Effects of Adjuvant Chemoradiotherapy on the Frequency and Function of Regulatory T Cells in Patients with Head and Neck Cancer

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Abstract

**Purpose:** Regulatory T cells (Treg) accumulate in tumor tissues and the peripheral blood of cancer patients and may persist after therapies. This cross-sectional study examines effects of adjuvant chemoradiotherapy (CRT) on Treg numbers and function in head and neck squamous cell carcinoma (HNSCC) patients.

**Experimental Design:** The frequency and absolute numbers of CD4\(^+\) T cells, and expression levels of CD39, CD25, TGF-\(\beta\)-associated LAP and GARP on Treg were measured by flow cytometry in 40 healthy donors (NC) and 71 HNSCC patients [29 untreated with active disease (AD); 22 treated with surgery; 20 treated with CRT]. All treated subjects had no evident disease (NED) at the time of phlebotomy. In an additional cohort of 40 subjects with AD (\(n = 15\)), NED (\(n = 10\)), and NC (\(n = 15\)), in vitro sensitivity of CD4\(^+\) T-cell subsets to cisplatin and activation-induced cell death (AICD) was tested in Annexin V–binding assays.

**Results:** CRT decreased the frequency of circulating CD4\(^+\) T cells (\(P < 0.002\)) but increased that of CD4\(^+\)CD39\(^+\) Treg (\(P \leq 0.001\)) compared with untreated or surgery-only patients. Treg frequency remained elevated for >3 years. CRT increased surface expression of LAP, GARP, and CD39 on Treg. In vitro Treg were resistant to AICD or cisplatin but conventional CD4\(^+\) T cells (Tconv) were not. CRT-induced Treg from AD or NC subjects upregulated prosurivival proteins whereas Tconv upregulated proapoptotic Bax.

**Conclusions:** Highly suppressive, cisplatin-resistant Treg increase in frequency and persist after CRT and could be responsible for suppression of antitumor immune responses and recurrence in HNSCC.

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Introduction

Standard of care therapy for patients with head and neck squamous cell carcinoma (HNSCC) includes radical surgery and various regimens of chemoradiotherapy (CRT; ref. 1). However, survival rates remain at less than 50% in HNSCC because of disease recurrence and the development of distant metastases (2). It has been suggested that CRT eliminates subsets of immune cells, thus reducing antitumor effector functions and contributing to disease recurrence (3). For this reason, current interest has been focused on the influence of CRT on the host immune system and on alterations in various hematopoietic cell populations that occur during CRT. We and others have reported that CD4\(^+\) T cells are especially sensitive to CRT, and that their absolute number decreases after treatment (3, 4). In contrast, the frequency of regulatory T cells (Treg) was reported to be increased after chemotherapy in some studies (5), and was linked to poor prognosis and shorter patient survival (6). In our earlier studies, we observed an increased frequency and elevated suppressor function of Treg in the peripheral blood of HNSCC patients and at the tumor sites. Furthermore, the Treg frequency was found to be elevated in the blood of patients treated with adjuvant CRT (7). These findings suggesting that in contrast to conventional CD4\(^+\) T cells (Tconv), Treg seemed to be resistant to CRT provided a rationale for a more detailed examination of Treg attributes in HNSCC patients treated with adjuvant CRT.

Recently, human Treg have been reported to express surface CD39, an ectonucleotidase, which is a component of the adenosine pathway (8–10) and is considered as a reliable marker for isolation of human Treg (11). We reported that the CD4\(^+\)CD39\(^+\) T-cell subset consists of 2
Translational Relevance

Despite radical surgery and various regimens of chemoradiotherapy (CRT), survival rates remain lower than 50% for head and neck squamous cell carcinoma (HNSCC). The patients initially responsive to therapy develop recurrent disease, second primaries, or metastases. Current interest has been focused on effects of CRT on the host immune system. In this cross-sectional study, absolute numbers, frequency, and functions of CD4<sup>+</sup>CD39<sup>+</sup> regulatory T cells (Treg) were monitored in patients with HNSCC and were compared in cohorts of patients who were either untreated or treated with surgery-only or adjuvant CRT. The data show that in HNSCC patients, CRT increases the frequency of highly suppressive Treg that are resistant to activation-induced cell death and to cisplatin. Mechanisms responsible for this resistance include enhanced expression of prosurvival proteins Bcl-2/Bcl-xl in Treg. The persistence of Treg in the patients’ circulation long after CRT is finished could be responsible for post-CRT immunosuppression, leading to cancer recurrence in HNSCC patients.

