

## The Sympathetic Nervous System Induces a Metastatic Switch in Primary Breast Cancer

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### Abstract

Metastasis to distant tissues is the chief driver of breast cancer–related mortality, but little is known about the systemic physiologic dynamics that regulate this process. To investigate the role of neuroendocrine activation in cancer progression, we used *in vivo* bioluminescence imaging to track the development of metastasis in an orthotopic mouse model of breast cancer. Stress-induced neuroendocrine activation had a negligible effect on growth of the primary tumor but induced a 30-fold increase in metastasis to distant tissues including the lymph nodes and lung. These effects were mediated by  $\beta$ -adrenergic signaling, which increased the infiltration of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages into primary tumor parenchyma and thereby induced a prometastatic gene expression signature accompanied by indications of M2 macrophage differentiation. Pharmacologic activation of  $\beta$ -adrenergic signaling induced similar effects, and treatment of stressed animals with the  $\beta$ -antagonist propranolol reversed the stress-induced macrophage infiltration and inhibited tumor spread to distant tissues. The effects of stress on distant metastasis were also inhibited by *in vivo* macrophage suppression using the CSF-1 receptor kinase inhibitor GW2580. These findings identify activation of the sympathetic nervous system as a novel neural regulator of breast cancer metastasis and suggest new strategies for antimetastatic therapies that target the  $\beta$ -adrenergic induction of prometastatic gene expression in primary breast cancers. *Cancer Res*; 70(18); 7042–52. ©2010 AACR.

### Introduction

Metastasis is a major factor in the morbidity and mortality of breast cancer. Multiple host cell types contribute to metastasis, and recent research has focused on the host microenvironment as a target for new antimetastatic therapies. Neuroendocrine dynamics have the potential to regulate gene expression in many cell types in the tumor microenvironment (1, 2), but their contribution to breast cancer metastasis remains largely unexplored. Nerve fibers from the sympathetic nervous system (SNS) are present in organs that serve as key targets for breast cancer metastasis, including the lymph nodes, lung, and bone (3, 4). However, little is

known about the effects of SNS neural activation on breast cancer progression and, in particular, its effects on metastasis to distant tissues.

Potential SNS regulation of tumor cell biology is suggested by the presence of  $\beta$ -adrenergic receptors in archival human breast cancer samples (5) and by the clinical relationships between  $\beta$ -blocker usage and reduced cancer risk (6, 7). Physiologic stress activates sympathetic nerve fibers to release micromolar concentrations of the neurotransmitter norepinephrine into the local tissue microenvironment and nanomolar concentrations into the systemic circulation (2, 8, 9). Norepinephrine ligation of cellular  $\beta$ -adrenergic receptors triggers a G-protein–coupled signaling cascade leading to cyclic AMP (cAMP) synthesis, protein kinase A phosphorylation, and transcription factor activation (10, 11). *In vitro* studies have shown that adrenergic signaling can regulate multiple cellular processes involved in cancer progression, including tumor cell proliferation, extracellular matrix invasion, angiogenesis, matrix metalloprotease activation, and expression of inflammatory and chemotactic cytokines (12–14). In a xenograft model of ovarian cancer metastasis, SNS activation by chronic stress increased vascular endothelial growth factor (VEGF)–mediated vascularization of intraperitoneal tumors (2). However, little is known about the effect of chronic stress on the dissemination of metastatic cancer cells from a primary tumor to distant target organs such as the lymph nodes and lung. In the present study, we sought to determine whether experimentally imposed chronic stress

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can influence the rate of distant metastasis from primary breast tumors and define the neuroendocrine, cellular, and molecular mechanisms involved.

## Materials and Methods

### *In vivo* metastasis model

Six-week-old female BALB/c and nu/nu mice (Charles River Laboratories) were housed under BSL2 barrier conditions. 66cl4 mammary adenocarcinoma cells (a gift from Prof. Robin Anderson, Peter MacCallum Cancer Centre, Melbourne, Australia) were transduced with the FUhucW lentiviral vector containing firefly luciferase under the control of the ubiquitin-C promoter (15) and were cultured as previously described (16). Tumor cells ( $1 \times 10^5$ ) were injected into the 4th mammary fat pad for spontaneous metastasis studies or into the tail vein for organ colonization studies. Mammary tumor size was measured using digital calipers and tumor volume was calculated as  $(\text{length} \times \text{width}^2)/2$ . The frequency and quantity of metastases were tracked in live mice by repeated noninvasive optical imaging of tumor-specific luciferase activity using the IVIS 100 system (Caliper). After anesthetization with 2% isoflurane and i.v. injection of 150 mg/kg luciferin, mice were photographed under bright-field illumination and images were overlaid with luminescence data gathered over the maximum exposure period without pixel saturation (0.5–60 seconds). Metastasis was measured by triplicate determination at each time point of total bioluminescence in a region of interest of constant size located distant from the primary tumor (usually the chest region containing the lung and axillary and brachial lymph nodes). Tissue-specific metastasis was measured *ex vivo* by bioluminescence immediately after sacrifice on day 28 or by microscopic evaluation on day 41 in experiments involving removal of the primary tumor. All procedures were carried out under protocols approved by the Institutional Animal Use and Care and University Institutional Review Boards at the University of California Los Angeles. Each experiment was repeated two to four times.

### Chronic stress

Mice were randomly assigned to home cage control conditions or 2 hours per day restraint for 20 days commencing 5 days before tumor cell inoculation or for 14 days commencing 2 days after surgical removal of the primary tumor. Mice were restrained in a confined space that prevented them from moving freely but did not press on them or induce pain (2). This paradigm has been shown to induce chronic stress as shown by neuroendocrine activation (2, 17), weight loss, and anxiety-like behaviors (18, 19) but does not cause pain or wounding (20).

### Pharmacologic studies

For  $\beta$ -adrenergic agonist studies, mice received isoproterenol (Sigma) at 10 mg/kg (a receptor-saturating dose; ref. 2) by daily s.c. injection commencing 5 days before tumor cell inoculation. For  $\beta$ -adrenergic antagonist studies, mice were

implanted intrascapularly with a 21-day-release pellet containing 0.5 mg of propranolol or placebo (Innovative Research of America) 2 days before commencing stress (or control conditions). This dose has been established to block peripheral  $\beta$ -adrenergic receptors (21). For macrophage inhibition studies, mice were treated with 160 mg/kg GW2580 [5-(3-methoxy-4-[(4-methoxybenzyl)oxy]benzyl)-pyrimidine-2,4-diamine; R.I. Chemical] or filtered diluent control (0.5% hydroxypropyl methylcellulose, 0.1% Tween20; Sigma) by daily oral gavage commencing 1 day after tumor cell inoculation (22). This dose was shown to have maximum bioavailability while maintaining substrate specificity (22).

