

Turning off methylglyoxal stress: an alternative approach to inhibit MDSC expansion and metastasis in triple-negative breast cancer

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ABSTRACT

Background Metabolic reprogramming through enhanced glycolysis is a hallmark of cancer that supports tumor progression and promotes protumor immune responses. Methylglyoxal (MG), a reactive by-product of glycolysis, has recently emerged as an oncometabolite implicated in cancer progression and therapy resistance. Our previous work demonstrated that an imbalance between MG production and detoxification by the glyoxalase system, referred to as MG stress, contributes to progression and metastatic dissemination in triple-negative breast cancer (TNBC). However, the impact of MG stress on the tumor immune microenvironment remains poorly understood.

Methods Using two preclinical breast cancer models, we investigated the relationship between MG stress and immune modulation, with a focus on granulocytic myeloid-derived suppressor cells (g-MDSCs), major mediators of immune evasion. In silico analyses were performed to assess correlations between MG stress-related gene signatures and transcriptional markers of MDSC infiltration in patients with TNBC, as well as associations with anti-programmed cell death protein 1 (PD-1) immunotherapy response in melanoma cohorts. In vivo experiments combined the MG scavenger carnosine with PD-1 blockade in the immunotherapy-resistant 4T1 breast cancer model.

Results MG stress was associated with the expansion of g-MDSCs in breast cancer models. Importantly, MG stress conferred metastatic potential to non-metastatic 67NR breast tumors, potentially through activation of the Nuclear Factor kappa B (NF-κB) pathway, increased granulocyte-macrophage colony-stimulating factor expression, and systemic expansion of g-MDSCs. In silico analyses revealed a positive correlation between MG stress-related gene signature and transcriptional markers of MDSC infiltration in patients with TNBC. Furthermore, this signature distinguished anti-PD-1 responder (low MG stress), from non-responders (high MG stress) in patients with melanoma. Therapeutically, combined targeting of MG stress with carnosine and PD-1 signaling significantly reduced g-MDSC accumulation in tumors, spleens, and

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Enhanced glycolysis promotes tumor progression and immune evasion. Methylglyoxal (MG), a by-product of glycolysis, induces cellular stress that has been linked to metastasis in triple-negative breast cancer (TNBC), but its impact on the immune microenvironment remains unclear.

WHAT THIS STUDY ADDS

⇒ This study identifies MG stress as a driver of immunosuppression in TNBC through the expansion of granulocytic myeloid-derived suppressor cells (g-MDSCs). MG stress promoted systemic g-MDSC expansion, NF-κB activation, granulocyte-macrophage colony-stimulating factor overexpression and metastatic dissemination in a non-metastatic TNBC model. MG stress gene signature correlated with transcriptional markers of MDSC infiltration in patients with TNBC and discriminated responders from non-responders to anti-programmed cell death protein 1 (PD-1) therapy in patients with melanoma. Combined targeting of MG stress using the MG scavenger carnosine together with PD-1 blockade reduced g-MDSC accumulation and lung metastatic burden in an immunotherapy-resistant TNBC model.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings identify MG stress as a driver of immunosuppression and metastatic progression, the blockade of which could potentially improve immunotherapy efficacy in TNBC.

lungs, and decreased lung metastatic burden in the 4T1 model.

Conclusions These findings identify MG stress as a driver of an immunosuppressive tumor microenvironment that may impair immunotherapy efficacy and promote metastatic progression in TNBC. Dual targeting of MG stress and PD-1 signaling represents a promising

therapeutic strategy to overcome immune suppression and limit metastasis in immunotherapy-resistant breast cancer.

INTRODUCTION

Aberrant metabolism is widely recognized as one of the hallmarks of cancer.¹ Recent studies have highlighted the role of tumor metabolic reprogramming in promoting cellular proliferation, metastatic dissemination, and resistance to therapy. One mechanism through which these processes may occur is the suppression of anti-tumor immune responses. Glycolytic reprogramming, a common feature of cancer cells, has been previously linked to impaired immune surveillance.²

An expanding body of evidence indicates that by-products arising from metabolic pathways contribute to tumor progression and survival. Among these emerging metabolites, methylglyoxal (MG) has recently garnered attention as a bona fide oncometabolite.³ In cells, MG mainly derives from the fourth step of glycolysis as it is produced from the spontaneous degradation of triose phosphate intermediates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.⁴ MG is a highly reactive and cytotoxic dicarbonyl compound that interacts with proteins to form advanced glycation end products (AGEs). MG-AGEs generated through the modification of arginine residues include hydroimidazolones and argpyrimidine adducts, both of which are detectable in cancer cells.⁵ MG is detoxified into D-lactate primarily through the activity of glyoxalase 1 (GLO1), an ubiquitously expressed enzyme in mammalian cells that maintains MG homeostasis and prevents MG-AGEs formation, collectively referred to hereafter as MG stress. Our studies have contributed to demonstrating that MG-derived glycation adducts are present at elevated levels in malignant tumors of the breast,^{6,7} colon,⁸ and pancreas⁹ compared with their respective normal tissues, suggesting that MG-induced stress is a common feature of tumorigenesis. Consistently, we showed that MG-scavenging compounds such as carnosine and aminoguanidine were able to reverse the protumorigenic phenotype induced by MG stress consisting of enhanced metastatic potential⁷ and resistance to therapy.^{8,9}

Although previous studies have established the involvement of MG in tumor progression, its specific effects on the immune landscape of the tumor microenvironment (TME) remain largely unexplored. Elucidating the role of MG in this context may uncover novel mechanisms by which glycolytic tumors establish a protumoral niche and evade immune surveillance. This is particularly pertinent in triple-negative breast cancer (TNBC), an aggressive breast cancer subtype associated with poor clinical outcomes and limited therapeutic options. Once metastasis occurs, patients with TNBC face a median overall survival of only 12–15 months.¹⁰ Like other highly aggressive malignancies, TNBC is characterized by elevated glycolytic activity to sustain its metabolic demands.¹¹ Notably, tumor-derived lactate has been strongly associated with

a heightened risk of metastatic dissemination in a TNBC preclinical model.¹²

Immune checkpoints, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4, are critical regulators of immune responses, essential for preventing excessive immune activation and maintaining self-tolerance.¹³ Tumor cells exploit these pathways to evade immune surveillance. Immune checkpoint inhibitors (ICIs) were initially developed and clinically validated in melanoma, where they have profoundly transformed patient outcomes, and have since been extended to multiple solid tumor types, including breast cancer.¹⁴ In TNBC, ICIs have demonstrated promising clinical efficacy.¹⁵ Pioneering clinical trials have shown encouraging results for immune checkpoint blockade in TNBC, initially in programmed death-ligand 1 (PD-L1)-positive metastatic cases (KEYNOTE-355)¹⁶ and subsequently in high-risk early-stage disease within the neoadjuvant setting (KEYNOTE-522).¹⁷ Although immunotherapy has demonstrated encouraging outcomes in TNBC, its clinical efficacy remains variable, partly due to the immunosuppressive nature of the TME.

The TME is often characterized by a complex network of cellular and molecular components that actively inhibit antitumor immune responses. These include the presence of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages, which secrete immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)- β . These populations of cells are also present in a healthy body, except for MDSCs which emerge predominantly under pathological conditions.¹⁸ Tumor development induces systemic immune alterations with immature neutrophils and monocytes accumulating in blood and immune organs, leading to the formation of MDSCs endowed with immunosuppressive activity.

In this study, in good alignment with recent nomenclature recommendation,^{18–20} MDSCs are classified into CD11b⁺Ly6C^{low}Ly6G⁺ granulocytic MDSCs (g-MDSCs) and CD11b⁺Ly6C⁺Ly6G[−] monocytic MDSCs (m-MDSCs).²¹ Along this phenotypic characterization, the identification of MDSCs requires the demonstration of their immunosuppressive activity.

MDSCs not only infiltrate the primary tumor but also home to distant organs to prepare pre-metastatic niches.^{22–23} Moreover, they inhibit T-cell activation through the production of reactive oxygen species, nitric oxide, and arginase-1, promote Treg cell expansion, and support angiogenesis, thereby being critically involved in the regulation of tumor progression and metastasis and contributing to immune evasion and resistance to immunotherapy.^{24,25}

The inconsistent response to immunotherapy in patients with TNBC is under active investigation. Current challenges include the identification of reliable predictive biomarkers²⁶ and the design of clinical trials to evaluate novel combinations of ICIs²⁷ with the aim of overcoming resistance. However, the interplay between tumor

glycolytic activity and the immunosuppressive microenvironment remains a significant obstacle to achieving effective immunotherapy.²⁸ Building on this problematic and our prior findings highlighting the pro-metastatic role of MG stress, we present a strong rationale to investigate whether MG modulates the immunosuppressive TME and its tight association with the metastatic potential of cancer cells.

In this study, we investigate whether MG-related stress contributes to the systemic accumulation of MDSCs and thereby promotes metastatic progression in preclinical models of breast cancer (transgenic mammary tumor virus (MMTV)-polyoma virus middle T-antigen (PyMT) and 4T1 models) that closely mimic human breast cancer in their growth, immunogenicity, and metastatic characteristics. Using complementary preclinical models and patient-derived datasets, we explore the mechanistic link between MG stress, NF- κ B-driven signaling, and MDSC-associated mediated immunosuppression. Given the variable efficacy of ICIs in TNBC, we further assess whether targeting tumor-associated MG stress can enhance responsiveness to anti-PD-1 therapy. Together, these approaches aim to clarify the role of MG stress in shaping the immunosuppressive TME and to evaluate its potential as a therapeutic target to improve ICI outcomes.

RESULTS

Carnosine treatment decreases tumor-infiltrating immunosuppressive cells in the breast tumor microenvironment

We used the MMTV-PyMT spontaneous mouse model of breast cancer to assess MG-related stress during breast cancer progression and metastasis development. Interestingly, immunohistochemistry (IHC) detection of cytoplasmic (figure 1a) and nuclear (online supplemental figure S1a) MG protein adducts showed their significant increased accumulation from adenoma to late metastatic carcinoma stages. In addition, spontaneous breast lesions exhibited a higher recruitment of CD45 positive immune cells (CD45⁺) in the PyMT model compared with normal adjacent mammary gland tissue (figure 1a).

When 5-week-aged mice were treated with carnosine, a potent MG scavenger, we observed a significant inhibitory effect on the tumor mass (figure 1b) and lung metastatic burden (figure 1c). The detection of MG adducts using western blot showed significant reduction on carnosine treatment in breast tumors from paired treated and untreated mice (figure 1d). On carnosine treatment the number of CD45⁺ cells in the tumors remained unchanged compared with untreated mice (figure 1e). Nevertheless, flow cytometry-based phenotyping of immune cells revealed that carnosine-treated PyMT mice exhibited a significant reduction in the frequency of g-MDSCs, detected as Ly6G^{high} Ly6C^{low}, at the tumor site (figure 1f). The recruitment and expansion of MDSCs is a well-established hallmark of tumor progression. Notably, the proportions of other immune cell subsets

analyzed remained unchanged under carnosine treatment (figure 1f) indicating a specific effect of carnosine on this immunosuppressive cell population. Specifically, the proportion of CD4 Treg immunosuppressive cells remained unchanged under carnosine treatment (online supplemental figure S1b). Overall, these observations highlight a potential role for carnosine in alleviating the immunosuppressive tumor environment driven by MG stress, thereby limiting tumor growth and metastasis in this model.

