Intratumoral Administration of Recombinant Human Interleukin 12 in Head and Neck Squamous Cell Carcinoma Patients Elicits a T-Helper 1 Profile in the Locoregional Lymph Nodes

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ABSTRACT
The objective of this Phase II study was to evaluate the pharmacodynamic and immune effects of intratumorally administered recombinant human interleukin-12 (IL-12) on regional lymph nodes, primary tumor, and peripheral blood. Ten previously untreated patients with head and neck squamous cell carcinoma were injected in the primary tumor two to three times, once/week, at two dose levels of 100 or 300 ng/kg, before surgery. We compared these patients with 20 control (non-IL-12-treated) patients. Toxicity was high, with unexpected dose-limiting toxicities at the 300 ng/kg dose level. Dose-dependent plasma IFN-γ and IL-10 increments were detected. These cytokine levels were higher after the first injection than after the subsequent injections. A rapid, transient reduction in lymphocytes, monocytes, and all lymphocyte subsets, especially natural killer cells, was observed, due to a redistribution to the lymph nodes. In the enlarged lymph nodes of the IL-12-treated patients, a higher percentage of natural killer cells and a lower percentage of T-helper cells were found compared with control patients. The same pattern was detected in the infiltrate in the primary tumor. Real-time semiquantitative PCR analysis of peripheral blood mononuclear cells in the peripheral blood showed a transient decrease of T-bet mRNA. Interestingly, the peripheral blood mononuclear cells in the lymph nodes showed a 128-fold (mean) increase of IFN-γ mRNA. A switch from the Th2 to a Th1 profile in the lymph nodes compared with the peripheral blood occurred in the IL-12-treated patients. In conclusion, in previously untreated head and neck squamous cell carcinoma patients, recombinant human IL-12 intratumorally showed dose-limiting toxicities at the dose level of 300 ng/kg and resulted in measurable immunological responses locoregionally at both dose levels.

INTRODUCTION
Head and neck squamous cell carcinoma (HNSCC) provides an excellent model to study the effects of locoregional immunotherapy such as cytokines. HNSCC is characterized by the occurrence of regional lymph node metastases. Therefore, a resection of the primary tumor, along with a regional lymph node dissection, is part of the standard treatment in limited disease. Furthermore, HNSCC is an immunogenic tumor, as demonstrated by a variable amount of infiltrating lymphocytes and other immune cells susceptible for activation by immunomodulatory cytokines (1). Several kinds of immune dysfunction, starting at the site of the tumor and eventually generalized, have been reported previously (2, 3). Locoregional immunotherapy given preoperatively allows study of the effects on the primary tumor and the lymph nodes in addition to the peripheral blood. Tumors in the oral cavity or oropharynx carcinoma permit local injection of cytokines.

Interleukin-12 (IL-12) is a heterodimeric cytokine that consists of two disulfide-linked subunits, i.e., IL-12p40 and IL-12p35 (4). IL-12 has a wide range of biological activity (4, 5). It stimulates the proliferation and activation of CTLs and natural killer (NK) cells and induces the production of a number of other cytokines, especially IFN-γ, but also tumor necrosis factor α and granulocyte macrophage colony-stimulating factor (6–8). IL-12 is the key cytokine in the induction of T-helper 1 (Th1) responses and thereby of cellular immunity (9). Furthermore, IL-12 inhibits angiogenesis (10, 11). In experimental murine tumor model, IL-12 has antineoplastic activity (12, 13). Several Phase I (14–19) and Phase II studies in various cancer types (20–22) have been performed with either i.v. or s.c. administration of recombinant human IL-12 (rhIL-12). Toxicity was strongly schedule dependent (20, 23). Some clinical responses were observed. However, IFN-γ production-induced in vivo by rhIL-12 was attenuated rapidly with consecutive cycles, suggesting down-regulation of the biological response to rhIL-12 during therapy (14–16).

