 tumour labelled with anti-TNFα antibody; *E. coli* K-12 (A, D), Hoechst-labelled nuclei (B, E), CD68⁺ macrophages (C), TNFα (F); the yellow box was used for Plot profile analysis and the red sector marked the b-zone; images and the Plot profiles in (C) and (F) showed an increased level of CD68⁺ macrophages and TNFα expressing cells within the b-zone.

G. Overview of TNFα (green) distribution in 3-days-colonized 4T1 tumours; b-zone (b), proliferative (p) and necrotic (n) tumour tissue.

H. Cell culture experiments with 4T1 tumour cells and murine J774A.1 macrophages, which were exposed for 6 h to 10 μg ml⁻¹ LPS, heat-inactivated or viable *E. coli* K-12. TNFα expression in the supernatants [S] was analysed by Western blotting; β-actin in whole-cell lysates [CL] served as an internal control for the used cell numbers of different preparations.

I. Representative example of TNFα expression in whole tumour lysates of a 3-days-colonized 4T1 tumour and a control tumour of equivalent size. β-Actin served as an internal control of the loaded protein amount. All images are representative examples. Scale bars represent 100 μm.
Discussion

Two of the primary characteristics of an ideal anticancer bacterium strain are the specific accumulation within tumours and the rapid clearance throughout the rest of the body. In these experiments, systemic injection of E. coli K-12 revealed highly specific accumulation within murine 4T1 breast tumours, demonstrated by the rapid elimination of bacteria in livers and spleens within 3 days. In comparison with S. typhimurium (Pawelek et al., 1997), we found that systemically administered E. coli K-12 showed enhanced tumour specificity. Previously, we compared different strains of E. coli with pathogenic Salmonella strains in the murine 4T1 tumour model and found, in general, that E. coli strains showed improved tumour targeting (Stritzker et al., 2007). The enhanced tumour selectivity of E. coli may be the result of insufficient bacterial immune evasion mechanisms in the spleen and liver. By comparison, S. typhimurium strains are able to invade host cells and thereby may escape from the humoral immune system. We also compared tumour colonization of E. coli K-12 in immunocompetent and immunocompromised athymic nude mice with impaired adaptive immunity and found no significant difference in bacterial cell counts and survival time within tumours. These results underline the central role of immune suppression in the tumour microenvironment, which may allow bacterial growth, as proposed by Yu et al. (2004).

Analysis of E. coli K-12 colonization within the 4T1 tumour tissue revealed dramatic differences in the distribution pattern over the first 3 days after injection. At day 1, only small patches of bacteria were present. By day 3, bacteria colonized the large necrotic area, mainly along the border region. We propose, in accordance with Forbes et al. (2003), that some bacteria become trapped in the chaotic tumour vasculature following systemic injection, and begin then to replicate exponentially, and are disseminated from these small patches, which are detectable 24 h p.i. During the initial growth phase, we propose that the bacteria compete with tumour and stroma cells for nutrients and oxygen, leading presumably to local sinks that finally inhibit survival of eukaryotic cells. These initially small, exhausted regions become larger and cells may die. In vitro studies using the tumour cylindroid model suggested that these dying cells are surrounded by quiescent cells which may attract bacteria by producing specific metabolic compounds (Kasinskas and Forbes, 2006). Whether chemotaxis or passive dissemination mechanisms such as interstitial fluid pressure are responsible for the bacterial distribution in vivo has to be investigated further. However, the concept that bacterial growth results in a sink for substrates followed by an active or passive movement of the bacteria to quiescent cells could explain the expansion of tumour necrosis from the centre towards the proliferating tumour edge over time.

The direct competition for nutrients and oxygen seems to be one possible driving force for the expansion of tissue necrosis. On the other hand, bacterial components like the outer membrane LPS have been implicated in direct induction of cell death. It was previously shown that LPS triggers apoptosis of endothelial cells (Bannerman and Goldblum, 2003), which could explain the lack of CD31-positive endothelial cells in the colonized tumour tissue. The absence of functional tumour vasculature impairs perfusion of the tumour tissue with blood-borne substances such as oxygen that leads to enlarged tumour hypoxia, which in turn has been shown to inhibit the phagocytic activity of macrophages (Lewis and Murdoch, 2005). This may also explain the notion that tumours are immunological sanctuaries.