ATP-hydrolyzing subsets: CD25<sup>+</sup>FOXP3<sup>+</sup> cells, which suppress cytokine expression and proliferation of autologous CD4<sup>+</sup> T<sub>conv</sub> and CD25<sup>(neg)</sup>FOXP3<sup>(neg)</sup> cells, which are not suppressive in CFSE-based assays, but are capable of mediating suppression via the adenosine pathway (12). These 2 CD4<sup>+</sup> T-cell subsets share the ability to hydrolyze exogenous (e)ATP to ADP and AMP (12, 13). Effects of CRT on these Treg subsets are unknown, and we considered the possibility that their functions and interactions with each other or with CD4<sup>+</sup> T<sub>conv</sub> are influenced by oncological therapies.

In this cross-sectional study, we prospectively examine the frequency and functions of these 2 Treg subsets in cohorts of HNSCC patients who were either untreated or treated with surgery alone or CRT. The objective was to evaluate short-term and long-term effects of CRT on these Treg subsets relative to CD4<sup>+</sup> T<sub>conv</sub> or CD8<sup>+</sup> T<sub>conv</sub> and to consider the mechanisms responsible for persistently elevated frequency of Treg after CRT and its potential impact on disease recurrence.

Materials and Methods

Patients and normal donors

Peripheral venous blood samples obtained from 20 normal donors (NC) and 71 HNSCC patients were used for phenotyping studies. An additional cohort of 25 patients and 15 NC donated peripheral blood for in vitro experiments with cisplatin. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh (IRB #991206). The first patient cohort included 23 females and 48 males with a mean age of 59.3 ± 10.1 years (range: 31–78 years). The 29 patients with active disease (AD) were untreated at the time of blood draws; 22 patients were treated with surgery alone (SRG); and 20 patients had received adjuvant CRT 14 ± 9 months (mean ± SD) before the phlebotomy for this study. All treated patients were no evident disease (NED) at the time of blood draws. Chemotherapy was platinum-based and consisted of cisplatin or carboplatin. Panitumumab and paclitaxel each were added in one case, respectively. All patients received radiotherapy, which ranged from 44 to 70 Gy. Clinicopathologic and demographic data for the patient cohorts are listed in Table 1. The age-matched NC cohort included 9 females and 31 males with a mean age of 51 ± 6 years (range 39–69 years).

The cohort of 25 patients who donated blood for the in vitro sensitivity/resistance studies included 17 males and 8 females with a mean age of 60 years (range 23–82 years); 10 patients had AD (7 of 10 had primary untreated tumors and 3 of 10 had a recurrent disease); all 15 NED patients underwent surgery and were treated with CRT. Their therapy was terminated from 3 weeks to 12 months before phlebotomy for this study.

Collection of peripheral blood mononuclear cells

Blood samples (20 mL) were drawn into heparinized tubes, and plasma was collected after centrifugation at low speed. The cell pellets were resuspended in 40 mL PBS and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Bioscience). Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments. A fraction of harvested cells was cryopreserved for cytokine expression assays by flow cytometry.

Flow cytometry reagents

The following antihuman monoclonal antibodies (mAb) were used for staining: anti-CD19-ECD, anti-CD4-PC5, anti-CD8-PE, anti-CD3-APC, anti-HLA-DR-ECD (all Beckman Coulter); anti-CD73-PE, anti-Bcl-2-FTTC, anti-Bcl-2-PE, anti-Bcl-xL-FTTC, and anti-Bax-FTTC (all BD Pharmingen); anti-CD39-FTTC, anti-CD38-FTTC, FOXP3-FTTC (Clone PCH101), LAP-PE, GARP-APC (all eBioscience), and anti-CD25-PE (Miltenyi). Isotype controls were included in all assays and served as negative controls for surface as well as intracellular staining. All Abs were pretitered using activated as well as nonactivated PBMC to determine the optimal staining dilution for each.

Surface and intracellular staining

Freshly isolated cells to be used for flow cytometry were incubated with mAbs specific for each surface marker in 50 μL PBS for 30 minutes at room temperature in the dark and washed with PBS before acquisition for surface marker detection. For intracellular staining of prosurvival Bcl-2 and Bcl-xL proteins or proapoptotic Bax, cells were first incubated with relevant mAbs specific for surface markers. After washing, cells were fixed with 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature, washed once with PBS, and permeabilized with PBS containing...
0.5% bovine serum albumin (BSA) and 0.1% (v/v) saponin. Next, pretitered antibodies specific for BcL-1, BcL-xL, or Bax were added and incubated with the cells for 30 minutes at room temperature. Cells were further washed twice with PBS containing 0.5% BSA and 0.2% saponin, resuspended in FACS flow solution and analyzed by flow cytometry.