Recently, we performed a Phase Ib study with rhIL-12 administered intratumorally (i.t.) in patients with recurrent or metastatic HNSCC at 100 or 300 ng/kg once/week (24). RhIL-12 was administered i.t. rather than systemically because of the expected higher locoregional concentration in the primary tumor and the draining lymph nodes, which may enhance efficacy with less systemic toxicity. RhIL-12 i.t. at these dose levels and schedule was well tolerated and resulted in measurable immunological responses in peripheral blood. Significant reductions in absolute numbers of peripheral blood lymphocytes in all...
subsets, especially NK and Tc cells, were detected. Most interesting was the significant decrease of 80% in T-bet mRNA, a Th1 transcription factor, 12 h after rhIL-12 injection, with normalization after 48–72 h. Also, the T-bet/c-Maf [a T helper 2 (Th2) transcription factor] ratio, an indicator for the Th1/Th2 balance, decreased.

The primary objective of this Phase II study was to evaluate the pharmacodynamic and immune effects of i.t. injected rhIL-12, administered before surgery, at two previously studied dose levels, on the regional lymph nodes, the primary tumor, and the peripheral blood, in previously untreated HNSCC patients. We were interested in the distribution of the different lymphocyte subsets locoregionally compared with the peripheral blood and in the expression levels of IFN-γ, IL-10, and the T-bet/c-Maf ratio locoregionally. The secondary objectives were the assessment of toxicity and clinical activity and the pharmacokinetics (PK) of rhIL-12 after i.t. administration.

PATIENTS AND METHODS

Patient Selection. All patients had histological proof of HNSCC, with the primary tumor in the oral cavity or oropharynx, staged as T1–4, N0–2, and M0, for which surgical resection, including a supraomohyoid or radical neck lymph node dissection, was planned. Patients were previously untreated, i.e., they did not have prior surgery, radiotherapy, or any systemic therapy. The tumor had to be accessible for local injection with a diameter not exceeding 5 cm. Additional eligibility criteria included ages between 18 and 75 years, WHO performance score 0–2, life expectancy > 3 months, adequate renal function (serum creatinine ≤ 1.5 times normal), adequate hepatic function (serum bilirubin ≤ 1.5 times normal; normal serum calcium (≤ 11 mg/dl), serum hemoglobin ≥ 9 g/dl), granulocytes ≥ 1500/µl, and platelets ≥ 100,000/µl. Systemic corticosteroids were not allowed. Patients with major concurrent disease were excluded, as were patients known to be positive for HIV or hepatitis B surface antigen.

To allow comparison of the immunological parameters of the rhIL-12-treated HNSCC patients, we collected also blood samples, lymph nodes, and primary tumor resection material from 20 control patients; these patients were eligible for the study but preferred not to receive the rhIL-12 injections.

The local regulatory committee approved the study. All patients gave written informed consent.

Study Design and Treatment Schedule. In this single-center study, open-label, nonrandomized Phase II study rhIL-12 was supplied by Genetics Institute (Cambridge, MA) and was administered at two dose levels of 100 and 300 ng/kg by single or multiple i.t. injections in the primary tumor only. Five patients/dose level were planned. Patients were treated once weekly in the normal waiting procedure before surgery, with a minimum of two and a maximum of four doses. Surgery was never postponed because of participation in this study. The last planned injection was administered 24 h before surgery. This was based on the results of the prior Phase Ib study (24) in which the most pronounced immunological effects were seen after 12–24 h. The first injection was given as inpatient treatment; others were on an outpatient basis, with an observation period of 1 h after each injection. The mean volume of the injected rhIL-12 was 0.51 ml (range, 0.37–1.1 ml), related to the dose level and the weight of the patient.

Toxicity was assessed using the National Cancer Institute-Common Toxicity Criteria, version 2. Dose-limiting toxicities were defined as drug-related grade 3 or 4 toxicity, with the exception of lymphopenia.