### Determination of cAMP synthesis

66cl4 tumor cells were treated with 0, 0.1, 1.0, or 10  $\mu\text{mol/L}$  norepinephrine  $\pm$  10  $\mu\text{mol/L}$  nadolol, and intracellular cAMP concentration was measured by cAMP-Glo Assay (Promega) according to the manufacturer's instructions.

### Analysis of macrophage infiltration and vascular density

For *in situ* analysis of macrophage infiltration, 5  $\mu\text{m}$  cryosections were fixed in  $-20^\circ\text{C}$  acetone and then incubated with antibodies against F4/80 (clone BM8 at 10  $\mu\text{g/mL}$ , eBioscience) and  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR; H-20 rabbit polyclonal diluted 1:120, Santa Cruz Biotechnology) for 16 hours at  $4^\circ\text{C}$ , followed by incubation with fluorescent Alexa-conjugated secondary antibodies (Invitrogen) and Hoechst 33342 nuclear staining (Sigma). Immunostaining was imaged using an inverted Axio Observer D1 microscope with fluorescence filters (Zeiss), coupled to a computer with Axiovision 4.7 software (Zeiss). The relative frequency of F4/80<sup>+</sup> and  $\beta$ 2AR<sup>+</sup> cells was determined by averaging the pixel density of positive cells in five randomly selected microscope fields in each of five tissue sections from each tumor using ImageJ software (NIH). CD31<sup>+</sup> vascular density was analyzed in tumor cryosections fixed with 4% paraformaldehyde (Sigma), pretreated with proteinase K (Invitrogen) for antigen retrieval, and incubated for 16 hours at  $4^\circ\text{C}$  with 10  $\mu\text{g/mL}$  anti-CD31 (MEC13.3, BD Bioscience) followed by anti-rat Alexa-568 (Invitrogen). Relative vascular density was determined by pixel threshold analysis using ImageJ software as described above.

For flow cytometric analysis of immune cell infiltration, single-cell suspensions were prepared from tumors harvested 28 days after mammary fat pad inoculation and incubated with directly conjugated antibodies against CD45, CD11b, Gr1, F4/80 (eBiosciences), and Ly6C (BD Bioscience) as previously described (22). Samples were analyzed using a LSRII flow cytometer (Beckman Coulter) and FlowJo software (TreeStar).

### Expression of metastasis-related genes

Quantitative real-time reverse transcription-PCR (RT-PCR) was used to quantify *Arg1*, *Csf1*, *Mmp9*, *Ptgs2*, *Tgfb*, *Vcam1*, and *Vegf* mRNA isolated from 5 mg of mammary tumor tissue using RNeasy kit (Qiagen) or CSF-1-differentiated bone marrow-derived macrophages treated with 0, 1, and 10  $\mu\text{mol/L}$  norepinephrine  $\pm$  nadolol. mRNA was assayed

by real-time RT-PCR reactions using gene-specific TaqMan Gene Expression assays (Applied Biosystems) and QuantiTect RT-PCR reagents (Qiagen) with 50 PCR amplification cycles of 15 seconds of strand separation at 95°C and 60 seconds of annealing and extension at 60°C. Triplicate determinations were quantified by threshold cycle analysis of FAM fluorescence intensity using iCycler software (Bio-Rad).

### Statistical analysis

Standard power calculations selected a sample size for *in vivo* experiments that had a power of 0.89 to detect a 1.5-SD effect at  $P < 0.05$  (23). Data are presented as mean  $\pm$  SE, and all statistical analyses were carried out using SAS version 9.2 (SAS Institute). Student's *t* test analyzed the effect of stress on the size and frequency of metastasis and the differences in gene expression and protein levels. To determine the effect of stress on the longitudinal growth trajectory of tumors and whether those effects were modified by pharmacologic interventions that targeted  $\beta$ -adrenoreceptors or macrophages, we examined the stress  $\times$  treatment interaction term in a 2 (control versus stress)  $\times$  2 (treatment versus placebo) experimental design in the context of mixed-effects linear model analysis (SAS PROC MIXED). Spearman's correlation coefficient quantified the relationships between macrophage infiltration and metastatic burden.

## Results

### Chronic stress enhances metastasis to distant tissues

To assess the effect of chronic physiologic stress on breast cancer progression, we used *in vivo* optical imaging to track metastasis of luciferase-tagged 66cl4 breast cancer cells from the mammary gland to distant target tissues (Fig. 1A). This approach can resolve as few as 3,000 cells (Supplementary Fig. S1A). BALB/c mice syngeneic to 66cl4 cells were randomly assigned to 2 hours per day restraint or home cage control conditions for 20 days, starting 5 days before tumor cell inoculation into the 4th mammary fat pad. Physical restraint is a standardized stressor that increases the circulation of catecholaminergic neurotransmitters and corticosterone but avoids physical pain or wounding (2, 17, 18, 20). We have previously documented a 2.5- to 3.5-fold increase in tissue catecholamine levels in stressed animals (2). Physiologic stress response was confirmed by a 6.3% weight loss ( $P = 0.004$ ), which was rapidly reversed at the conclusion of restraint (Supplementary Fig. S1B; ref. 19).

Chronic stress increased the metastasis of primary breast tumor cells to distant tissues by 38-fold versus controls ( $P = 0.04$ ; Fig. 1B). In control mice, luciferase signal from metastatic tumor cells that had disseminated to the chest region (lung and lymph nodes) increased steadily and then reached a plateau at 21 days after tumor cell inoculation (Fig. 1B, inset). Stress increased metastasis in clinically relevant tissues, with a 37-fold increase in the lung ( $P = 0.034$ ) and a 67% increase in the lymph nodes ( $P = 0.009$ ; Fig. 1C). These effects were driven by both increased numbers of metastatic masses [control versus stress:  $1.4 \pm 0.74$  metastatic foci in lung versus  $4.6 \pm 0.75$  ( $P = 0.016$ ), shown by microscopic anal-

