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Inhibition of Breast Cancer Metastasis by Resveratrol-Mediated Inactivation of Tumor-Evoked Regulatory B Cells

Catalina Lee-Chang,* Monica Bodogai,* Alejandro Martin-Montalvo,† Katarzyna Wejksza,* Mitesh Sanghvi,‡ Ruin Moaddel,§ Rafael de Cabo, † and Arya Biragyn*

We reported previously that tumor-evoked regulatory B cells (tBregs) play an essential role in breast cancer lung metastasis by inducing TGF-β–dependent conversion of metastasis-promoting Foxp3+ regulatory T cells (Tregs). In this article, we show that resveratrol (RSV), a plant-derived polyphenol, at low and nontoxic doses for immune cells, can efficiently inhibit lung metastasis in mice. The mechanism of this process is that RSV inactivates Stat3, preventing the generation and function of tBregs, including expression of TGF-β. As a result, it frees antitumor effector immune responses by disabling tBreg-induced conversion of Foxp3+ Tregs. We propose that low doses of RSV may also benefit humans by controlling cancer escape–promoting tBregs/Tregs without nonspecific inactivation of effector immune cells. The Journal of Immunology, 2013, 191: 000–000.

Successful metastasis is an active process controlled by primary tumors to use regulatory immune cells to suppress antitumor effector immune responses. For example, murine mammary 4T1 adenocarcinoma, which represents a highly aggressive model of human breast carcinoma (1), produces GM-CSF, IL-1β, and TGF-β to expand and activate various myeloid-derived suppressor cells (MDSCs) and M2 macrophages (2–4). They impair antitumor immune responses and promote metastases, acting either directly or indirectly via induction/expansion of regulatory T cells (Tregs) (5–7). As such, the increase in MDSCs and Tregs is often associated with a poor disease outcome in mice and humans with cancer (8–10). However, our recent attempts to link myeloid cells with the induction of immune suppression needed for the successful lung metastasis in mice with 4T1 cancer (4T1 adenocarcinoma, such as 4T1 or 4T1.2 cancer cells, established in mammary gland) failed (11–14). Instead, we found that cancer metastasis requires an additional player, a unique subset of TGF-β–producing regulatory B cells designated tumor-evoked regulatory B cells (tBregs) (13, 14). The role of tBregs is to induce TGF-β–dependent conversion of metastasis-promoting Foxp3+ Tregs from non-Treg CD4+ T cells (13) to inactivate antitumor NK cells and effector CD8+ T cells and, thereby, protect metastasizing cancer cells (11, 14). This process is actively controlled by the primary tumor, in particular, by nonmetastatic cancer cell subsets that induce the generation of tBregs from normal B cells using metabolites of 5-lipoxygenase (15). The clinical implication of this is that, as long as cancer persists, tBregs will continue to be induced, initiating the chain of suppressive events. In the absence of B cells, such as in mice with B cell deficiency, 4T1 cancer can only progress at the primary site (mammary gland) and will fail to metastasize into the lungs, unless replenished with wild-type B cells or tBregs (11, 13, 14). Thus, tBregs need to be inactivated to block cancer metastasis. However, the lack of available tBreg-specific markers makes this task difficult. tBregs substantially differ from the immune tolerance–inducing IL-10–producing B cells and Bregs (16–18) and even from a handful of B cells/Tregs that induce carcinogenesis-supporting inflammation (19, 20) or promote cancer by disabling CD4+ T cell help in mice (21). Although tBregs express CD25 and IL-10, as in recently found human granzyme B–expressing human CD19−CD38−CD1d+IgM−CD147+ Bregs (22), phenotypically they resemble poorly proliferative B2-like cells (IgDhigh) that express constitutively active Stat3 and surface markers CD25high, B7-H1high, CD81high, CD86high, CCR6high, and CD62Llow, IgMint/Low, and CD20low (13, 14). Although lung metastasis can be abrogated by injecting anti-B220 Ab (targets B cells and plasmacytoid dendritic cells) and PC61 Ab (depletes CD25+ cells, such as Tregs and tBregs), they are not specific and may also deplete lymphocytes required for the elimination of cancer cells. Similarly, rituximab (anti-CD20 Ab widely used in humans with B cell malignancies) cannot be used, because it worsens metastasis in mice with 4T1 cancer (14), enhances tumor burden in mice injected i.v. with B16-F10 melanoma (23), and, importantly, fails to benefit patients with renal cell carcinoma and melanoma (24). At least in mice with 4T1 cancer, we linked these effects with the anti-CD20 Ab–induced enrichment of tBregs expressing low levels of CD20 due to the depletion of beneficial B cells (14).

Resveratrol (RSV; 3,5,4′-trihydroxystilbene) is a phytoalexin found in grapes, mulberries, and peanuts. As a potential anticancer therapeutic drug and inhibitor of cancer angiogenesis, RSV controls mammalian cell apoptosis via multiple molecular pathways, such as by targeting p53, Rb, and cell cycle kinases (25). This probably explains why RSV acts differentially on various cells, depending on their activation and differentiation state, and exhibits both estrogenic and antiestrogenic properties on mammary...
cancer cells (26). Although it can inhibit initiation and progression of tumors in mice (26–29), it fails to suppress breast cancer metastasis in mice (30) or, instead, worsens survival of mice with prostate cancer xenografts (31) and human patients with refractory multiple myeloma (32). RSV can also either inhibit or induce expression of TGF-β and, thereby, affect downstream functions mediated by TGF-β (33–36). This probably explains why only Foxp3+ Tregs were inhibited in mice treated with RSV, whereas MDCS were expanded (29, 37). RSV is also a potent inhibitor of Stat3 phosphorylation and acetylation (38, 39) and, as such, it specifically induces apoptosis of malignant cells expressing activated Stat3 (40). Because Tregs also express activated Stat3 and induce conversion of metastasis-promoting Foxp3+ Tregs in a TGF-β–dependent way (13), RSV may also inactivate tBregs and, thereby, block breast cancer lung metastasis.