The localization of systemically injected IgGs in colonized tumours revealed an accumulation of IgGs around bacterial accumulations. This correlates with the site of functional vasculature termination and also with the distribution of hypoxic areas. Further, we found that the increased deposition of collagen in the colonized tumour tissue impeded the diffusion of blood-borne particles (Jain, 1987; Zaharoff et al., 2002) and may be the reason for the observed restricted IgG extravasation pattern. The limited diffusion within colonized tumours may have...
far-reaching implications for therapeutic strategies that combine bacteria with other systemically administered chemotherapeutics or therapeutic antibodies. Therefore, the effective diffusion of blood-borne therapeutics should be included in the experimental design of future combined therapies.

The enlarged necrotic tissue destruction we observed in colonized tumors may also be the result of host defence against the invading microbes and due to indirect tissue destruction by specific immune cells. Microscopic analysis showed a high density of macrophages within the hypoxic b-zone and in the colonized, necrotic tumour tissue. It is well established that the LPS-mediated activation of macrophages via Toll-like receptor 4 (TLR-4) leads to the induction of TNFα expression (Cross et al., 2004; Lin and Yeh, 2005). Pro-inflammatory TNFα itself acts on many different cell types and is a potent inducer of apoptosis (Rath and Aggarwal, 1999). TNFα has also been described as the sole factor responsible for the success of Coley’s vaccine in bacterium-mediated cancer therapy (Hoption Cann et al., 2003). The observed tissue necrosis induced by E. coli K-12 was comparable to the localized Shwartzman reaction described by Guiducci et al. (2005). Such treatment used bacterial CpG in combination with an anti-IL-10 receptor antibody and a CCL16 chemokine to promote accumulation of macrophages within murine tumors. The authors described a rapidly triggered tumor necrosis in large tumors due to enhanced TNFα production that was initiated by changing the resident M2 macrophage population into an activated, cytotoxic M1 macrophage population. Moreover, blocking the immunosuppressive IL-10 pathway plays an important role in redirecting macrophages and activation of the type I antitumour immune response. In our syngeneic model system, the large necrosis is presumably also triggered by bacterium-activated TNFα-producing M1 macrophages. However, this reaction seems to be restricted to the colonized areas and is not sufficient to destroy the fast-proliferating tumor tissue. We hypothesize that only a part of the tumoral macrophage population may have switched to an activated M1 macrophage population, while a tumor-promoting TAM population may remain within the proliferating tumor tissue supporting tumor growth. In turn, the M1 macrophages possess higher microbicidal activity than M2 macrophages (Mantovani et al., 2002) and could be responsible for eliminating >75% of the bacteria within 14 days. We propose that, in our model system, one major reason for the failure of tumor rejection is the limited or incomplete immune response due to the restricted distribution of bacteria, which thereby trigger only local immune responses. Repeated injections of bacteria may lead to multiple colonization foci of different stages distributed throughout the tumor and may help boost the immune response.