Before the start of the study, all patients underwent a complete medical history, physical examination, electrocardiography, hematology and blood chemistry tests, dipstick urinalysis, test for occult blood in stool, and chest X-ray. Patients were monitored by physical examinations, complete blood counts, and serum chemistry tests before each weekly injection. The duration between the first injection and the surgery (8–15 days) was too short to use computed tomography scans for clinical response evaluation. In 2 IL-12-treated patients, an ultrasonic examination to measure the lymph nodes was performed.

PK and Pharmacodynamic Sampling and Parameters. Venous blood samples were collected after the first injection and just before the last injection. Whole blood samples anticoagulated in EDTA and citrate were obtained for determination of total and differential leukocyte counts and the separation of peripheral blood mononuclear cells (PBMCs) by Percoll density centrifugation. Immunophenotypic analysis and semi-quantitative PCR were performed before rhIL-12 injection, after 8 and 24 h in both weeks, and after 72 h in the first week. Plasma samples were obtained for determination of serum cytokine levels before rhIL-12 injection, after 0.5, 1, 2, 4, 8, and 24 h in both weeks, and after 72 h in the first week. Twenty-four h before surgery, venous blood samples were collected anticoagulated in EDTA and citrate from the control patients for the same determinations.

Immediately after the resection of the primary tumor and the lymph nodes, the resected material was put on ice. The lymph node specimen was freshly cut out by the pathologist (P. de Wilde). The neck was divided in six lymph node regions (I–VI), from which all lymph nodes were collected. Each lymph node was fixated in unifix for routine histology, and depending on the size of the lymph node, parts were taken for flow cytometry or reverse transcription-PCR. If the primary tumor was sufficiently large, samples were taken for flow cytometry. Before performing flow cytometry or reverse transcription-PCR, a single-cell suspension was made of the lymph nodes and primary tumor by using an open filter chamber (NPBI). The lymph nodes regions were designated as draining or nondraining based on the expected anatomical draining pathway.

Plasma IL-12p70 and IL-12p40 were measured in an ELISA as described previously (25). To measure IFN-γ and IL-10, commercial ELISA kits were used (Sanquin Reagents, Amsterdam, the Netherlands). They were standardized with the standards of the National Institute for Biological Standards and Control.

The total number of leukocytes and differential leukocyte counts was determined on the Advia 120 (Bayer). Whole blood samples (100 µl) and the single-cell suspension of the lymph nodes and primary tumor were incubated with directly staining monoclonal antibodies. The following monoclonal antibodies (and clones) were used: CD2 (MT910; DAKO); CD3 (UCHT1; DAKO); CD4 (MT310; DAKO); CD8 (DK25; DAKO); CD19
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normalizing to

The expression of the markers on the PBMCs was analyzed by flow cytometry using a FACScan of Becton Dickinson (Mountain View, CA).

In 6 selected IL-12-treated patients, all of whom received more than one injection, and 6 control patients, comparable with regard to all patient characteristics, total RNA of PBMCs was extracted using Trizol Reagent (Life Technologies, Inc.). Reverse transcription was performed using 5 μg of total RNA, random hexamers, and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Predeveloped assay reagents (PE Biosystems) were used for determination of IFN-γ, IL-10, IL-12p35, and IL-12p40. A predeveloped assay reagent (PE Biosystems) was used for determination of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Taqman probes for T-bet, c-Maf, and phosphorhobilinogen deaminase, a housekeeping gene, were synthesized by PE Biosystems (Branchburg, NJ). The sequence of the primers and probes of T-bet, c-Maf, and PBGD used in this study have been described previously (24). Real-time sequence quantitative analysis was performed as described previously (26). To compare the amount of mRNA of cytokines and transcription factors in the peripheral blood and the lymph nodes of the IL-12-treated and the control patients, the quantity was normalized to glyceraldehyde-3-phosphate dehydrogenase and calculated relative to the mean of the control group. In the IL-12-treated patients, the pharmacodynamic effect of rhIL-12 in the peripheral blood on the amount of mRNA of cytokines or transcription factors in time was determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase and calculating relative to the amount of target present before the start of rhIL-12 administration. A cycle time (Ct) of <35 cycles was established as meaningful.

