Objective: To investigate the role of Toll-like receptor (TLR) signaling and T-regulatory (T-reg) cells in patients with head and neck squamous cell carcinoma (HNSCC).

Design: Multicolor flow cytometry was used to study the frequency and phenotype of CD4+CD25+CD127− T-reg cells and CD4+CD25−CD127+ T-effector (T-eff) cells in peripheral blood mononuclear cells (PBMCs).

Setting: All patients were seen at the outpatient clinic at the Department of Otorhinolaryngology at the University of Duisburg–Essen from March 1, 2009, through December 31, 2009.

Patients: Eleven patients with HNSCC and 10 healthy donors (HDs) were studied. T-reg and T-eff cells were isolated from PBMCs using a magnetic bead–activated cell-sorting technique.

Main Outcome Measures: Proliferation of T-eff cells and suppressor activity of T-reg cells were assessed in functional assays after preincubation with the TLR4 ligand heat shock protein 60 or lipopolysaccharide in the presence or absence of neutralizing antibody against TLR4.

Results: Frequency of T-reg cells in PBMCs was strongly increased in patients with HNSCC vs HDs. Isolation of T-reg cells from PBMCs of patients with HNSCC showed a significantly higher expression of TLR4, TLR6, TLR9, and TLR10 compared with HDs, whereas TLR2 was not detectable. After incubation with heat shock protein 60 or lipopolysaccharide, the suppressive function of T-reg cells was significantly increased (1.14- and 1.44-fold, respectively), whereas the proliferation capacity of T-eff cells remained unchanged. This effect was reversed after TLR4 inhibition on T-reg cells.

Conclusion: The TLR ligation on T-reg cells may contribute to tumor-mediated immune suppression by enhancing their suppressive activity.


HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) is an aggressive malignant neoplasm with a 5-year patient survival rate of less than 50%, which has remained unchanged for the past 30 years despite advances in diagnostics and treatment.1 Although the origin of the process of tumorigenesis is clearly linked to environmental carcinogens (eg, tobacco and alcohol) ensuing in a plethora of epigenetic events, which govern the development of HNSCC, its progression is strongly influenced by the host immune system and results in emergence of a tumor that is resistant to immune effector cells.2,3 A variety of strategies to evade immune detection and elimination have evolved in HNSCC. A mechanism of immune suppression that has recently been emphasized involves T-regulatory (T-reg) cells. Although T-reg cells maintain tolerance against their own antigens and prevent development of autoimmunity in healthy individuals, tumor cells take advantage of T-reg cells to protect themselves by suppressing antitumor immune responses.4,7 T-reg cells are currently classified into 2 main subsets according to their origin and suppressive activity.3 Natural T-reg cells originate in the thymus and have been described to suppress T-effector (T-eff) cell responses in a contact-dependent and cytokine-independent manner. They are mainly characterized by expression of interleukin (IL) 2 receptor α (CD25) and the transcription factor forkhead box P3 (FoxP3). Adaptive or induced T-reg cells are antigen-specific suppressor cells that are activated during inflammatory processes in peripheral tissues and mediate their suppressive activity through secretion of IL-10 and transforming growth factor β, respectively.6

One of us and others7-9 have reported on the presence of natural T-reg cells and induced T-reg cells in patients with HNSCC. Those works have demon-
gested that activation of T-reg cells through TLR4 ligation, which in turn recruit T-reg cells via secreting soluble factors that activate T-eff cell activity were analyzed. The rationale for recruitment and function of TLR4 ligands on T-reg and T-eff cells was based on the hypothesis that tumor cells recruit T-reg cells via secreting soluble factors that activate T-reg cells through TLR4 ligation, which in turn act against an antitumor immune response. Our results suggest that activation of T-reg cells through TLR4 ligation in patients with HNSCC cells may contribute to tumor-mediated immune suppression.

### METHODS

**Biocoll separating solution was used for density centrifugation of peripheral blood and fetal calf serum was inactivated by heating for 30 minutes to 56°C (Biochrom AG, Berlin, Germany). We also used RPMI 1640 culture medium, l-glutamine, streptomycin, and penicillin (Invitrogen, Karlsruhe, Germany). Complete medium (Rxx10) consisting of RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and l-glutamine (2 mmol/L) was used for cell culture assay.** The following monoclonal antibodies (mAbs) were used for flow cytometry: anti–CD4-peridinin chlorophyll, anti–CD25-allophycocyanin, anti–TLR2-Alexa Fluor 488, and 7–amino actinomycin D (7-AAD) (FITC), and functional grade purified anti–TLR4 (eBioscience Corp, Frankfurt am Main, Germany); and anti–TLR6-FITC, anti–TLR9-PE (Imgenex Corp, San Diego, California), anti–TLR4-PE (Abcam, Cambridge, England) and anti–TLR10-FITC (Santa Cruz Biotechnology Inc, Heidelberg, Germany).

**COLLECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH HNSCC AND HEALTHY DONORS**

Blood samples were obtained from 11 patients with HNSCC and 10 healthy donors (HDs) as control individuals. Peripheral venous blood (50-150 mL) was drawn into heparinized tubes, diluted (1:1, vol/vol) with phosphate-buffered saline (PBS), and subjected to density gradient centrifugation (room temperature, 300 g, 30 minutes). Peripheral blood mononuclear cells (PBMCs) were recovered, washed with PBS, counted (CasyCounter; Innovatis-Roche, Bielefeld, Germany), and immediately used for experiments.

The study was approved by the institutional review board of the University of Duisburg–Essen, and all participants signed an informed consent form. All patients were seen at the outpatient clinic at the Department of Otorhinolaryngology at the University of Duisburg–Essen from March 1, 2009, through December 31, 2009. The patient cohort included men and women with a mean age of 68 years (range, 55-82 years). The HD group was sex matched and included 6 men and 4 women with a mean age of 36 years (range, 22-46 years), all of whom were non-smokers. All patients had untreated primary tumors. The age, sex, and clinicopathologic characteristics of the patients are listed in the Table.

**ISOLATION OF CD4+CD25+ T-REG CELLS**

For magnetic bead purification, a CD4+CD25+ T-reg isolation kit was used according to the manufacturer’s protocol (Miltenyi Biotec, Monchengladbach, Germany). In brief, all non-CD4 cells were depleted over a magnetic cell separator (MACS) LD column (Miltenyi Biotech) after incubation with a cocktail of biotinylated mAbs against CD8, CD14, CD16, CD19, CD36, CD56, CD123, CD127, T-cell receptor γ/δ, and CD235a. The unlabeled CD4+ T cells were incubated with CD25 magnetic beads and subsequently separated over 2 MACS MS columns (Miltenyi Biotech) into CD4+CD25+ T-reg cells and CD4+CD25- T-eff cells. Purity of cell subsets was routinely tested and ranged from 90% to 97%.

**Table. Clinicopathologic Characteristics of Study Patients With Head and Neck Squamous Cell Carcinoma**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Patients a</th>
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<td>M1</td>
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</table>

*Data are presented as number of patients unless otherwise indicated.*
FLOW CYTOMETRY

Freshly isolated PBMCs were stained for TLR expression. Therefore, cells were incubated in sodium azide–PBS containing 3% human serum with the optimal dilution of each mAb for 25 minutes at 4°C in the dark. The following antibodies were used for surface staining: anti–CD4-perdinin chlorophyll (TH1 cells), anti–CD25-allophycocyanin (IL-2 receptor chain; CD25), anti–CD127-PE-Cy7 (IL-7 receptor α), anti–TLR2-Alexa Fluor 488, anti–TLR4-PE, anti–TLR6-PE, and anti–TLR10-FITC. Cells were subsequently washed and fixed with 2% (vol/vol) paraformaldehyde or proceeded to intracellular staining (FoxP3, TLR9) (eBioscience Corp). For this purpose, cells were incubated with fixation and permeabilization working solution in the same 30 minutes at 4°C in the dark, washed twice with permeabilization buffer, and incubated with mAbs diluted in the same buffer for 25 minutes at 4°C in the dark. All mAbs were used on fresh PBMCs to determine their optimal working dilutions. Respective isotype controls were used in all experiments (all corresponding isotypes were purchased from BD Biosciences except for ratlgG2a–FITC, which was obtained from Dianova Inc, Hamburg, Germany). After staining, cells were immediately subjected to acquisition in a flow cytometer (FACSCanto II; BD Biosciences). The collected data were analyzed with Diva6 software (Becton Dickinson, Heidelberg, Germany).

