ARTICLE

STAT3 Modulation of Regulatory T Cells in Response to Radiation Therapy in Head and Neck Cancer

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Abstract

Background: Radioresistance represents a major problem in the treatment of head and neck cancer (HNC) patients. To improve response, understanding tumor microenvironmental factors that contribute to radiation resistance is important. Regulatory T cells (Tregs) are enriched in numerous cancers and can dampen the response to radiation by creating an immune-inhibitory microenvironment. The purpose of this study was to investigate mechanisms of Treg modulation by radiation in HNC.

Methods: We utilized an orthotopic mouse model of HNC. Anti-CD25 was used for Treg depletion. Image-guided radiation was delivered to a dose of 10 Gy. Flow cytometry was used to analyze abundance and function of intratumoral immune cells. Enzyme-linked immunosorbent assay was performed to assess secreted factors. For immune-modulating therapies, anti-PD-L1, anti-CTLA-4, and STAT3 antisense oligonucleotide (ASO) were used. All statistical tests were two-sided.

Results: Treatment with anti-CD25 and radiation led to tumor eradication (57.1%, $n = 4$ of 7 mice), enhanced T-cell cytotoxicity compared with RT alone (CD4 effector T cells [Teff]: RT group mean = 5.37 [0.58] vs RT + α CD25 group mean = 10.71 [0.67], $P = .005$; CD8 Teff: RT group mean = 9.98 [0.81] vs RT + α CD25 group mean = 16.88 [2.49], $P = .01$) and induced tumor antigen-specific memory response (100.0%, $n = 4$ mice). In contrast, radiation alone or when combined with anti-CTLA4 did not lead to durable tumor control (0.0%, $n = 7$ mice). STAT3 inhibition in combination with radiation, but not as a single agent, improved tumor growth delay, decreased Tregs, myeloid-derived suppressor cells, and M2 macrophages and enhanced effector T cells and M1 macrophages. Experiments in nude mice inhibited the benefit of STAT3 ASO and radiation.

Conclusion: We propose that STAT3 inhibition is a viable and potent therapeutic target against Tregs. Our data support the design of clinical trials integrating STAT3 ASO in the standard of care for cancer patients receiving radiation.

Despite aggressive treatment involving chemotherapy and radiotherapy (RT), the overall survival rate for head and neck squamous cell carcinoma (HNSCC) remains below 50% at 5 years (1, 2). RT represents standard of care for a majority of these patients (3) and has been largely viewed to exert its effects through direct DNA damage and indirect damage from free radical formation. However, RT can also induce antitumor immune responses that contribute to indirect tumor cell kill (4–6). Data

over the last decade have shown that RT's antitumor immune effects can be blunted by mechanisms of immune evasion and immune-suppression including upregulation of programmed-death ligand 1 (PD-L1) on tumor cells and secretion of immunosuppressive factors that promote infiltration of regulatory T cells, myeloid-derived suppressor cells (MDSCs), and macrophages (4, 6–10). These mechanisms potentially limit the antitumor effects of RT. To develop better therapeutic strategies,

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understanding tumor microenvironmental (TME) factors that contribute to radioresistance is important.

Here, we specifically evaluate the role of regulatory T cells (Tregs). This is a unique subpopulation of CD4 T cells characterized by expression of the forkhead box P3 (FOXP3) transcription factor and high levels of CD25 (11,12). Tregs play a major role in dampening spontaneous tumor-associated antigen (TAA)-specific immune responses (13,14). Moreover, RT can increase the recruitment of Tregs to the local TME and attenuate radiation-induced tumor death (15). Tregs were shown to be increased in the tumor and blood of HNSCC patients compared with healthy donors and their presence correlated with low CD8/Treg ratio (16). We previously demonstrated that Tregs are highly enriched in orthotopic models of HNSCC and contribute to treatment resistance (17). Given that administration of anti-CD25 in established tumors fails to demonstrate therapeutic activity or deplete intratumoral Tregs (18), therapeutically targeting Tregs remains a major challenge. We assessed modulation of Tregs by STAT3 using a murine selective antisense oligonucleotide (ASO). STAT3 is a necessary transcription cofactor for FOXP3 (19), but the effects of its inhibition on Treg function have remained elusive. In this study, we hypothesized that targeting Tregs through STAT3 inhibition can enhance RT response.

Materials and Methods

Cell lines and cell culture methods, materials and methods for flow cytometry, and all other experimental procedures are described in detail in the [Supplementary Methods](#) (available online).

Mouse Model

Orthotopic HNSCC mouse models were established as previously described (6). Murine MOC2 and LY2 squamous cell carcinoma cells were implanted into the buccal mucosa of C57BL/6 and Balb/c mice, respectively. Seven to ten female mice ages 6–7 weeks were used per experimental group. Cell suspensions were mixed with equal volumes of Matrigel and injected via the intraoral route into the buccal mucosa. All protocols for animal tumor models were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Irradiation

Irradiation was performed using X-RAD image-guided irradiator at 225 kVp. Mice were positioned in the prone orientation and a computerized tomography (CT) scan was acquired. Radiation was delivered at a dose rate of 5.6 Gy/min.

The Cancer Genome Atlas (TCGA) Analysis

The HNSCC data set was downloaded from TCGA, and gene-expression profiles were sorted according to the combined average expression of FOXP3, CD25, and TGFB1. Patients on this spectrum were divided into quartiles. Patients in the first and fourth quartile ($n = 36$ patients in Q1 and $n = 37$ patients in Q4) were assessed for overall survival (OS) and disease-free survival (DFS). Statistical significance in survival was assessed using the log-rank (Mantel-Cox) test. Differences in the number of patients between OS and DFS analyses are due to patients lost

to follow-up. All patient identifiers as provided by the TCGA are provided in [Supplementary Table 1](#) (available online).

