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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 noncytotoxic 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: 4141–4151.

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 immunosuppression 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/ Bregs 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 recently found human granzyme B–expressing human CD19–CD38–CD11c+IgM+IgD+CD62L+ B cells (22), phenotypically they resemble poorly proliferative B2-like cells (IgDHigh) that express constitutively active Stat3 and surface markers CD25High, B7-H1High, CD81High, CD28High, CD69High, CCR6High, and CD62LLow, IgMint/Low, and CD2D1Low (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

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The online version of this article contains supplemental material.

Abbreviations used in this article: Breg, regulatory B cell; cRPMI, complete RPMI; LN, lymph node; MDSC, myeloid-derived suppressor cell; NIA, National Institute on Aging; NIH, National Institutes of Health; RSV, resveratrol; tBreg, tumor-evoked regulatory B cell; TIS, Turbo Ion Spray; Treg, regulatory T cell.

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adoptively transferred with B cells from donor tumor-bearing BALB/c mice treated with RSV or mock. Specifically, the donor mice with 10^6 4T1.2 cells were injected i.p. with RSV (50 µg/mouse) or mock every other day from days 3 to 11. Subsequently, their B cells (4 x 10^7) were isolated and transferred i.v. into host BALB/c mice at 13 and 15 d postchallenge with 5 x 10^5 4T1.2 cells. The host mice were pretreated i.p. with RSV (50 µg/mouse) 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 (Biovision), 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 antiphosphorylated Stat3 (Ty705, 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 (Ty705; 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 anticancer mechanisms of RSV could indirectly suppress 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 (detection 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 tBregs (CD25+CD19high 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 (noncytotoxic 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 replenished 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 I. Detection of RSV and its metabolite (RSV 3-O-sulfate) in the plasma of mice

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Groups</th>
<th>RSV 3-O-sulfate (ng/ml; Mean ± SEM)</th>
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<tbody>
<tr>
<td>1 h</td>
<td>RSV 50 µg</td>
<td>45.5 ± 7.5</td>
</tr>
<tr>
<td>3 h</td>
<td>RSV 50 µg</td>
<td>141.1 ± 4.4</td>
</tr>
<tr>
<td>6 h</td>
<td>RSV 50 µg</td>
<td>BQ</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-glucoronides 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 nmol).
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 posttumor challenge (B) in four to five mice/group. Experiment was repeated three times. Proportion (%) of tBregs (CD81HIGHCD25+ 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 CD81HIGHCD25+ 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
tBregs (11, 13). 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
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 affect function of tBregs by blocking Stat3 and, thereby, disabling TGF-β-induced Foxp3+ Treg conversion (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 noncytotoxic 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 Stattic. 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 tBregs 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 Stattic or RSV (Fig. 7E). Taken together, the mechanism of ant metastatic activity of a noncytotoxic 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-β1-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+...
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

![Graph showing RSV inhibits TGF-β production from tBregs](image)
FIGURE 7. 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 Static (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, Static, 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 tTregs 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 augmented or abrogated metastasis in mice with 4T1 cancers, respectively (11, 13–15). Thus, it is tempting to speculate that, although MDSCs play important regulatory and tumorigenic roles, they may be required for other steps 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 noncytotoxic 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 noncytotoxic 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 therapeutic benefit in cancer patients via blockage of the tBreg–Treg axis and the concurrent induction of antitumor effector responses.

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Disclosures

The authors have no financial conflicts of interest.

References


**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+CD4+ 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 (CD25CD4+ 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+ 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+ CD4+ 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+ gated on total CD4+ cells) in blood
(middle panel) and draining LN (right panel, C). (D) However, RSV did not affect proportion of MDSCs (CD11b^+Gr1^+) 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^+ 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^+ 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^+ 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