The macrophage accumulation within colonized tumors morphologically resembles granulomas formed after Mycobacterium tuberculosis infection of lung tissue. These granulomas tend to encapsulate and locally restrict the focus of infection (Price et al., 2003). In the case of tuberculosis, the early accumulation of TLR-activated macrophages and the production of TNFα around the infection focus result in the formation of granulomas (Russell, 2007). Taylor et al. (2006) have described the importance of MMP-9 for tissue remodelling and formation of tight, fibrotic granulomas, which both contribute to resistance against pulmonary mycobacterial infections. In colonized tumours, we observed an enhanced expression of MMP-9, located primarily in the b-zone, as well as in the colonized, necrotic tissue that is accompanied by the deposition of type IV collagen. We would expect that an increase in MMP-9 would result in a decrease of the MMP-9 substrate collagen IV. In contrast, we have observed an increased fibrosis. The total matrix collagen content is a product of both synthesis and degradation of matrix compounds, and therefore degraded products may further serve as stimulus of collagen synthesis (Li et al., 2000). Emerging evidence indicates that matrix degradation is neither the sole nor the main function of these proteases. Rather, these proteases may play an important role in regulation of inflammation and immunity (Parks et al., 2004). Further, MMP-9 was also shown to proteolytically activate latent TGF-β and cleave IL-2Rα, representing immunosuppressive mechanisms (Sheu et al., 2001). In addition, MMP-9 was shown to proteolytically generate tumstatin, a fragment of collagen IV, which suppresses angiogenesis via α5β3 integrins (Hamano et al., 2003). This may also explain the herein described lack of tumour vasculature. Recent findings demonstrate a TNFα-dependent upregulation of MMP-9 production in macrophages (Hagemann et al., 2004). These support our findings that enhanced TNFα and MMP-9 expression is localized to the b-zone and colonized, necrotic tumour tissue. To better understand the role of MMP-9 in the colonization process of 4T1 tumors by E. coli K-12, mmp-9−/− knockout mice will be used in future colonization studies.

We found that, in spite of dramatic inflammatory changes in the tumor no tumor shrinkage occurred within 2 weeks. In fact, the accumulation of TAMs (Leek et al., 1996), as well as the increased expression of MMP-9 (Deryugina and Quigley, 2006), correlate more often with poor prognosis and with enhanced metastasis. In contrast to such predictions, the colonization of 4T1 tumors by E. coli K-12, which in turn leads to an accumulation of TAMs and to an increased MMP-9 expression, did not enhance metastasis in colonized tumor-bearing mice. As 4T1 cells metastasize via the haematogenous route (Heppner et al., 2000), the reduced vascularization...
of colonized 4T1 tumours may have reduced the probability of metastatic events. However, the exact underlying mechanism responsible for prolonged lung metastasis-free survival of tumorous mice will have to be investigated in future studies.

In conclusion, we described here that colonization of murine breast tumours by *E. coli* K-12 leads to dramatic changes within the tumour microenvironment, such as the site-specific accumulation of TAMs, the generation of an extended fibrotic granuloma-like structure with central necrosis and the upregulation of TNF-α and MMP-9. These findings may help to design more effective tumour therapies using bacteria in combination with immune modulators, chemotherapeutics or radiation.

**Experimental procedures**

**Bacteria and plasmids**

*Escherichia coli* K-12 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

The dsRed-containing plasmid pMW211 (Sorensen et al., 2003) was kindly provided by T. Ölschläger (Institute for Molecular Infection Biology, Wuerzburg, Germany).

The plasmid pMW-Pxy-luxABCDE containing the luxABCDE operon from *Photobacterium luminescens* was constructed as described in Appendix S1. All plasmid-transformed *E. coli* K-12 strains were cultured in LB medium containing 100 μg ml⁻¹ ampicillin.

**Cell lines**

The murine 4T1 mammary carcinoma cell line (ATCC Accession No.: CRL-2539) and the murine (BALB/c) macrophage cell line J774A.1 (ATCC-No: TIB-67) were cultured in RPMI/10% FBS; 4T1 cells expressing firefly luciferase (4T1-Luc) were kindly provided by P. Casey (Duke University Medical Center, Durham, NC) and maintained in RPMI/10% FBS containing 2 μg ml⁻¹ puromycin.

**Tumour inoculation and bacterial administration**

Five-week-old female BALB/c and athymic BALB/c nude mice were obtained from Harlan (Harlan Winkelmann GmbH, Borchen, Germany) and used for tumour cell implantation of 5 × 10⁴ 4T1 cells or 4T1-Luc cells. Tumour cells were injected subcutaneously into the abdominal right flank and volume was calculated as (length × width²)/2. For all experiments, tumours were grown up to 200–400 mm³ in size (12–14 days) before bacterial injection. Bacteria were grown in LB medium and harvested at the mid-logarithmic phase, washed and diluted with endotoxin-free PBS. Mice were injected with 5 × 10⁶ cfu of *E. coli* K-12 into the tail vein with 100 μl of the bacterial suspension.