Statistical Analysis

Analysis of variance (ANOVA) was performed to assess differences in expression of markers across groups. For multiple comparisons between pairs of groups, the Tukey test was applied. Unpaired *t* test was used to compare means between two groups. Fisher exact test was used to compare response rate between pairs of groups. For survival analysis, Kaplan-Meier curves were analyzed based on the log-rank (Mantel-Cox) test for comparison of all groups. Hazard ratios were generated between pairs of groups. All statistical tests were two sided and a *P* value of less than .05 was considered statistically significant.

Results

Effect of Treg Depletion on Response to Radiation

We hypothesized that high baseline Tregs contribute to radioresistance. To test this hypothesis, we used anti-CD25-depleting antibody, which has been shown to deplete Tregs (20), or anti-CTLA4, which has been shown to block the inhibitory activity of Tregs (21). We tested a dose of 10 Gy in single-fraction or five fractions to determine which fractionation schedule is more immunogenic. We observed an increase in effector CD4 and CD8 T cells with single-dose 10 Gy but not fractionated 10 Gy ([Supplementary Figure 1](#), available online). We therefore chose single-dose 10 Gy for subsequent studies. Only the combination of RT with anti-CD25 resulted in smaller tumors when compared with RT alone (RT alone mean tumor volume = 223 [80.63] mm³ vs RT + α CD25 mean tumor volume = 12.40 [7.84] mm³, $P = .01$) (Figure 1, F and I). Long-term follow-up showed that RT + anti-CD25 resulted in tumor eradication in 57.1% of mice ($n = 4$ of 7) (Figure 1F) and improved survival compared with all other groups (Figure 1J). In contrast, RT + anti-CTLA4 did not lead to durable tumor control ($n = 0$ of 7 mice). Tumor eradication was achieved only in sufficiently Treg-depleted tumors. Mice that progressed despite RT + anti-CD25 treatment showed higher levels of Tregs compared with cured or naïve mice ([Supplementary Figure 2B](#), available online). Analysis of circulating transforming growth factor (TGF)- β 1 levels showed a decrease only in the RT + anti-CD25 group compared with RT alone ($P = .003$) and RT + anti-CTLA4 ($P = .01$; [Supplementary Figure 3](#), available online). To determine if immunologic memory was induced, we re-challenged cured mice in the contralateral buccal: 100.0% ($n = 4$ of 4) of re-challenged mice did not demonstrate tumor growth (Figure 1G). To demonstrate tumor antigen-specific responses, we isolated T cells from draining lymph nodes (DLNs) of cured mice and injected them into naïve donor mice, which were implanted with tumor cells 24 hours later (Figure 1H). Mice receiving T cells from cured mice developed statistically significantly smaller tumors compared with mice that received naïve T cells, indicating transferred T cells mounted an effective recall response (Figure 1H; [Supplementary Figure 4](#), available online). To determine if cured mice show LY2 tumor-specific immunity, we inoculated four cured mice with the CT26 colon cancer cell line. All mice developed CT26 tumors ([Supplementary Figure 5](#), available online). We further tested the effect of Treg depletion in MOC2 HNSCC tumors in C57BL/6 mice. Analysis of circulating and intratumoral Tregs showed a statistically significant reduction in Tregs in the RT + anti-CD25

Figure 1. Effect of regulatory T-cell (Treg) depletion on response to radiation. **A–F)** α CD25 or α CTLA4 were administered alone or in combination with RT (10 Gy) delivered on day 8 post-tumor implantation in LY2 tumor-bearing mice. IgG and RT groups were used as control. Eradicated tumors are represented by number of complete responders observed in each group. **F)** Mouse IDs are shown to highlight mice with complete response. **G)** Tumor growth of cured mice that were re-challenged in the contralateral buccal (mouse identifiers are indicated). **H)** Schematic showing isolation of T cells from DLNs and their introduction into recipient mice. Graphs show tumor growth analysis of recipient mice that were injected intravenously with T cells from cured mice compared with mice that were injected with T cells from naive mice. **I)** Analysis of tumor volume on the last day mice in all groups were alive (day 22). Bars represent standard deviation. Statistical significance was assessed with one-way analysis of variance, and the Tukey test was applied. **J)** Log-rank survival plot for mice in each group. Table shows median survival times and statistical significance of each group compared to the IgG control group (Mantel-Cox test). All statistical tests were two-sided. For all experiments seven or eight mice per group were used. CR = complete response; DLN = draining lymph node; IgG = immunoglobulin G; RT = radiotherapy.

group compared with the RT + immunoglobulin G (IgG) group (Supplementary Figure 6, A and B, available online). Treatment with anti-CD25 in combination with RT resulted in a statistically significant delay in tumor growth compared with RT alone (Supplementary Figure 6C, available online). However, in contrast to LY2 tumors treated with RT + anti-CD25, we did not observe tumor rejection in any MOC2 tumors (Supplementary Figure 6D, available online). To identify factors that could explain these data, we performed RNA sequencing (RNAseq) on

cultured LY2 and MOC2 cells and compared basal expression of immunosuppressive chemokines. Consistent with a Treg-enriched microenvironment in both tumors, we observed in vitro expression of CCL20, an established Treg chemoattractant (Supplementary Figure 6E, available online). MOC2 cells, however, also expressed high levels of CCL2, which was absent in LY2 cells (Supplementary Figure 6E, available online). CCL2 is a chemoattractant for MDSCs and macrophages and may therefore contribute to an additional mechanism of resistance to RT.