In this study, we show that low and noncytotoxic doses of RSV can indeed preferentially inhibit tBregs and concurrently block lung metastasis in mice with highly metastatic 4T1.2 cancer. The mechanism of this process is that RSV blocks phosphorylation of Stat3 (i.e., inhibiting its activity), such as production of TGF-β in tBregs. Because TGF-β is a key factor used by tBregs to convert metastasis-promoting Foxp3+ Tregs (13), its RSV-induced inactivation in tBregs is sufficient to almost completely block lung metastasis by releasing the suppressed state of effector cellular immune responses. Hence, to our knowledge, this is the first mechanistic proof of principle that underscores the therapeutic relevance of noncytotoxic low doses of RSV to combat cancer escape mediated by tBregs.

Materials and Methods

Reagents, cells, and mice

RSV (ResVida, >98% pure) was a gift from DSM Nutraceuticals (Aurangabad, India). Stat3 inhibitor V (Static) and V1 (S31-201) were purchased from Calbiochem (EMD Millipore, San Diego, CA). 4T1 adenocarcinoma cells and B16-F10 melanoma cells were purchased from American Type Culture Collection. 4T1.2 cells, a subset of 4T1 cells, were a gift from Dr. Robin L. Anderson (Peter McCallum Cancer Center, Melbourne, Australia). Nontumorigenic 4T1.2-PE cells were generated from 4T1.2 cells using TARC-PE38 chemotoxin (11). Female BALB/c and C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). B-cell–deficient μMT mice in BALB/c background were a gift from Dr. Thomas Blankenstein (Max-Delbrück-Centrum, Berlin, Germany). pmel mice were a gift from Dr. Nicolas P. Restifo (National Cancer Institute, Bethesda, MD) and were described elsewhere (41).

Separation of RSV and its O-sulfated metabolite by HPLC

The extraction of trans-RSV and its metabolites was carried out as described elsewhere (42). Briefly, 70 μl methanol and 10 μl hexistrol (internal standard) were added to 20 μl serum samples collected at different time points, which were vortex mixed and centrifuged at 20,800 relative centrifugal force at 4°C for 10 min. The supernatant was analyzed in a Shimadzu Prominance HPLC system (Shimadzu, Columbia, MD) using a Shim-pack XRP-C18 guard column (4.6 × 12 μm) and an analytical column Discovery C18 (150 × 4.6 mm ID, 5 μm; Supelco). The mobile phase consisted of water containing 0.1% acetic acid and 0.07% triethylamine as component A and acetonitrile as component B. A linear gradient was run as follows: 0–3 min 20% B, 3–25 min 20–60% B, and 25–30 min 60–20% B at a flow rate of 1.0 ml/min. The total run time was 30 min/sample.

Mass spectrometry analysis was performed using a triple quadrupole mass spectrometer model ABI 4000 system from Applied Biosystems/MDMS SCIEX equipped with a Turbo Ion Spray (TIS) (Applied Biosystems, Foster City, CA). The data were acquired and analyzed using Analyst version 1.4.2 (Applied Biosystems). Negative electrospray ionization data were acquired using multiple reaction monitoring, and the standards were characterized using the following multiple reaction monitoring transitions: RSV (227–185), RSV-Sulf (307–227), and hexestrol (269–134). The TIS instrumental operating conditions for declustering potential, collision energy, and collision cell exit potential were 72 V, 20 V, and 20 V, respectively. The data were acquired and analyzed using Analyst version 1.4.2 (Applied Biosystems). Negative electrospray ionization data were acquired using multiple reaction monitoring, and the standards were characterized using the following multiple reaction monitoring transitions: RSV (227–185), RSV-Sulf (307–227), and hexestrol (269–134). The TIS instrumental operating conditions for declustering potential, collision energy, and collision cell exit potential were 72 V, 20 V, and 20 V, respectively.

In vitro immune cell manipulations

tBreg generation, T cell suppression, and Treg conversion assays were performed following our previously described methods (11, 13). In brief, tBregs were generated from murine splenic B cells (>95% purity, isolated by negative selection using the RoboSep system, Stem Cell Technologies, Vancouver, Canada) by incubating for 2 d in 50% conditioned medium of 4T1-PE cells in complete RPMI (cRPMI; RPMI 1640 with 10% heat-inactivated FBS, 10 mM HEPES–sodium Pyruvate, 1 mM sodium pyruvate, 0.01% 2-ME, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, all from Invitrogen) at 37°C in humidified atmosphere with 5% CO2. Control B cells were treated with 100 ng/ml recombinant mouse BAFF (R&D Systems) and 0.5 μM ibaflurin (Sigma-Aldrich). B cells and BAFF were generated in the presence or absence of different concentrations of RSV (stock solution of 200 mM in EtOH). tBregs were characterized as CD11b+CD25+ CD69− cells gated within CD19+ cells using the respective mAbs (CD19-PerCP/Cy5.5, CD81-PE, and CD25-Pacific Blue; all from BioLegend). To test the suppressive activity of B cells, splenic CD3+ T cells were isolated using the cell T Cell Enrichment Column (R&D Systems) and labeled with the cell proliferation dye eFluor 670 (eBioscience) before incubating with B cells at a 1:1 ratio for 4–5 d in the presence of 1.5 μg/ml anti-mouse CD3 Ab (BD Biosciences). Cells were stained with anti-mouse CD4+ PerCP and CD8-FITC (BioLegend). A decrease in eFluor 670 expression of CD4+ and CD8+ T cells correlates with the proportion of cells that underwent divisions. To test the ability of tBregs to convert Tregs, non-Treg CD25+ CD4+ T cells (isolated with CD3+ T Cell Enrichment Column [R&D Systems] and CD4+ T Cell Isolation Kit II [Miltenyi Biotec]) and depleted of CD25+ cells [Dynabeads, Invitrogen]) were mixed with B cells at a 2:1 ratio and cultured for 5 d in the presence of bead-conjugated anti-CD3/CD28 Abs (Invitrogen) and 500 U/ml mouse rIL-2. 4T1.2 cell proliferation was assessed using the cell WST-1 proliferation reagent (Roche), following the manufacturer’s instructions. Briefly, cells were plated in 96-wells plates in triplicate per sample, and 72 h after the addition of RSV. After 72 h, supernatants were replaced with fresh cRPMI + 10% WST and incubated for 30 min at 37°C. The formazan dye conversion/viable cells were quantified using an ELISA plate reader (Bio-Rad) at 450 nm.