To determine bacterial cfu in tumour, liver and spleen tissues, mice were sacrificed, and then lungs were excised and imaged for 1 min. Clearly separated bioluminescent pulmonary metastases were counted. For statistical analysis of metastatic events a Fisher’s exact test was performed.

**Bioluminescence imaging**

For monitoring studies of the distribution of bioluminescent *E. coli* K-12 in tumour-bearing mice, animals were initially anaesthetized with 4% Isoflurane (Forene™, Abbott, Ludwigshafen, Germany) in a knockout box and were then maintained in an anaesthesia module aerated with 1.5% Isoflurane/oxygen. The mice were imaged using the CCD-Camera based NightOWL LB 981 Imaging System (Berthold Technologies, Bad Wildbad, Germany). Photons were collected for 1 or 5 min.

To determine the number of pulmonary metastases, mice were intraperitoneally injected with 150 μg g⁻¹ D-Luciferin. Ten minutes later, mice were sacrificed, and then lungs were excised and imaged for 1 min. Clearly separated bioluminescent pulmonary metastases were counted.

**In vitro infection and protein expression analysis**

A total of 5 × 10⁶ 4T1 and J774A.1 cells were incubated with either RPMI, 10 μg ml⁻¹ *E. coli* LPS (Sigma Aldrich, Taufkirchen, Germany) or 1 × 10⁻⁵ *E. coli* K-12 harvested at mid-log phase and re-suspended as a viable culture or as heat-killed *E. coli* cells (10 min, 80°C) in RPMI. The cell layers were treated with 50 μg ml⁻¹ gentamicin after 45 min to kill live bacteria. After 6 h of incubation, the supernatants were collected and sterile-filtered and the proteins were precipitated with 15% Trichloroacetic acid. The cell layers were lysed, the chromosomal DNA was sheared and, after centrifugation, the supernatants were collected. Both the cell lysates and the proteins from supernatants were separated in 10–12% SDS-polyacrylamid gels and blotted onto nitrocellulose membranes. The membranes were blocked and incubated with the primary antibody in TBT buffer for 2 h, washed three times with TBT buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody.

**Protein isolation from solid tumours**

For protein analysis, tumours were freshly excised and homogenized in Lysis buffer containing Lysing Matrix D tubes with 1.4 mm ceramic spheres (Qiogene, Heidelberg, Germany) with a Fastprep FP120 (Thermo, Dreieich, Germany). After centrifugation, the supernatants were collected and re-suspended in sample buffer.

**Antibodies, reagents and treatment of tumorous animals**

Endothelial cells were labelled with monoclonal rat anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA). Basement membrane was labelled using polyclonal rabbit anti-mouse collagen IV antibody (Abcam, Cambridge, UK). Macrophages were labelled using rat anti-mouse CD68 antibody (Serotec, Düsseldorf, Germany). TNF-α and MMP-9 were detected by immunohistochemistry in tumour sections and by Western blotting using goat anti-mouse TNF-α, mixture of anti-N- and anti-C-terminal antibody (Santa Cruz, Santa Cruz, CA), and goat anti-mouse MMP-9 antibody (Neuromics, Edina, MN). Actin was detected in Western blots using monoclonal anti-mouse actin antibody (Abcam).
Horseradish peroxidase-conjugated secondary antibodies were obtained from Abcam. The Cy2- or Cy5-conjugated secondary antibodies (donkey) were obtained from Jackson ImmunoResearch (West Grove, PA).

The Hypoxyprobe™-1 Plus Kit (Chemicon International, Temecula, CA) was used for the detection of tissue hypoxia. Pimonidazole hydrochloride (60 mg per kg body weight) was injected intravenously; 24 h after injection, the mice were sacrificed and the tumour tissues were excised and snap-frozen in liquid N₂ for histological preparations. Pimonidazole-positive hypoxic tissues were visualized after preparation of tissue sections using MAb1-FITC antibody.

Phalloidin-FITC (Sigma) was used to label actin and Hoechst 33342 to label nuclei in tissue sections. Non-specific rat IgG from Jackson ImmunoResearch was used in extravasation studies and injected intravenously into tumour-bearing mice (11 mg per kg body weight) as described by Nakahara et al. (2006). After 6 h of incubation, the treated tumours were excised and used for histological analysis.