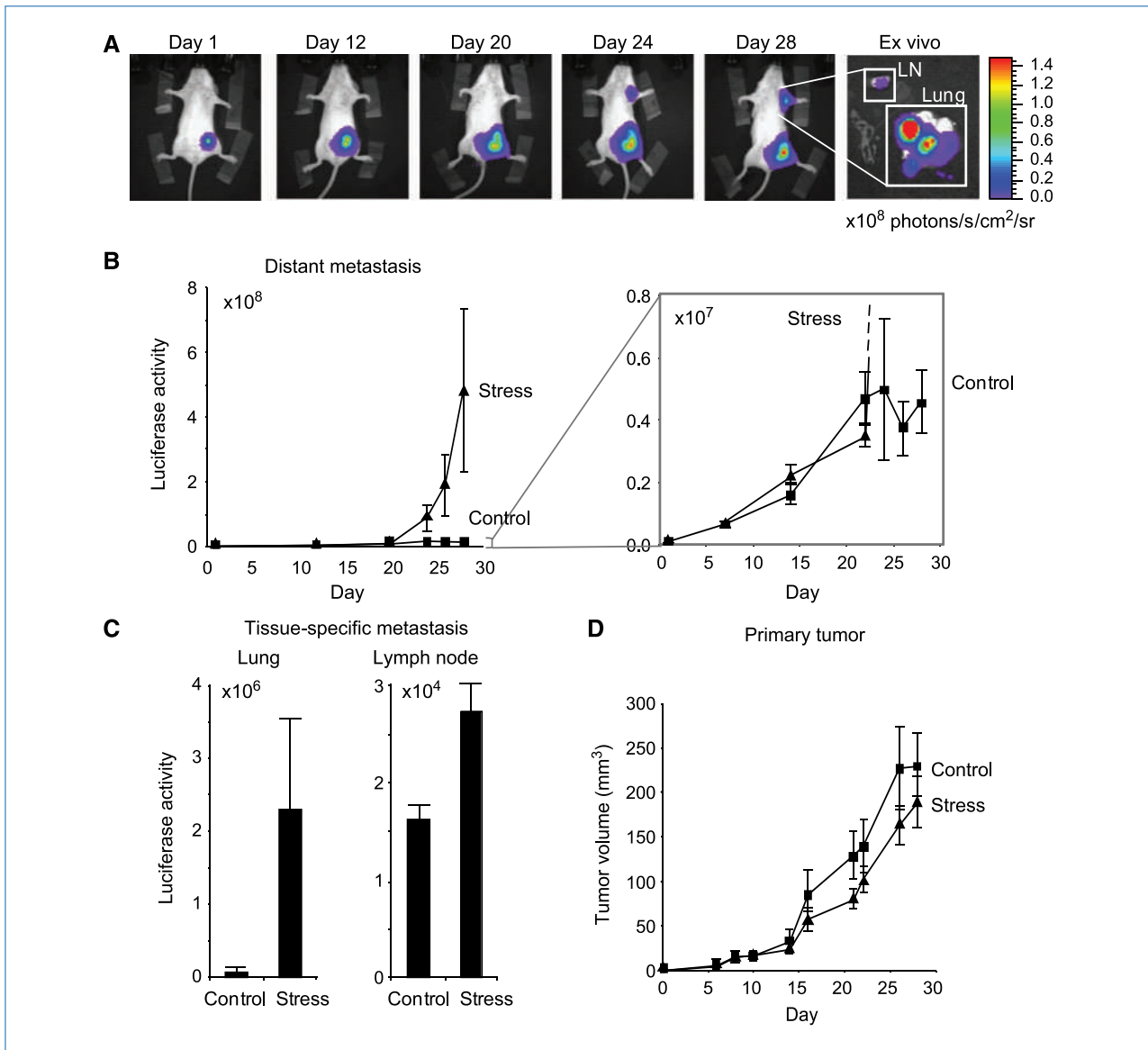
ysis] and increased size of metastatic masses (9.9-fold increase in metastatic luciferase signal,  $P = 0.04$ ). Despite greater rates of metastasis, primary tumor growth rate was not significantly altered by stress (Fig. 1D;  $P = 0.41$ ).

To identify the mechanisms of stress-induced metastatic upregulation, we injected tumor cells into the blood stream and assayed the bioluminescent tumor signal in distant tissues over time. This direct assay of metastatic colonization (which avoids variance in primary tumor escape and intravasation) showed a 3.3-fold increase in colonization rates as a function of stress (Fig. 2A). *Ex vivo* analyses of tissue harvested at 27 days after injection found that stress increased lung tumor colonization by 2.5-fold ( $P = 0.038$ ) and lymph node colonization by 2.1-fold ( $P = 0.037$ ; Fig. 2B). To examine the effect of stress on metastatic development independently of effects on the primary tumor, daily restraint was initiated only after surgical removal of the primary tumor. Results showed that stress increased metastasis by 2.7-fold ( $P = 0.05$ ; Fig. 2C). However, these 2- to 3-fold increases in colonization efficiency were not sufficient in magnitude to account for the total  $\sim$ 30-fold increase in spontaneous metastasis that occurred from primary tumors (Fig. 1B). Additional analyses thus focused on the dynamics within the primary tumor microenvironment occurring before intravasation.

### The SNS regulates distant metastasis

Physiologic stressors activate the SNS to release norepinephrine both systemically and within common metastatic target organs (3, 9). Norepinephrine ligation of  $\beta$ -adrenergic receptors activates the associated cAMP/protein kinase A signaling pathway, and  $\beta$ 2ARs have been documented in both breast cancer cells and immune cells (e.g., T lymphocytes and macrophages) present in the breast cancer microenvironment (5, 9, 24, 25). To investigate the role of SNS activation in stress-enhanced tumor progression, we treated stressed and control mice with the  $\beta$ -adrenergic antagonist propranolol starting 2 days before tumor cell inoculation and continuing throughout the 20-day stress period. Immunostaining against  $\beta$ 2AR showed the expected compensatory increase in  $\beta$ 2AR cell surface expression in tumors from propranolol-treated animals (placebo versus propranolol:  $1.9 \pm 0.1\%$  tissue area versus  $6.7 \pm 1.1\%$ ,  $P < 0.001$ ; see also Supplementary Fig. S3B), confirming functional penetration of  $\beta$ 2AR antagonism throughout the tumor parenchyma (26). Propranolol treatment had no significant effect on metastatic burden in nonstressed control mice ( $P = 0.08$ ; Fig. 3A). However, propranolol completely blocked stress-enhanced metastasis in animals subjected to chronic restraint stress ( $P < 0.0001$ ; Fig. 3A and B). Propranolol treatment had no effect on primary tumor growth in the mammary fat pad for either stressed ( $P = 0.73$ ) or nonstressed control animals ( $P = 0.89$ ).

To determine whether  $\beta$ -adrenergic activation without concomitant activation of other receptor types is sufficient to enhance metastasis, unstressed mice were treated with the  $\beta$ -agonist isoproterenol to pharmacologically activate  $\beta$ 2AR. Isoproterenol increased metastasis to distant tissues by 22-fold compared with saline-treated controls ( $P = 0.03$ ;



**Figure 1.** Effect of chronic stress on breast cancer metastasis to distant tissues. A and B, metastasis was quantified by periodic imaging of tumor-specific bioluminescence signal in the chest region in BALB/c mice (A) and quantified over multiple mice (five per group; B). Inset shows a higher-resolution image of the control condition. Luciferase activity: photons per second. C, tissue-specific metastasis was quantified by *ex vivo* bioluminescent imaging of tumor masses in the lung and lymph nodes (axillary and brachial). D, primary tumor volume was derived from two-dimensional caliper measurements. Data are mean  $\pm$  SE.