To generate Tregs, in vitro–generated tBregs or B cells isolated from lymph nodes (LNs) of BALB/c mice with 4T1.2 cancer were incubated with non-Treg cells CD25-CD4+ cells from BALB/c or C57BL/6 mice with Foxp3-GFP mice, purity >99.5% at a 1:1 ratio and cultured for 5 d in the presence of bead-conjugated anti-CD3/CD28 Abs (Invitrogen) and 500 U/ml mouse rIL-2 in the absence or presence of DMSO or SB431542 (Tocris Bioscience, Ellisville, MO).

In vivo manipulations

Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] Publication No. 86-23, 1985). The experiments were conducted in microisolator cages with respect to syngeneic tumor cells, such as 5 × 106 4T1 cells (in the fourth mammary gland) or 1 × 106 B16-F10 melanoma cells, and euthanized by CO2 asphyxiation. To assess effects of RSV on in vivo tumor growth, 4T1-bearing BALB/c mice were injected i.p. with RSV (20 or 50 μg/mouse) or mock every other day. Animals were euthanized at 13–20 d posttumor challenge. To assess the effect of systemic administration of RSV, 4T1-tumor bearing BALB/c mice were injected i.p. with RSV (20 or 50 μg/mouse) or mock every day. Animals were euthanized at 13–20 d posttumor challenge. To assess in vivo effects of RSV on blocking tBreg generation, 4T1 tumor–bearing BALB/c mice were injected i.p. with RSV (50 μg/mouse) or mock every other day. In vivo effects of RSV on in vivo tumor growth, 4T1-bearing BALB/c mice were injected i.p. with RSV (20 or 50 μg/mouse) or mock every other day. Animals were euthanized at 13–20 d posttumor challenge. To assess effects of RSV on in vivo tumor growth, 4T1-bearing BALB/c mice were injected i.p. with RSV (20 or 50 μg/mouse) or mock every other day. Animals were euthanized at 13–20 d posttumor challenge. To assess in vivo effects of RSV on blocking tBreg generation, 4T1 tumor–bearing BALB/c mice were injected i.p. with RSV (50 μg/mouse) or mock every other day. Animals were euthanized at 13–20 d posttumor challenge.
Because tBregs also express activated Stat3 and induce conversion other day from days 3 to 11. Subsequently, their B cells (4 \times 10^9) were isolated and transferred i.v. into host BALB/c mice at 13 and 15 d postchallenge with 5 \times 10^5 4T1.2 cells. The host mice were pretreated i.v. with RSV (50 \mu g/mouse) or mock every other day from days 3 to 11. Donor mouse LN B cells were isolated at days 13 and 15 using magnetic separation with anti-mouse CD19-FITC (BioLegend) and anti-FITC MicroBeads (Miltenyi Biotec). All B cell samples were tested in parallel for the ability to suppress T cell proliferation and to convert naive T cells into Foxp3^+ T cells, as described above.

**TGF-β production and pStat3 expression**

Production of TGF-β (active form) was quantified in supernatants using an ELISA Ready-SET Go kit for human/mouse TGF-β1 (eBioscience), following the manufacturer’s instructions. To evaluate intracellular expression, cells were treated with 1/1000 monensin (eBioscience) for 2 h before permeabilization and staining with Foxp3 and IFN-γ (eBioscience) or TGF-β–PE (BioLegend). TGF-β expression from splenocytes, LNs, and tumor B cells from tumor-bearing mice (with or without i.p RSV treatment) were analyzed by stimulating cells for 4 h with PMA (5 ng/ml) and ionomycin (50 ng/ml) (both from R&D). Stat3 expression was assessed in 10 μg whole-cell lysate with Western blotting using anti-Stat3 (9132) and anti-phosphorylated Stat3 (Tyrof705, 9138- mAbs (Cell Signaling). For intracellular expression, cells were fixed with 2% paraformaldehyde in PBS for 10 min at 37°C and chilled on ice for 1 min. Cells were then spun down, resuspended in prechilled 90% methanol (in water), and incubated for 30 min on ice. The cells were stained with anti-mouse CD19-PerCP/Cy5.5 (BioLegend) and rabbit anti-mouse pStat3–Alexa Fluor 647 (Tyrof705; Cell Signaling) at 1/200 dilution.

**Statistical analysis**

The results are presented as the mean ± SEM. Differences were tested using the Student t test, and a two-sided p value < 0.05 was considered statistically significant.

**Results**

**RSV blocks cancer progression and metastasis**

High doses of RSV (>100 mg/kg) suppress cancer progression in mice (37, 43) via direct induction of apoptosis of phosphorylated Stat3–expressing malignant cells (40) and indirectly by blocking the generation of cancer escape-promoting Foxp3^+ Tregs (29, 37).