Figure 2. Analysis of response to radiotherapy treatment based on regulatory T-cell (Treg) gene-expression signature. **A)** Gene-expression signature composed of the average expression of *FOXP3*, *IL-2RA*(*CD25*), and *TGFB1* shows these genes are highly expressed in patients identified as having high-Treg tumors compared with patients with low-Treg tumors based on upper and lower quartiles of expression. Unpaired *t* test was performed to assess statistical significance. **B)** Analysis of overall survival and disease-free survival in head and neck squamous cell carcinoma patients receiving radiotherapy based on Treg gene signature. Log-rank (Mantel-Cox) test was used to determine statistical significance and derive hazard ratios (HRs) (and 95% confidence intervals). Number of individuals at risk is presented in tables. All statistical tests were two sided. RSEM = RNA-Seq by Expectation Maximization.

Both cell types, we further validated, were present in MOC2 tumors treated with RT, but unlike LY2 tumors, addition of anti-CD25 to RT did not reduce MDSCs or macrophages (Supplementary Figure 6F, available online).

Because Treg depletion was previously shown to synergize with anti-PD-L1 therapy in preclinical tumor models (18,22), we tested whether addition of anti-PD-L1 to anti-CD25 or to RT and anti-CTLA-4 could provide durable tumor control. Our data show that addition of anti-PD-L1 does not provide durable tumor control (Supplementary Figure 7, available online). The addition of anti-PD-L1 to RT + anti-CD25 resulted in tumor eradication in six of seven mice, consistent with the potent effect of RT + anti-CD25 shown in Figure 1F. The effect of RT + anti-CD25 + anti-PD-L1 was not statistically significantly different from the effect of RT + anti-CD25 ($P = .60$, Fisher exact test).

To examine the effect of Tregs on response to RT in HNSCC patients, we analyzed the HNSCC cohort of patients receiving RT in the TCGA. A total of 140 patients were identified who received RT for curative intent (defined as >60 Gy). Based on a comparison of upper quartile vs lower quartile, all three selected genes (*FOXP3*, *CD25*, and *TGFB1*) were statistically significantly higher in the Treg-high group compared with the Treg-low group (Figure 2A). A statistically significant gain in OS in the Treg-low group was observed compared with the Treg-high group (hazard ratio [HR] = 2.31, 95% CI = 1.12 to 5.13, $P = .03$). Median survival in the Treg-low group was 76.2 months (range = 8.8–65.5 months) compared with 26.5 months (range = 10.7–76.2 months) in the Treg-high group (Figure 2B). DFS was not statistically significant between the Treg-low and Treg-high groups (HR = 1.68, 95% CI = 0.88 to 3.41, $P = .12$) (Figure 2B).

Effect of Treg Depletion on Effector T Cells (Teff) When Combined With Radiation

Mice were allowed to develop LY2 tumors for 2 weeks with and without anti-CD25 treatment. Tumors were then exposed to RT and harvested 72 hours later (Figure 3A). Mean (SD) proportion of intratumoral CD4+FoxP3+ Tregs was 35.36 (1.62)%, 29.48 (0.70)%, 3.54 (1.09)%, and 3.81 (3.23)% in the control IgG, RT, anti-CD25, and RT + anti-CD25 groups, respectively (Figure 3B). Assessment of Teff (CD44 high IFN γ +) showed statistically significant induction of CD4 and CD8 Teff in the RT alone and RT + anti-CD25 groups (Figure 3, C and D). RT + anti-CD25 treatment induced higher CD4 and CD8 Teffs compared with RT alone (CD4 Teff: RT group mean = 5.37 [0.58] vs RT + α CD25 group mean = 10.71 [0.67], $P = .005$; CD8 Teff: RT group mean = 9.98 [0.81] vs RT + α CD25 group mean = 16.88 [2.49], $P = .01$) (Figure 3, C and D). Analysis of absolute T-cell numbers showed higher CD4 and CD8 T-cell infiltration in the RT + anti-CD25 group relative to all other groups (Figure 3E).

Effect of Treg Depletion on Myeloid Cell Populations When Combined With Radiation

Tregs can influence myeloid cell populations (23–25) and their depletion can induce antitumor M1 macrophage infiltration (26). We assessed myeloid populations in tumors from Treg-depleted mice with and without RT. Treg depletion alone and in combination with RT resulted in a statistically significant reduction in the proportion of macrophages (F4/80+) relative to all myeloid cells (CD11b+) (Figure 4A). Phenotypic characterization of macrophages revealed that combination of RT and Treg

Figure 3. Effect of radiation and regulatory T-cell (Treg) depletion on T-cell numbers and function. **A)** Schematic of experimental timeline. **B)** Proportion of Tregs in tumors treated with radiotherapy (RT) and α CD25 in comparison with α CD25, RT, and control groups. **C–D)** Analysis of effector CD8 and CD4 T cells (CD44 high IFN γ +) across groups. Representative scatter plots are shown. **E)** Quantification of absolute numbers of CD8 and CD4 T cells normalized to tumor weight across groups. Bars represent SD of four or five independent tumor samples. P values are shown for statistical significance in comparison with all groups (two-way analysis of variance). All statistical tests were two-sided. FITC = fluorescein isothiocyanate; IgG = immunoglobulin G; SD = standard deviation; FITC = Fluorescein isothiocyanate.

depletion resulted in a statistically significant shift in the M1 to M2 ratio that is attributed to increased proportion of M1 macrophages (F4/80 + inducible nitric oxide synthase+) and reduced M2 macrophages (F4/80+CD163+) (Figure 4, B–D). Interestingly, RT increased the proportion of M2 macrophages when compared with IgG control ($P = .02$; Figure 4D). This is consistent with previous reports demonstrating RT-mediated macrophage infiltration in various tumor models (27–29). In addition, a statistically significant decrease in MDSCs (CD11b+Gr1+) was observed in the RT + anti-CD25 group relative to all other groups (Figure 4E).