**Histology and microscopy**

For histological studies, tumours were excised and snap-frozen in liquid N₂, followed by fixation in 4% paraformaldehyde/PBS pH 7.4 for 16 h at 4°C. Fixed tumours were rinsed with PBS and embedded in 5% (w/v) low-melting agarose (AppliChem, Darmstadt, Germany).

Tissue sectioning (100 μm) was performed using the Leica VT1000S Vibratom (Leica, Heerbrugg, Switzerland). Tissue specimens were permeabilized and blocked in PBS containing 0.3% Triton X-100 and 5% normal donkey serum for 1 h. The tissue sections were labelled with the primary antibody and/or Phalloidin-FITC for 12–15 h. After washing with PBS, specimens were incubated with the secondary antibody for 5 h. Sections were washed with PBS, prior to incubation for 30 min in 60% (v/v) glycerol/PBS. In a final step, the tissue sections were mounted onto glass slides in 80% (v/v) glycerol/PBS.

For Nuclear Fast Red staining, 100 μm tissue sections were incubated for 5 min in Nuclear Fast Red solution (Sigma), washed with PBS, mounted on glass slides and analysed immediately.

For haematoxilin/eosin (H&E) staining, fixed tissue was processed through routine histopathology.

The fluorescent-labelled preparations were examined using the MZ16 FA Stereo-Fluorescence microscope (Leica) equipped with a digital CCD camera (DC500, Leica) and the Leica TCS SP2 AOBS confocal microscope equipped with an argon, helium-neon and UV laser. H&E-stained sections were analysed using the Axiosvert 200M microscope (Zeiss) equipped with the AxioCam MRc5 camera (Zeiss, Oberkochen, Germany). Digital images (1300 × 1030 and 1024 × 1024 pixel RGB-colour images) were processed with Photoshop 7.0 (Adobe Systems, Mountain View, CA) and merged to yield pseudo-coloured images. Digital images were further analysed using the Plot Profile function of ImageJ software (http://rsb.info.nih.gov/ij/).

**Acknowledgements**

The authors thank T. Tietze and E. Bachmann for excellent technical assistance, M. Adellfinger for animal care, A. Feathers for editorial assistance, T. Hagood for help with graphics and A. Tresch for statistical analysis.

The research was supported by Genelux Corporation, San Diego. S. W. is the recipient of a graduate fellowship from the Competence Network PathoGenoMik (0313134) of the German Federal Ministry of Education and Research, awarded to W. G. and A. A. S., and also the recipient of a graduate fellowship from Genelux Corporation.

**References**

Agrawal, N., Bettegowda, C., Cheong, I., Geschwind, J.F., Drake, C.G., Hipkiss, E.L., et al. (2004) Bacteriolytic therapy can generate a potent immune response against experimental tumors. *Proc Natl Acad Sci USA* 101: 15172–15177.

Bannerman, D.D., and Goldblum, S.E. (2003) Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *Am J Physiol Lung Cell Mol Physiol* 284: L899–L914.

Bingle, L., Brown, N.J., and Lewis, C.E. (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 196: 254–265.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* 72: 3666–3670.

Coussens, L.M., and Werb, Z. (2002) Inflammation and cancer. *Nature* 420: 860–867.

Cross, M.L., Ganner, A., Teilab, D., and Fray, L.M. (2004) Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. *FEMS Immunol Med Microbiol* 42: 173–180.

Deryugina, E.I., and Quigley, J.P. (2006) Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 25: 9–34.

Forbes, N.S., Munn, L.L., Fukumura, D., and Jain, R.K. (2003) Sparse initial entrapment of systemically injected *Salmonella typhimurium* leads to heterogeneous accumulation within tumors. *Cancer Res* 63: 5188–5193.

Guiducci, C., Vicari, A.P., Sangaletti, S., Trinchieri, G., and Colombo, M.P. (2005) Redirecting *in vivo* elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res* 65: 3437–3446.