INCORPORATION ASSAY

The T-eff cells were preincubated for 2 hours with LPS (Microbiology and Infection Biology, Division of Biophysics, Research Center Borstel, Borstel, Germany) at different concentrations and extensively washed before adding CD2/CD3/CD28 MACS bead particles (MACS Bead; Miltenyi Biotec), according to the manufacturer’s instructions. Assays were conducted in quadruplicate on a 96-microtiter well plate (Greiner Bio-One, Frickenhausen, Germany) in a total volume of 0.2 mL for 3 days. For the last 16 hours cells were labeled with 1 µCi thymidine labeled with tritium per well (TRA.120; specific activity, 5 Ci/mmol; Amersham, Buckinghamshire, England). Cells were then harvested (Micro96 Harvester; Skatron Instruments, Transby, Norway) onto glass fiber filtermates and followed by T-reg identification using IL-2 receptor chain (CD25) and IL-7 receptor chain (CD127) (Figure 1A). The percentages of circulating CD4+CD25+CD127− T-reg cells were significantly increased in patients with HNSCC (7.7% [1.6%]; P < .001) (Figure 1B). FoxP3 as another eminent functional marker for T-reg cell identification was also assessed by flow cytometry. On average, 71.3% (7.4%) of CD4+CD25+CD127− T-reg cells from HDs showed positive results for FoxP3, whereas 86.3% (2.1%) showed positive results in the HNSCC cells (data not shown).

CARBOXYFLUORESCEIN DIACETATE SUCCINIMIDYL ESTER–BASED SUPPRESSION ASSAY AND FLOW CYTOMETRY–BASED CYTOTOXICITY ASSAY

Responder CD4+CD25− T cells were MACS isolated from PBMCs, stained with 5-µM carboxyfluorescein diacetate succinimidyl ester (CFSE), and cocultured with autologous suppressor cells. For that purpose, T-reg cells were preincubated with 100-ng/mL LPS or 10-ng/mL HSP60 (Lionex GmbH, Braunschweig, Germany) in Rxs10 for 2 hours. To analyze the role of TLR4 signaling, neutralizing mAb (20 µg/mL) and corresponding monoclonal lgG2a isotype control, which were previously titrated, were added to T-reg cells in some experiments 25 minutes before LPS stimulation. Afterward, cells were extensively washed with complete medium and activated over-night with CD2/CD3/CD28 MACS bead particles. On the following day, 5 × 10^5 autologous CFSE-labeled T-eff cells were seeded into 96 round-bottom plates. T-reg cells were again washed and added at various ratios (ratio of responder to suppressor cells, 1:1, 3:1, 10:1) to the wells supplemented with CD2/CD3/CD28 MACS bead particles as recommended in the manufacturer’s instructions. This assay was performed in triplicate. Cocultures were incubated for 5 days at 37°C in an atmosphere of 5% carbon dioxide in air.

To assess cytotoxic lysis of T-eff cells induced by T-reg cells, a flow cytometry–based cytotoxicity assay was performed as previously described. Briefly, directly before analysis, cells were stained in PBS with the vital dye 7-AAD for 15 minutes at room temperature. The 7-AAD intercalates into double-stranded DNA and emits red fluorescent light. The 7-AAD cannot pass through intact cell membrane and thus is a helpful surrogate parameter for late cell death or apoptosis. Cells were resuspended in 200 µL of PBS and were immediately subjected to acquisition in a flow cytometer (FACSCanto II). Flow cytometry analysis and CFSE data analysis were performed using Diva6 software.

STATISTICAL ANALYSIS

All data are presented as mean (SD) of at least 3 experiments. Data were analyzed using the t test and Mann-Whitney rank sum test for comparisons across outcome groups (HDs vs patients with HNSCC). Data are presented in box plots showing the mean, median, and interquartile range. A significance level of .05 was assumed.

RESULTS

FREQUENCY OF CD4+CD25+CD127− T-REG CELLS IN PATIENTS WITH HNSCC

The PBMCs obtained from the 11 patients with HNSCC and the 10 HDs were first investigated for the frequency of CD4+CD25+CD127− T-reg cells. For this purpose, 6-color flow cytometry was used. A previously reported gating strategy to identify highly functional T-reg cells was used to assess the frequency of T-reg cells. Briefly, after gating on the lymphocyte fraction as to their front scatter and side scatter properties, CD4+ cells were gated and followed by T-reg identification using IL-2 receptor chain (CD25) and IL-7 receptor chain (CD127) (Figure 1A). The percentages of circulating CD4+CD25+CD127− T-reg cells were significantly increased in patients with HNSCC (7.7% [1.6%]; P < .001) (Figure 1B). FoxP3 as another eminent functional marker for T-reg cell identification was also assessed by flow cytometry. On average, 71.3% (7.4%) of CD4+CD25+CD127− T-reg cells from HDs showed positive results for FoxP3, whereas 86.3% (2.1%) showed positive results in the HNSCC cells (data not shown).