Expression of intracellular FOXP3 was evaluated using a staining kit available from eBioscience as previously described (14). Flow cytometry

Flow cytometry was performed using an EPICS XL-MCL or Gallios flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the live cells based on forward and side scatter properties of the cells. At least 10^5 events were acquired for analysis and, where applicable, gates were restricted to the CD4^+ and CD4^+CD39^+ T-cell subsets. The cell frequency and mean fluorescence intensity (MFI) were determined. Absolute numbers of CD4^+ T cells were determined using the whole blood counts; absolute numbers of Treg were calculated by multiplying the Treg frequency values by the absolute number of CD4^+ T cells.

**Isolation of Treg**

CD4^+ T cells were isolated from buffy coats of NC by negative magnetic bead separation according to the manufacturer’s protocol (AutoMACS, Miltenyi). CD4^+CD39^+ T cells were then isolated by magnetic immunobeads as previously described (11). Briefly, CD4^+ T cells were incubated with anti-CD39 biotinylated Ab and anti-biotin magnetic beads before isolation by AutoMACS. The purity of isolated cell populations was >85%.

**Ex vivo activation of T cells**

PBMC obtained from NC or patients with HNSCC were incubated in anti-CD3/antiCD28 Abs-coated wells in flat-bottom 96-well plates in the presence of interleukin (IL)-2 (150 IU/mL) for 48 hours at 37°C in the atmosphere of 5% CO2 in air. In some experiments, PBMC were pre-treated with physiologic doses of cisplatin determined as described by van den Bongard and colleagues (15) for 4 hours before further incubation with activating Abs and IL-2.

**Detection of Annexin V-binding (ANXV^+) to activated T cells**

PBMC immediately after their isolation; PBMC after ex vivo activation as described earlier; or PBMC preincubated with cisplatin ± activating Abs and IL-2 were analyzed for ANXV binding to Treg or Tconv by flow cytometry. Samples were stained with FITC-conjugated or PE-conjugated ANXV (Molecular Probes), according to instructions provided by the manufacturer. Flow cytometry was performed with gates restricted to CD3^+CD4^+CD23^high and CD3^+CD4^+CD25^low T-cell subsets.

*Table 1. Clinicopathologic characteristics of patients studied for the Treg frequency*

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<th>Control</th>
<th>Untreated</th>
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<th>Treated with CRT</th>
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<td>408 ± 279 (1–975)</td>
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^a^Two patients had received adjuvant radiation without chemotherapy.

^b^Age at diagnosis.

^c^The patients’ TNM status was based on clinical/pathological diagnoses. In the CRT cohort, diagnostic biopsies were performed in 12 of 20 patients.

^d^The patients who donated blood for the Treg studies were NED at the time of phlebotomy.
In vitro sensitivity of T-cell subsets to cisplatin

CD4⁺CD39⁻ and CD4⁺CD39⁺ T-cell subsets were isolated from PBMC of NC using magnetic beads as described earlier. These T-cell subsets were then incubated with cisplatin (3–5 μg/mL) in the presence of Staphylococcal Protein B (SEB, 1 μg/mL; Sigma-Aldrich) or activating Abs and IL-2 in RPMI media. On day 2 and 5, cells were harvested and stained for Annexin-FITC, 7AAD (Invitrogen), and CD4-PE (Beckman-Coulter) for 20 minutes on ice as previously described (16).

Statistics

The data are presented as mean values ± SD. For samples with nonparametric distribution, P-values were calculated by Kruskal–Wallis and two-tailed exact Wilcoxon–Mann–Whitney tests. Correlations were calculated by the Spearman test. P-values < 0.05 and R² values > 0.5 were considered to be significant.

Results

Frequency and absolute numbers of T-cell subsets

The frequency and absolute numbers of CD4⁺ and CD8⁺ T-cells in the peripheral blood specimens obtained from HNSCC patients who were not yet treated (i.e., with AD before surgery) as well as patients treated with surgery alone (SRG) or with CRT were compared (Fig. 1). The frequency and absolute numbers of both CD4⁺ and CD8⁺ T-cells were significantly decreased in the blood of patients with AD as well as those treated with surgery alone relative to NC values. In the cohort of CRT patients, percentages and absolute numbers of CD4⁺ T cells were dramatically decreased (P < 0.002 for both; Fig. 1A). However, the frequency of CD8⁺ T cells increased following CRT in this patient cohort relative to NC values, although the absolute number of CD8⁺ T cells significantly decreased (P < 0.001) as shown in Fig. 1B. The data suggest that although CRT adversely affects both T-cell subsets, it has especially detrimental effects on the CD4⁺ T cells. CD8⁺ T cells seem to be less sensitive to CRT or they recover faster than CD4⁺ T cells after CRT.