Because tBregs also express activated Stat3 and induce conversion of Foxp3^+ Tregs (13), we hypothesized that some antitumor benefits of RSV could be through the inactivation of tBregs. To test this idea, we screened for lower doses of RSV to avoid the involvement of its nonspecific cytotoxicity. As reported by other investigators (30, 40), we also found that, at doses >12 μM, RSV was cytotoxic for most cells tested, such as 4T1.2 cancer cells (Supplemental Fig. 1A) and naive mouse B cells and T cells (B+BAFF and CD4^+CD25^−, respectively; Supplemental Fig. 1B, 1C), although consistently yielding a higher cytotoxicity for Tregs (CD4^+CD25^+) and tBregs (Supplemental Fig. 1B, 1C). In vivo, RSV is usually metabolized quickly (44, 45), and its plasma levels were reported to drastically decrease from 95 to 1 μM within 480 min post-i.v. injection of 20 mg/kg RSV (46). In concordance, we did not detect RSV in plasma of mice (detetection threshold 39 ng/ml, ~170 nm) injected i.p. with lower-dose RSV (5 mg/kg, 100 μg/mouse), although its metabolite RSV 3-O-sulfate was only transiently present at a maximum of 450 nM by 60 min after injection (140 ± 48 ng/ml, Table I). We failed to detect RSV or its metabolite after 60 min in mice injected with 50 μg RSV (Table I). Thus, RSV injected at doses <100 μg probably will not generate systemic levels in mice sufficient to elicit nonspecific cytotoxicity to cancer cells or T cells and B cells. Despite this, progression of s.c. challenged B16-F10 melanoma was substantially decreased in C57BL/6 mice treated with 50 μg RSV (Supplemental Fig. 2A). Similarly, it also reduced 4T1.2 breast cancer growth in the mammary gland (Fig. 1A) and its lung metastasis (Fig. 1B) in BALB/c mice. Hence, considering the importance of tBregs in inducing metastasis-promoting Tregs in 4T1 cancer lung metastasis (11, 13), the low dose of RSV could probably disable cancer escape by inactivating tBregs/Tregs. In concordance, RSV–treated mice also had significantly reduced Tregs (CD25^+CD19^hi cells within CD19^+ B cells, Fig. 1C, 1D) and Foxp3^+ Tregs (Fig. 1E, Supplemental Fig. 2B, 2C). In contrast, and in support of a recent report by other investigators (29), RSV did not affect MDSCs, because they were comparably expanded in both RSV- and mock-treated tumor-bearing mice (Supplemental Fig. 2D).

**RSV blocks metastasis by inactivating tBregs**

To confirm the role of RSV on tBregs, we tested whether it affects in vitro generation of tBregs from naive B cells treated with conditioned media from breast cancer cells (13). RSV significantly blocked the generation of tBregs (Fig. 2), even at noncytotoxic doses (Supplemental Fig. 1B). It reduced expression of tBreg–associated surface markers (Fig. 2A) and disabled tBregs to suppress T cell proliferation stimulated with anti-CD3 Ab (both CD4^+ and CD8^+ T cells, respectively, Fig. 2B, 2C). Importantly, 1–3 μM RSV (nontoxic dose, Supplemental Fig. 1B) was sufficient to almost completely block a second feature of tBregs: the induction of Foxp3^+ Tregs from naive CD4^+CD25^− T cells (p < 0.05, Fig. 2D). In contrast, RSV did not affect control B cells (incubated with BAFF, B+BAFF), which neither inhibited T cell proliferation (Fig. 2A–D) nor converted Foxp3^+ Tregs (Fig. 2D). Hence, low doses of RSV can block in vitro generation of tBregs, suggesting that it could also do so in vivo to abrogate metastasis.

To test this idea, we performed adoptive-transfer experiments in 4T1.2 cancer-bearing mice pretreated with 50 μg RSV every other day from days 3 through 11 posttumor challenge to eliminate endogenous tBregs and Tregs (Fig. 3A, see also Fig. 1C, 1D). At day 13 when circulating RSV presumably fully disappeared, mice were randomized and adoptively transferred with in vitro–generated tBregs treated with either RSV (tBreg–RSV) or ethanol (tBreg–Mock, Fig. 3A). As expected for tBregs (13), mice repleted with tBreg-mock cells had significantly increased lung metastasis compared with PBS-injected RSV-pretreated mice (p < 0.05, PBS versus tBreg-Mock, Fig. 3A). In contrast, transfer of tBregs–RSV completely failed to augment metastasis (p < 0.05, tBregs-Mock versus tBreg–RSV, Fig. 3A), confirming our in vitro conclusion that RSV inactivates tBregs. To further confirm these results and to rule out artifacts of in vitro manipulations, we performed similar experiments using B cells isolated from 4T1 cancer–bearing mice treated with RSV (Fig. 3B). As we reported previously (13, 14), purified B cells from mice with 4T1.2 cancer (Mock B) contained cancer-induced tBregs, because they readily suppressed proliferation of T cells (Fig. 3C) and converted Foxp3^+ Tregs

<table>
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<th>Time Point</th>
<th>Groups</th>
<th>RSV 3-O-sulfate (ng/ml; Mean ± SEM)</th>
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<tr>
<td>1 h</td>
<td>RSV 50 μg</td>
<td>45.5 ± 7.5</td>
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<tr>
<td>3 h</td>
<td>RSV 50 μg</td>
<td>141.1 ± 4.4</td>
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<td>6 h</td>
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Mice were injected i.p. with 50 or 100 μg RSV in 200 μl sterile PBS, and serum samples were collected at 1, 3, and 6 h post-RSV injection. Neither RSV nor RSV 3-O-glucuronides were detected in samples. RSV 3-O-sulfate was the only RSV metabolite present at measurable levels. Data are from two mice/group/experiment. BQ, below the detection sensitivity (<39 ng/ml).
from non-Treg CD4+ T cells (Fig. 3D) ex vivo. Importantly, adoptive transfer of these Mock B cells (isolated from untreated cancer-bearing mice) significantly increased cancer progression (Fig. 3E) and lung metastasis (Fig. 3F) in RSV-pretreated mice with 4T1.2 cancer. In contrast, B cells from RSV-treated cancer-bearing donor mice did not contain tBregs, because they failed to suppress T cells and induce Treg conversion (Fig. 3C, 3D), and they did not enhance cancer progression and metastasis (RSV B, Fig. 3E, 3F).