STAT3 Modulation on Tregs in Response to Radiation

Because STAT3 is a cotranscription factor with FOXP3 (19, 30), we performed flow cytometry on isolated T cells and assessed STAT3 phosphorylation. Tregs expressed the highest level of pSTAT3 followed by CD8 T cells and CD4+FOXP3– T cells (Supplementary Figure 8A, available online). The majority of pSTAT3+ Tregs were highly proliferative as evidenced by more than 80.0% Ki67+ staining, whereas less than 10.0% of pSTAT3-negative Tregs expressed Ki67 (Supplementary Figure 8B, available online). These data corroborate previous studies implicating STAT3 signaling in enhanced Treg function and conversion (31). We further examined whether RT affects the conversion of CD4 T cells to Tregs and the role STAT3 plays in mediating such

conversion. CD4 T cells were isolated from mouse spleens and exposed to 10Gy in vitro. Expression of pSTAT3 increased at 3 hours and remained elevated compared with nonirradiated CD4 T cells at 24 hours (1.7-fold and 1.8-fold increase, respectively) (Figure 5A). This was associated with a statistically significant increase in Tregs (1.7-fold) and TGF- β 1 (2.5-fold) after RT (Figure 5, B and C). To evaluate the implication of this RT-induced increase in STAT3 phosphorylation on CD4s, we utilized the murine surrogate of the third-generation STAT3 ASO, AZD9150, which has been shown to decrease STAT3 (32). STAT3 ASO decreased the proportion of pSTAT3+ CD4+ T cells from a mean of 5.53 (0.62)% to 1.76 (0.13)%. When combined with RT, the proportion of pSTAT3+ CD4+ T cells decreased from a mean of 5.81 (1.11)% to 0.65 (0.23) % (Figure 5D). In addition, STAT3 ASO resulted in a statistically significant reduction in the proportion of Tregs at baseline and when combined with RT (mean proportion at baseline = 5.43 [0.50]% compared with 1.57 [1.19]%, $P = .01$; mean proportion when combined with RT = 9.17 [1.27] % compared with 1.99 [2.43] %) (Figure 5E).

Effect of RT and STAT3 ASO on Tumor Growth and Tumor Immune Microenvironment

To test the therapeutic efficacy of STAT3 ASO in combination with RT, we administered STAT3 ASO in established tumors and delivered RT 72 hours later. In LY2 mice, average tumor

Figure 4. Assessment of changes in myeloid cell populations in response to radiation and regulatory T-cell (Treg) depletion using flow cytometry. **A)** Proportion of macrophages in tumors treated with radiotherapy (RT) and αCD25 in comparison with αCD25, RT, and control groups. **B–D)** Analysis of M1 and M2 macrophages across groups. Representative scatter plots are shown. **E)** Analysis of MDSCs. Bars represent SD of 4–5 independent tumor samples. Statistical significance comparing all groups (two-way analysis of variance) is shown. All statistical tests were two sided. IgG = immunoglobulin G; MDSCs = myeloid-derived suppressor cells.

volume in the RT + STAT3 ASO group on day 22 when all mice were alive was 53.0 (5.6) mm³ compared with 176.7 (23.1) mm³ in the RT group, 277.4 (53.8) mm³ in the STAT3 ASO group, and 622.1 (72.3) mm³ in the control ASO group (Figure 6A). In MOC2 mice, average tumor volume in the RT + STAT3 ASO group on day 19 when all mice were alive was 254.8 (81.6) mm³ compared with 920.7 (550) mm³ in the RT alone group, 1042.9 (326.8) mm³ in the STAT3 ASO alone group, and 1527.9 (370.3) mm³ in the ASO control group (Figure 6B). Median survival in LY2 and MOC2 tumor-bearing mice treated with RT + STAT3 ASO was improved compared with all other groups (Figure 6, C and D). The majority of mice (n = 7 of 8 in LY2 and n = 8 of 8 in MOC2) had tumors at the time of terminating STAT3 ASO dosing (2 weeks post-initial dose) indicating that continuous STAT3 ASO dosing is necessary to maintain local control (Supplementary Figure 9,

A and B, available online). In one LY2 mouse, tumor eradication was achieved with RT + STAT3 ASO and re-challenge of the mouse in the contralateral buccal showed tumor rejection (Supplementary Figure 9C, available online). The addition of anti-PD-L1 to RT + STAT3 ASO did not further improve tumor control or survival (Supplementary Figure 10, available online).

We performed flow cytometry on established tumors treated with STAT3 ASO alone or in combination with RT (Figure 7A). pSTAT3 was statistically significantly decreased in STAT3 ASO and RT + STAT3 ASO-treated tumors relative to control in CD8 T cells, CD4+FoxP3⁻ T cells and CD4+FoxP3⁺ Tregs (Supplementary Figure 11, available online). STAT3 ASO statistically significantly reduced the proportion of intratumoral and circulating Tregs relative to control ASO (Figure 7, B and C). When combined with RT, STAT3 ASO statistically significantly

Figure 5. Assessment of STAT3 phosphorylation on T cells in response to radiation and the effect of STAT3 inhibition using antisense oligonucleotide (ASO) AZD9150. **A)** Analysis of STAT3 phosphorylation in isolated CD4 T cells in the presence and absence of STAT3 ASO when irradiated with 10 Gy by flow cytometry. **B)** Assessment of the proportion of Tregs in CD4 T cells that were exposed to 10 Gy radiation. **C)** Analysis of TGFβ1 from conditioned media of CD4 T cells that were exposed to 10 Gy RT. **D)** Analysis of the proportion of CD4 T cells that express pSTAT3 when combined with RT in the presence of STAT3 ASO or control ASO. **E)** Analysis of the proportion of Tregs in CD4 T cells when exposed to 10 Gy radiation and treated with STAT3 ASO. Bars represent standard deviation from three to five independent experiments. Statistical significance between pairs of groups (unpaired t test) was assessed. All statistical tests were two sided. CTL = control; h = hours; RT = radiation therapy; Tregs = regulatory T cells.