Fig. 3C and D). *Ex vivo* analysis of tissues confirmed the increased metastasis (Fig. 3D).

### T lymphocytes are not essential for stress-enhanced metastasis

To determine whether stress-induced alterations in T cell-mediated immune responses might contribute to the metastatic dynamics observed, we repeated the studies in T cell-deficient *nu/nu* mice. Similar to immune-intact BALB/c mice, chronic stress increased the total metastatic mass by 28-fold in *nu/nu* mice ( $P = 0.05$ ; Supplementary Fig. S2A). *Ex vivo* analysis confirmed significant increases in metastasis

to the lung ( $P = 0.03$ ) and lymph nodes ( $P < 0.001$ ; Supplementary Fig. S2B). Again, stress did not alter primary tumor growth (Supplementary Fig. S2C). These results indicate that T lymphocytes do not mediate the observed stress effect, and our subsequent studies thus focused on other cellular mediators.

### Macrophages are an intratumoral target of SNS signaling

To define the cellular pathway by which  $\beta$ -adrenergic signaling might regulate prometastatic signals in primary tumors, we conducted two-color immunofluorescence analyses



of cellular differentiation markers and  $\beta 2AR$  in sectioned primary tumors. Weak  $\beta 2AR$  staining was observed in primary tumor cells (Fig. 4A, i). To verify the functional activity of these receptors, we treated 66cl4 cells with norepinephrine and assayed the activity of the second messenger molecule cAMP. Results showed a significant cAMP flux in response to  $\beta 2AR$  ligation, which was inhibited by the  $\beta$ -blocker nadolol (Supplementary Fig. S3), consistent with other studies documenting catecholamine regulation of breast cancer cell functional activity (14). Within the tumor parenchyma, we also observed a strong colocalization of  $\beta 2AR$  staining with the macrophage marker F4/80 (Fig. 4A, overlay).  $\beta 2AR$  activation is known to functionally regulate gene expression in macrophages (9, 27–29), raising the possibility that SNS activity may regulate primary tumor dynamics by recruiting or modifying the activity of tumor-associated macrophages.

### Stress increases tumor infiltration by macrophages

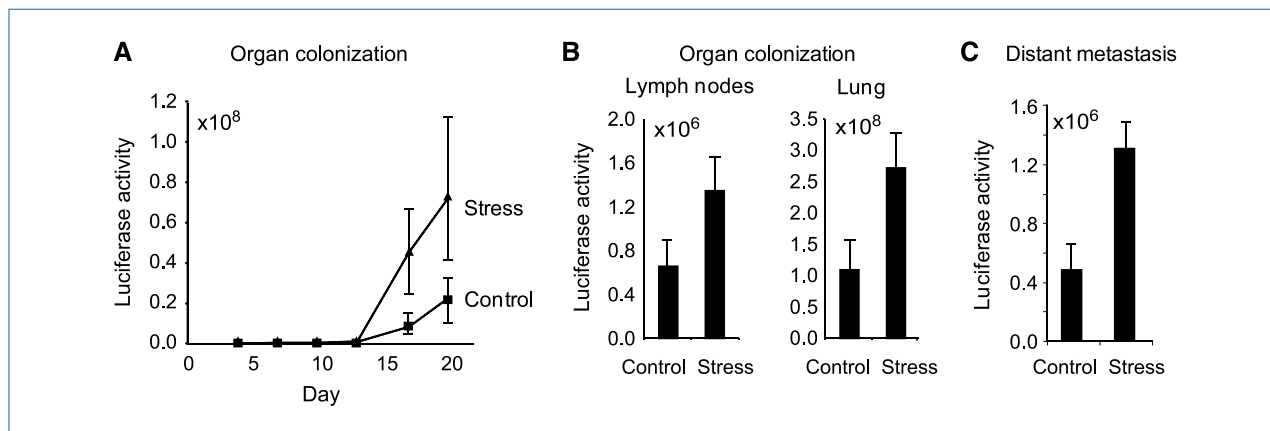
To determine whether chronic stress affects macrophage infiltration into primary mammary tumors, we used flow cytometry to quantify cell composition in digested 66cl4 primary tumors harvested from mammary fat pads (Fig. 4B). Stress increased the mammary tumor infiltration of  $CD11b^+F4/80^+$  macrophages by 53% (control versus stress:  $7.23 \pm 0.49\%$  of live cells versus  $11.04 \pm 1.22\%$ ,  $P = 0.013$ ; Fig. 4B). Stress also marginally increased the tumor infiltration of  $CD11b^+Gr^loLy6C^hi$  cells (myeloid-derived suppressor cells; ref. 22; 43% increase,  $P = 0.073$ ). Stress-induced recruitment of immune cells was specific to macrophages, as no significant increase was observed for other myeloid cell types such as  $CD11b^+Gr^hiLy6C^lo$  polymorphonuclear neutrophil-like suppressor cells ( $P = 0.76$ ; ref. 22) or  $CD45^+CD11b^-$  lymphocytes ( $P = 0.15$ ).

*In situ* analysis of F4/80<sup>+</sup> immunostaining confirmed a 47% increase in macrophage recruitment within primary tumors from stressed animals ( $P < 0.006$ ; Fig. 4A). Isoproterenol stimulation of  $\beta$ -adrenergic signaling similarly increased macrophage infiltration into primary tumor parenchyma (saline

versus isoproterenol:  $7.12 \pm 1.0\%$  tissue area versus  $20.2 \pm 2.2\%$ ,  $P < 0.001$ ; Fig. 4C; Supplementary Fig. S4). To confirm that the stress-induced infiltration of macrophages was mediated by SNS activation, we mapped the distribution of F4/80<sup>+</sup> cells in day 28 tumor parenchyma from stressed animals treated with the  $\beta$ -adrenergic antagonist propranolol (Fig. 4D; Supplementary Fig. S4). As shown in Fig. 4D, propranolol largely abrogated the stress-induced increase in F4/80<sup>+</sup> cells within the primary tumor.