Methylglyoxal-related stress increases g-MDSCs in 4T1 tumor-bearing mice

To explore the direct effect of MG stress on the immune TME and metastasis development, we generated 4T1 breast cancer cells stably depleted for Glo1, the limiting enzyme of the MG detoxifying system²⁹ to induce an endogenous accumulation of MG.^{7,8} After orthotopic injection of Glo1-depleted 4T1 breast cancer cells in syngeneic BALB/c immunocompetent mice, we validated the efficient knock-down of Glo1 and the accumulation of MG protein adducts in 4T1 tumors depleted for Glo1 (shGlo1) in comparison with control tumors (shnt) (online supplemental figure S1e). While Glo1 depletion in 4T1 cells did not affect tumor growth (figure 1g), it significantly enhanced their metastatic capacity (8 mice out of 10) (figure 1h) and lung tumor burden (figure 1i).

In line with the seminal study of DuPre and Hunter,³⁰ which correlated tumor growth in 4T1 tumor-bearing mice with a prominent expansion of immature granulocytes, our data further support the relevance of this model for studying g-MDSCs accumulation in tumor-bearing mice compared with naive mice (online supplemental figure S1c). We observed a pronounced increase in the proportion of g-MDSCs cells (figure 1k) within the primary tumor starting from the third week post-graft, which persisted into the fourth week at the time of tumor resection. We also monitored m-MDSCs but found no significant changes in their abundance (figure 1k), reinforcing our focus on g-MDSCs in the context of MG stress.

Induction of endogenous MG stress via Glo1 depletion in 4T1 cells led to an increased proportion of tumor-infiltrating granulocytes, as identified by CD11b⁺ Ly6G⁺ Ly6C⁺ double staining, compared with control cells (figure 1l), with no significant change in tumor CD45⁺ cell spatial density (figure 1j). Further Flow cytometry-based phenotyping (FACS) revealed that this increase was specific to g-MDSCs (figure 1m). Indeed, m-MDSCs levels remained unchanged in Glo1-depleted tumors (figure 1m). As expected, g-MDSCs isolated from the lungs and spleens of 4T1 tumor-bearing mice exhibited significant immunosuppressive activity, as demonstrated by their inhibition of CD8⁺ T cell proliferation (online supplemental figure S1d).

Taken together, the findings from carnosine-treated PyMT and MG-stressed 4T1 tumors are complementary to establish a link between MG stress and the selective expansion of g-MDSCs in breast tumors.

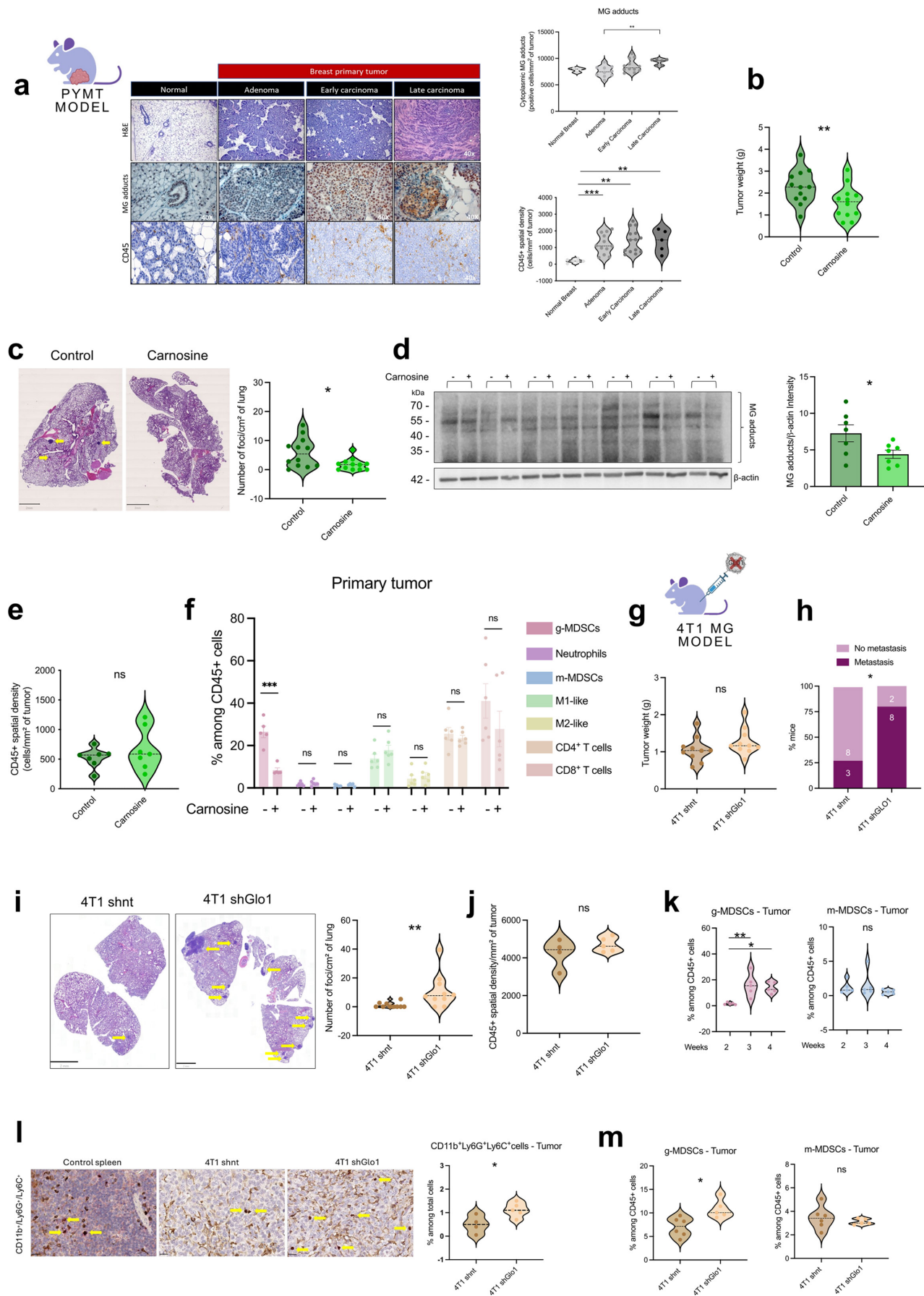


Figure 1 (Continued)

Figure 1 Methylglyoxal-related stress alters tumor immune microenvironment in breast cancer mouse models. (a) Left panel: Representative images of H&E, cytoplasmic MG adducts, and CD45 immunohistochemical staining of MMTV-PyMT tumors, comparing normal mammary gland adjacent to breast lesions (adenoma, early carcinoma, and late carcinoma). Magnification $\times 40$; right panel: Quantification of cytoplasmic MG adducts positive cells and CD45⁺ immune cells per mm² in MMTV-PyMT mice from normal mammary gland (n=3–6), adenoma (n=10–12), early carcinoma (n=12–14), and late carcinoma (n=5–6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (b) Primary tumor weight in control (n=12) and carnosine-treated (n=12) mice. Data were analyzed using a two-tailed paired t-test. (c) Left panel: Representative H&E-stained lung sections from control and carnosine-treated mice. Yellow arrows indicate metastatic foci. Scale bar=2 mm; right panel: Quantification of lung metastatic burden in control (n=12) and carnosine-treated (n=12) mice. Statistical significance was assessed using the Wilcoxon matched pairs signed-rank test. (d) Left: Western blot analysis of MG protein adducts in tumor extracts from control (n=7) and carnosine-treated (n=7) mice; right: Quantification of MG adducts normalized to β -actin expression. Statistical analysis was performed using a two-tailed paired t-test. (e) Quantification of CD45⁺ immune cells per mm² in the tumor of control (n=7) and carnosine-treated (n=7) MMTV-PyMT mice. Statistical analysis was performed using a two-tailed paired t-test. (f) Flow cytometry-based abundance of g-MDSCs, m-MDSCs, neutrophils, M1 macrophages (F4/80⁺CD80⁺), M2 macrophages (F4/80⁺CD206⁺), CD4⁺ T cells, and CD8⁺ T cells in tumors from control (n=5–6) and carnosine-treated (n=5–6) MMTV-PyMT mice. Statistical analysis was performed using a two-tailed paired t-test. (g) Tumor weight comparison between Glo1-depleted 4T1 tumors (shGlo1, n=10) and control 4T1 tumors (shnt, n=10). Data were analyzed using a two-tailed t-test. (h) Lung metastatic incidence in MG-stressed (shGlo1, n=11) versus control (shnt, n=10) 4T1 tumor-bearing mice. Proportions and number of mice with lung lesions are indicated. Metastatic incidence was assessed via H&E staining and compared using Fisher's exact test. (i) Left panel: Representative H&E-stained lung sections from 4T1 shnt and shGlo1 tumor-bearing mice. Yellow arrows indicate metastatic foci. Scale bar=2 mm. Right panel: Quantification of metastatic foci per cm² of lung tissue in 4T1 shnt (n=10) and shGlo1 (n=10) mice. Statistical analysis was performed using a two-tailed Mann-Whitney test. (j) Quantification of CD45⁺ immune cells per mm² in Glo1-depleted (n=10) and control (n=10) 4T1 tumors. Statistical analysis was performed using a two-tailed paired t-test. (k) Flow cytometry-based quantification of g-MDSCs and m-MDSCs in 4T1 primary tumors at 2, 3, or 4 weeks post-grafting (n=5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (l) Left panel: Representative immunohistochemical detection of CD11b⁺Ly6G⁺Ly6C⁺ cells in Glo1-depleted and control 4T1 tumors. Spleen tissue is shown as a positive control; right panel: Quantification of positive cells as a percentage of total cells in Glo1-depleted (n=5) and control (n=4) 4T1 tumors. Statistical analysis was performed using a two-tailed t-test. (m) Flow cytometry-based quantification of g-MDSCs and m-MDSCs in Glo1-depleted (n=6) and control (n=5) 4T1 tumors. Statistical significance was assessed using a two-tailed t-test. Error bars show the SEM. Statistical significance is shown as ns or *p<0.05, **p<0.01, ***p<0.001. The exact p value is indicated when a trend was observed (p value between 0.05 and 0.1). ANOVA, analysis of variance; GLO1, glyoxalase 1; g-MDSC, granulocytic myeloid-derived suppressor cell; MG, methylglyoxal; m-MDSC, monocytic myeloid-derived suppressor cell; MMTV, mammary tumor virus; ns, non-significant; PyMT, polyoma virus middle T-antigen.

Methylglyoxal-related stress increases systemic g-MDSC levels in non-metastatic 67NR model

To study more in depth the connection between MG stress, g-MDSCs and breast cancer metastatic potential, we took advantage of the 67NR cell line, derived from the same spontaneous mouse breast tumor as 4T1 and representing its non-invasive counterpart. First, we evaluated MG stress status in the two cell lines. Consistent with previous comparative studies,³¹ we observed that metastatic 4T1 cells were more glycolytic than non-metastatic 67NR cells (figure 2a). This characteristic of 4T1 cells likely impacted the production of MG, as indicated by the elevated levels of MG detectable in these cells when compared with 67NR isogenic cells (figure 2b).