Figure 6. Effect of combining radiation with STAT3 ASO. Schematic shows experimental timeline. **A and B)** Tumor growth in LY2 and MOC2 tumor-bearing mice when treated with STAT3 ASO alone and in combination with RT ($n = 7-10$ mice per group). **C and D)** Survival analysis of LY2 and MOC2 tumor-bearing mice. Log-rank (Mantel-Cox) test was used to determine statistical significance and derive hazard ratios (and 95% confidence intervals). All statistical tests were two sided. ASO = antisense oligonucleotide; RT = radiotherapy.

reduced the proportion of intratumoral Tregs relative to all other groups and statistically significantly reduced the proportion of circulating Tregs relative to control ASO and RT alone groups (Figure 7, B and C). Mice treated with RT + STAT3 ASO demonstrated the most statistically significant increase in effector T cells compared with all other groups (Figure 7D). Multiplex analysis of secreted factors in the plasma showed

near complete elimination of TGF-β1, IL-6, and IL-10 levels (Figure 7, E-G) and a statistically significant increase in IL2 in the RT + STAT3 ASO group, which may be related to the abolishment of Tregs, a major consumer of IL-2 (Figure 7, E-H). Importantly, we observed near complete elimination of plasma CCL20, a major chemoattractant of Tregs (Figure 7I). Assessment of intratumoral myeloid populations showed a

Figure 7. Analysis of intratumoral and systemic effects of radiotherapy (RT) and STAT3 antisense oligonucleotide (ASO) shows downregulation of regulatory T cells (Tregs) and associated chemokines and enhanced effector T cells (Teffs). **A** Schematic illustration of experimental timeline. Tumors were allowed to grow for 14 days without intervention to ensure similar tumor size at harvesting for flow cytometry. **B**) Proportion of Tregs in tumors treated with RT and STAT3 ASO in comparison with STAT3 ASO, RT, and control groups. **C**) Proportion of circulating Tregs in response to different treatments. **D**) Analysis of Teffs (CD44 high IFN γ +) across groups. Bars represent SEM of four or five independent tumor samples. **E–I**) Analysis of the secreted chemokines TGF- β 1, IL-6, IL-10, IL-2, and CCL20 by multiplex enzyme-linked immunosorbent assay. Bars represent SEM from four to six mice per group. **J–L**) Analysis of intratumoral myeloid populations by flow cytometry. Two-way analysis of variance was performed to assess statistical significance between groups. All statistical tests were two-sided.

statistically significant decline in MDSCs and M2 macrophages in the RT + STAT3 ASO group compared with all other groups (Figure 7, J and K). This was at the expense of increased M1 macrophages (Figure 7L).

Because STAT3 ASO targets multiple cell populations including tumor cells, T cells, and myeloid cells, we investigated whether response to STAT3 ASO and RT is dependent on T cells by testing its effect in nude mice. We could not observe any benefit from RT + STAT3 ASO in nude mice (Figure 8, A–D), indicating that the response to STAT3 ASO is dependent on T cells. Collectively, our findings demonstrate that the RT and STAT3 ASO combination reprograms the immune TME, resulting in a potent antitumor immune response that is dependent on Teffs.

Discussion

Immune parameters that determine response to RT in HNSCC have largely remained elusive. In previous work, we showed that RT enhances T-cell infiltration and sensitizes PD-L1 refractory tumors to immunotherapy, but adaptive resistance develops (6). Treg accumulation in tumors and increased levels of circulating Tregs correlate with increased risk of recurrence, metastasis, and worse survival in several cancers (33–47). Tregs were shown to be highly enriched in patients with HNSCC, and elevated serum levels of IL-10 and TGF- β 1 correlated with worse survival (48,49). A recent TCGA analysis showed that Tregs were prognostic for improved survival, but only on univariate analysis (50). In our analysis we focused on the subset of patients

Figure 8. Efficacy of combination STAT3 antisense oligonucleotide (ASO) and radiotherapy (RT) is lost in Balb/c nude mice. **A)** Assessment of tumor volume between groups on days 10, 14, and 17 post-tumor inoculation. Two-way analysis of variance was performed to assess statistical significance between the groups. All statistical tests were two sided. **B)** Comparison of average tumor growth between the groups. **C-D)** Assessment of mouse survival in each experimental group. Number of mice at risk is shown below the graph and median survival is presented.

receiving RT. Our data showed that high expression of a Treg genomic signature was associated with statistically significant reduction in OS of HNSCC patients.

Tregs can induce immunosuppression through contact-dependent mechanisms or through contact-independent mechanisms, including sequestration of IL-2 and production of soluble immunosuppressive molecules including IL-10, TGF β , and adenosine (51–54). In mouse models of lung carcinoma, Tregs were shown to inhibit T-cell-mediated cytotoxicity in a TGF β -dependent manner, and Treg depletion enhanced T-cell antitumor responses (55). Our data show that Treg depletion alone is not sufficient to induce an antitumor immune response. This is likely because the tumor models we selected are poorly infiltrated with Teffs, which could explain their intrinsic resistance to immune checkpoint blockade, but also suggests that in these tumors a local insult to the tumor is necessary to allow Teffs to orchestrate an antitumor immune response. Thus, combining Treg depletion strategies with immunotherapy in poorly immunogenic tumors will likely not work because the absence of Tregs is not sufficient to expose tumor neoantigens and attract cytotoxic immune cells. RT is well suited for transforming poorly immunogenic tumors because it can expose tumor neoantigens (56), increase secretion of effector T-cell chemokines including CXCL9 and CXCL10 (6, 57), and activate stimulator of interferon genes (58). These immune-boosting properties of RT are primarily observed with high single-doses (>10 Gy) or hypofractionated doses (59–63). Compared with conventionally fractionated RT, Morisada et al. showed that in HNSCC models, hypofractionated RT enhanced peripheral and tumor-infiltrating CD8 T lymphocyte numbers and function and decreased peripheral MDSC accumulation (59). Similarly, we observed a statistically significant increase in effector CD8 and CD4 T cells with single-dose 10 Gy, but not with 10 Gy in five fractions. Our observation of improved tumor response when RT is combined with Treg depletion highlights that the two modalities act on distinct aspects of antitumor immunity, which

on their own are not sufficient to provide tumor control. Importantly, this response was not observed with anti-CTLA4 treatment. Anti-CTLA4 works by blocking the interaction between CTLA4 on Tregs and soluble B7 ligands on antigen-presenting cells (64). Although CTLA4 has been shown to be associated with Tregs proliferation (65), other studies showed that anti-CTLA4 does not deplete Tregs and is not required by Tregs to exert immunosuppressive activity (66–68).