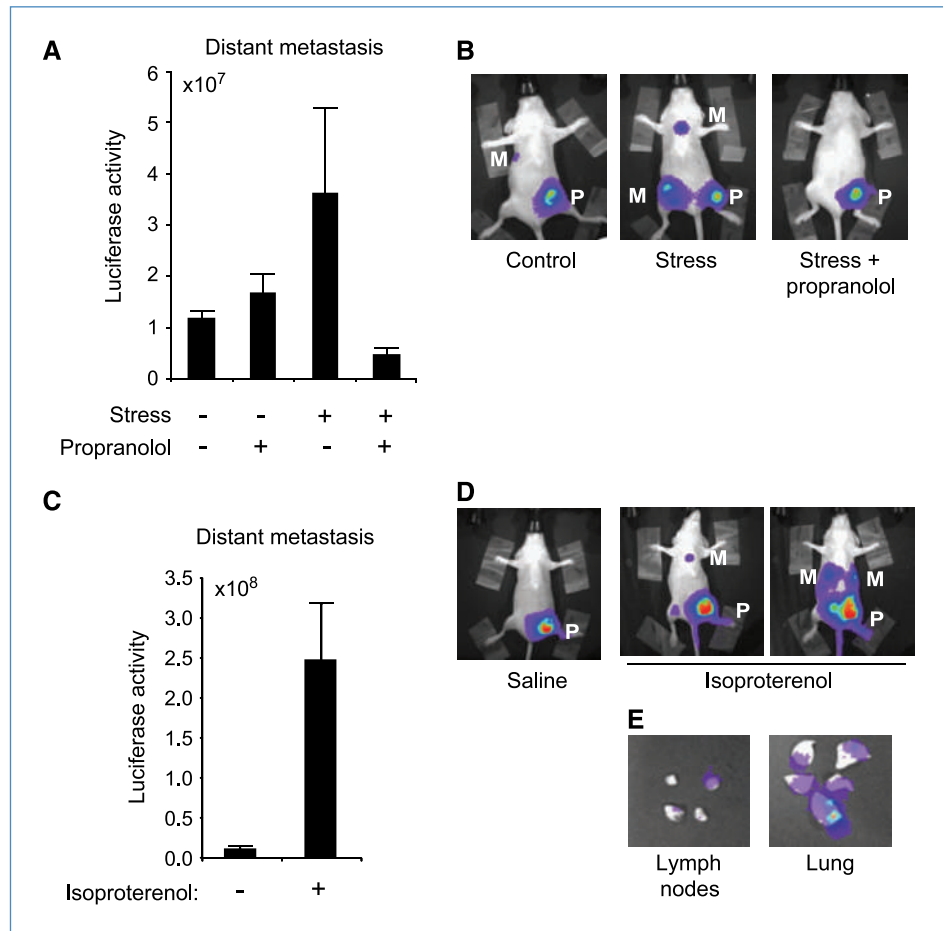
### Stress induces the expression of macrophage-derived prometastatic molecules

Previous studies indicate that macrophages contribute to breast cancer metastasis by expressing prometastatic genes within the tumor microenvironment (30). Myeloid cells including tumor-associated macrophages may also develop an immunosuppressive phenotype, mediated in part through local transforming growth factor- $\beta$  activity and characterized by arginase-1 production (30). Consistent with this dynamics, stress induced a 5.3-fold increase in *Tgfb* gene expression ( $P < 0.001$ ) and a 3.9-fold increase in *Arg1* expression ( $P < 0.001$ ) in primary tumors (Fig. 5A). In contrast, stress reduced *Ifnb* gene expression by 3.3-fold, consistent with a protective role for type I IFNs in cancer progression (31). Stress also increased the expression of proinflammatory and prometastatic genes including *Cox2* (*Ptgs2*; 3.0-fold increase,  $P < 0.001$ ), *Mmp9* (54% increase,  $P = 0.02$ ), *Vegf* (30% increase,  $P = 0.046$ ), and *Vcam1* (9.4-fold increase,  $P < 0.001$ ; Fig. 5A), as well as the macrophage chemoattractant and growth factor *Csfl* (25% increase,  $P = 0.02$ ; Fig. 5A). To investigate the effect of adrenergic signaling on macrophage phenotype, bone marrow-derived macrophages were treated with norepinephrine and gene expression was assayed by quantitative RT-PCR 6 hours later. Adrenergic signaling increased the expression of genes characteristic of an M2 phenotype (*Arg1*: 24-fold,  $P < 0.001$  and *Tgfb*: 2.5-fold,  $P = 0.02$ ) and decreased the expression of the key M1 gene *Nos2* (44%



**Figure 2.** Effect of stress on colonization of metastatic target tissues. A, organ colonization by  $1.0 \times 10^5$  i.v. injected 66cl4 tumor cells was quantified by repeated imaging of tumor-specific bioluminescence signal in the chest region. B, tissue-specific colonization was quantified by *ex vivo* imaging of lymph nodes and lung on day 27 after i.v. inoculation of tumor cells. C, distant metastasis was quantified by bioluminescence imaging at day 41 after surgical removal of primary tumors on day 14. Stress was commenced only after surgical removal of primary tumors. Data are mean  $\pm$  SE.

**Figure 3.** Role of the SNS in stress-induced metastasis. A, distant metastasis was quantified in the lung and lymph node regions by *in vivo* imaging at day 28 after tumor cell inoculation into stressed versus control mice treated with propranolol or placebo. B, representative images of mice in the control (+ placebo), stress (+ placebo), and stress +  $\beta$ -blocker groups. C, distant metastasis was quantified 28 d after primary tumor inoculation in mice treated with saline versus isoproterenol. D and E, representative *in vivo* images of saline- and isoproterenol-treated mice (D) and *ex vivo* lung and lymph nodes showing tumor-specific bioluminescent signal ( $\times 10^6$  photons/s/cm<sup>2</sup>/sr; E). P, primary tumor; M, distant metastasis.



decrease,  $P = 0.05$ ). Consistent with transcriptional alternations in whole mammary tumors (Fig. 5A), norepinephrine also increased macrophage expression of *Vegf* (3.2-fold,  $P = 0.003$ ) and *Mmp9* (3.0-fold,  $P = 0.04$ ). To determine if increased *Vegf* expression was sufficient to modulate angiogenesis *in vivo*, we mapped primary tumor blood vessel density through CD31 immunostaining and found a 2.8-fold increase in stressed animals ( $P < 0.001$ ; Fig. 5B and C). Consistent with the SNS regulation of angiogenesis (2), these studies showed that propranolol inhibited the stress-induced increase in vascularization of mammary tumors ( $P < 0.001$ ).

#### Macrophage infiltration mediates stress-enhanced metastasis

To determine whether SNS-mediated macrophage recruitment might contribute to metastatic spread, we examined the relationship between the extent of F4/80<sup>+</sup> macrophage infiltration at 4 weeks and the total cellular burden of distant metastasis at 4 weeks. This correlation was highly significant in stressed mice (Spearman correlation coefficient,  $r = 0.66$ ,  $P = 0.007$ ) but not in controls ( $r = -0.12$ ,  $P = 0.67$ ). To confirm that macrophage recruitment functionally mediated stress effects on metastasis, we used a CSF-1 receptor antagonist to suppress macrophage recruitment and function *in vivo*.