Beyond their distinct profiles in invasiveness and metastatic potential, 4T1 tumors are notably associated with robust activation of the host immune system, as evidenced by splenomegaly and a pronounced granulocytic reaction.³⁰ In contrast, such immune responses are only modestly observed in the 67NR tumor model. 67NR-bearing mice do not show splenomegaly and fail to develop lung metastases after orthotopic inoculation.³² We generated 67NR clones stably depleted for Glo1 that were injected orthotopically in BALB/c mice. The detection of accumulated MG protein adducts in tumors validated endogenous MG stress on Glo1 depletion (online

supplemental figure S2a). In direct relation to the onset of immune changes, we noticed that mice injected with Glo1-depleted 67NR cells showed a significant increase in spleen mass compared with control mice (figure 2c), while primary tumors were comparable (figure 2d). An increase of g-MDSCs, with no change of m-MDSCs, was measured in the enlarged spleen and in the peripheral blood compartment of mice bearing Glo1-depleted 67NR tumors (figure 2f,h), reminiscent of the granulocytic hyperplasia reported in the 4T1 model³⁰ and in patients with cancer.^{33 34} Under MG stress conditions, the immunoregulatory character of the spleen in tumor-bearing mice was further accentuated by a significant reduction of CD8⁺ lymphocytes (figure 2g), which was also reflected in decreased levels of circulating CD4⁺ and CD8⁺ lymphocytes (figure 2i).

At the tumor site, infiltration of CD45⁺ cells showed no difference between Glo1-depleted and control 67NR conditions (figure 2e). However, under MG stress, a significant increase in g-MDSCs was observed in tumors (figure 2j) supporting the establishment of an immunosuppressive TME. Consistently, mean arginase-1 level in g-MDSCs was considerably increased at the primary site (figure 2k), which was paralleled by a significant decrease of interferon (IFN)- γ expression by CD8⁺ tumor-infiltrating lymphocytes (figure 2m) occurring with no

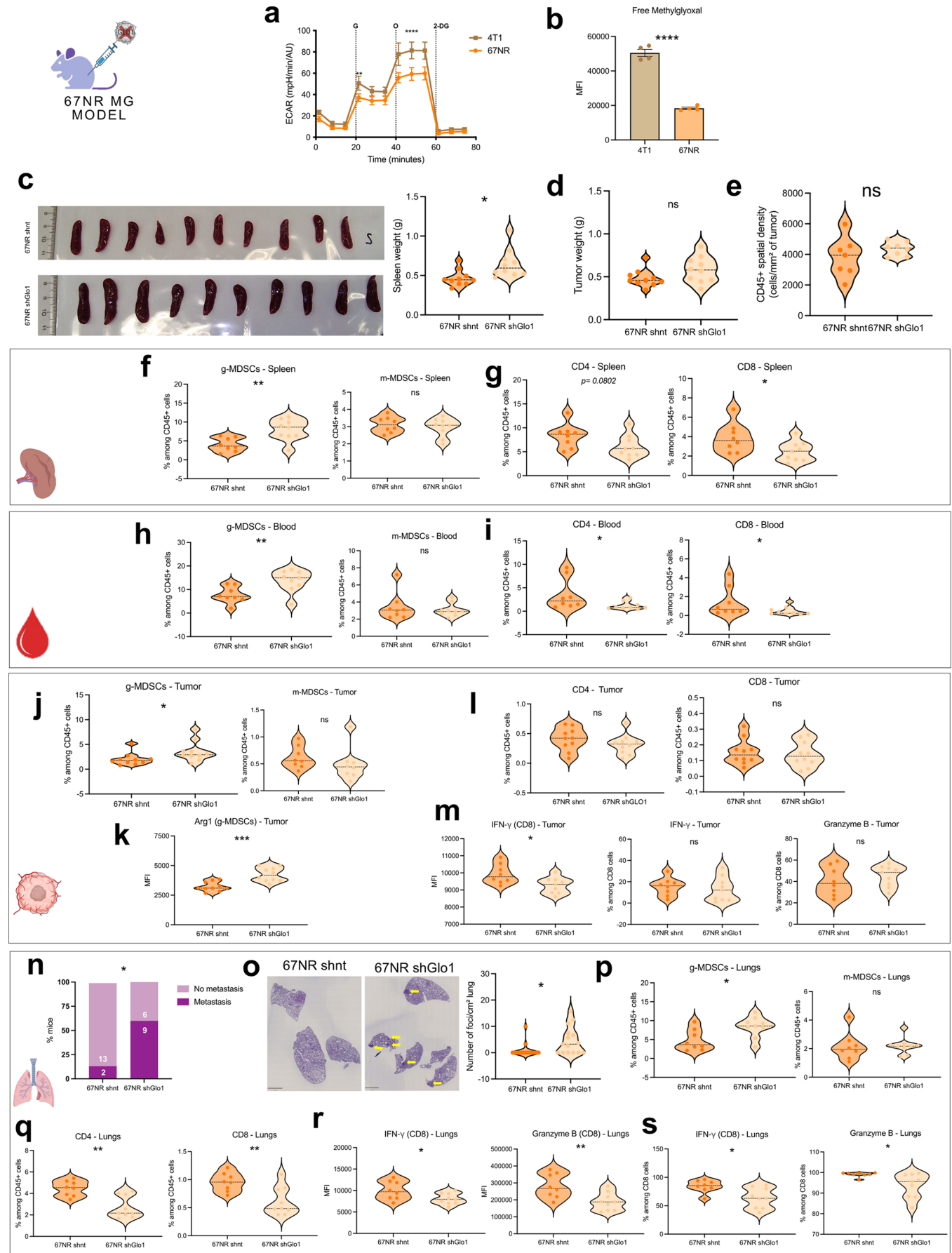


Figure 2 (Continued)

Figure 2 Endogenous MG stress induces an immunosuppressive microenvironment and metastasis formation in 67NR-tumor bearing mice. (a) ECAR measured in 4T1 and 67NR cells using the Seahorse glycolysis stress test and normalized with DNA quantity assessed by Hoechst incorporation. Sequential injections included glucose, oligomycin, and 2-deoxy-glucose. Error bars show the SD, $n=2$. (b) MFI of intracellular free methylglyoxal in 4T1 and 67NR cells, quantified by flow cytometry using MBo fluorescent probe. Error bars show the SD, $n=4$. (c) Left: Images of spleens from mice bearing Glo1-depleted (shGlo1, $n=10$) or control (shnt, $n=10$) 67NR tumors; right: Spleen weights corresponding to the left panel. Statistical analysis was performed using a two-tailed t-test. (d) Tumor weight comparison between Glo1-depleted (shGlo1, $n=10$) and control (shnt, $n=10$) 67NR tumors. Data were analyzed using a two-tailed t-test. (e) Quantification of CD45⁺ immune cells per mm² in Glo1-depleted ($n=10$) and control ($n=10$) 67NR tumors. Statistical analysis was performed using a two-tailed t-test. (f) Abundance of g-MDSCs and m-MDSCs in the spleens of mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (g) Abundance of CD4⁺ and CD8⁺ T cells in the spleens of mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (h) Abundance of circulating g-MDSCs and m-MDSCs in mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (i) Abundance of circulating CD4⁺ and CD8⁺ T lymphocytes in mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (j) Abundance of g-MDSCs and m-MDSCs in Glo1-depleted (shGlo1, $n=10$) and control (shnt, $n=10$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (k) Intracellular arginase-1 expression in g-MDSCs, assessed by flow cytometry as MFI in Glo1-depleted (shGlo1, $n=10$) and control (shnt, $n=10$) 67NR tumors. Statistical significance was calculated using a two-tailed t-test. (l) Abundance of CD4⁺ and CD8⁺ T cells in Glo1-depleted (shGlo1, $n=10$) and control (shnt, $n=10$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (m) Intracellular IFN- γ expression in CD8⁺ T cells, assessed by flow cytometry as median fluorescence intensity (MFI) and as percentage of cells expressing the protein in Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors. Statistical significance was calculated using a two-tailed t-test. (n) Lung metastatic incidence in MG-stressed (shGlo1, $n=15$) versus control (shnt, $n=15$) 67NR tumor-bearing mice. The proportion and number of mice with lung lesions are indicated. Metastatic incidence was assessed by H&E staining and compared using Fisher's exact test. (o) Left panel: Representative H&E-stained lung sections from 67NR shnt and shGlo1 tumor-bearing mice. Yellow arrows indicate metastatic foci. Scale bar=2 mm; right panel: Quantification of metastatic foci per cm² of lung tissue in control (shnt, $n=10$) and Glo1-depleted (shGlo1, $n=10$) mice. Statistical significance was calculated using a two-tailed Mann-Whitney test. (p) Abundance of g-MDSCs and m-MDSCs in the lungs of mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (q) Abundance of CD4⁺ and CD8⁺ T cells in the lungs of mice bearing Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors, assessed by flow cytometry. Statistical significance was calculated using a two-tailed t-test. (r) Intracellular IFN- γ (left) and granzyme B (right) expression in CD8⁺ T lymphocytes, assessed by flow cytometry as MFI in Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors. Statistical significance was calculated using a two-tailed t-test. (s) Intracellular IFN- γ (left) and granzyme B (right) expression in CD8⁺ T lymphocytes, assessed by flow cytometry as percentage of cells expressing the protein in Glo1-depleted (shGlo1, $n=8$) and control (shnt, $n=8$) 67NR tumors. Statistical significance was calculated using a two-tailed t-test. Statistical significance is shown as ns or * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. The exact p value is indicated when a trend was observed (p value between 0.05 and 0.1). ECAR, extracellular acidification rates; g-MDSC, granulocytic myeloid-derived suppressor cell; IFN, interferon; MFI, mean fluorescence intensity; MG, methylglyoxal; m-MDSC, monocytic myeloid-derived suppressor cell; ns, non-significant.

changes in CD4⁺ and CD8⁺ proportions (figure 2l). The proportion of m-MDSCs (figure 2j) and Treg cells (online supplemental figure S2d) was not significantly affected indicating that MG stress impacted the immunosuppressive landscape of the tumor essentially through the accumulation of g-MDSCs.

Methylglyoxal-related stress triggers metastasis in the 67NR non-metastatic model

Having shown that MG stress induces an MDSC-based immunosuppressive environment in the non-metastatic 67NR tumor and given the major contribution of MDSCs to the metastatic potential of breast cancer cells, we reasonably hypothesized that MG stress could promote the acquisition of a metastatic phenotype in 67NR cells. In accordance with this assumption, MG-stressed 67NR tumor-bearing mice exhibited a significantly higher incidence and severity of lung metastatic lesions compared with their control counterparts (figure 2n,o). At the lung metastatic site, we found an elevated proportion of g-MDSCs whereas that of

m-MDSCs remained constant (figure 2p), with no change in total CD45⁺ cell density (online supplemental figure S2b). Remarkably, the exploration of T-cell mediated antitumor immunity showed a significant reduction of CD8⁺ and CD4⁺ T cells in the lungs of mice bearing MG-stressed tumors (figure 2q). Further analysis showed a marked decrease of both IFN- γ and granzyme B, mean levels (figure 2r) and proportion of expressing cells (figure 2s), consistent with reduced CD8⁺ T-cell activity residing in lungs colonized by MG-stressed 67NR cells. Altogether, these data indicate that MG-stressed tumor-bearing mice develop a major imbalance between their immune defense capacity and the immunosuppressive status at both tumor and metastatic sites.