**FIGURE 1.** RSV attenuates the development of 4T1 breast cancer and lung metastasis. Female BALB/c mice were injected i.p. with either 20 or 50 μg RSV every other day starting from day 3 post s.c. challenge with 4T1 cancer cells (5 × 104). Control tumor-bearing mice (mock) were injected with empty vehicle. Tumor size (mm² ± SEM) measured at indicated times (A) and number of lung metastatic foci (+ SEM) at day 28 post tumor challenge (B) in four to five mice/group. Experiment was repeated three times. Proportion (%) of tBregs (CD81 HighCD25+ gated on CD19+ cells) in spleen (C) and LNs (D) and proportion (%) of splenic Tregs (Foxp3+ gated on CD4+ cells) (E) were measured in mice shown in (A) and (B). Dot blots in (C)–(E) are representative of the data shown in the bar graphs (lower panels). *p < 0.05, **p < 0.01.

**FIGURE 2.** RSV abrogates tBreg generation. To test effects of RSV on in vitro generation of tBregs, titrated doses of RSV were incubated from the beginning of tBreg generation. Controls were BAFF-treated B cells (B+BAFF) cultured with the indicated doses of RSV (A) Expression of tBreg-like surface markers on B cells (% ± SEM of CD81 HighCD25+ gated on CD19+ cells). Percentage (+ SEM) of proliferated (eFluor 670–diluted) CD4+ T cells (B) and CD8+ T cells (C). (D) Percentage (+ SEM) of Treg conversion from non-Treg CD4+ T cells (Foxp3+ gated on CD4+ cells). In (B–D), T cells were stimulated with 3 μg/ml anti-CD3 mAb for 5 d in the presence of tBregs and B cells from (A). Dot plot (A) and graphs (B–D) show representative flow cytometry data. All data are from experiments repeated four times in triplicate. *p < 0.05, **p < 0.01.
when transferred into RSV-pretreated mice. Thus, low doses of RSV block tBregs and, thereby, inhibit 4T1 breast cancer escape and metastasis. The mechanism of this process could be the inhibition of metastasis-promoting Foxp3+ Tregs induced by

FIGURE 3. RSV inhibits tBreg function in vivo. To confirm tBreg inactivation by RSV, we performed a series of adoptive-transfer experiments with either in vitro (A) or in vivo (B–F) inactivated donor tBregs/B cells [see schemas in (A) and (B)]. Host female BALB/c mice with 4T1 cancer were “depleted” of tBregs by treating every other day with RSV (50 µg) from days 3 through 11 after tumor challenge. (A) At day 13, mice were randomized and adoptively transferred with ex vivo–generated tBregs in the presence of 12.5 µM RSV or mock-treated tBregs. In (B) and (F), B cells were isolated from LNs of donor tumor-bearing mice treated with RSV (50 µg) or mock [see schema in (B)] and used for adoptive transfer. These B cells were also tested for the ability to suppress CD4+ T cell proliferation (C) and convert Foxp3+ Tregs (D), as in Fig. 2B and 2D. The results also were compared with activity of naive mouse B cells (naive, C, D). B cells/tBregs from mock-treated, but not RSV-treated, mice augment 4T1.2 cancer growth (E) and lung metastasis (F) when adoptively transferred into host RSV-pretreated mice compared with control mice injected with PBS (E, F). Graphs (upper panels) in (C) and (D) show representative flow cytometry data for the bar graphs (mean ± SEM) (lower panels). Every result was reproduced at least twice in experiments using four to five mice/group. *p < 0.05, **p < 0.01.
Although high doses of RSV are cytotoxic for Tregs in vitro (Supplemental Fig. 1C) and in vivo (29, 37), Tregs also were significantly reduced in tumor-bearing mice treated with a low dose of RSV (Fig. 1E). Of note, RSV appears to affect their generation, but not function, because Tregs surviving RSV treatment readily suppressed target T cells (Supplemental Fig. 1D).

**RSV inhibits tBreg–Treg axis**

To test the indirect effect of a low dose of RSV on Tregs, we quantified Treg numbers in mice adoptively transferred with tBregs (Fig. 3B). Although the numbers of Foxp3+ Tregs infiltrating the secondary lymphoid organs and tumor (Fig. 4A–D) were significantly increased in tumor-bearing mice adoptively transferred with Mock B cells (isolated from untreated tumor-bearing mice), we failed to detect any enhancement of Tregs when mice were replenished with B cells from RSV-treated donor tumor-bearing mice (RSV B, Fig. 4A–D). In fact, the numbers of Tregs in mice transferred with RSV B cells and RSV-pretreated control (PBS) mice were almost indistinguishable (Fig. 4A–D). Compared with naive mice, Tregs were still increased in these two groups of mice (p < 0.05, naive versus PBS, Fig. 4A–D), suggesting the tumor-induced restoration of Tregs and tBregs after 2 wk of cessation of RSV treatment. Thus, the low dose of RSV reduces Foxp3+ Tregs by inhibiting tBregs. As a result, it may release the immunosuppressed state of antitumor effector immune cells.

To test this possibility, we evaluated CD8+ T cells in RSV-pretreated tumor-bearing mice adoptively transferred with tBregs (Fig. 3B). Although the role of CD8+ T cells in 4T1.2 cancer-bearing mice is not known, RSV significantly increased the numbers of IFN-γ-producing CD8+ T cells in mice with 4T1.2 cancer (p < 0.05, naive versus PBS, Fig. 4E). This increase was almost completely reduced to the levels of naive mice if RSV-treated mice were adoptively transferred with B cells/tBregs from 4T1.2 cancer–bearing mice (Fig. 4E). In contrast, transfer of B cells/tBregs from RSV-treated cancer-bearing donor mice failed to affect or inhibit CD8+ T cells (p < 0.05, Mock B versus RSV B, Fig. 4E). Thus, RSV releases suppression of antitumor effector immune responses by inhibiting tBregs. To further support this conclusion, we used a different tumor model with defined CD8+ T cell responses: pmel mice transgenic with CD8+ T cells specific for B16 melanoma-expressed gp10025–32 epitope (41). In these mice, adoptive transfer of tBregs (generated from congenic wild-type C57BL/6 mice) also significantly enhanced progression of B16-F10 melanoma (Fig. 5A). Importantly, the enhanced tumor