Frequency and absolute numbers of CD4⁺CD39⁻ Treg

Because CD4⁺ T cells contain Treg, we were especially interested in therapy-induced changes in the CD4⁺ T-cell subset. We have previously shown that in humans, functional CD4⁺ Treg are CD39⁺CD25⁺, express FOXP3 and hydrolyze (e)ATP to 5'AMP (12). Within the CD4⁺ T-cell compartment, the frequency of these cells was found to be increased (P < 0.01) in the AD and SRG patient cohorts relative to that seen in NC (Fig. 1C). However, the most dramatic increase in the frequency, although not the absolute number (Fig. 1C), of Treg was seen in the CRT patients (P < 0.001). These results suggest that in the shrinking CD4⁺ T-cell compartment, CD4⁺CD39⁺CD25⁺ Treg are relatively resistant to CRT as compared with CD4⁺ Tconv. Their frequency increases whereas that of CD4⁺ T cells declines following CRT. We have also calculated the CD8⁺/Treg ratio for all patient cohorts, because this ratio has been often used in evaluating changes in the immune profile in situ and in the peripheral circulation of cancer patients treated with

Figure 1. The frequency and absolute numbers of CD4⁺ and CD8⁺ T lymphocyte subsets in the peripheral blood of HNSCC patients. Box plots show median, 25 and 75 percentiles, and the highest and lowest values. NC, normal control (n = 40); AD, untreated active disease (n = 29); SRG, treated with surgery alone (n = 22); CRT, treated with adjuvant chemoradiotherapy (n = 20). *, P < 0.05; **, P < 0.01.
various therapies (6, 17). As shown in Fig. 1D, the CD8+/Treg ratio was lower in all patient groups than in NC, but it was most prominently decreased in the CRT patient cohort, which is consistent with the significant increase in the frequency of Treg after CRT and the resistance of these cells to CRT as described earlier. Here, the significantly decreased frequency of Treg after CRT and the resistance of these cells, which is consistent with the significant increase in the Treg ratio, was most prominently decreased in the CRT patient cohort, Treg was lower in all patient groups than in NC, but it also be resistant to CRT.

A decline in the size of the CD4+ T-cell compartment following oncological therapies was further evaluated, and Fig. 2A shows that it is accompanied by increasing percentages of CD4+CD39+ Treg in representative patients selected from each of the cohorts. Importantly, CD4+CD39+CD25+ Treg seem as a distinct, well-defined population among CD4+ T cells, which can be easily distinguished from CD4+CD39+CD25+ Tconv by flow cytometry. For patients in the SRG and CRT cohorts, the frequency and absolute numbers of total circulating CD4+ T cells negatively correlate with the percentage of CD4+CD39+CD25+ Treg at P < 0.001 and P < 0.002, respectively (Fig. 2B). We have previously shown that based on expression of surface markers and functional characteristics, human Treg can be divided into (i) strongly suppressive CD4+CD39+CD25+ T cells that are FOXP3+ and (ii) CD4+CD39+CD25neg T cells that do not express FOXP3 and mediate little suppression (12). Within the Treg subpopulation, a strong positive relationship exists between these 2 subsets in NC (12) and, as shown in Fig. 2C, this relationship is not altered in untreated patients (AD) or in patients with NED who were treated with SRG or CRT. The 2 Treg subsets track each other and the frequency of both was found to be equally increased in all patient cohorts compared with NC (Fig. 2C). As expected, the frequency of CD4+ CD39+CD25+ Treg significantly correlated with that of

Figure 2. Changes in the CD4+ T-cell compartment after oncological therapies. A, decreased total CD4+ T-cell frequency is accompanied by increased frequency of CD4+CD39+ Treg. Density plots show data from representative HNSCC subjects selected from each study group. Gates are set on the lymphocyte window (left) and on the CD4+ T-cell subset (right). B, negative associations between percentages (left) or absolute numbers (right) of total CD4+ T cells and the percentages of CD4+CD39+CD25+ Treg in the circulation of HNSCC patients treated with SRG or CRT. C, a positive correlation between the frequency of CD4+CD39+CD25+ Treg and CD4+CD39+CD25neg cells in the peripheral circulation. D, a positive correlation between the frequency of CD4+CD25neg and CD4+CD39+CD25+ Treg subsets in the peripheral circulation of NC as well as all HNSCC patients.
CD4+CD25high T cells in the peripheral blood of HNSCC patients, indicating that these 2 subsets of Treg closely overlap (Fig. 2D).