The importance of CD11b⁺Gr1⁺ MDSCs has been thoroughly documented in 4T1 and 67NR models in relation to their metastatic capacity.³⁵ The increase of g-MDSCs at the primary and metastatic sites in Glo1-depleted 67NR cells made us compare their basal level with that of 4T1 metastatic cells. Given the established role of MDSCs in the formation

of the premetastatic niche within the lungs, we initially hypothesized that g-MDSC abundance would be lower in the lungs of 67NR tumor-bearing mice compared with those bearing metastatic 4T1 tumors. However, our data revealed no significant difference in g-MDSC levels between the metastatic and non-metastatic models (online supplemental figure S2c), suggesting that pulmonary g-MDSC abundance was not the primary determinant of acquired metastatic potential in the Glo1-depleted 67NR model. In contrast, at the tumor site, g-MDSC levels were significantly lower in 67NR tumor-bearing mice relative to 4T1-bearing mice but were elevated on induction of MG stress (online supplemental figure S2c). These findings suggest that increased intratumoral g-MDSC infiltration may contribute, at least in part, to the acquisition of metastatic capacity under conditions of MG stress. We next examined whether endogenous MG stress could reprogram the transcriptomic landscape of cancer cells, thereby promoting a transition from low to overt metastatic potential.

Glo1-depleted 67NR non-metastatic model gains metastatic features and cytokine expression profile, through *Tnfα*/NF-κB signaling, that are found in 4T1 metastatic cells

Building on the isogenic background shared by 67NR and 4T1 cells, we sought to investigate transcriptional alterations in MG-stressed 67NR cells that might underlie their enhanced metastatic potential to the lungs. Principal component analysis (PCA) was performed to assess global transcriptomic differences among the cell lines under study. The resulting plot displays distinct clustering along PC1, which likely reflects the variance between 4T1 and 67NR cells (figure 3a). Interestingly, 67NR shGlo1 cells are positioned between 4T1 and 67NR shnt cells, suggesting that Glo1 knockdown may shift the transcriptomic profile of 67NR cells toward a more 4T1-like state (figure 3a). RNA sequencing (RNA-seq) enabled the identification of 5,720 genes differentially expressed between 4T1 and 67NR cells (metastatic vs non-metastatic status), and 3,098 genes differentially expressed between control and Glo1-depleted 67NR cells (basal vs MG-stressed condition). Comparative analysis intersecting the two datasets resulted in 2,336 common genes (online supplemental table S1) of which 2,245 were modulated in the same direction (figure 3b). Common transcriptional changes associated with the transition from a non-metastatic to a metastatic phenotype were most effectively illustrated with a heatmap, which revealed that Glo1-depleted 67NR cells adopted a gene expression profile resembling that of metastatic 4T1 cells (figure 3c). Remarkably, MG stress-related transcriptomic changes occurring in 67NR cells are characterized by the coordinated loss or acquisition of specific genes shifting their transcriptomic profile toward that of 4T1 cells (figure 3c). Genes that were differentially expressed in each dataset were found to be significantly enriched in several hallmark pathways, as revealed by Gene Set Enrichment Analysis (GSEA) (figure 3d). Notably, 20 out of the top 25 hallmark pathways were commonly enriched across both datasets, indicating a

substantial overlap in the underlying biological processes. Signaling cascades mediated by Kras and NF-κB along with EMT were among the processes associated with metastatic progression (figure 3d). Given the ambivalent role of NF-κB in cancer, balancing immune regulation and metastatic promotion, we selected this pathway for further investigation. By integrating transcriptomic data of shnt versus shGlo1 67NR cells using Ingenuity Pathway Analysis (IPA), we constructed a hypothesis-generating regulatory network implicating NF-κB, Glo1, MG oncometabolite and cytokines known to impact MDSCs (figure 3e). Within this network, a subset of genes was identified for further validation in cell lines. Indicative of NF-κB pathway activation under MG stress in 67NR cells, the NF-κB2 gene showed an increased expression that reached the basal level of 4T1 cells (figure 3f). Interestingly, the IPA diagram suggested that tumor necrosis factor (TNF) may serve as a molecular link between MG and NF-κB2 signaling. However, TNF expression remained unchanged in Glo1-depleted 67NR cells (online supplemental figure S3a), indicating that this connection may not be functionally relevant in this cellular context. Next, when we explored NF-κB targets, granulocyte-macrophage colony-stimulating factor (Gm-csf), a key mediator of MDSC expansion in cancer,³⁶ was significantly upregulated in MG-stressed 67NR cells, reaching expression levels similar to those observed in metastatic 4T1 cells (figure 3f). No comparable increase was observed for other key regulators of MDSCs such as granulocyte colony-stimulating factor and Cxcl5 indicating a selective cytokine regulatory mechanism under MG stress in 67NR cells (online supplemental figure S3a). Further interrogation of the NF-κB/MG-associated network in Glo1-depleted cells revealed elevated expression of matrix metalloproteinase-9 (MMP9) that is strongly in support of the acquired metastatic phenotype (figure 3f). In contrast, no significant changes were observed for other NF-κB targets under study such as Vegf and Lcn2 (online supplemental figure S3a), indicating a certain degree of specificity in the transcriptional response to MG stress.

Methylglyoxal signature score correlates with tumor MDSC status and immunosuppressive signature in patients with TNBC

Based on our previously published RNA-seq performed on MDA-MB-231 TNBC cells silenced for GLO1,⁶ we generated a robust MG stress signature as described in Material and methods. This signature was then used to calculate an MG score for each patient that allowed us to segregate The Cancer Genome Atlas (TCGA) (n=195) and METABRIC (n=355) TNBC cohorts into low, intermediate, and high MG score tumors (figure 4a,b). These cohorts showed a significant positive correlation with TNF-α signaling via NF-κB genes, which points to a connection between the activation of this pathway with MG stress in patients with TNBC. Accordingly, MMP9 expression that is mainly regulated by NF-κB correlated positively with MG stress in these tumors (figure 4a,b).

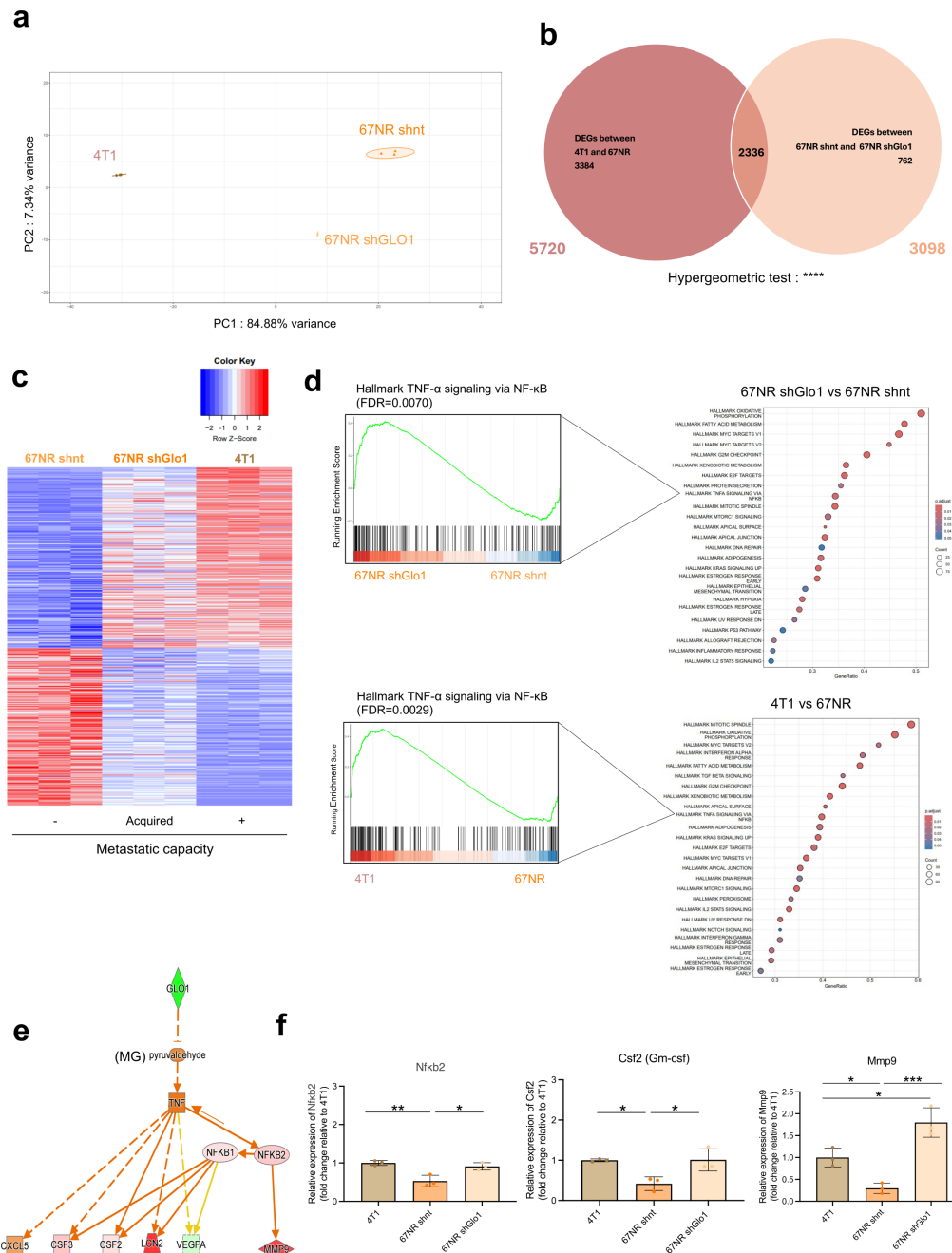


Figure 3 Endogenous MG stress in the 67NR model mirrors features of the metastatic 4T1 model. (a) Principal component analysis of 4T1, 67NR shnt, and 67NR shGlo1 cells based on the top 500 most variable genes from RNA sequencing data. Each point represents an individual sample; ellipses indicate the 95% CI for each group. (b) Venn diagram illustrating DEGs between 4T1 and 67NR cells, and between 67NR shnt and shGlo1 cells, highlighting the shared DEGs. Statistical significance of the overlap was assessed using a hypergeometric test. (c) Heatmap showing transcriptomic profiles based on the shared DEGs between 4T1 versus 67NR and 67NR shGlo1 versus shnt comparisons. (d) Top 25 hallmark pathways significantly enriched in 67NR shGlo1 versus shnt and 4T1 versus 67NR cells, identified by Gene Set Enrichment Analysis. Highlighted is the enrichment plot for the TNF-α signaling via NF-κB pathway. The plot displays the running enrichment score across the ranked gene list, with vertical black lines indicating gene positions. FDR q values are reported. (e) Custom pathway diagram generated using the “My Pathways” tool in IPA, integrating RNA-sequencing data from 67NR shnt and shGlo1 cells with curated molecular interactions. Colors indicate gene expression changes in 67NR shGlo1 versus shnt: red (upregulated), green (downregulated), orange (IPA predicted activation), and yellow (inconsistent with the state of the downstream molecule). Dashed lines indicate indirect relationships and solid lines indicate direct relationships. (f) mRNA expression of selected genes in 67NR shGlo1 and shnt cells, assessed by RT-qPCR and expressed relative to mRNA levels of 4T1 cells. Statistical significance was determined using one-way ANOVA followed by Tukey’s multiple comparisons test. Error bars show the SD. Statistical significance is shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ANOVA, analysis of variance; FDR, false discovery rate; Gm-csf, granulocyte-macrophage colony-stimulating factor; IPA, Ingenuity Pathway Analysis; MG, methylglyoxal; MMP9, matrix metalloproteinase-9; mRNA, messenger RNA; RT-qPCR, reverse-transcription quantitative PCR; TNF, tumor necrosis factor.