**FIGURE 4.** Modulation of Foxp3+ Tregs and effector CD8+ T cells in tumor-bearing mice adoptively transferred with tBregs. Mice were treated and adoptively transferred with B cells as in Fig. 3B, and the presence of Foxp3+ Tregs and IFN-γ/CD8+ T cells was evaluated in the host mice. As expected, B cells from mock-treated tumor-bearing mice (mock B) had increased proportions (%, A) and numbers of Foxp3+ Tregs in spleen (B), draining LNs (C), and tumor (D) compared with naive and tumor-bearing PBS-treated host mice. (E) The tBreg transfer also reduced the numbers of splenic IFN-γ/CD8+ T cells. Conversely, B cells/tBregs from RSV-treated tumor-bearing mice (RSV B) did not increase Tregs (A–D) and failed to reduce IFN-γ/CD8+ T cells (E). Representative data are shown from experiments using four mice/group and repeated twice. *p < 0.05, **p < 0.01.
growth was accompanied by a significant increase in Foxp3+ Tregs (Fig. 5B–D) and a substantial reduction in IFN-γ-expressing CD8+ T cells specific to gp10025–32 peptide (Fig. 5E, 5F). Conversely, these effects (the increase in tumor growth and Tregs and the decrease in CD8+ T cells) were completely reversed if mice were transferred with tBregs pretreated with RSV (Fig. 5). Taken together, the low dose of RSV inactivates tBregs, thereby abrogating the generation of cancer escape–promoting Tregs and concurrently activating antitumor effector CD8+ T cells.

**RSV inhibits tBreg-mediated Treg conversion by blocking TGF-β1**

A unique feature of tBregs is that they constitutively express activated (phosphorylated) Stat3 (13). Because RSV is a potent inhibitor of phosphorylation and acetylation of Stat3 (38, 39), it may activated (phosphorylated) Stat3 (13). Because RSV is a potent inhibitor of phosphorylation and acetylation of Stat3 (38, 39), it may irreversibly inactivate Stat3 (13). To test this possibility, first we confirmed the importance of TGF-β in tBreg-mediated conversion of Foxp3+ Tregs (13, 14) by incubating naive mouse CD25−CD4+ T cells (non-Tregs) with in vitro–generated syngeneic tBregs or B cells (tumor B) isolated from BALB/c mice with 4T1.2 cancer for 5 d in the presence or absence of ALK5 inhibitor SB431542, a selective inhibitor of TGF-β type I receptor activity. Unlike control B cells from naive BALB/c mice, which did not induce Foxp3 in non-Tregs, regardless of the presence or absence of the inhibitor (Fig. 6A), tBregs or tumor B cells only converted Foxp3+ Tregs in the absence of SB431542 (Fig. 6B). As shown by ELISA (Fig. 6B) and intracellular staining (Fig. 6C), TGF-β expression in tBregs was significantly, and in dose–dependent manner, reduced by treatment with a low nontoxic dose of RSV (<12 μM). Similarly, in vivo, B cells from tumor-bearing mice treated with 50 μg RSV also failed to secrete significant amounts of TGF-β1 (RSV, Fig. 6D), whereas B cells from mock-treated mice with 4T1.2 cancer produced abundant TGF-β (p < 0.01, Mock versus RSV, Fig. 6D). Confirming this, we found significant loss of TGF-β expression in tBregs (Fig. 6E–G) and B cells (Supplemental Fig. 3) infiltrating the secondary lymphoid organs (Fig. 6E, 6F, Suppl. Fig. 3A–C) and in the tumor (Fig. 6G, Supplemental Fig. 3D).

Next, to prove that this reduction is a result of Stat3 inhibition, we compared pStat3 in tBregs after treatment with RSV and specific Stat3 inhibitors: S31-201 and Stat3. As shown by Western blotting of total-cell lysates (Fig. 7A) and confirmed by intracellular staining (Fig. 7B), pStat3 was significantly reduced in Bregs after treatment with RSV and Stat3 inhibitors, respectively. Importantly, like RSV, Stat3 inhibitors also inhibited TGF-β1 expression in tBregs (Fig. 7C), a key factor required for tBreg-mediated conversion of Foxp3+ Tregs from non-Treg cells (13). In support, Stat3 inhibitor–treated Bregs also failed to convert in vitro (Fig. 7D) and expand in vivo Foxp3+ Tregs (Supplemental Fig. 4) after adoptive transfer into B cell–deficient mice with 4T1 cancer. As a result, unlike adoptive transfer of mock-treated tBregs, which significantly increased lung metastasis in these mice (tBreg-Mock, Fig. 7E), metastasis failed to increase if mice were replenished with tBregs treated with Stat3 or RSV (Fig. 7E). Taken together, the mechanism of antitumor activity of a nontoxic low dose of RSV is in the inactivation of Stat3 in tBregs, which blocks TGF-β production, thereby disabling conversion of metastasis-promoting Foxp3+ Tregs.