After random assignment to control or stress conditions, 50% of mice in each condition received either 160 mg/kg of GW2580 (a small-molecule inhibitor of CSF-1 receptor kinase) or placebo (22). Treatment with GW2580 largely reversed stress-enhanced metastasis (Fig. 6A). Longitudinal bioluminescent imaging and *ex vivo* analyses found little colonization of distant tissue in stressed mice treated with GW2580, whereas stressed mice treated with placebo continued to show increased rates of metastasis to distant target tissues (Fig. 6A and B). Flow cytometric analysis of primary tumors showed that treatment with GW2580 reduced the infiltration of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages by 2.9-fold (placebo versus stress:  $7.8 \times 10^3 \pm 0.8 \times 10^3$  CD11b<sup>+</sup>F4/80<sup>+</sup> cell/mg tissue versus  $2.7 \times 10^3 \pm 0.35 \times 10^3$ ,  $P < 0.001$ ) and thereby blocked the stress-induced increase in macrophage recruitment (Fig. 6C and D, middle). GW2580-treated mice also showed reduced CD11b<sup>+</sup>F4/80<sup>+</sup> cells in the bone marrow (45% decrease,  $P = 0.05$ ), as expected, by CSF-1 receptor inhibition. Consistent with the reduced myeloid cell infiltration of mammary tumors, GW2580 largely reversed the stress-induced upregulation of *Arg1* gene expression (Supplementary Fig. S5). Inhibition of macrophage infiltration by GW2580 had no effect on primary tumor growth (mean volume at week 4: 395 mm<sup>3</sup> in stressed animals treated with placebo

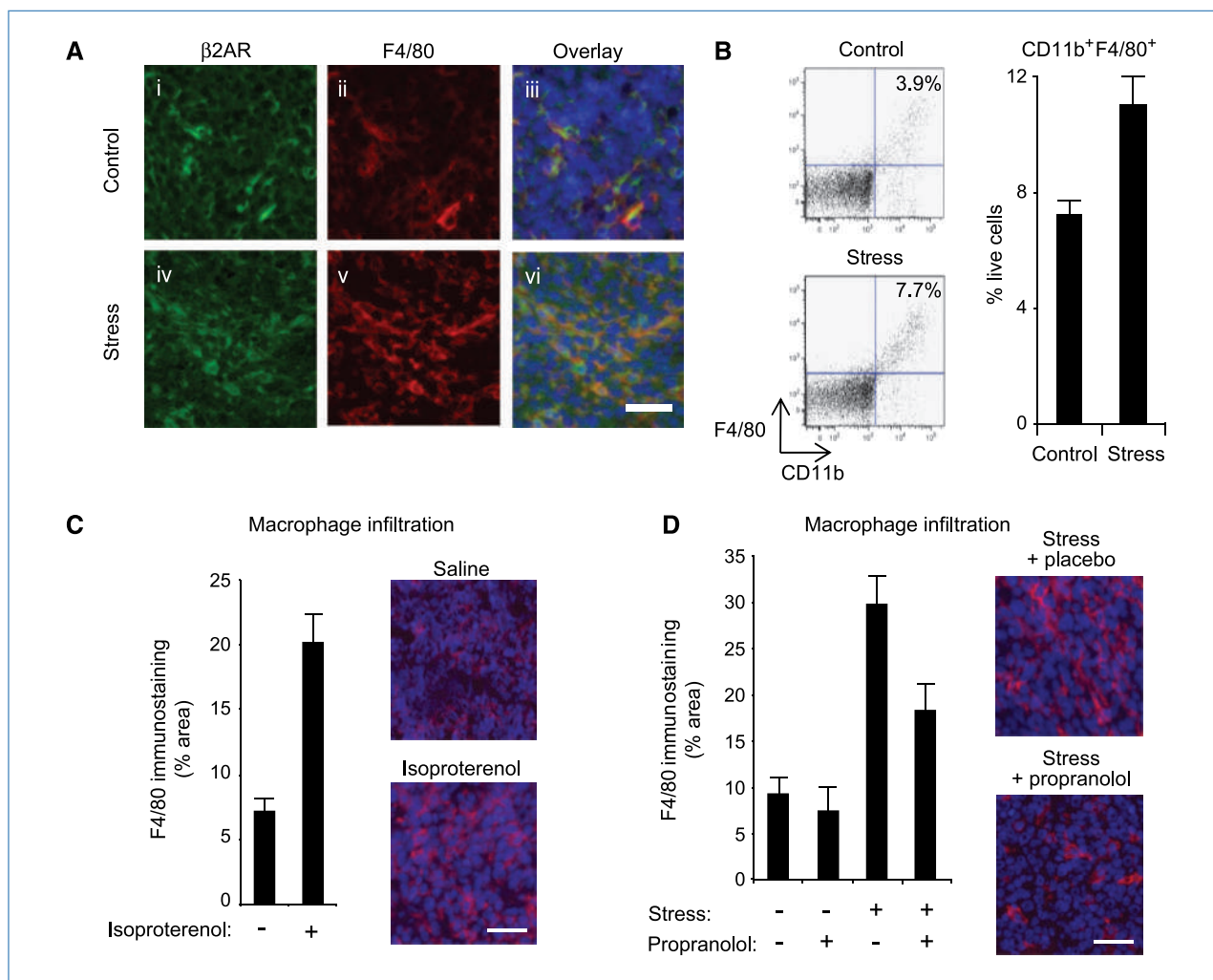
versus 397 mm<sup>3</sup> in stressed animals treated with GW2580,  $P = 0.88$ ). However, analyses of CD31 expression showed that GW2580 reversed the stress enhancement of blood vessel density (Fig. 6D, bottom), which indicates a central role for macrophages in the SNS regulation of vascularization.

## Discussion

These studies identify SNS activation as a novel physiologic regulator of breast cancer metastasis to distant tissue sites including the lymph nodes and lung. SNS effects were mediated through  $\beta$ -adrenergic signaling, which acted to recruit alternatively activated macrophages into the primary tumor parenchyma and thereby induce a prometastatic gene expression signature. These effects occurred in syngeneic tumors in immunocompetent mice, as well as in mice lacking a func-