**Functional characteristics of Treg in patients treated with CRT**

Suppressor cell assays based on proliferation inhibition require large cell numbers and, therefore, they are not useful for monitoring of patient specimens, which are often limited in volume. For this reason, we used flow cytometry–based assays to measure expression of functionally relevant proteins, such as the latency-associated peptide (LAP) and the glycoprotein A repetitions predominant (GARP), known to be expressed on the surface of cells that produce and release TGF-β (18). Because Treg utilize TGF-β as a suppressive mechanism (19), and LAP as well as GARP are established surrogate markers for TGF-β (20, 21), we evaluated expression of LAP and GARP on the surface of CD4+ CD39+ Treg, as surrogate markers for Treg suppressor activity in all patient cohorts (Fig. 3A and B). We have previously reported that suppressive CD4+CD39+FOXP3+ Treg expressed LAP and GARP, whereas CD4+ CD39+FOXP3neg T cells did not (12). The highest percentages of LAP+ or GARP+ Treg were seen in CRT-treated patients (Fig. 3C). Also, expression of these markers was highly and significantly upregulated on Treg in the CRT patient cohort (Fig. 3A and B). In addition, the MFI of CD39 was upregulated on Treg in all the HNSCC groups relative to that in NC, and the highest MFI for CD39 was observed in Treg of patients in the CRT cohort (Fig. 3D). These data suggest that Treg of the CRT-treated patients have a better ability to hydrolyze exogenous ATP to 5′-AMP than Treg of patients in the other cohorts. We have previously shown that CD39 expression levels on the cell surface positively correlate with enzymatic hydrolysis of exogenous ATP by Treg (12).

**Figure 3.** Analysis of functional surface markers on Treg. Expression of TGF-β–associated LAP and GARP on the surface of CD4+CD39+FOXP3+ T cells stimulated with SEB as described in Materials and Methods. A, density plots are gated on CD4+CD39+ T cells of one representative subject from each study group. B, surface expression (MFI) of LAP and GARP in Treg of patients or NC (*, P = 0.01). C, box plots show percentages of LAP+ or GARP+ Treg among total CD4+CD39+ Treg in NC and HNSCC patient cohorts (*, P = 0.02). D, box plots show CD39 expression (MFI) in Treg obtained from HNSCC patients (*, P = 0.04).
Persistent upregulation of the Treg frequency in CRT-treated patients

Blood specimens were obtained from NED patients treated with CRT at various times after the end of therapy (n = 20). The average time period elapsed between the end of treatment and phlebotomy for this study was 14 ± 9 months (mean ± SD). The increased Treg frequency observed in the CRT-treated patients was independent of the time of blood draws after the end of therapy (Fig. 4A and B). This suggests that the increase in the Treg frequency represents a long-lasting effect in these patients. A subset of HNSCC patients (N = 22) treated with surgery alone also showed an increase in the Treg frequency shortly after surgery (1–6 months). However, in patients tested 6 to 15 months after surgery, the Treg frequency was normal, suggesting that in contrast to CRT, surgery was not associated with persistent elevation of Treg (Fig. 4). Importantly, in some of the NED patients who were treated with CRT, the frequency of Treg was elevated even after 3 years after end of therapy. In 3 NED patients treated with CRT, serial blood specimens were obtained at 6, 8, and 12 months after termination of therapy. The Treg frequency remained elevated, ranging between 12 and 16%, in these patients’ blood, further suggesting that a stable and persistent increase in Treg characterizes this patient cohort. Figure 4 shows that the percentages of total CD4+ T cells remained within the normal range in patients treated with surgery, whereas CRT-treated patients had low percentages of CD4+ T cells months after therapy.

Clinical significance of persistently high Treg

The cohort of 20 CRT patients was followed for 37 ± 13 months (mean ± SD from the end of treatment) to determine whether anyone experienced disease recurrence. During this period of time, 4 patients recurred and 3 others developed second primary HNSCC (35%). Two of the second primary tumors occurred at 25 months and the third at 45 months after CRT ended, and all 3 patients had elevated percentages of Treg (6–19%) in the peripheral circulation. Within the CRT cohort, there was no significant