**Discussion**

We previously reported that cancer metastasis requires active involvement of regulatory immune cells, such as Foxp3+CD4+ Tregs and TGF-β–expressing tBregs (11, 13, 14). Breast cancer, by producing metabolites of 5-lipoxygenase, induces the generation of tBregs from normal B cells (13, 15) to convert Foxp3+CD4−

![FIGURE 5.](image-url)
Tregs from non-Treg CD25^+CD4^+ T cells using TGF-β (13). The Tregs, in turn, protect metastasizing cancer cells by inactivating antitumor NK cells using galectin-1/b-galactoside–binding protein (11) and downregulating effector CD8^+ T cells (14). Thus, Tregs and tBregs need to be controlled to successfully combat lung metastasis. Although the inactivation of tBregs alone appears to be sufficient (13–15), there are no simple and specific strategies that inactivate tBregs. They cannot be depleted with anti-CD20 Ab/rituximab, a current clinical strategy to combat B cell malignancies, because it worsens cancer escape by enriching tBregs expressing low levels of CD20 (14). In this study, we demonstrate that, unlike the majority of studies that used relatively high doses of RSV (up to 100 mg/kg) that are cytotoxic for both cancer cells and potentially beneficial effector immune cells in vitro and in vivo (26–29, 37, 39, 43), low and noncytotoxic doses of RSV (1–5 mg/kg) can inhibit the progression of B16 melanoma and 4T1.2 breast cancer cells and abrogate lung metastasis by inactivating tBregs. High and low doses of RSV differentially act on target cells. High amounts of RSV (300 μM) reduce cellular energy and activate AMPK (47), whereas, at concentrations <10

FIGURE 6. RSV inhibits TGF-β production from tBregs. (A) For in vitro conversion assay of Tregs, CD25^+CD4^+ T cells from naive BALB/c mice were incubated with B cells treated with PBS, in vitro–generated tBregs, or B cells isolated from naive or tumor (4T1.2)-bearing BALB/c mice in the presence or absence of 10 μM SB431542. T cells were stimulated with anti-CD3 Ab and 500 U/ml IL-2 for 5 d, and Foxp3 expression was detected by intracellular staining (% of Foxp3 in CD4^+ T cells ± SEM of a triplicate experiment). (B) As shown in the schema (upper panel), RSV was added after the generation of tBregs. Control B cells were BAFF-treated B cells (B+BAFF) incubated with RSV (lower panel, B, C). X-axis shows concentration of RSV used, and Y-axis shows levels of secreted TGF-β1 ± SEM (pg/ml, lower panel, B) and percentage ± SEM of intracellular TGF-β1 expression in B cells (lower panel, C) after 12 h of incubation. (C) Dot plot (upper panel) are representative data of triplicate experiments (lower panel) repeated three times. (D) To assess in vivo effects of RSV, BALB/c mice were treated with RSV (50 μg) or mock every other day starting 3 d after challenge with 4T1.2 cancer cells. At day 10, B cells isolated from LNs were tested for the ability to secrete TGF-β by ELISA after 48 h of stimulation with PMA/ionomycin. Similarly, numbers of TGF-β–expressing tBregs (CD8^+CD25^+CD19^+ B cells) were assessed via intracellular staining in freshly isolated B cells from spleen (E), draining LNs (F), and tumor (G). Representative data are shown for experiments using four mice/group performed three times. *p < 0.05, **p < 0.01.
RSV inactivates Stat3 in tBregs and disables conversion of Tregs via downregulation of TGF-β. tBregs were treated overnight with RSV (12.5 μM, A–D) or specific Stat3 inhibitor Stat3i (1 μM, A–D) and S31-201 (5 μM, B–D) to test phosphorylation of Stat3 (A, B) and TGF-β expression (C) in tBregs using Western blotting (A) and intracellular staining (B, C). Data in (A–C) (upper panels) are representative (mean ± SEM) of triplicate experiments (lower panels) independently repeated at least five times. Data in (A) (upper panel) are for two independent tBreg lysates (Exp 1 and Exp 2). (D) The loss of Stat3 prevents tBregs from converting Foxp3+ Tregs from non-Treg CD4+ T cells in vitro. Shown are representative data of triplicate experiment repeated four times. (E) To confirm the inability of Stat3-inactivated tBregs to promote lung metastasis, 4T1.2 cancer-bearing μMT mice genetically deficient in B cells (five/group) were adoptively transferred with congenic BALB/c tBregs pretreated with RSV, Stat3i, or mock and compared with untreated tumor-bearing mice (PBS). Lung metastasis (number of metastatic foci) was assessed at day 30 posttumor challenge. *p < 0.05, **p < 0.01. The p values in (D) are for comparisons of Mock versus treated tBregs.
μM it activates AMPK without decreasing energy (48). In rats and humans, a high-dose of RSV (>3000 mg/kg) also induces renal toxicity (32, 49), limiting its potential clinical use.

Our data from this study indicate that the immunological mechanism underlying low-dose RSV use is that it inactivates tBregs, thereby disabling their ability to convert non-Tregs into Foxp3⁺ Tregs. For example, in vitro low-dose RSV-induced inactivation of tBregs almost completely disabled Treg conversion, and in vivo inactivation of tBregs reduced the endogenous pool of Foxp3⁺ Tregs in tumor-bearing mice. As a result, although control tBregs (either ex vivo generated or isolated from donor tumor-bearing mice) increased Tregs and concurrently lung metastasis upon adoptive transfer into tumor-bearing mice, RSV-treated Bregs failed to affect this process. In concordance with our recent finding that the loss of tBregs (due to the inhibition of cancer-produced metabolites of 5-lipoxygenase) also leads to the disappearance of cancer-associated Foxp3⁺ Tregs (15), these data further underscore the sufficiency of the inactivation of tBregs alone to abrogate breast cancer lung metastasis. Thus, although high doses of RSV are cytotoxic for normal immune cells and other regulatory immune cells, such as Foxp3⁺ Tregs (29, 37), we show that cancer escape-supporting Tregs can also be reduced by inactivating their key inducers: tBregs. In contrast, RSV does not appear to affect MDSCs, although they are reported to promote 4T1 cancer escape (2–4) and expand Tregs (5–7). In support of this and confirming the ability of RSV to expand MDSCs to protect endothelial cells from a high-dose IL-2-induced injury (29), we did not detect any change in MDSCs in tumor-bearing mice after treatment with low-dose RSV. Instead, to underscore the primary role of tBregs in 4T1 cancer escape, we consistently observed that the proportion of MDSCs was increased by adoptive transfer of mock-treated, but not RSV-treated, tBregs in tumor-bearing B cell-deficient mice (A. Biragyn, personal communication) (13–15). Moreover, despite the presence and expansion of MDSCs, only the loss or the restoration of tBregs abrogated or augmented metastasis in mice with 4T1 cancers, respectively (11, 13–15). Thus, it is tempting to speculate that, although MDSCs play important roles in cancer progression, such as the induction of Tregs via an arginase-dependent, but TGF-β-independent manner, and promoting Th2-skewed responses, survival, and cancer angiogenesis (50–52).