INCREASED TLR EXPRESSION ON CD4+CD25+CD127− T-REG CELLS IN PATIENTS WITH HNSCC

To identify the potential role of TLR on T-reg cells in patients with HNSCC, we investigated the phenotypic TLR profile on CD4+CD25+CD127− T-reg cells using flow cytometry. Except for TLR2, all TLRs tested were detected on peripheral T-reg cells, 15. On average, 71.3% (7.4%) of CD4+CD25+CD127− T-reg cells from HDs showed positive results for FoxP3, whereas 86.3% (2.1%) showed positive results in the HNSCC cells (data not shown).
TLR9, and TLR10 was significantly increased (P < .001) on the T-reg cells of patients with HNSCC compared with those of HDs: TLR4, 163.3 vs 803.0; TLR6, 160.9 vs 386.4; TLR9, 305.0 vs 1001.9; and TLR10, 13.3 vs 69.9. Box plots in Figure 2A summarize the mean, median, and interquartile range of the TLR expression on T-reg cells. Representative flow cytometry histograms show the difference in the expression levels of TLR4, TLR6, TLR9, and TLR10 on T-reg cells of HDs vs those of patients with HNSCC (Figure 2B). Flow cytometry analysis of TLR on T-eff cells revealed similar expression levels (data not shown).

EFFECT OF LPS ON T-EFF CELL PROLIFERATION

To assess the role of TLR on T-eff and T-reg cells, we chose LPS as a paradigmatic TLR4 ligand. MACS-isolated CD4+ CD25+ T-eff cells were preincubated for 2 hours with 0.01-, 0.1-, or 1-µg/mL LPS at 37°C in an atmosphere of 5% carbon dioxide in air. Cells were washed and allowed to proliferate for 3 days on anti-CD2/CD3/CD28 activation. This short exposure to LPS did not alter proliferation capacity of T-eff cells in either an enhancing or an inhibiting manner. Counts per minute of proliferated T-eff cells ranged from 50,000 to 55,000, as shown in Figure 3.

EFFECT OF LPS ON THE SUPPRESSIVE FUNCTION OF T-REG CELLS

Next, we sought to determine whether LPS as an exogenous ligand or HSP60 as an endogenous ligand modulates the suppressive function of T-reg cells. In previous experiments, different LPS or HSP60 concentrations ranging from 0.001 to 1 µg/mL were tested in preliminary runs of CFSE suppression assays. A concentration of LPS at 0.1 µg/mL and a concentration of HSP60 at 0.01 µg/mL showed the strongest results. Therefore, MACS-isolated CD4+ CD25+ T-reg cells were preincubated for 2 hours with 0.1-µg/mL LPS or 0.01-µg/mL HSP60 and activated overnight with CD2/CD3/CD28 MACS bead particles. Next, T-reg cells were washed and added to autologous T-eff cells, which were labeled with CFSE. Cocultures were activated in presence of CD2/CD3/CD28 MACS bead particles for 5 days. In these runs, T-reg cells, which were preactivated with HSP60, exhibited a 1.14-fold (±0.17-fold) increase of suppressor activity compared with untreated T-reg cells. Furthermore, T-reg cells pretreated with LPS showed a 1.44-fold (±0.19-fold) increase of suppressing T-cell proliferation compared with untreated T-reg cells (Figure 4).

To assess TLR4-dependent suppressor activity of T-reg cells on inhibition of T-cell proliferation, flow cytometry was used with gates set on CFSE-positive cells. At the responder to suppressor cell ratio of 1:1, the mean percentage of proliferation was 28.3% (4.55%) for autologous CD4+ CD25+ T-eff cells compared with proliferation of T-eff cells alone. When T-reg cells were preincubated with LPS, proliferation of autologous T-eff cells was further significantly impaired with a mean of 22.3% (7.2%) (Figure 5A). Inhibition of TLR4 using a functional-grade neutralizing antibody against human TLR4 epitopes resulted in abrogation of TLR4-specific enhancement of T-reg cell suppressor activity. Figure 5B (left column) shows a representative example of 4 experiments.