Total IgG was measured on the Beckman Coulter (Mijdrecht, the Netherlands) Image Nefelometer using Beckman Coulter reagents. Calibration was on the international standard CRM470. IgG subclasses were measured on a BNII Nefelometer Dade Behring (Brussels, Belgium) using the reagents of The Binding Site (Birmingham, United Kingdom). Calibration was performed on a CLB (Amsterdam, the Netherlands) standard H1234.

Statistics. The clinicopathological characteristics of the IL-12-treated and control patients were analyzed by the use of a contingency table; statistical significance was evaluated using the Fisher exact test. Overall survival and disease-free survival were calculated from the date of surgery and estimated using the Kaplan-Meier method (27). Disease-free survival was defined as the length of time after surgery that a patient experienced a complete remission. The comparison of survival parameters in IL-12-treated versus control patients was performed using the log-rank test (28). Nonparametric tests according to Mann-Whitney and Wilcoxon signed-rank tests were performed to compare independent and related samples. Linear regression analyses were performed to compare the monocytes, lymphocytes, and lymphocyte subsets in time after IL-12 injection and to compare the first versus the second or third injection.

For correlation analysis between variables, Spearman’s correlation coefficient was calculated. For all tests, P < 0.05 was considered to be statistically significant. All statistical analysis were two-tailed. Statistical analysis was performed with SPSS 11.0 for Windows.

RESULTS

Patient Characteristics. A total of 10 patients was entered in the study: 4 men and 6 women, with a median age of 55 years (range, 46–69 years; Table 1). The median WHO performance score was 1 (range, 0–1). Tumor sites were the oral cavity (n = 8) and oropharynx (n = 2). The T and N tumor stages are summarized in Table 1. Radical modified neck dissections or supraomohyoidal neck dissections were performed in 8 and 2 patients, respectively. The characteristics of the IL-12-treated patients were comparable with the 20 control patients (Table 1).

Treatment Schedule, Toxicity, Clinical Response, and Survival. As planned, the first 5 patients received two to three injections with 100 ng/kg rhIL-12 i.t. In 1 patient, the injected rhIL-12 flew out of the tumor immediately, and therefore, this patient was omitted from the PK analysis. One patient developed a grade 3 increase of transaminases after the third injection (Table 2). Besides fever and transient lymphopenia, nausea grade 2, vomiting grade 2, and myalgia grade 2 were seen. The next four patients received 300 ng/kg. Two of the 4 patients received only one injection because of grade 3 fatigue and grade 3 liver toxicity, respectively. In 1 of the 4 remaining patients, the dose for the second injection was decreased to 100 ng/kg because of grade 2 liver toxicity. The 1 patient who received three
full doses of 300 ng/kg developed grade 3 metabolic acidosis after surgery, possibly related to rhIL-12 injection, and had a prolonged stay at the intensive care unit for 24 h. Thus, grade 3 toxicity at the 300 ng/kg dose level was seen in 3 of 4 patients, and therefore, this dose level showed dose-limiting toxicity. Consequently, in the tenth patient, the dose level was decreased to 100 ng/kg. He received three injections without serious toxicity.

We did not observe measurable changes in the primary tumor or the lymph nodes in the neck by physical examination during the short treatment period. However, in 2 tested IL-12-treated patients, we measured a significant increase in the diameter of the lymph nodes in the neck by ultrasonic examination after 24 h compared with pretreatment measurements (data not shown). The number of patients who were down- or up-staged from clinical Tumor-Node-Metastasis stage to pathological Tumor-Node-Metastasis stage were not different between the IL-12-treated and control patients. Seven of 10 IL-12-treated patients and 16 of 20 control patients received radiotherapy after surgery according to the standard protocol.

The median overall survival has not yet been reached in the IL-12-treated group and is 2.3 years in the control group (P = 0.09). The median disease-free survival has not been reached in the IL-12-treated group until now and is 2.9 years in the control group (P = 0.14). The median follow-up of the IL-12-treated patients is 2.9 years (1.7–4.8 years) and of the control patients 3.5 years (1.7–5.0 years). In the IL-12-treated group, 2 of 10 patients developed a local recurrence and three a second primary tumor. In the control group, 9 of 20 patients developed a local recurrence and three a second primary tumor.