In this study, we also showed that the underlying mechanism of this process is that, at a low and nontoxic dose (3–10 μM) (38, 39), RSV inhibits the generation and function of tBregs by inactivating Stat3 phosphorylation and acetylation. Although the molecular mechanism of this process is not fully understood and is the focus of a different study, the inactivation of Stat3 in tBregs presumably leads to the inhibition of TGF-β expression, a downstream target of Stat3 (53). Confirming this, the specific inhibitors of Stat3 also blocked tBregs and inhibited production of TGF-β, thereby disabling tBregs’ ability to convert Foxp3⁺ Tregs, a process that we reported to require TGF-β (13). As a result and as we reported previously (11, 13, 14), the loss of tBregs/Tregs, in turn, “releases” the suppressed state of effector immune cells, such as antitumor IFN-γ-producing CD8⁺ T cells and NK cells (11), thus protecting the lungs from metastasizing cancer cells. Using two tumor models, we show that, unlike its high and cytotoxic dose (>10 μM) for cancer (39, 40) and immune cells, RSV at low and nontoxic concentrations does not prevent activation of effector IFN-γ–expressing CD8⁺ T cells. Importantly, the low-dose RSV–induced inactivation of tBregs was sufficient to reduce Tregs and abrogate lung metastasis in mice with highly aggressive breast cancer. Taken together, we propose that low doses of RSV can be safely used to provide beneficial treatment in cancer patients via blockade of the tBreg–Treg axis and the concurrent induction of antitumor effector responses.

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Disclosure
The authors have no financial conflicts of interest.

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**SUPPLEMENTARY FIGURES LEGEND** (Catalina Lee Chang et al. 2013)

**Suppl. Figure 1. High doses of RSV are cytotoxic.**

To test cell viability with WST assay titrated amounts of RSV (μM, X-axis) or vehicle (ethanol, mock) were incubated with exponentially growing 4T1.2 cells for 72 h (A). Apoptosis of tBregs (ex vivo generated, B) and Tregs (CD25<sup>+</sup>CD4<sup>+</sup> cells purified from spleen and incubated with 500 U/ml IL-2, C) was assessed using annexin V + propidium iodine staining after 6, 12 and 24 h (B) or 24h (C) after incubation with titrated amounts of RSV (μM). Control cells were mouse splenic B cells incubated with BAFF (B+BAFF) and purified non-Treg T cells (CD25<sup>−</sup>CD4<sup>+</sup> cells). To test regulatory activity of RSV treated Tregs (D), normalized for viable Tregs and non-Tregs used in C were incubated for 5 days with CFSE-labeled murine CD8<sup>+</sup> T cells stimulated with 1.3 μg/ml anti-CD3 Ab. Shown representative data of triplicate experiment repeated twice. From hereon, *p<0.05, ** p<0.01, *** p<0.001 are for statistical significance and NS is for non-significance, respectively.

**Suppl. Figure 2. RSV-induced inhibition of tumor progression is associated with the loss of Tregs.** (A) Females C57BL/6 mice with B16F10 melanoma were i.p. treated with RSV (50 or 500 μg in PBS) or mock 3 days post tumor challenge. Y-axis shows mean tumor weight ± SEM (g) of 4 mice per group experiment. The results were reproduced three times using 4-5 mice per group experiments. (B) In BALB/c mice with 4T1.2 cancer, cancer is associated with the increase in Foxp3<sup>+</sup> CD4<sup>+</sup>Tregs in blood, spleen and draining LN of three per group BALB/c mice. (C) RSV treatment (20 μg and 50 μg) significantly reduces proportion of Tregs (Foxp3<sup>+</sup> gated on total CD4<sup>+</sup> cells) in blood.
(middle panel) and draining LN (right panel, C). (D) However, RSV did not affect proportion of MDSCs (CD11b<sup>+</sup>Gr1<sup>+</sup>) in blood (middle panel) and spleen (right panel, D). Left panels in C and D are representative dot plots of data of triplicate experiments shown in middle and right panels (C and D). Every result was repeated at least twice with 4-5 mice per group experiments.

**Suppl. Figure 3. RSV blocks TGFβ production from B cells of tumor-bearing mice.**

Expression of TGFβ in B cells were isolated from spleen (A,B), or LN (A,C), or tumor (A,D) of mock or 50 μg RSV treated BALB/c mice with 4T1.2 cancer (Mock and RSV). RSV was injected every other day starting day 3 after challenge with 4T1.2 cancer cells. Control B cells were isolated from spleen and LN of untreated naïve mice (Naïve, A-C). Y-axis shows intracellular TGFβ1 ± SEM (%) ± SEM) expression in CD19<sup>+</sup> B cells at day 10. Dot plot in C is representative data of four mice per group experiments shown in D-F. Experiment was reproduced three times with 4 per group mice.

**Suppl. Figure 4. Inactivation of Stat3 in tBregs disables FoxP3<sup>+</sup> Treg conversion in vivo.** Confirming effects on lung metastasis (see Fig.7E), RSV or static-induced inactivation of Stat3 and concurrent down regulation of TGFβ in tBregs disables conversion of FoxP3<sup>+</sup> Tregs in vivo. Tregs were tested in LN of μMT mice (5 per group) were adoptively transferred with congenic BALB/c tBregs pretreated with RSV (12.5 μM), static (1 μM) or mock one day before and 5 days after challenge with 4T1.2 cancer. Control Tregs were from naïve untreated μMT mice (Naïve).
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4