Furthermore, to determine the effect of T-reg cell suppression on T-eff cells, we used flow cytometry–based cytotoxicity assay. Flow cytometry–based cytotoxicity assay discriminates between CFSE-labeled, 7-AAD–positive (dead) responder cells and unlabeled, 7-AAD–negative (live) responder cells and simultaneously measures the extent of proliferation inhibition (CFSE dye dilution) in responder cell populations. In a coculture (responder cell plus T-reg cells) established with HD cells, T-reg cells induced little apoptosis (5.9% of 7-AAD–-negative responder cells). When pretreated with LPS, proliferation of responder cells was 19.8% compared with responder cell proliferation alone, whereas the proportion of dead responder cells remained steady at 5.3%. The LPS-treated T-reg cells in the presence of neutralizing antibody against TLR4 medi-
ated an inhibition of proliferation (28.2%) and a percentage of apoptotic cells (6.1%) comparable with untreated T-reg cells (Figure 5B). These results suggest that suppressor activity of T-reg cells is in part dependent on TLR4-mediated signaling but does not confer cytotoxic effects on proliferating T-eff cells.

**COMMENT**

It is now well recognized that TLRs play a pivotal role in the activation of innate immunity against invading pathogens, induction of cytokine production, and development of adaptive immune responses. In contrast with the protective role of TLRs against pathogen infections, this study suggests that TLRs might play an oppositional role biologically in the context of tumor progression in patients with cancer. Consistent with the findings of previous studies by some of us, we now confirm that the frequency of T-reg cells with a high suppressor phenotype is significantly elevated in patients with HNSCC. Previously, some of us had further reported that CD4\(^+\)CD25\(^{hi}\)FoxP3\(^+\) T-reg cells in patients with HNSCC suppress proliferation of CD4\(^+\) T cells by a granzyme/perforin-dependent mechanisms and kill CD8\(^+\) T-eff cells by inducing Fas-mediated apoptosis. In aggregate, this emphasizes the contribution of T-reg cells to a highly immunosuppressive tumor microenvironment. Within this microenvironment, orchestration of the soluble factors secreted by the tumor cells or altered stromal cells may not only inhibit immune cell function directly or by induction of apoptosis of T-eff cells but also might be perceived by TLRs expressed on T-reg cells. Furthermore, it has been demonstrated that HNSCC cells themselves express TLR4 among other TLRs, and LPS promotes tumor cell proliferation. This would further contribute to the induction of an immunosuppressive environment, which, at least in part, may be used by T-reg cells recruited by the tumor.

For the first time, to our knowledge, we present evidence of the expression of TLRs on T-reg cells in patients with HNSCC. This allows a presumption that TLR signaling on T-reg cells in patients with cancer might play a significant role and it is worthwhile to further elucidate this idea by future studies.

Recent findings indicate that TLRs modulate T-reg cell activity directly in infection biology. Netea et al\(^{19}\) demonstrated that TLR signaling on T-reg cells in patients with cancer might play a significant role and it is worthwhile to further elucidate this idea by future studies.
onstrated that TLRs directly promote the suppressive activity of T-reg cells in fungal infections. Using TLR4 as an example, we demonstrated that its exogenous ligand, LPS, or its endogenous ligand, HSP60, augments suppressive function of T-reg cells. This finding agrees with that of the study by Caramalho et al., who reported that LPS treatment of T-reg cells increases their suppressor efficiency by 10-fold. In contrast, we observed that LPS has no effect on T-eff cell proliferation. Several strategies have been proposed to combine T-reg cell neutralization with conventional antitumor therapies. Among those, linking TLR signaling to the functional control of T-reg cells may offer new opportunities to improve the outcome of cancer immu-
nototherapy by coadministration of certain TLR ligands. For example, it has been shown that the suppressive activity of T-reg cells could be reversed by poly-G oligonucleotides, which bind to TLR8 expressed on T-reg cells, although this inhibiting effect is only temporary. Apetoh et al\(^2\) described a contribution of the immune system to anticancer radiochemotherapy as a clinically relevant immunoadjuvant through a TLR4-dependent activation of tumor antigen–specific T-cell immunity. However, further murine in vivo studies are needed to further delineate the relevance of TLR4 signaling in tumor-mediated immune suppression mediated by T-reg cells and thus evaluate the clinical potential of TLR ligands to reverse T-reg cell function and improve the efficacy of cancer vaccines. Finally, the development and clinical application of combined chemotherapy and immunotherapy may promise enduring success in the fight against cancer.\(^3\)

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Author Contributions: Dr Bergmann had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Wild, Brandau, Hoffmann, Lang, and Bergmann. Acquisition of data: Wild, Lindemann, and Lotfi. Analysis and interpretation of data: Wild, Brandau, Lotfi, and Bergmann. Drafting of the manuscript: Wild and Bergmann. Critical revision of the manuscript for important intellectual content: Brandau, Lindemann, Lotfi, and Bergmann. Administrative, technical, and material support: Brandau, Lindemann, Lotfi, and Bergmann. Study supervision: Brandau and Bergmann.

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