Table 2  Toxicity of the interleukin 12-treated patients (n = 10)

<table>
<thead>
<tr>
<th></th>
<th>Grade 3</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 2</th>
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</thead>
<tbody>
<tr>
<td>Elevation of ALT&lt;sup&gt;a&lt;/sup&gt;/AST</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Fatigue</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Metabolic acidosis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vomiting</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Myalgia</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Fever</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Lymphopenia</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> ALT, amino alanine transferase; AST, aspartate amino transferase.
<sup>b</sup> Possibly related.

The rhIL-12 PKs. Results of the PK studies are shown in Table 3. PK analysis was performed in 5 of 6 patients at the 100 ng/kg dose level, both after the first injection and the last (second or third) injection. The area under the curve of IL-12p70 (0–24 h) was decreased after the second or third injection compared with the area under the curve after the first injection (P < 0.05). At the 300 ng/kg dose level in 3 of 4 patients, PK analysis was performed only after the first injection. These patients received either only one injection (n = 2) or had a dose reduction to 100 ng/kg (n = 1) in the subsequent injection. In 1 of the 4 patients a complete PK analysis was not possible because of a reluctant attitude to all of the blood collections.

In all patients, IL-12 was detected in plasma 30 min after rhIL-12 injection.

Pharmacodynamics

Effects on Plasma Cytokine Levels. The effect of rhIL-12 on the plasma levels of IFN-γ (Fig. 1A) and IL-10 was analyzed (Fig. 1B). An increase of IFN-γ was seen after 4 h with peak concentration occurring at 24 h. After 72 h, the level of IFN-γ was nearly normalized. After the first injection at the 100 ng/kg dose level, the mean maximal concentration was 143 pg/ml (range, 65–323 pg/ml) and was increased at the 300 ng/kg dose level to 435 pg/ml (range, 369–506 pg/ml; P < 0.05). At the 100 ng/kg dose level, the mean maximal concentration of IFN-γ was higher after the first injection than after the subsequent injections (P < 0.05).

IL-10 production followed the same pattern as IFN-γ in each patient. An increase of IL-10 was first detected after 4–8 h and reached its maximum after 24 h. After 72 h, the IL-10 level was normalized. In week 1 at the 100 ng/kg dose level, the mean maximal concentration was 27 pg/ml (range, 22–33 pg/ml) and at the 300 ng/kg dose level 71 pg/ml (range, 32–107 pg/ml; P = 0.07). At the 100 ng/kg dose level, the mean maximal concentration of IL-10 was higher after the first injection than after the subsequent injections (P < 0.05).

Effects on the Lymph Nodes in the Lymph Node Dissection Specimen. In the lymph node dissection specimen, the mean diameters of the lymph nodes were 7.9 mm (range, 7–8.7 mm) in the IL-12-treated group and 6.1 mm (range, 4.6–8.5 mm) in the control group (P < 0.001). Eventual differences in the number of lymph nodes were only analyzed in patients who underwent a radical lymph node neck dissection. The mean

Table 3  Pharmacokinetic data: mean (±SD) pharmacokinetic parameters on dose levels 100 and 300 ng/kg after the first injection (week 1) and the last injection (week 2 or 3), 24 h before surgery (only on the 100 ng/kg dose level)

<table>
<thead>
<tr>
<th></th>
<th>100 ng/kg</th>
<th>300 ng/kg</th>
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<tbody>
<tr>
<td></td>
<td>Week 1 (n = 5)</td>
<td>Week 2/3 (n = 5)</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (pg/ml)</td>
<td>600 (668.8)</td>
<td>350 (294.8)</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>6.5 (3.4)</td>
<td>4.9 (3.2)</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>7.8 (3.5)</td>
<td>9.8 (8.3)</td>
</tr>
<tr>
<td>Area under the curve (pg × h/ml) (0–24 h)</td>
<td>9,523 (6383.8)</td>
<td>15,870 (4800.5)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Difference between area under the curve (0–24 h) in week 1 and week 2 or 3 at 100 ng/kg (P < 0.05).
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number of the lymph nodes was 42 (range, 31–50) in the IL-12 group (n = 7) versus 28 (range, 15–42) in the control group (n = 14; P = 0.001).