Figure 4. Persistence of Treg in the circulation of NED patients after therapy and in vitro resistance of Treg to cisplatin. A, percentages of CD4+CD39+CD25+ Treg in the peripheral circulation of HNSCC patients who are NED after CRT. The elevated Treg frequency in patients was independent of the time period elapsed between the last treatment and phlebotomy for this study. B, in HNSCC patients treated with SRG alone, the Treg frequency was increased shortly after treatment but later seemed to normalize. C, percentages of total CD4+ T cells were persistently decreased in CRT-treated patients. A–B, the solid line represents the mean Treg (3.2%) or CD4+ T-cell (42%) frequency established for NC, and the dotted lines show ±1 SD of the mean. D, isolated CD4+CD39- and CD4+CD39+ T cells obtained from 4 different NC were incubated in the presence of physiological concentrations of cisplatin for 48 hours. At the cisplatin concentration of 3 µg/mL, the increase in frequency of ANX+ cells was greater (P < 0.03) among CD4+CD39- Tconv than among CD4+CD39+ Treg.
difference in the Treg frequency \( (P = 0.18) \) between patients who recurred or developed new primary tumors \( (12 \pm 4\%) \) and those who did not recur \( (9 \pm 5\%; \text{means} \pm SD) \). In the similarly followed surgery-only cohort, 4 of 22 patients experienced recurrence \( (18\%) \), and there were no second primary cancers observed. There was also no significant difference in the Treg frequency \( (P > 0.2) \) between patients who had recurrence and those who did not.

**In vitro sensitivity of CD4^{+} T-cell subsets to cisplatin or AICD**

Our data suggest that the high frequency of Treg seen in the blood of HNSCC patients treated with CRT could be because of resistance of these cells induced by therapy. To test the hypothesis that the long-term Treg survival after CRT is a characteristic of these cells, sensitivity/resistance to cisplatin of CD4^{+} CD39^{+} Treg freshly isolated from the PBMC of NC and activated \textit{ex vivo} (i.e., incubated either with SEB or anti-CD3/CD28 Abs and IL-2) was tested in ANXV-binding assays. CD4^{+} CD39^{+} Treg and CD4^{+} CD39^{+/-} T\textsubscript{conv} were incubated with physiologic concentrations of cisplatin as previously described by van den Bongard and colleagues \((15)\). The CD4^{+} CD39^{+} Treg subset was found to be more resistant \( (P < 0.03) \) to cisplatin than CD4^{+} CD39^{+/-} T\textsubscript{conv} \((\text{Fig. 4D})\).

Using PBMC obtained from NC and HNSCC patients treated or not treated with CRT, we studied resistance/sensitivity of Treg to activation-induced cell death (AICD) following \textit{ex vivo} activation of PBMC with anti-CD3/CD28 Abs and IL-2 \((150 \text{ IU/mL})\). After 48-hour incubation, we used flow cytometry to measure ANXV binding to CD4^{+}. CD25^{high} versus CD4^{+} CD25^{+/-} T\textsubscript{cell} subsets and to compare their resistance/sensitivity of these cells to AICD or cisplatin. Figure 5A shows that after \textit{ex vivo} activation, only CD4^{+} CD25^{high} Treg of patients treated with CRT were resistant to activation-induced cell death, as evidenced by the lack of ANXV binding \( (\text{left}) \), whereas activated Treg of untreated patients or of NC were highly sensitive to AICD. In contrast, no difference in ANXV binding to CD4^{+} CD25^{+/-} T\textsubscript{conv} was seen among the 3 cohorts: only 20–40\% of T\textsubscript{conv} bound ANXV \( (\text{right}) \). Furthermore, as shown in Fig. 5B, preincubation with cisplatin \( (4 \text{ hours}) \) followed by PBMC activation for 48 hours induced resistance of CD4^{+} CD25^{high} Treg obtained from NC or untreated HNSCC patients to AICD \( (\text{left}) \), and this resistance was also evident in Treg of patients treated with CRT. In contrast, pretreatment with cisplatin induced greater sensitivity to AICD in CD4^{+} CD25^{+/-} T\textsubscript{conv} of NC as well as HNSCC patients, as nearly all of these cells became ANXV\textsuperscript{+} as shown in Fig. 5B \( (\text{right}) \).

**Differential effects of cisplatin on Treg versus CD4^{+} T\textsubscript{conv}**

To investigate the mechanisms responsible for resistance of Treg in CRT-treated patients to AICD, expression of prosurvival Bcl-2/Bcl-xl and of proapoptotic Bax was compared in Treg versus T\textsubscript{conv} preincubated with or without cisplatin before \textit{ex vivo} activation (Fig. 5C). Cisplatin upregulated expression of the prosurvival proteins in Treg, whereas it significantly downregulated prosurvival protein expression in T\textsubscript{conv} \( (\text{Fig. 5C, right}) \). Also, the pretreatment with cisplatin decreased expression of Bax in Treg, whereas it dramatically upregulated expression of this proapoptotic protein in T\textsubscript{conv}. These CD4^{+} CD25^{+} Treg in the blood of HNSCC patients treated with CRT \( (\text{POST}) \) upregulated expression levels of survival proteins relative to those in PRE Treg \( (P < 0.001) \) and were highly resistant to \textit{in vitro} cisplatin, perhaps because of cumulative direct or indirect \textit{in vivo} effects of CRT. In contrast, CD4^{+} CD25^{+} Treg in the blood of NC incubated \textit{in vitro} with cisplatin for 4 hours \( (\text{Fig. 4D}) \) showed a relatively small, albeit statistically significant, increase in resistance versus that observed in CD4^{+} CD25^{+/-} T\textsubscript{conv}.