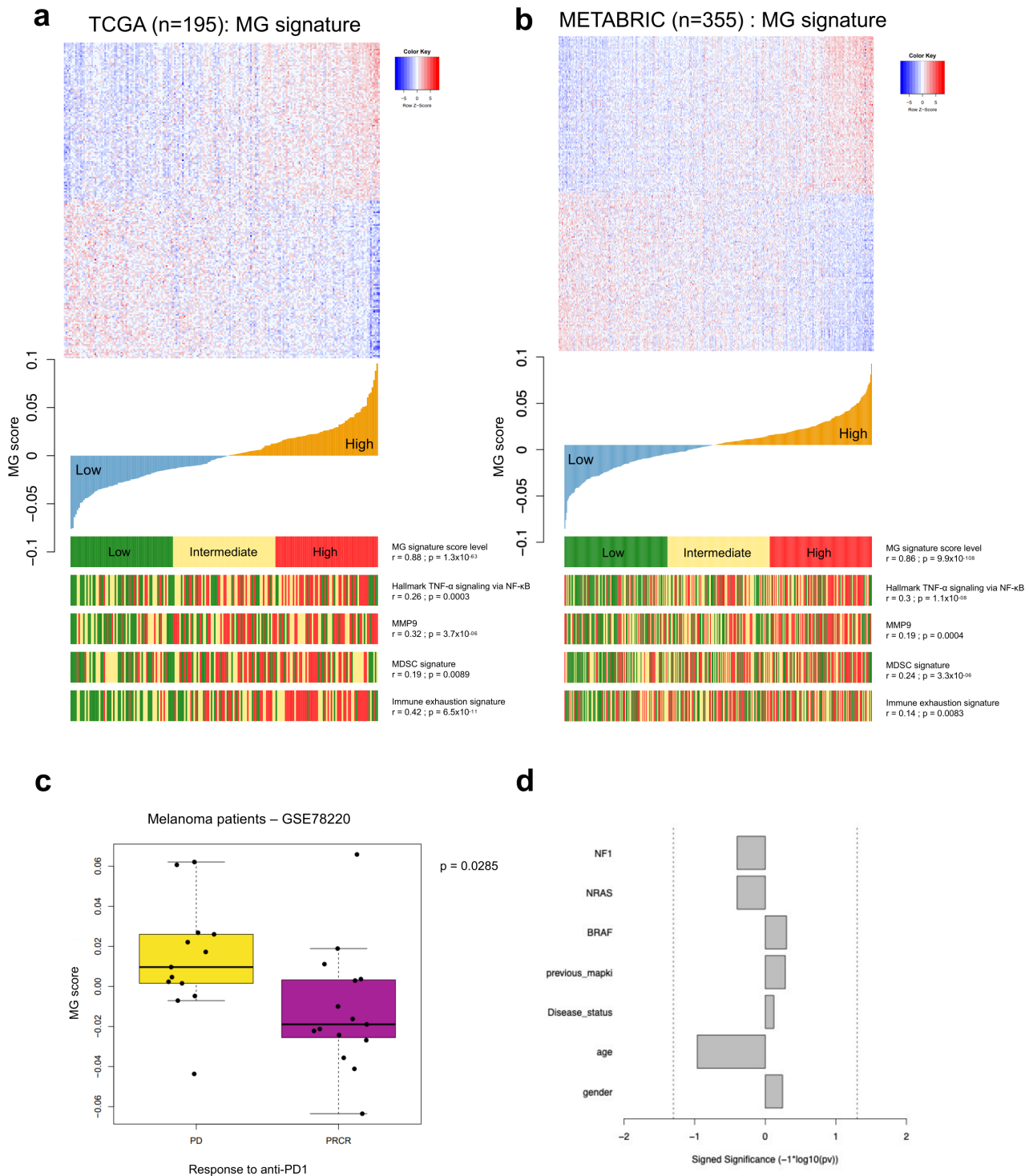


Figure 4 MG stress positively correlates with MDSC-associated features in patients with breast cancer. (a and b) Patients with TNBC from the TCGA and METABRIC cohorts, respectively, were stratified based on their MG signature score. Top panels: Heatmaps displaying the top 100 upregulated and downregulated genes comprising the MG signature across patients in each cohort; middle panels: Waterfall plots ranking patients with TNBC from low to high MG signature scores; bottom panels: Classification of patients into low (green), intermediate (yellow), and high (red) MG score groups, with corresponding Pearson correlation coefficients (r) and p values comparing immune-related features, as indicated, to MG signature scores. (c) Comparison of MG signature scores between patients with melanoma with PD and those with PRCR to anti-PD-1 therapy. Statistical significance was assessed using a two-tailed unpaired t -test. (d) Forest plot showing the differences between patients with PD and PRCR melanoma, analyzed in panel c, in terms of mutational status (NF1, NRAS, BRAF), prior to MAPK inhibitor treatment, disease status, age and gender. Statistical significance was assessed using hypergeometric test or Student's t -test as indicated in the Material and methods section. Vertical dotted lines correspond to significance threshold (p value < 0.05). MDSC, myeloid-derived suppressor cell; MG, methylglyoxal; PD, progressive disease; PD-1, programmed cell death protein 1; PRCR, partial or complete response; TNBC, triple-negative breast cancer.

Next, we used an MDSC-specific gene signature originally developed from a single-cell RNA sequencing performed on MDSCs isolated from the spleen of tumor-bearing PyMT mice and shown to be translatable into the human context.³⁷ We observed a significant positive correlation between this MDSC signature and MG score, suggesting that an MDSC-mediated suppressive tumor environment is associated with MG stress in TNBC tumors (figure 4a,b).

Next, we specifically examined a previously defined 79-gene signature established in TNBC, which enabled the identification of a subgroup of immunosuppressed patients resistant to ICI therapy.³⁸ Interestingly, this molecular signature of the TME correlates positively with MG score in patients with breast cancer, suggesting a link between MG-related stress and the components used to generate this signature, such as the expression of inhibitory checkpoints, high proportion of immunosuppressive cell types and enhanced cytokine expression³⁸ (figure 4a,b). As relevant datasets are not yet available for TNBC, we used a publicly available dataset from metastatic melanoma (GSE78220) to further investigate the potential connection between MG stress and response to anti-PD-1 therapy. In this melanoma dataset, more than 90% (12/13) of non-responder patients were either scored intermediate or high for MG-related stress when compared with patients showing partial or complete response, supporting the correlation between high MG score and resistance to ICI ($p=0.0285$) (figure 4c). Importantly, we have excluded that these data could be explained by differences in clinical and molecular variables between the responder and non-responder groups of patients (figure 4d).

Taken together, these results support the translational relevance of our observations from preclinical models, although larger patient cohorts with documented responses to ICI therapy will be needed to confirm and extend these findings.

Anti-PD-1 and carnosine combined treatment abrogates the metastatic capacity of 4T1 cells

Several studies have reported that 4T1 cells are poorly sensitive to immunotherapies.^{39,40} Combining ICIs with agents targeting MDSCs represents a promising strategy to enhance cancer immunotherapy. To test this approach, we treated 4T1 tumor-bearing mice with anti-PD-1 and carnosine (figure 5a), aiming to neutralize the systemic g-MDSCs increase associated with MG stress. Tumor growth (figure 5b), metastatic incidence (figure 5d) and lung tumor burden (figure 5e) were significantly impacted by the combined treatment, as compared with vehicle (figure 5b). Most remarkably, none of the mice treated with combined anti-PD-1 and carnosine developed lung metastases (figure 5d). Assessment of the combined treatment on the immune TME revealed a significant decrease in g-MDSCs in the lungs and spleen, while only a trend toward reduction was observed at the primary tumor site ($p=0.0789$) (figure 5f, i and l). Total CD45+cells abundance remained unchanged in the

primary (figure 5c) and metastatic lesions (online supplemental figure S4a). Regarding m-MDSCs, the combined treatment had no consistent impact on their abundance (online supplemental figure S4b).

Analysis of T-cell populations following the combined treatment revealed that CD4⁺ and CD8⁺ lymphocyte levels remained stable within both tumor and metastatic sites (figure 5g,j). In contrast, their frequencies were significantly elevated in the spleen, suggesting the onset of immune repopulation and restoration of anti-cancer defense mechanisms in this secondary lymphoid organ (figure 5m). Additionally, we observed a consistent decrease in PD-1 mean expression level in CD8⁺ lymphocytes, denoting an alleviation of their exhausted state⁴¹ at both primary and metastatic sites (figure 5h,k), but not in the spleen (figure 5n). The expression of other activity-associated markers in T lymphocytes did not show a uniform variation across sites and treatment conditions (online supplemental figure S4b).

Overall, this investigation highlights the systemic action of the anti-PD-1 and carnosine combination in reducing g-MDSCs, thereby supporting the observed eradication of lung metastases in the 4T1 model. Consequently, the immunosuppressive activity of g-MDSCs was attenuated, as evidenced by the decreased frequency of exhausted CD8⁺ cells.