**Effects on Leukocyte Subsets.** In the peripheral blood of all 10 patients after the first rhIL-12 injection, a rapid and significant absolute decrease of lymphocytes, monocytes, CD2+CD3+ cells (T cells), CD3+CD4+ cells (Th cells), CD3+CD8+ cells (Tc cells), CD3-CD16+CD56+ cells (NK cells), CD19+CD45+ cells (B cells), CD45RA+CD4+ cells, CD45RO+CD4+ cells, CD45RA+CD8+ cells, and CD45RO+CD8+ cells was detected (Table 4). The observed decreases were maximal after 24 h and were also detected to the same extent after the second or third injection. An exception were the monocytes, which did not show a decrease after repeated injections. No differences in the absolute amounts of lymphocytes, monocytes, and subsets of lymphocytes were found between the start of treatment or before the second or third injection. After 24 h, hardly any NK cells were detected. The Th/Tc ratio increased significantly in the first 24 h (4–10; P < 0.005), indicating that the CD3+CD8+ cells decreased faster than the CD3+CD4+ cells. Seventy-two h after the rhIL-12 injection, all subclasses were rising but were not fully normalized. However, the T-cell/B-cell ratio was increased compared with the pretreatment values (P < 0.05) because of a stable number of B cells and an increasing number of T cells between 24 and 72 h after the rhIL-12 injection. After 8 h, the percentage of the CD8+ cells that were CD28+ (63–80%; P < 0.02) or CD62L+ (54–73%; P < 0.02) increased significantly. The expression of these activation markers on the CD4+ cells remained stable.

The lymph nodes of 9 of 10 evaluable IL-12-treated patients were compared with 15 control patients. A significantly higher percentage of NK cells (1.6 versus 0.7%; P < 0.001) and a lower percentage of T cells (56 versus 64%; P < 0.05) and Th cells (46 versus 55%; P < 0.05) were found in the enlarged lymph nodes of the IL-12-treated patients (Table 5). The percentage of B cells (41 versus 34%) was not significantly higher and the percentage of Tc cells (8.7 versus 8.2%) did not differ between the IL-12-treated and control patients. The increase in NK cells in the lymph nodes fits well with the observed decrease in NK cells in the blood. The Th/Tc ratio in the lymph nodes was lower in the IL-12-treated versus control patients (6.1 versus 7.6; nonsignificant). This corresponds well with the observation in the blood of an increase in this ratio after rhIL-12. There were no differences between the two dose levels of rhIL-12. A direct comparison of the absolute numbers of leukocyte subclasses in the lymph nodes between the IL-12-treated and control patients was not possible because only parts of the lymph nodes were available for analysis.

Analysis of draining versus nondraining lymph nodes and metastases-containing lymph nodes in IL-12-treated and control patients demonstrated essentially the same effects on redistribution of lymphocyte subsets. In 3 IL-12-treated and 7 control patients, we were able to isolate the infiltrating immune cells from the primary tumor (Table 5). Although the number was small, a trend toward a higher percentage of NK cells and B cells and a lower percentage of T cells was seen in the IL-12-treated patients, as in the lymph nodes.

Because of the increase of B cells in the lymph nodes, the total IgG and the IgG subclasses were determined in plasma in 3 patients before treatment and after 14 (n = 2) or 7 (n = 1) days of treatment. No IgG subclass shifting was found (data not shown).