**Discussion**

Effects of oncologic therapies on lymphocyte subsets have been of considerable interest, because of the possibility that therapy-induced changes in immune-cell homeostasis might interfere with antitumor activity. In this respect, \textit{in vivo} studies in mice indicate that radiation and chemoradiation exert strong effects on the host immune system, including Treg \( (22) \). The limited data that are available for patients with cancer suggest that CRT reduces the number of circulating lymphocytes \( (23) \). In HNSCC patients, CD4^{+} T helper cells were previously reported to be particularly sensitive to CRT, as they were found to be significantly depleted relative to other T-cell subsets \((3,24)\). Recently, attention has focused on Treg, a subset of CD4^{+} T cells responsible for mediating suppression of antitumor immune responses \( (25) \). The presence and accumulations of Treg at tumor sites and in the peripheral circulation of cancer patients, including HNSCC patients, have been widely reported \((6,7,26)\). These high Treg frequencies have been negatively or positively correlated with disease outcome in various malignancies \((6,27,28)\). Studies also indicate that the Treg frequency tends to increase after oncologic therapies \((23)\), introducing a possibility that Treg are more resistant to CRT than CD4^{+} T\textsubscript{conv}. This prospective cross-sectional study in a large cohort of HNSCC patients was performed to examine this possibility and to provide insights into potential mechanisms responsible for Treg resistance to oncological therapies.

Confirming our previous observations, we show that in comparison to NC, untreated HNSCC patients or those treated with surgery or surgery plus CRT have reduced percentages and absolute numbers of CD4^{+} T cells but increased percentages of CD4^{+} CD39^{+} Treg. As the CD4^{+} T-cell compartment shrinks in disease, the proportion but not absolute number of circulating CD4^{+} CD39^{+} Treg increases. This contraction was especially magnified in the CRT patient cohort, implying that Treg, unlike CD4^{+} T\textsubscript{conv}, are resistant to CRT. Treg resistance to radiation and drugs has been reported \((29,30)\). However, the exact mechanisms responsible for Treg resistance to CRT remain unclear. This resistance might reflect the slow proliferation rate of Treg in the periphery or existence of protective mechanisms, for
example, multidrug transporter pumps, and the enhanced capability for DNA repair (31). A short exposure of Treg to cisplatin before activation seems to protect them from AICD and is mediated by elevated expression levels of survival proteins, Bcl-2 and Bcl-xL, and lower levels of proapoptotic Bax than those observed in Tconv (32) and signaling in response to ligands released by

Figure 5. In vitro resistance of CD4+CD25<sup>high</sup> Treg to cisplatin and upregulated expression of the prosurvival proteins in Treg treated with cisplatin. A, ANXV binding to Treg (CD4+CD25<sup>high</sup>) (left) or Tconv (CD4+CD25<sup>neg</sup>) (right) in the in vitro activated PBMC obtained from NC (n = 15), untreated HNSCC patients (pre; n = 10) or HNSCC patients treated with CRT (post; n = 15). PBMC were incubated with anti-CD3/CD28 Abs and 150 IU/mL of IL-2 for 48 hours and studied for the evidence of AICD (ANXV binding) by flow cytometry, gating on Treg and Tconv cells. Only Treg obtained from the CRT-treated patients (post) were resistant to AICD. B, ANXV binding to Treg (CD4+CD25<sup>high</sup>) (left) or Tconv (CD4+CD25<sup>neg</sup>) (right) in PBMC, which were pretreated in vitro with cisplatin for 4 hours before incubation of the cells in the presence of anti-CD3/CD28 Abs and IL-2 for 48 hours. Only Treg became resistant to AICD as determined by measuring ANXV binding. C, expression of Bcl-2, Bcl-xL, and Bax are shown as MFI ± SD in CD4+CD25<sup>high</sup> Treg and CD4+CD25<sup>neg</sup> Tconv. PBMC were obtained from the blood of NC, untreated, or CRT-treated patients and were preincubated or not with cisplatin and then activated as described earlier in A. The solid lines (PRE to POST panels) show increased expression levels of Bcl-2 and Bcl-xL in POST Treg as compared with those in PRE Treg that were incubated with in vitro cisplatin. *, P < 0.001; **, P < 0.005.
radiation-induced tissue damage activate the PI3K/Akt survival pathway, protecting Treg from effects of CRT. Such TLR-mediated chemoresistance has been previously described for tumor cells (33–35). Others report that platinum-based chemotherapies decrease immune suppression acting not on Treg but on dendritic cells (DC) or tumor cells, reducing expression of the T-cell inhibitory molecule PD-L2 on these cells and upregulating recognition of tumor targets by T cells (36).