DISCUSSION

Glycolytic solid tumors, irrespective of tissue origin, generate a metabolically hostile microenvironment that facilitates immune evasion.⁴² This metabolic reprogramming not only supports rapid tumor proliferation but also contributes to immunosuppression. While competition for glucose and other essential nutrients was initially seen as the main barrier to immune activity,⁴³ recent findings highlight that tumor-derived metabolites, such as lactate⁴⁴ and cholesterol,⁴⁵ can directly suppress both innate and adaptive immune responses, further promoting immune escape. Being a very reactive aldehyde, MG has been previously reported to directly affect immune cells, that is, macrophages and lymphocytes, in the context of infection-induced glycolysis and in tumor-bearing mice.^{46,47}

Here, we show that endogenous MG stress in breast cancer tumors promotes the development of an immunosuppressive microenvironment characterized by the expansion of MDSCs, thereby enhancing the tumors' intrinsic metastatic potential to the lung. The critical involvement of MDSCs during cancer progression has been well established for over a decade. In fact, depletion of circulating g-MDSCs has been shown to nearly abolish tumor formation and mitigate resistance to ICI therapy.⁴⁸ Consistently, elevated levels of g-MDSCs are positively correlated with increased metastatic potential, and co-injection of breast cancer cells with g-MDSCs significantly promotes lung metastasis.⁴⁹

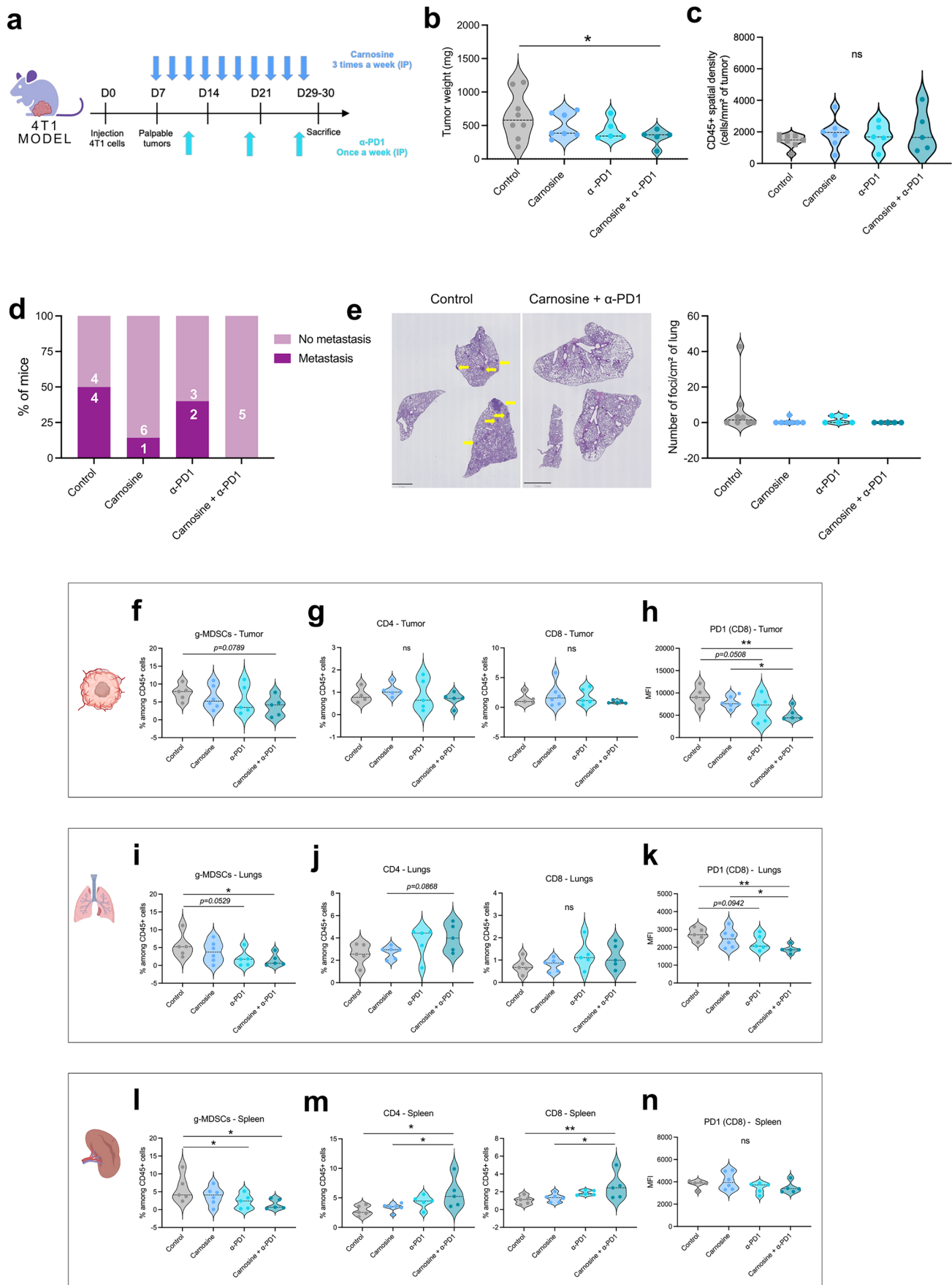


Figure 5 (Continued)

Figure 5 Combination of immunotherapy with methylglyoxal scavenging impedes tumor progression and modulates immune cell populations. (a) Schematic representation of the treatment schedule administered to 4T1 tumor-bearing mice. Tumor cells were implanted on day 0, followed by initiation of treatment at day 7 once tumors were palpable. Mice received vehicle, carnosine, anti-PD-1 (α -PD-1) or carnosine and α -PD-1 at defined intervals. (b) Primary tumor weight of 4T1 tumor-bearing mice treated with vehicle control (n=8), carnosine (n=7), α -PD-1 (n=5), or a combination of carnosine and α -PD-1 (n=5). Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (c) Quantification of CD45⁺ immune cells per mm² in the tumors of control (n=8), carnosine (n=7), α -PD-1 (n=5), and combination-treated (n=5) mice. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (d) Lung metastatic incidence in control (n=8), carnosine-treated (n=7), α -PD-1-treated (n=5), and combination-treated (n=5) mice. The proportion and number of mice with lung lesions are indicated. Metastatic incidence was determined based on H&E staining. (e) Left panel: Representative H&E staining of lung sections from untreated and carnosine+ α -PD-1-treated 4T1 tumor-bearing mice. Yellow arrows indicate metastatic foci. Scale bar=2 mm; right panel: Quantification of metastatic foci per cm² of lung tissue from control (n=8), carnosine (n=7), α -PD-1 (n=5), and combination-treated (n=5) mice. (f) Flow cytometry-based quantification of g-MDSCs in tumors from control (n=8), carnosine-treated (n=7), α -PD-1-treated (n=5), and combination-treated (n=5) mice. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (g) Flow cytometry-based quantification of CD4⁺ and CD8⁺ T cells in tumors from control, carnosine, α -PD-1, and combination-treated mice. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (h) Intracellular expression of PD-1 in CD8⁺ T cells was assessed in tumors from control, carnosine, α -PD-1, and combination-treated mice. Data are presented as MFI. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (i) Quantification of g-MDSCs in lungs from the same treatment groups by flow cytometry. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (j) Quantification of CD4⁺ and CD8⁺ T cells in lungs from the same treatment groups. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD or uncorrected Dunn's multiple comparisons tests. (k) Intracellular expression of PD-1 in CD8⁺ T cells was assessed in lungs from the same treatment groups. Data are presented as MFI. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (l) Quantification of g-MDSCs in spleens from the indicated treatment groups. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (m) Quantification of CD4⁺ and CD8⁺ T cells in spleens from the same treatment groups. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD multiple comparisons test. (n) Intracellular expression of PD-1 in CD8⁺ T cells was assessed in spleens from the same treatment groups. Data are presented as MFI. Statistical significance was assessed using one-way ANOVA followed by Fisher's LSD or uncorrected Dunn's multiple comparisons tests. Statistical significance is shown as ns, *p<0.05 or **p<0.01. The exact p value is indicated when a trend was observed (p value between 0.05 and 0.1). ANOVA, analysis of variance; g-MDSC, granulocytic myeloid-derived suppressor cell; IP, intraperitoneal; LSD, least significant difference; MFI, mean fluorescence intensity; MG, methylglyoxal; ns, non-significant; PD-1, programmed cell death protein 1.

A key finding of our study is incontestably that *Glo1* depletion in 67NR-bearing mice recapitulates several phenotypic features observed in 4T1-bearing mice, including splenomegaly, elevated intratumoral g-MDSC levels, and the acquisition of metastatic capacity to the lungs. *Glo1*-deficient 67NR tumor-bearing mice exhibited a significant increase in g-MDSCs not only at the primary tumor site but also the spleen, peripheral blood, and lungs. These findings strongly support the notion of a systemic expansion of g-MDSCs, whose well-established role in promoting tumor cell invasion and metastatic dissemination aligns with our observations. From the early characterization of the 4T1 and 67NR sister cell lines, it became clear that 67NR cells were unable to leave the primary site as they were not detected in the blood, lymph nodes or lungs, even after extended implantation in the mammary fat pad.⁵⁰ Intravenous injection of 67NR cells results in the development of pulmonary metastases, reinforcing the notion that their failure to metastasize in vivo is primarily due to an impairment at the intravasation stage of the metastatic cascade.⁵¹

The increase of g-MDSCs at the primary and metastatic sites in MG-stressed 67NR tumor-bearing mice made us compare their basal levels in 67NR non-metastatic and 4T1 metastatic tumors. Based on the known role of MDSCs in the preparation of the premetastatic niche in

the lungs, we expected to see a lower g-MDSCs abundance in the lungs of non-metastatic compared with metastatic tumor-bearing mice. However, there was no significant change suggesting that g-MDSCs level in the lungs is not the main alteration associated with acquired metastatic capacity of *Glo1*-depleted 67NR tumors. In contrast, when we considered the tumor site, we observed a significantly lower basal level of g-MDSCs in 67NR compared with 4T1 tumor-bearing mice. Consistently, g-MDSC level was brought to the level of 4T1 metastatic cells in *Glo1*-depleted 67NR tumors. These data suggest that, under MG stress, increased g-MDSCs in the tumor rather than in the lung plays a major role in metastasis formation, likely by increasing cancer cell intravasation capacity. Sustaining this hypothesis, it is worth to consider the link between MDSCs and dysfunctional vasculature with MDSCs releasing VEGF and this latter, in turn, further enhancing MDSC recruitment and activity.⁵² In breast cancer, MDSCs are known to secrete TGF- β , which induces lysyl oxidase expression, thereby increasing extracellular matrix stiffness and facilitating tumor cell movement and vascular infiltration via integrin-mediated signaling.^{53 54} Another mechanism supporting increased local invasive potential is suggested by our observation that non-invasive 67NR cells, when subjected to MG stress, overexpress MMP9 at levels even higher than the basal expression observed

in 4T1 metastatic cells. This key MMP is notably overexpressed in aggressive breast cancer subtypes in patients.⁵⁵

MDSCs suppress T-cell activity through several biochemical mechanisms, especially by producing high levels of arginase, nitric oxide, and reactive oxygen species.⁵⁶ Notably, although Tregs represent a well-established immunosuppressive axis downstream of g-MDSCs, their abundance was not affected by MG stress in our models, suggesting that MG-driven g-MDSC activity operates independently of Treg expansion.

Remarkably, g-MDSCs within MG-stressed tumors exhibited elevated arginase levels, compared with control tumors, indicating an enhanced immunosuppressive capacity. Although the overall number of tumor-infiltrating T cells remained unchanged, the expression of IFN- γ and PD-1 in CD8⁺ cells was significantly reduced, denoting a state of functional exhaustion. Interestingly, the proportions of CD8⁺ and CD4⁺ lymphocytes were markedly diminished in the spleen, peripheral blood, and lungs of MG-stressed 67NR tumor-bearing mice. More critically, both the number and functional activity of T cells, assessed by granzyme B and IFN- γ expression, were reduced in the lung thus signifying the diminished cytotoxic defense at the metastatic site.