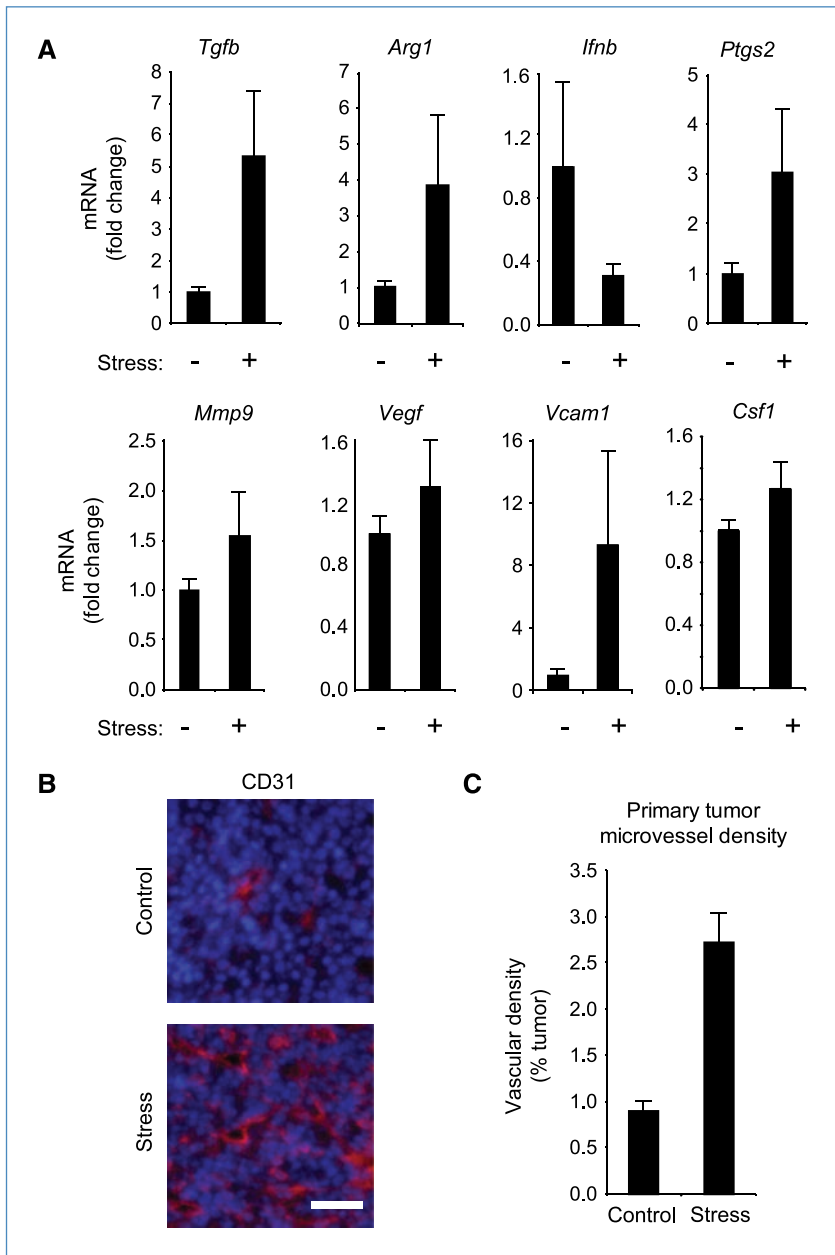
tional T cell compartment. Thus, direct regulation of macrophage biology by the SNS seems to constitute a previously unrecognized pathway by which external conditions affecting the autonomic nervous system can activate a metastatic switch within a growing primary tumor. These findings expand our understanding of the physiologic processes that regulate breast cancer metastasis and suggest novel strategies for inhibiting metastatic spread through targeted inhibition of SNS-regulated macrophage dynamics.

The current findings confirm previous indications that macrophage infiltration can influence breast cancer metastasis (30), and extend those findings to identify a novel SNS/ $\beta$ -adrenergic signaling pathway that can drive changes in macrophage recruitment and differentiation and thereby alter gene expression within the primary tumor. SNS regulation of prometastatic macrophage dynamics provides an alternative mechanism to Th2 lymphocyte regulation of tumor-associated



**Figure 4.** Role of macrophage infiltration. A, mammary tumor cryosections from control and stressed mice were immunostained with anti- $\beta$ 2AR (green; i and iv) and anti-F4/80 (red; ii and v) and nuclear counterstained with Hoechst 33324 (blue; iii and vi). B, CD11b<sup>+</sup>F4/80<sup>+</sup> cells were quantified by flow cytometry in disaggregated 66cl4 primary tumors harvested 28 d after inoculation. C and D, macrophage infiltration of mammary tumors was visualized by immunostaining with anti-F4/80 (red) and nuclear counterstaining (blue) of tumors harvested at day 28 from saline- or isoproterenol-treated mice (C) or stressed mice treated with placebo versus propranolol (D), and quantified across sections from multiple mice ( $n = 5$  per group). Bar, 30  $\mu$ m.

**Figure 5.** Effect of stress on prometastatic gene expression and primary tumor vascularization. A, gene expression was quantified by RT-PCR in mammary tumors from control versus stressed mice ( $n = 5$  per group). B, mammary tumor cryosections were immunostained with anti-CD31 antibody (red) and nuclear counterstained (blue). Bar, 30  $\mu\text{m}$ . C, CD31<sup>+</sup> blood vessel density was quantified in five sections from multiple tumors ( $n = 5$  per group).