Although the role of Tregs in immunosuppression and tumor progression is well established, mechanisms by which Tregs infiltrate tumors remain elusive. Curiel et al. showed that CCL22 induces migration of Tregs into ovarian tumors (33). In our model, CCL22 was not detectable. Instead, CCL20 was highly expressed in our HNSCC tumor cell lines. CCL20 is a regulator of Treg infiltration and the sole chemokine ligand for CCR6, which is expressed predominantly by Tregs (69, 70). In addition to CCL20, our RNAseq analysis revealed important differences between HNSCC cell lines, which potentially highlight their differential response to treatment. Only MOC2 cells expressed CCL2, a known chemoattractant for macrophages and MDSCs (71). This may explain why tumor eradication was observed in LY2 tumors but not MOC2 tumors in response to RT + anti-CD25 treatment. It is therefore conceivable that multiple immune-suppressive populations may need to be targeted to allow RT to orchestrate a robust antitumor immune response.

Targeting Tregs represents a major challenge. Although TGF- β inhibition can decrease Tregs, it can also inhibit the proliferation and functional differentiation of T cells, natural killer cells, and macrophages (72, 73). Cyclophosphamide can have Treg-depleting properties by acting on glutathione (74). However, it has been shown to induce infiltration of MDSCs, which can reverse the beneficial effects of Treg depletion (75). In contrast, our data with STAT3 ASO and RT demonstrate a statistically significant decrease in Tregs and MDSCs in orthotopic HNSCC tumors. STAT3 is a member of the signal transduction and activator of transcription family known to mediate tumorigenesis and immunity (76, 77). STAT3

ASO (AZD9150) has been shown to deplete STAT3 at the RNA and protein levels (32) and has shown efficacy in reducing tumor growth in animal models of neuroblastoma, lymphoma, and lung cancer (32, 78). In addition, AZD9150 has been tested in Phase Ib trials and was shown to have a good safety profile (32, 79).

One of the limitations of this study is that we cannot exclude the possibility that STAT3 ASO may act directly on tumor cells. The complete absence of a response to RT and STAT3 ASO in nude mice provides evidence that T cells are indispensable for the therapeutic efficacy of RT and STAT3 ASO but does not preclude the possibility of a cross-talk between T cells and myeloid cells. It remains to be explored whether STAT3 ASO acts directly on Tregs, which subsequently modulate myeloid populations, or whether STAT3 ASO also targets myeloid cells.

Our data support a model in which RT induces tumor secretion of inflammatory cytokines, which play paradoxical roles in anti- and pro-tumor immunity. We propose that suppression of pro-tumorigenic immune factors is necessary for improved efficacy of RT and durable tumor control. Our study provides the first evidence that targeting Tregs by antagonizing STAT3 enhances therapeutic response to RT. These findings have direct clinical implications for improving patient tumor control and survival.