Collectively, these data demonstrate that a strong decrease in absolute numbers of lymphocytes, monocytes, and all subsets of lymphocytes, especially NK cells, occurred in the peripheral blood after rhIL-12 injection. This decrease was maximal at 24 h and reached approximate normalization after 72 h. In the IL-12-treated patients, the larger lymph nodes contained a higher percentage of NK cells and, surprisingly, a lower percentage of T cells (especially Th cells, with a stable percentage of Tc cells) and a nonsignificant higher percentage of B cells.

**Effects on mRNA of Cytokines and Transcription Factors.** A semiquantitative PCR was performed in 5 IL-12-treated patients to determine the effects of rhIL-12 on the expression of IFN-γ, IL-10, IL-12p35, IL-12p40, T-bet, and c-Maf at different time points after rhIL-12 injection. In the peripheral blood, the mRNA encoding IFN-γ showed no differences in time before and 8, 24, or 72 h after rhIL-12 injection. The expression of IL-10 showed a mean maximal increase of 2.2 times after 24 h relative to the pretreatment sample in week 1 and no differences in week 2 or 3 (data not shown). The mRNA expression of IL-12p35 showed a mean maximal increase of 2.9 after 8 h, both in week 1 and week 2 or 3. After 24 h in both weeks, an increase of 2.1 was still detected, which returned to baseline values after 72 h (Fig. 2A). T-bet expression decreased in all 5 patients. During the first week 8 and 24 h after injection, only 30 and 27%, respectively, of the initial amount of mRNA of T-bet was present. The T-bet mRNA levels returned to normal
investigated patients. The T-bet/c-Maf ratio decreased in time to 0.3 and 0.29 after 8 and 24 h, respectively (Fig. 2C). The expression of IL-12p40 was too low for reliable measurements.

Strikingly, a 128 times higher IFN-γ mRNA expression was found in the lymph nodes of the IL-12-treated patients compared with control patients (mean 139 in IL-12-treated patients and 1.08 in control patients; P < 0.005; Fig. 3A). T-bet expression was 1.7 times (nonsignificant) higher in the IL-12-treated patients compared with control patients. The 2 IL-12-treated patients with the largest tumor load (patients 2 and 8) had the lowest T-bet and IFN-γ expression of the group of 6 investigated patients. The T-bet/c-Maf ratio was not significantly higher in the IL-12-treated patients. The 2 patients (patients 2 and 4) with no up-regulation of the T-bet/c-Maf ratio were the same patients who developed a local recurrence of their disease after 6 months and died because of the recurrence (Fig. 3C). Of the other 4 IL-12-treated patients with an up-regulated T-bet/c-Maf ratio, none had a local recurrence or metastases. No correlation was found between the expression of T-bet and IFNγ in the IL-12-treated or control patients. No differences were found in IL-10, IL-12p35 or IL-12p40 expression.

The expression levels of the cytokines and transcription factors were compared between the lymph nodes and the peripheral blood in both the control patients, collected the day before surgery, and in the IL-12-treated patients, collected 1 h before surgery. These expression levels were compared because of the observed lymphocyte redistribution after rhIL-12 injection. The analysis revealed that in control patients, IFN-γ and T-bet expression was higher in the peripheral blood and IL-10, and c-Maf expression was higher in the lymph nodes (Fig. 4). Strikingly, the expression profile was completely reversed in the IL-12-treated patients: IFN-γ and T-bet expression was higher in the lymph nodes, and IL-10 expression was higher in the peripheral blood. The c-Maf expression was somewhat higher in the lymph nodes but much less than in the control patients. The T-bet/c-Maf ratio (Th1/Th2 balance) was approximately the same in the blood as in the lymph nodes in the control patients but was higher in the lymph nodes compared with blood in the IL-12-treated patients. These observations are a strong indica-