Here, we advance the hypothesis that CRT induces tissue changes that have indirect effects on Treg, favoring their survival and suppressor functions. For example, increased TGF-β levels in irradiated tissues could promote Treg differentiation (12, 22). The persistently enlarged post-CRT Treg compartment includes cells with increased immuno-suppressive capabilities (e.g., upregulated LAP, GARP, and CD39). These CRT-induced Treg produce more 5'-AMP and ADO, acquire and maintain the ‘killer phenotype’ associated with expression of Fas, Fasl, and GrB/perforin, as previously reported by us (37), and upregulate prosurvival proteins. We show here that activated CD4+CD39+B Treg are more resistant to cisplatin than CD4+Tconv and that CD4+CD25+B B Treg obtained from HNSCC patients with NED after CRT become resistant to AICD, whereas CD4+CD25+B+ Tconv remain sensitive (Fig. 5). Together, our data support the hypothesis that Treg induced in the CRT environment acquire new characteristics that make them into long-surviving and potentially more dangerous immune regulator cells.

In preclinical models, radiotherapy has been reported to either interfere with antitumor immune responses by the elimination of immune effector cells (38) or promote these responses by enhancing the processing and presentation of tumor antigens by APC (39, 40). These opposing radiotherapy-induced effects are related to alterations in tissues and cells, which seem to be dependent on the dose and type of radiotherapy (38–40). For example, the ATP concentration is increased in damaged irradiated tissues (22), providing therapy-induced Treg with an opportunity to produce 5'-AMP and ADO. These factors acting via the adenosine receptors potentially influence survival and activity of Treg. Because isolated CD4+25+B T cells contain significantly higher levels of mRNA coding for A1R, A2AR, and A3R than CD4+CD25+B+B T cells (our unpublished data), we surmise that autocrine and paracrine signaling via these receptors has profound effects on functions and survival of CRT-induced Treg. Our experiments with CD4+CD39+ or CD4+CD25+B+B Treg subsets suggest that environmentally generated signals, as exemplified by TGF-β, ATP, or ADO, regulate functions of CRT-induced Treg and might contribute to their resistance and survival after CRT. Thus, resistance of these Treg to cisplatin or AICD might not be their intrinsic characteristic but rather reflects an environmentally regulated requirement for their activity to regulate immune homeostasis disturbed by therapy that alters the immune cell numbers and balance. To what extent this induced resistance to drugs such as cisplatin is related to immune escape mechanisms, which are responsible for the promotion of tumor growth in cancer patients, remains to be determined. Our data suggest that human Treg are able to utilize various survival mechanisms to protect themselves from exogenous stress signals. Treg survival seems to be critically important for the host, emphasizing the significant role these T cells play in health and disease.

The observed elevations in the Treg frequency and functions in patients with active (pretherapy) and inactive (posttherapy) disease were independent of the time elapsed between the treatment and blood draws. In fact, the Treg frequency remained elevated for weeks and months in many, although not all, HNSCC patients who were NED after CRT. The suppressive potential of Treg is known to be related to the Treg/Tconv ratio. The functional consequence of an increase in the proportion of Treg after CRT is that this ratio changes in favor of Treg. These Treg acquire stronger suppressor function after CRT and express more TGF-β-associated LAP and GARP as well as ATP-hydrolyzing CD39 on their surface. Our data are provocative, as they suggest that persistent elevations in the CD4+CD39+B Treg frequency induced in the blood of cancer patients by potentially curative therapies, such as surgery and adjuvant CRT, result in suppression of antitumor immune effector cells and thus promote disease recurrence or the development of secondary cancers. The fact that in our study, 3 of the CRT-treated patients with elevated Treg percentages developed second primary cancers within 2 to 4 years of therapy suggests that the imbalance in immune regulation might promote disease progression.

It has also been suggested that CRT induces a state of chronic inflammation in tissues, which has been linked to cancer development (41). The presence of iTreg in this setting could be beneficial to the host by normalizing T-cell responses and maintaining immune responses in balance. The role of Treg that accumulate after CRT in the promotion or inhibition of tumor progression is currently unclear. Prospective serial monitoring will be necessary to determine the biologic and clinical consequences of increased Treg frequency in HNSCC patients treated with CRT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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