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Notes

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References

- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005;55(2):74–108.
- Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol*. 2006;24(14):2137–2150.
- Colevas AD, Yom SS, Pfister DG, et al. NCCN guidelines insights: head and neck cancers, Version 1.2018. *J Natl Compr Canc Netw*. 2018;16(5):479–490.
- Wu CT, Chen WC, Chang YH, Lin WY, Chen MF. The role of PD-L1 in the radiation response and clinical outcome for bladder cancer. *Sci Rep*. 2016;6(1):19740.
- Wang X, Schoenhals JE, Li A, et al. Suppression of type I IFN signaling in tumors mediates resistance to anti-PD-1 treatment that can be overcome by radiotherapy. *Cancer Res*. 2017;77(4):839–850.
- Oweida A, Lennon S, Calame D, et al. Ionizing radiation sensitizes tumors to PD-L1 immune checkpoint blockade in orthotopic murine head and neck squamous cell carcinoma. *Oncimmunology*. 2017;6(10):e1356153.
- Derer A, Spiljar M, Baumler M, et al. Chemoradiation increases PD-L1 expression in certain melanoma and glioblastoma cells. *Front Immunol*. 2016;7(12):610.
- Liu S, Sun X, Luo J, et al. Effects of radiation on T regulatory cells in normal states and cancer: mechanisms and clinical implications. *Am J Cancer Res*. 2015;5(11):3276–3285.
- Vatner RE, Formenti SC. Myeloid-derived cells in tumors: effects of radiation. *Semin Radiat Oncol*. 2015;25(1):18–27.
- Seifert L, Werba G, Tiwari S, et al. Radiation therapy induces macrophages to suppress T-cell responses against pancreatic tumors in mice. *Gastroenterology*. 2016;150(7):1659–1672.e1655.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057–1061.
- d’Hennezel E, Piccirillo CA. Analysis of human FOXP3+ Treg cells phenotype and function. *Methods Mol Biol*. 2011;707(1):199–218.
- Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol*. 2005;6(4):345–352.
- Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol*. 2006;6(4):295–307.
- Muroyama Y, Nirschl TR, Kochel CM, et al. Stereotactic radiotherapy increases functionally suppressive regulatory T cells in the tumor microenvironment. *Cancer Immunol Res*. 2017;5(11):992–1004.
- Lechner A, Schlößer H, Rothschild SI, et al. Characterization of tumor-associated T-lymphocyte subsets and immune checkpoint molecules in head and neck squamous cell carcinoma. *Oncotarget*. 2017;8(27):44418–44433.
- Oweida AH, Phan A, Binder D, et al. Resistance to radiotherapy and PD-L1 blockade is mediated by TIM-3 upregulation and regulatory T-cell infiltration. *Clin Canc Res*. 2018;24(21):5368.
- Arce Vargas F, Furness AJS, Solomon I, et al. Fc-optimized anti-CD25 depletes tumor-infiltrating regulatory T cells and synergizes with PD-1 blockade to eradicate established tumors. *Immunity*. 2017;46(4):577–586.
- Hossain DM, Panda AK, Manna A, et al. FoxP3 acts as a cotranscription factor with STAT3 in tumor-induced regulatory T cells. *Immunity*. 2013;39(6):1057–1069.
- Setiady YY, Coccia JA, Park PU. In vivo depletion of CD4+FOXP3+ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcγRIIIb+ phagocytes. *Eur J Immunol*. 2010;40(3):780–786.
- Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008;322(5899):271–275.
- Liu J, Blake SJ, Harjunpaa H, et al. Assessing immune-related adverse events of efficacious combination immunotherapies in preclinical models of cancer. *Cancer Res*. 2016;76(18):5288–5301.
- Ugel S, De Sanctis F, Mandruzzato S, Bronte V. Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages. *J Clin Invest*. 2015;125(9):3365–3376.
- Fujimura T, Kambayashi Y, Aiba S. Crosstalk between regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) during melanoma growth. *Oncimmunology*. 2012;1(8):1433–1434.
- Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology*. 2013;138(2):105–115.
- Galani IE, Wendel M, Stojanovic A, et al. Regulatory T cells control macrophage accumulation and activation in lymphoma. *Int J Cancer*. 2010;127(5):1131–1140.
- Chiang CS, Fu SY, Wang SC, et al. Irradiation promotes an m2 macrophage phenotype in tumor hypoxia. *Front Oncol*. 2012;2(8):89.
- Teresa Pinto A, Laranjeiro Pinto M, Patricia Cardoso A, et al. Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. *Sci Rep*. 2016;6(1):18765.
- Wu Q, Allouch A, Martins I, Modjtahedi N, Deutsch E, Perfettini JL. Macrophage biology plays a central role during ionizing radiation-elicited tumor response. *Biomed J*. 2017;40(4):200–211.
- Chaudhry A, Rudra D, Treuting P, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*. 2009;326(5955):986–991.
- Hsu P, Santner-Nanan B, Hu M, et al. IL-10 potentiates differentiation of human induced regulatory T cells via STAT3 and foxo1. *J Immunol*. 2015;195(8):3665–3674.