Our transcriptomic profiling of MG-stressed 67NR and 4T1 cells revealed a phenotypic convergence driven by shared molecular programs implicated in cancer progression and metastasis. These include KRAS signaling, epithelial-to-mesenchymal transition and the NF- κ B pathway, a key regulator of innate immunity. MG-stressed 67NR cells exhibited elevated expression of the Nf- κ B2 gene. Several downstream targets of the NF- κ B pathway may contribute to the systemic expansion of g-MDSCs observed in Glo1-depleted 67NR tumors. This effect appears to be, at least partially, mediated by Gm-csf, a hematopoietic growth factor upregulated in MG-stressed 67NR cells. In breast cancer, Gm-csf is recognized as one of the key tumor-derived soluble factors driving the differentiation of bone marrow progenitor cells into MDSCs.⁵⁷ While our findings support a model in which MG stress promotes an Nf- κ B-Gm-csf-g-MDSC axis, our data primarily establish associations between these components, and further studies will be required to determine the extent to which each step is mechanistically linked.

We established an MG-associated gene signature derived from Differentially Expressed Genes (DEGs) between MG-stressed and control human TNBC cell lines. Interrogation of patients with TNBC datasets revealed a positive correlation between this signature and activation of the NF- κ B pathway, elevated MMP9 expression, and increased MDSC infiltration, thus providing translational support for our experimental findings.

Beyond TNBC, melanoma represents another tumor type characterized by a predominant reliance on glycolysis. In a cohort of melanoma patients treated with anti-PD-1 therapy, the MG-associated gene signature effectively stratified responders from non-responders, suggesting that MG-related stress may influence ICI responsiveness

outside the breast cancer context. However, given that our experimental work is centered on breast cancer models, these findings should be interpreted as hypothesis-generating rather than evidence of broad pan-cancer applicability. Larger, independent patient cohorts will be required to determine whether this relationship extends beyond TNBC and to establish its clinical significance.

g-MDSCs suppress T-cell activation and proliferation, contributing to resistance against PD-1 inhibitors. MG blockade, using carnosine, combined with anti-PD-1 proved to be efficient at reversing tumor and lung immunosuppressive environment in the 4T1 tumor model that is highly recalcitrant to most therapeutic agents, including immunotherapy.⁵⁸ The effect of this original combination showed striking effects on the metastatic capacity of 4T1 cells resulting in a complete inhibition of lung metastases formation. The marked decrease in g-MDSCs within the lungs was associated with a reduced proportion of exhausted PD1⁺ CD8⁺ T cells. Importantly, the dual targeting of MG stress and PD-1 triggered CD4⁺ and CD8⁺ T-cell populations in the spleen, indicative of an immune overall reorganization that may contribute to enhanced systemic antitumor defense. These observations align with previous studies reporting that PD-1 blockade can reinvigorate progenitor exhausted T cells, particularly when combined with therapies that alleviate immunosuppressive signals. A compelling recent example of such combinatorial strategies is the use of all-trans retinoic acid, which promotes the differentiation of MDSCs into more mature and less immunosuppressive myeloid cells.⁵⁹ Whether carnosine similarly reprograms g-MDSCs toward a non-suppressive phenotype remains an intriguing question for future investigation. Beyond functional reprogramming, additional approaches have been developed to interfere with MDSC biology, including direct depletion and inhibition of trafficking to tumors. Within this broader framework, our findings suggest that MG stress is a mechanistically distinct yet convergent driver of g-MDSC accumulation and activity. Targeting MG stress may therefore complement existing MDSC-directed strategies by reducing the metabolic (ie, Warburg effect) and inflammatory (ie, NF- κ B) signals that sustain MDSC recruitment and suppressive function. However, despite substantial preclinical evidence supporting MDSC-targeting combinations with ICI, clinical validation remains limited, and the translational potential of MG stress modulation will require evaluation in larger patient cohorts.

Targeting the metastatic potential of tumors remains a major challenge in TNBC. Advances in this area are likely to stem from a better understanding of tumor metabolic plasticity associated with the production of oncometabolites. Using relevant preclinical animal models of metastatic breast cancer, we demonstrated that the combined inhibition of immune checkpoints and MG stress effectively mitigates the immunosuppressive TME and restores T-cell functionality. Nevertheless, further investigation is needed to elucidate the mechanisms underlying systemic

MDSC expansion under MG stress condition and, critically, to assess the translational relevance of these findings to human pathology.

MATERIAL AND METHODS

Cell culture and reagents

Murine 4T1 and 67NR breast cancer cell lines were kindly provided by the Laboratory of Experimental Pathology at the University of Liège (Belgium) and authenticated in 2022 by Single Nucleotide Polymorphism (SNP) genotyping. Cells were used within ≤ 10 passages for all experiments and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 2 mM L-glutamine (Lonza), non-essential amino acids, 1 mM sodium pyruvate, and 50 μ M β -mercaptoethanol. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and routinely screened for *Mycoplasma* contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Lentiviral transduction of 4T1 and 67NR cells was performed at the Viral Vectors Platform of the GIGA Institute (University of Liège). Short hairpin RNAs targeting Glo1 (shGlo1) or a non-targeting control against enhanced Green Fluorescent Protein (eGFP) (shnt) were designed by the same platform and sourced from Sigma-Aldrich. Transduced clones were selected using 5 μ g/mL puromycin (P9620, Sigma-Aldrich), and the most efficiently silenced clones were used for subsequent experiments. L-carnosine (C9625) was obtained from Sigma-Aldrich. Anti-PD-1 antibody used for in vivo experiments (InVivoPlus anti-mouse PD-1, CD279, clone RMP1-14) was purchased from Bio X Cell.

In vivo metastatic breast cancer models

All animal experimental procedures were performed in accordance with the guidelines of the Federation of European Laboratory Animal Sciences Associations and were approved by the Institutional Animal Care and Ethics Committee of the University of Liège (#2347). Animals were housed in the GIGA-accredited animal facility (University of Liège) maintained on a 12-hour day/night cycle. Female FVB/N transgenic mice carrying the PyMT transgene under the control of the MMTV promoter were sacrificed from 6 to 15 weeks of age for the progression model (adenoma to late carcinoma). The MMTV-PyMT mice treated with carnosine received intraperitoneal (IP) injection of carnosine (100 mg/kg) three times per week starting at 5 weeks until 13–14 weeks of age.

6-week-old female Balb/c mice from Janvier-Labs were anesthetized with an IP injection of 75 mg/kg of ketamine and 10 mg/kg of xylazine prior to orthotopic injection of 4T1, 4T1 shnt, 4T1 shGlo1, 67NR shnt or 67NR shGlo1 cells (50,000 cells in 50 μ L of phosphate-buffered saline (PBS)) into the right abdominal mammary fat pad. Mice were monitored daily, and body weight was measured three times per week, or daily when a weight loss of 10% was observed. From this point, a well-being score was

assessed every day, including appearance, spontaneous behavior, response to cage handling, and body weight. Mice showing a weight loss of 20% or an elevated well-being score (≥ 6) were euthanized by cervical dislocation. Mice were sacrificed before tumors reached 1,000 mm³, 4 weeks post-implantation (day 28–30 post-implantation). Mice received IP injection of carnosine (100 mg/kg) three times per week and/or IP injection of anti-PD-1 (10 mg/kg) once weekly starting when tumors were palpable (7 days post-implantation of 4T1 cells) and until the end of the experiment (figure 5a). Anti-PD-1 treatment has been previously reported to be fatal to mice-bearing 4T1 tumors at the fourth injection.⁶⁰ Consequently, our treatment strategy was limited to three anti-PD-1 injections. Nevertheless, the third injection caused noticeable discomfort in a subset of mice, necessitating their euthanasia in accordance with ethical guidelines. Tumors, lungs, spleens and blood samples (intracardiac puncture) were collected and processed as described for downstream specific analyses. Tumors were snap-frozen for protein extraction. The number of animals per experimental group is indicated in the figure legends.

Immunohistochemistry analysis

Tumors, lungs and spleens were fixed in 4% formaldehyde (11699408, VWR chemicals) for less than 24 hours, then transferred to 70% ethanol before dehydration and paraffin embedding. Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through successive baths of decreasing alcohol concentrations. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide diluted in methanol for 30 min, followed by a 20-minute rinse in PBS. Antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 40 min. To block non-specific binding, slides were incubated with 1.5% normal goat serum (Vector Laboratories) for 30 min. CD45 antibody (described in online supplemental table S2) incubation was carried out overnight at 4°C. Detection of bound antibodies was achieved using biotinylated secondary antibodies (anti-rabbit; Vector Laboratories) for 30 min at room temperature, followed by incubation with an avidin-biotin-peroxidase complex (Vectastain ABC Kit) for 30 min. Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin, dehydrated, and mounted. Negative controls processed without primary antibodies showed no detectable staining. H&E staining was performed according to standard protocols. Double IHC staining for CD11b and Ly6G was conducted by Histowiz (New York, USA) and antibodies are described in online supplemental table S2.

Evaluation of IHC staining

High-resolution digital scans of tissue sections were analyzed to quantify metastatic foci in lung samples, defined as clusters of at least 10 malignant cells. CD45, MG adducts, CD11b, and Ly6G positive cells were

quantified using automated image analysis with QuPath software (V.0.3.2, open source). Results were normalized to the tissue area and expressed either as the number of positive cells per square millimeter or as the percentage of positive cells relative to the total cell count. Staining was assessed in anatomically comparable regions of the lung or the tumor and statistical analysis using a t-test confirmed no significant differences in the analyzed areas between experimental groups.

Seahorse analysis

The extracellular acidification rate (ECAR) was measured using the Seahorse XFp extracellular flux analyzer (Agilent), following the manufacturer's protocol and as we described previously.^{61 62} A total of 20,000 4T1 and 67NR cells were seeded per well in XFp mini-plates (Agilent) and incubated overnight to allow for attachment. Prior to the assay, cells were equilibrated for 1 hour at 37°C in unbuffered, serum-free Dulbecco's Modified Eagle Medium (DMEM) (Basal DMEM, Agilent) supplemented with 2 mM glutamine, pH 7.4. During the assay, glucose (20 mM), oligomycin (1 µM), and 2-deoxyglucose (50 mM) were sequentially injected to induce metabolic stress. ECAR values were normalized to cell number, which was determined by Hoechst dye incorporation following fixation with cold methanol/acetone.

Protein extraction and western blot analysis

Proteins from cells and tissues were extracted using a 1% SDS buffer containing protease and phosphatase inhibitors (Roche). Protein concentrations were determined via the bicinchoninic acid assay (Pierce). Protein samples were separated using 7.5–12.5% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and transferred onto Polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 0.1% TBS-Tween containing 5% non-fat dry milk (Bio-Rad) and incubated overnight at 4°C with primary antibodies (described in online supplemental table S2). The following day, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody. Detection of immunoreactive bands was performed using the enhanced chemiluminescence western blotting substrate (Pierce). Band intensities were quantified by densitometric analysis using ImageJ software⁶³ and normalized to β-actin levels.

RNA extraction and quantitative reverse transcription PCR

Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized by reverse transcription using the cDNA Synthesis Kit (biotechrabbit). Quantitative PCR was carried out using 25 ng of cDNA mixed with each primer (described in online supplemental table S3) and SYBR Green from Takyon Low ROX SYBR 2X MasterMix blue dTTP (Eurogentec). Amplification was performed using the QuantStudio 3 Real-Time PCR System (Thermo Fisher

Scientific). Relative gene expression levels were normalized to 18S rRNA. All primers were synthesized by Eurogentec.