Hagemann, T., Robinson, S.C., Schulz, M., Trumper, L., Balkwill, F.R., and Binder, C. (2004) Enhanced invasive-ness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteinases. *Carcinogenesis* 25: 1543–1549.

Hamano, Y., Zeisberg, M., Sugimoto, H., Lively, J.C., Mae-shima, Y., Yang, C., *et al.* (2003) Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. *Cancer Cell* 3: 589–601.

Heppner, G.H., Miller, F.R., and Shekhar, P.M. (2000) Nontransgenic models of breast cancer. *Breast Cancer Res* 2: 331–334.
Hopton Cann, S.A., van Netten, J.P., and van Netten, C. (2003) Dr William Coley and tumour regression: a place in history or in the future. Postgrad Med J 79: 672–680.

Jain, R.K. (1987) Transport of molecules in the tumor interstitium: a review. Cancer Res 47: 3039–3051.

Jain, R.K., and Forbes, N.S. (2001) Can engineered bacteria help control cancer? Proc Natl Acad Sci USA 98: 14748–14750.

Kasinskas, R.W., and Forbes, N.S. (2006) Salmonella typhimurium specifically chemotax and proliferate in heterogeneous tumor tissue in vitro. Biotechnol Bioeng 94: 710–721.

Kelly, P., Moeller, B.J., Juneja, J., Booden, M.A., Der, C.J., Daaka, Y., et al. (2006) The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis. Proc Natl Acad Sci USA 103: 8173–8178.

van Kempen, L.C., de Visser, K.E., and Coussens, L.M. (2006) Inflammation, protesases and cancer. Eur J Cancer 42: 728–734.

Kimura, N.T., Taniguchi, S., Aoki, K., and Baba, T. (1980) Selective localization and growth of Bifidobacterium bifidum in mouse tumors following intravenous administration. Cancer Res 40: 2061–2068.

Leek, R.D., Lewis, C.E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A.L. (1996) Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56: 4625–4629.

Lewis, C., and Murdoch, C. (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 167: 627–635.

Lewis, C.E., and Pollard, J.W. (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66: 605–612.

Li, Y.Y., Feng, Y.Q., Kadokami, T., McTiernan, C.F., Draviam, R., Watkins, S.C., and Feldman, A.M. (2000) Myocardial extracellular matrix remodelling in transgenic mice overexpressing tumor necrosis factor alpha can be modulated by anti-tumor necrosis factor alpha therapy. Proc Natl Acad Sci USA 97: 12746–12751.

Lin, W.J., and Yeh, W.C. (2005) Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. Shock 24: 206–209.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23: 549–555.

Mueller, M.M., and Fusenig, N.E. (2004) Friends or foes – bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4: 839–849.

Murdoch, C., Giannoudis, A., and Lewis, C.E. (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood 104: 2224–2234.

Nakahara, T., Norberg, S.M., Shalinsky, D.R., Hu-Lowe, D.D., and McDonald, D.M. (2006) Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. Cancer Res 66: 1434–1445.

Opdenakker, G., Van den Steen, P.E., and Van Damme, J. (2001) Gelatinase B: a tuner and amplifier of immune functions. Trends Immunol 22: 571–579.

Parks, W.C., Wilson, C.L., and Lopez-Boado, Y.S. (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4: 617–629.

Pawelek, J.M., Low, K.B., and Bermudes, D. (1997) Tumor-targeted Salmonella as a novel anticancer vector. Cancer Res 57: 4537–4544.

Pawelek, J.M., Low, K.B., and Bermudes, D. (2003) Bacteria as tumour-targeting vectors. Lancet Oncol 4: 548–556.

Pollard, J.W. (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4: 71–78.

Price, N.M., Gilman, R.H., Uddin, J., Recavarren, S., and Friedland, J.S. (2003) Unopposed matrix metalloproteinase-9 expression in human tuberculous granuloma and the role of TNF-alpha-dependent monocyte networks. J Immunol 171: 5579–5586.

Rath, P.C., and Aggarwal, B.B. (1999) TNF-induced signaling in apoptosis. J Clin Immunol 19: 350–364.

Russell, D.G. (2007) Who puts the tubercle in tuberculosis? Nat Rev Microbiol 5: 39–47.