32. Hong D, Kurzrock R, Kim Y, et al. AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3 with early evidence of clinical activity in lymphoma and lung cancer. *Sci Transl Med*. 2015;7(314):314ra185.
33. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10(9):942–949.
34. Maj T, Wang W, Crespo J, et al. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nat Immunol*. 2017;18(12):1332–1341.
35. Granville CA, Memmott RM, Balogh A, et al. A central role for Foxp3⁺ regulatory T cells in K-Ras-driven lung tumorigenesis. *PLoS One*. 2009;4(3):e5061.
36. Ganesan AP, Johansson M, Ruffell B, et al. Tumor-infiltrating regulatory T cells inhibit endogenous cytotoxic T cell responses to lung adenocarcinoma. *J Immunol*. 2013;191(4):2009–2017.
37. Wang W, Hodgkinson P, McLaren F, et al. Small cell lung cancer tumour cells induce regulatory T lymphocytes, and patient survival correlates negatively with FOXP3⁺ cells in tumour infiltrate. *Int J Cancer*. 2012;131(6):E928–E937.
38. Su S, Liao J, Liu J, et al. Blocking the recruitment of naive CD4⁺ T cells reverses immunosuppression in breast cancer. *Cell Res*. 2017;27(4):461–482.
39. Shevchenko I, Karakhanova S, Soltek S, et al. Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. *Int J Cancer*. 2013;133(1):98–107.
40. Jang JE, Hajdu CH, Liot C, Miller G, Dustin ML, Bar-Sagi D. Crosstalk between regulatory T cells and tumor-associated dendritic cells negates antitumor immunity in pancreatic cancer. *Cell Rep*. 2017;20(3):558–571.
41. Peng DJ, Liu R, Zou W. Regulatory T cells in human ovarian cancer. *J Oncol*. 2012;345164 (11):1–7.
42. Shimizu K, Nakata M, Hirami Y, Yukawa T, Maeda A, Tanemoto K. Tumor-infiltrating Foxp3⁺ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. *J Thorac Oncol*. 2010;5(5):585–590.
43. Petersen RP, Campa MJ, Sperlaza J, et al. Tumor infiltrating Foxp3⁺ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer*. 2006;107(12):2866–2872.
44. Shou J, Zhang Z, Lai Y, Chen Z, Huang J. Worse outcome in breast cancer with higher tumor-infiltrating FOXP3⁺ Tregs: a systematic review and meta-analysis. *BMC Cancer*. 2016;16(8):687.
45. Flammiger A, Weisbach L, Huland H, et al. High tissue density of FOXP3⁺ T cells is associated with clinical outcome in prostate cancer. *Eur J Cancer*. 2013;49(6):1273–1279.
46. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3⁺ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res*. 2006;12(18):5423–5434.
47. Frey DM, Droese RA, Viehl CT, et al. High frequency of tumor-infiltrating FOXP3⁺ regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer*. 2010;126(11):2635–2643.
48. Alhamarneh O, Agada F, Madden L, Stafford N, Greenman J. Serum IL10 and circulating CD4⁺ CD25⁺ regulatory T cell numbers as predictors of clinical outcome and survival in patients with head and neck squamous cell carcinoma. *Head Neck*. 2011;33(3):415–423.
49. Gasparoto TH, de Souza Malaspina TS, Benevides L, et al. Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. *Cancer Immunol Immunother*. 2010;59(6):819–828.
50. Mandal R, Şenbabaoglu Y, Desrichard A, et al. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight*. 2016;1(17):e89829.
51. Halvorsen EC, Mahmoud SM, Bennewith KL. Emerging roles of regulatory T cells in tumour progression and metastasis. *Cancer Metastasis Rev*. 2014;33(4):1025–1041.
52. Campbell DJ. Control of regulatory T cell migration, function, and homeostasis. *J Immunol*. 2015;195(6):2507–2513.
53. Vignali DA. Mechanisms of T(reg) suppression: still a long way to go. *Front Immunol*. 2012;3:191.
54. Liu Z, McMichael EL, Shayan G, et al. Novel effector phenotype of Tim-3⁺ regulatory T cells leads to enhanced suppressive function in head and neck cancer patients. *Clin Cancer Res*. 2018;24(18):4529–4538.
55. Smyth MJ, Teng MW, Swann J, Kyparissoudis K, Godfrey DI, Hayakawa Y. CD4⁺CD25⁺ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol*. 2006;176(3):1582–1587.
56. Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 2015;520(7547):373–377.
57. Lee Y, Auh SL, Wang Y, et al. Therapeutic effects of ablative radiation on local tumor require CD8⁺ T cells: changing strategies for cancer treatment. *Blood*. 2009;114(3):589–595.
58. Deng L, Liang H, Xu M, et al. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity*. 2014;41(5):843–852.
59. Morisada M, Clavijo PE, Moore E, et al. PD-1 blockade reverses adaptive immune resistance induced by high-dose hypofractionated but not low-dose daily fractionated radiation. *Oncoimmunology*. 2018;7(3):e1395996.
60. Morisada M, Moore EC, Hodge R, et al. Dose-dependent enhancement of T-lymphocyte priming and CTL lysis following ionizing radiation in an engineered model of oral cancer. *Oral Oncol*. 2017;71:87–94.
61. Frey B, Ruckert M, Weber J, et al. Hypofractionated irradiation has immune stimulatory potential and induces a timely restricted infiltration of immune cells in colon cancer tumors. *Front Immunol*. 2017;8(3):231.
62. Zhang X, Niedermann G. Abscopal effects with hypofractionated schedules extending into the effector phase of the tumor-specific T-cell response. *Int J Radiat Oncol Biol Phys*. 2018;101(1):63–73.
63. Darragh L, Oweida A, Karam SD. Overcoming resistance to combination radiation-immunotherapy: a focus on contributing pathways within the tumor microenvironment. *Front Immunol*. 2019;9(1):3154.
64. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 costimulation: from mechanism to therapy. *Immunity*. 2016;44(5):973–988.
65. Tang AL, Teijaro JR, Njau MN, et al. CTLA4 expression is an indicator and regulator of steady-state CD4⁺ Foxp3⁺ T cell homeostasis. *J Immunol*. 2008;181(3):1806–1813.
66. Quezada SA, Peggs KS, Curran MA, Allison JP. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Invest*. 2006;116(7):1935–1945.
67. Kataoka H, Takahashi S, Takase K, et al. CD25⁺CD4⁺ regulatory T cells exert in vitro suppressive activity independent of CTLA-4. *Int Immunol*. 2005;17(4):421–427.
68. Sharma A, Subudhi SK, Blando J, et al. Anti-CTLA-4 immunotherapy does not deplete FOXP3⁺ regulatory T cells (Tregs) in human cancers. *Clin Cancer Res*. 2019;25(4):1233–1238.
69. Yamazaki T, Yang XO, Chung Y, et al. CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol*. 2008;181(12):8391–8401.
70. Zhang CY, Qi Y, Li XN, et al. The role of CCL20/CCR6 axis in recruiting Treg cells to tumor sites of NSCLC patients. *Biomed Pharmacother*. 2015;69(2):242–248.
71. Sierra-Filardi E, Nieto C, Domínguez-Soto A, et al. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J Immunol*. 2014;192(8):3858–3867.
72. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4⁺CD25⁺ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J Exp Med*. 2003;198(12):1875–1886.
73. Cazac BB, Roes J. TGF- β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity*. 2000;13(4):443–451.
74. Zhao J, Cao Y, Lei Z, Yang Z, Zhang B, Huang B. Selective depletion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells by low-dose cyclophosphamide is explained by reduced intracellular ATP levels. *Cancer Res*. 2010;70(12):4850–4858.
75. Becker JC, Schrama D. The dark side of cyclophosphamide: cyclophosphamide-mediated ablation of regulatory T cells. *J Invest Dermatol*. 2013;133(6):1462–1465.
76. Hillmer EJ, Zhang H, Li HS, Watowich SS. STAT3 signaling in immunity. *Cytokine Growth Factor Rev*. 2016;31(10):1–15.
77. Johnson DE, O’Keefe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat Rev Clin Oncol*. 2018;15(4):234–248.
78. Odate S, Veschi V, Yan S, Lam N, Woessner R, Thiele CJ. Inhibition of STAT3 with the generation 2.5 antisense oligonucleotide, AZD9150, decreases neuroblastoma tumorigenicity and increases chemosensitivity. *Clin Cancer Res*. 2017;23(7):1771–1784.
79. Reilly MJ, McCoon P, Cook C, et al. STAT3 antisense oligonucleotide AZD9150 in a subset of patients with heavily pretreated lymphoma: results of a phase 1b trial. *J Immunother Cancer*. 2018;6(1):119.