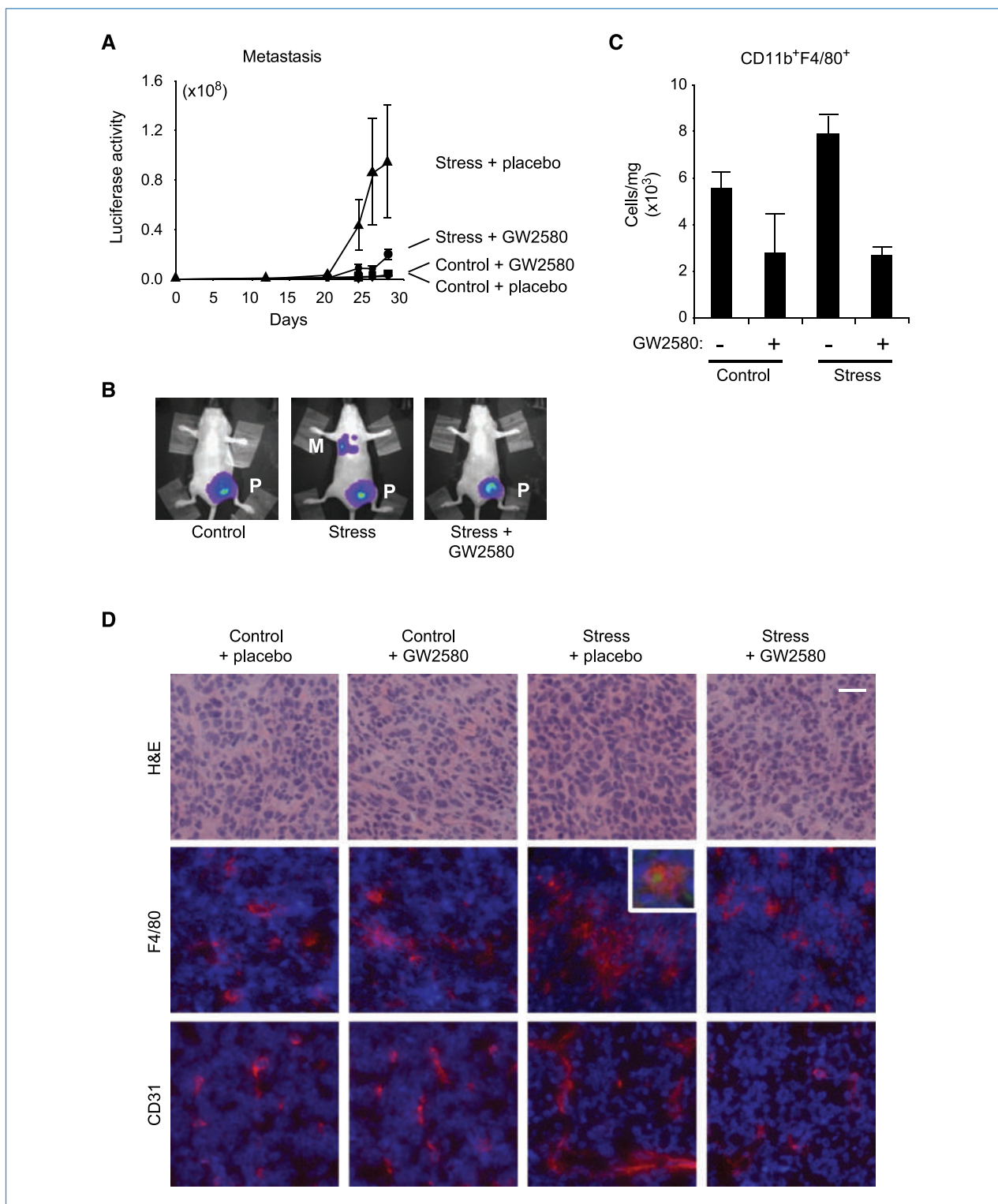


macrophage activity (32), and suggests that therapeutic strategies that seek to promote a tumoricidal M1 macrophage phenotype by orienting T helper cell response toward Th1 cytokine production (33) may be insufficient to modulate metastasis in the presence of chronic SNS activation. Furthermore, SNS regulation of macrophages in the absence of a T lymphocyte population suggests an antigen-independent mechanism of tumor-associated inflammation that need not invoke exogenous triggers (e.g., tumor-associated viruses; ref. 33) but instead occurs through direct neurotransmitter signaling to myeloid cells. SNS regulation of the physiologic dynamics via altered macrophage/monocyte communication would extend the influence of the peripheral nervous system beyond the dis-

tance of neurotransmitter diffusion from primary neural fibers (10–100  $\mu\text{m}$ ; ref. 34), and suggests a mechanism for transmitting the effects of autonomic nervous system activation into noninnervated or poorly innervated tissues such as solid epithelial tumors.

The present studies clearly indicate a role for macrophages in mediating SNS effects on metastasis, and they rule out any requirement for T lymphocytes in such dynamics; however, it remains conceivable that other immune cells act upstream of macrophages to regulate their recruitment and polarization (e.g., natural killer cells might potentially play a role, as suggested in recent studies; ref. 35). Defining the tumor and microenvironmental dynamics that ultimately shape





**Figure 6.** Effect of macrophage inhibition on stress-enhanced metastasis. A, metastasis was quantified using live *in vivo* imaging in control versus stressed mice treated with the CSF-1 receptor inhibitor GW2580 or placebo. (Control + placebo and control + GW2580 curves are indistinguishable.) B, representative images of mice in control (+ placebo), stress (+ placebo), and stress + GW2580 conditions. C, frequency of CD11b<sup>+</sup>F4/80<sup>+</sup> cells in 66c14 primary tumors from control versus stressed mice treated with GW2580 or placebo. D, mammary tumor cryosections were stained with H&E to show general morphology or immunostained with anti-F4/80 (red; middle) or anti-CD31 (red; bottom) and nuclear counterstained (blue). Bar, 20  $\mu$ m. Inset shows representative F4/80 macrophages positive for  $\beta$ 2AR (green). P, primary tumor; M, distant metastasis.

SNS modulation of tumor-associated macrophage biology represents an important topic for future research. Particularly important would be the identification of the cell types and signaling pathways that regulate macrophage recruitment and the functional effect of stress-induced M2 macrophage phenotype. The current study shows that SNS signaling modulates both the extent of tumor infiltration by macrophages and the associated intratumoral expression of prometastatic genes, including *Cox2* (*Ptgs2*), *Mmp9*, *Arg1*, *Tgfb*, and *Vegf*. Consistent with the SNS regulation of prometastatic macrophage gene expression, SNS activation has been shown to promote peritoneal growth of metastatic ovarian tumors in nude mice through increased VEGF-mediated vascularization (2). The present studies extend those observations into the domain of metastatic dissemination to distant tissues (including vascular-mediated colonization of target organs) and identify macrophage modulation within the primary tumor as a central molecular mechanism for alterations in primary tumor metastatic seeding.

Neural regulation of macrophage prometastatic activity may provide potential cellular and molecular mechanisms for clinical observations linking chronic stress to increased breast cancer progression in humans (1, 36, 37). Although these observations remain controversial, recent analyses have begun to define the circumstances in which such relationships are most likely to be observed. Few consistent relationships have been found between stress and the initial incidence of breast cancer (38–41). However, several epidemiologic studies and a large meta-analysis of 131 prospective studies have linked chronic stress to increased progression of established breast cancers (36, 37, 42–45). These results are consistent with the data from the present experimental model in which stress-induced activation of the SNS showed no significant effect on the growth of primary tumors but reliably enhanced metastatic spread via effects on both primary tumor seeding of metastasis and tumor cell extravasation/colonization of distant target tissues. The present findings also suggest that other physiologic or pharmacologic influences on SNS activity besides stress might potentially influence cancer progression. Such results would be consistent with epidemiologic findings linking  $\beta$ -blocker usage to reduced cancer risk (6, 7). Two case-control studies of advanced breast cancer did not find a link between  $\beta$ -blocker use and breast cancer incidence (46, 47), but a recent study has linked  $\beta$ -blockers to reduced metastasis and breast cancer-specific mortality (48).

The present data suggest that pharmacologic inhibition of SNS activity (e.g., with  $\beta$ -antagonists or anti-nerve growth factor antibodies; ref. 49) could potentially constitute a novel adjunctive strategy for minimizing breast cancer metastasis. Localized targeting of tumor-associated macrophages (e.g., with GW2580) might also block adverse effects of neuroendocrine activation on macrophage recruitment into primary tumors and thereby reduce prometastatic gene expression. Macrophage recruitment into the primary tumor might also serve as a biomarker for early detection of disease progression and/or therapeutic impact in clinical and intervention studies.

The present results show that systemic physiologic conditions can significantly shape the primary tumor microenvironment and may alter conditions in distant tissues in ways that facilitate metastasis to distant organs. This raises the intriguing possibility that systemic interventions (e.g., that target neural or immune compartments) may provide new adjunctive strategies to complement existing anticancer therapies (50). These findings highlight the importance of considering the patient's overall physiology in the development of new therapeutic approaches to limit cancer progression and minimize metastatic rates in breast cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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