Ryan, R.M., Green, J., and Lewis, C.E. (2006) Use of bacteria in anti-cancer therapies. Bioessays 28: 84–94.

Sheu, B.C., Hsu, S.M., Ho, H.N., Lien, H.C., Huang, S.C., and Lin, R.H. (2001) A novel role of metalloproteinase in cancer-mediated immunosuppression. Cancer Res 61: 237–242.

Sorensen, M., Lippuner, C., Kaiser, T., Misslitz, A., Aebscher, T., and Bumann, D. (2003) Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. FEBS Let 552: 110–114.

Stamenkovic, I. (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 200: 448–464.

Stritzker, J., Weibel, S., Hill, P.J., Oelschlaeger, T.A., Goebel, W., and Szalay, A.A. (2007) Tumor-specific colonization, tissue distribution, and gene induction by probiotic Escherichia coli Nissle 1917 in live mice. Int J Med Microbiol 297: 151–162.

Taylor, J.L., Hattle, J.M., Dreitz, S.A., Troud t, J.M., Izzo, L.S., Basaraba, R.J., et al. (2006) Role for matrix metalloproteinase 9 in granuloma formation during pulmonary Mycobacterium tuberculosis infection. Infect Immun 74: 6135–6144.

Trinchieri, G., and Sher, A. (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 7: 179–190.

Wei, M.Q., Ellem, K.A., Dunn, P., West, M.J., Bai, C.X., and Vogelstein, B. (2007) Facultative or obligate anaerobic bacteria have the potential for multimodality therapy of solid tumours. Eur J Cancer 43: 490–496.

Yu, Y.A., Shabahang, S., Timiryasova, T.M., Zhang, Q., Beltz, R., Gentschev, I., et al. (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nat Biotechnol 22: 313–320.

Zaharoff, D.A., Barr, R.C., Li, C.Y., and Yuan, F. (2002) Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. Gene Ther 9: 1286–1290.
Zhao, M., Yang, M., Li, X.M., Jiang, P., Baranov, E., Li, S., et al. (2005) Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing Salmonella typhimurium. Proc Natl Acad Sci USA 102: 755–760.

Supplementary material

The following supplementary material is available for this article online:

**Appendix S1.** Plasmid construction. The luxABCDE-encoding plasmid pUC18-Not-gfpmut3-luxABCDE was provided by P. Hill (University of Nottingham, Nottingham, UK) and used as a template for PCR amplification of the luxABCDE operon using primers containing restriction enzyme sites (forward-Sacl: 5’-CAAGTGAGCTCCAGGAGGACTCTCTATGAAATTTG-3’, reverse-EcoRI: 5’-CTATGGAATTCATATCAACTATCAAACGCTTCGG-3’) for subcloning into pMW-MCS, a derivate of pMW211. The xylA promotors of Bacillus megaterium was amplified from chromosomal DNA (forward-SalI: 5’-GATCTGTCGAC CATTGAAATAACATTTATTTG-3’, reverse-Sacl: 5’-TACATGAGCTCTTAGGAAAGTTATCC-3’) and inserted into pMW-MCS to generate pMW-PxyA. Insertion of SacI/EcoRI-digested luxABCDE operon generating the lux-reporter plasmid designated pMW-PxyluxABCDE.

**Fig. S1.** Monitoring of 4T1 tumour colonization by E. coli K-12 during a period of 14 days. Representative low-light images of a colonized athymic nude mouse (A) and BALB/c mouse (B) bearing 4T1 tumours at the right flank; mice were intravenously injected with $5 \times 10^6$ E. coli K-12 pMW-PxyluxABCDE expressing bacterial luciferase and photons were collected for 1 and 5 min.

**Fig. S2.** E. coli K-12 colonization led to extended tumour necrosis in solid 4T1 tumours. Nuclear Fast Red staining of tumour sections of control (A) and 3-days-colonized (B) 4T1 tumours. Nuclear Fast Red-stained nuclei appeared red; necrotic areas (n) appeared white (karyolysis). Scale bars represent 5 mm.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1462-5822.2008.01122.x

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.