Intracellular free methylglyoxal quantification

The intracellular free MG was quantified using a fluorescent BODIPY (MBo, Methyl diaminobenzene-BODIPY, exc. 495, em. 521),⁶⁴ as we described previously.⁷ Briefly, cells were incubated with MBo at 37°C for 1 hour and fluorescence was assessed by flow cytometry (CytoFLEX, Beckman Coulter).

Immunophenotyping by flow cytometry

Tumors and lungs were digested with 1 mg/mL of collagenase IA (Sigma C2674) and 0.02 mg/mL of DNase I (Sigma D4513) for 30 min at 37°C, then filtered through a 70 µm cell strainer and resuspended in PBS containing 2 mM of EDTA and 0.5% of Bovine Serum Albumin (BSA). The resulting cell suspension was processed to isolate CD45 positive cells by incubation with the MagniSort Mouse CD45 positive selection kit (Invitrogen). The cell suspension is then placed on LS columns (Miltenyi) on a MACS magnetic separator. After three PBS washes, columns are removed from the device and CD45 enriched suspensions are collected. The spleens were mechanically dissociated between two glass slides to obtain a cell suspension and filtered through a 70 µm cell strainer. Blood was mixed with 500 mM EDTA to prevent clotting, then processed for immunophenotyping. Blood and spleen suspensions were incubated with red blood cell lysis buffer, composed of ammonium chloride (NH₄Cl), sodium bicarbonate (NaHCO₃) and EDTA for 10 min, centrifuged and resuspended in 3% BSA. Cells isolated from all sites were blocked with Fc block CD16/CD32 (BD Biosciences) for 5 min and then incubated in 50 µL of PBS with fluorochrome-conjugated antibodies (described in online supplemental table S2) targeting surface markers corresponding to the immune cell populations of interest (described in online supplemental table S4). For intracellular staining, cells were incubated with a fixation/permeabilization solution (eBioscience FcγR3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent, Invitrogen) for 40 min and labeled in 50 µL of PBS for 40 min for intracellular markers of interest (described in online supplemental tables S2, S4). Finally, cells were fixed with 1% paraformaldehyde (11699408, VWR chemicals) and analyzed with the corresponding gating strategy by flow cytometry (FACSCanto II, BD biosciences or CytoFLEX, Beckman Coulter). Immune cell populations were quantified as a proportion among CD45 positive cells.

g-MDSC T-cell immunosuppression assay

Spleens and lungs were harvested from 4T1 tumor-bearing mice and processed as described above. g-MDSCs were isolated from the lungs and spleens by magnetic enrichment followed by FACS sorting. Briefly, CD11b⁺ myeloid cells were enriched using positive magnetic selection with

CD11b MicroBeads (mouse/human; Miltenyi Biotec). Following enrichment, g-MDSCs were FACS-sorted on a BD FACSAria using the same antibody panel (online supplemental table S4) and gating strategy as described above. CD8⁺ T cells were isolated from spleens by negative selection using the CD8a⁺ T Cell Isolation Kit (130–104-075, Miltenyi Biotec). Purified CD8⁺ T cells were labeled with CellTrace Violet (CTV; Invitrogen) according to the manufacturer's instructions and activated using Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) at a 1:1 bead-to-cell ratio. T cells were cultured alone or in the presence of g-MDSCs (2:1 ratio) in round-bottom 96-well plates in 150 µL of RPMI medium supplemented with 10% FBS (Gibco), 50 U/mL penicillin-streptomycin (Gibco), 1% MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), and 0.05 mM 2-mercaptoethanol (Gibco) at 37°C for 48 hours in a humidified CO₂ incubator. Following culture, T cells were harvested and stained with a viability dye and anti-CD3 antibody prior to acquisition on a BD LSRFortessa flow cytometer. CTV dilution histograms and percentages of proliferating cells were analyzed using FlowJo software.

RNA sequencing and gene expression profiling

Three biological total RNA extracts per cell line were sent to the GIGA-Genomics platform (ULiège) for RNA-seq. RNA-seq was performed using Illumina Stranded mRNA Prep Ligation. Samples were sequenced on the NovaSeq equipment (Illumina), with a sequence coverage goal of 20 Mb 150 bp paired-end reads (median achieved was 23.05M total reads). Raw data processing: Raw reads were demultiplexed and adapter-trimmed using Illumina bcl2fastq conversion software V.2.20 and were processed within our custom Nextflow RNA-seq pipeline inspired by nf-core/rnaseq. TrimGalore V.0.6.1 was used to trim a single base at 5' and 3' ends of each read and to perform a quality-based trimming after removing poly-G tails. Reads were aligned and quantified using STAR V.2.6.1 aligner and Salmon V.1.4.0 quantification, respectively. The alignment and quantification were done to the *Mus musculus* reference genome GRCm39 and the annotations from Ensembl release 112. DE analysis: The normalization of gene expression counts, as well as the identification of differentially expressed genes, was conducted using the R package DESeq2 V.1.48.1, which accounts for library depth by calculating a normalization factor for each sample (<https://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html>).

PCA was applied taking the 500 most variable genes across the samples to confirm stratification in the dataset regarding the groups.

Gene Set Enrichment analysis

Hallmark mouse gene sets were first obtained from mouse MSigDB (<https://www.gsea-msigdb.org/gsea/msigdb/mouse/collections.jsp>). Then, Wald statistic from the DESeq2⁶⁵ analysis comparing 67NT shGlo1 and 67NR shnt or 4T1 and 67NR shnt were extracted and used to

rank mouse genes. “fgsea” (V.1.30.0) implementation of GSEA was applied using the “GSEA” function from “clusterProfiler” package (V.4.12.6)⁶⁶ with the following parameters: nmin=5, nmax=300, p value cut-off=0.2, pAdjustMethod=“BH”, seed=TRUE, by=“fgsea”. The top 25 out of 50 hallmarks pathways are visualized using “dotplot” function from “enrichplot” package (V.1.24) and pathways with adjusted p value<0.05 were kept for further analysis and individual visualization using “gseaplot2” function from “enrichplot” package. Note that the aforementioned R tools were obtained from: <https://packagemanager.posit.co/bioconductor/2024-09-11>

Ingenuity Pathway Analysis

RNA-seq differential expression data were analyzed using QIAGEN IPA (July 2025 release). RNA-seq data were uploaded into the IPA environment and integrated with manually curated information from the scientific literature to custom an interaction network using the “My Pathway” feature.

MG score generation and computation from public databases

Differential analysis of our previously published RNA-seq data on MDA-MB-231 GLO1-depleted cells along with their controls⁶ was done using Sleuth⁶⁷ as described in.⁶⁸ All the genes differentially expressed between the two MDA-MB-231 GLO1-depleted clones and their controls were used as a large MG signature and a weight was associated to each gene to reflect its importance (described in online supplemental table S5). The weighting method is adapted from.⁶⁹ Briefly, the significance (–log₁₀ of the Likelihood Ratio Test (LRT) q value) was multiplied by the Wald beta-value (used as a proxy of the log₂ fold-change). To avoid over-representing the contribution of highly differential genes, the centered-rank of each gene-weight was computed by taking the rank of each gene-weight minus the number of negative weights, which ensures to keep the same sign as the Wald beta-values.

METABRIC and TCGA public data were obtained as described by Bareche *et al.*⁷⁰ For each patient with TNBC, an MG score was generated as the weighted mean expression of MG signature derived from MDA-MB-231 cells. Specifically, the expression of each gene in common with the MG signature was first z-score normalized and then multiplied by its aforementioned gene-weight. Finally, the mean of the weighted normalized expression of each signature-gene has been taken as MG score for each patient with TNBC.

Metastatic melanoma data were obtained from GEO (<https://www.ncbi.nlm.nih.gov/geo/>; GSE78220), response status were obtained from the “series matrix” and stratified as responders (“Partial Response” or “Complete Response”) or non-responder (“Progressive Disease”). RNA sequencing data were retrieved as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) and log₂ transformed after adding one FPKM pseudo count. MG scores were generated as described for METABRIC and TCGA and then stratified between responder

and non-responder patients. A t-test was applied for differential analysis.

Statistical association of GSE78220 covariates with response

Sample description of the GSE78220 cohort was retrieved from the GEO Series Matrix. “gender”, “age”, “Disease_status”, “previous_mapki” and mutation statuses were considered for association with response. Mutation statuses were summarized by three features (ie, presence/absence of BRAF, NRAS or NF1 mutation) while “Disease_status” was converted into a continuous feature (ie, M0=1, M1a=2, M1b=3, M1c=4). For continuous values (ie, “age” and “Disease_status”), t-test was applied comparing “Progressive disease” (PD) and “Partial Response or Complete Response” (PRCR). For binary features (“gender”, “previous_mapki”, “BRAF”, “NRAS” and “NF1” mutations), a hypergeometrical test was applied two times, intersecting the positive category (“F” for “gender”, “Y” for “previous_mapki” and “mutated” for mutation status) with either the PD set or the PRCR set. Then, the smallest p value from those two hypergeometrical tests was kept. For visualization, “signed significance” was computed as $-\log_{10}$ (p value) multiplied by the sign of the t-statistic for the t-test (with PRCR as reference). For the hypergeometrical test, the sign was set as positive if the intersection with PD was more significant and negative otherwise.

Statistical analysis

GraphPad Prism software (V.8.3) was used to perform statistical analysis. Data were checked for normal distribution and if they did not satisfy normality, they were transformed in log or considered not normal. Statistical analysis was performed as indicated in figure legends for each panel. Comparisons between groups were performed using Student’s t-test or one-way analysis of variance (ANOVA). Paired or unpaired Student’s t-tests were performed for parametric data and Mann-Whitney U tests or paired Wilcoxon matched pairs signed-rank tests for non-parametric data. One-way ANOVA was used for parametric data and Kruskal-Wallis test for non-parametric data. Tukey’s, Dunn’s or Fisher’s least significant difference tests were applied for multiple comparisons. Correlations were calculated using the Pearson correlation coefficient for parametric data and Spearman’s rank correlation for non-parametric data. Incidence data were analyzed using Fisher’s exact test. Contingency tables were constructed to compare observed frequencies between groups, and exact p values were computed to assess whether differences in incidence were statistically significant. The hypergeometric test was used to assess the significance of the probability of observing the overlap between the studied set and a reference set, based on the total population size, the number of elements in the reference set, the number of elements in the studied set, and the observed overlap. Exact p values were computed to evaluate whether the observed incidence was greater than expected by chance.

The ROUT method was employed to identify outliers with a $Q=1\%$. A bilateral p value of <0.05 was considered statistically significant with a CI of 95%. Statistical significance is shown as non-significant or significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). The exact p value is indicated when a trend is observed (p value between 0.05 and 0.1). Number of replicates are indicated in figure legends. Mouse in vivo data are presented as mean $\pm$ SEM, whereas in vitro data are reported as mean $\pm$ SD. Error bars represent the corresponding SEM or SD as indicated in the figure legends. Median is shown as a dashed line in violin plots.

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