| Table 4 Absolute numbers of lymphocytes, monocytes, and lymphocyte subsets and the T/B cell ratio in the peripheral blood before, 24, and 72 h after the first intratumoral administration of recombinant human interleukin 12 (mean/µl; SD; n = 7) |
|-----------------|-----------------|-----------------|------------------|------------------|
|                  | t = 0 h         | t = 24 h        | t = 72 h         | Difference between t = 0 and t = 24 | Difference between t = 0 and t = 72 |
| Lymphocytes     | 1767 (775)      | 471 (243)       | 1244 (694)       | <0.001           | <0.001           |
| Monocytes       | 404 (368)       | 181 (101)       | 246 (112)        | <0.005           | <0.005           |
| CD2+CD3+        | 1097 (592)      | 386 (249)       | 937 (618)        | <0.001           | <0.001           |
| CD3+CD4+        | 784 (444)       | 282 (189)       | 686 (414)        | <0.001           | <0.001           |
| CD3+CD8+        | 227 (176)       | 54 (51)         | 159 (120)        | <0.005           | <0.005           |
| CD3–CD16+CD56+  | 277 (115)       | 10 (4)          | 148 (43)         | <0.001           | <0.001           |
| CD19+CD45+      | 165 (70)        | 60 (36)         | 87 (32)          | <0.005           | <0.005           |
| CD45RA+CD4+     | 348 (227)       | 111 (57)        | 309 (205)        | <0.005           | <0.005           |
| CD45RO+CD4+     | 466 (249)       | 244 (166)       | 409 (272)        | <0.01            | <0.01            |
| CD45RA+CD8+     | 136 (113)       | 29 (23)         | 91 (72)          | <0.001           | <0.001           |
| CD45RO+CD8+     | 95 (91)         | 32 (25)         | 67 (62)          | <0.005           | <0.005           |
| T/B cell ratio  | 6.9 (2.8)       | 5.7 (2.3)       | 10.7 (4.4)       | <0.05            | <0.05            |

| Table 5 Lymphocyte subsets in the lymph nodes and primary tumors of the interleukin (IL) 12-treated and control patients [mean (SD)] |
|------|-----------------|-----------------|------------------|------------------|
|      | IL-12 treated   | Control         | IL-12 treated    | Control          |
|      | n = 9           | n = 15          | n = 3            | n = 7            |
| T cells (CD2+CD3+) (%)  | 56.4 (8.5)       | 64.2 (7.2)      | 67.2 (10.1)      | 77.8 (15.3)      |
| Th cells (CD3+CD4+) (%)  | 46.3 (7.8)       | 54.8 (9.3)      | 48.1 (13.3)      | 49.5 (9.6)       |
| Te cells (CD3+CD8+) (%)  | 8.7 (3.8)        | 8.2 (2.7)       | 18.1 (14.5)      | 18 (8.7)         |
| Natural killer cells (CD3–CD16+CD56+) (%) | 1.6 (0.8)   | 0.7 (0.3)       | 5 (3.5)          | 2.9 (1.9)        |
| B cells (CD19+CD45+) (%) | 40.6 (9.8)       | 34.3 (7.1)      | 29.6 (6.3)       | 19.6 (11.1)      |
| T-cell/B-cell ratio (CD2+CD3+/CD19+CD45+) | 1.6 (0.6)       | 2.1 (0.7)       | 2.4 (0.8)        | 5.1 (2.3)        |
| CD28+Th cells (CD28+CD4+/CD4+) (%) | 97.4 (0.8)       | 95.2 (9.7)      | 93.1 (4.7)       | 93.1 (3.9)       |
| CD28+Tc cells (CD28+CD8+/CD8+) (%) | 93.7 (2.5)       | 88.9 (19)       | 86.6 (12.1)      | 70.3 (14.8)      |
| CD62L+Th cells (CD62L +CD4+/CD4+) (%) | 69.5 (16.8)     | 69.1 (16.2)     | 49.4 (24.4)      | 53.1 (12.7)      |
| CD62L+Tc cells (CD62L +CD8+/CD8+) (%) | 60 (14.1)        | 53.8 (15.4)     | 33.1 (28.3)      | 21 (12.1)        |
| CD62L+Br cells (CD62L +CD19+CD19+) (%) | 65.2 (13.1)     | 68.1 (14.3)     | 46.0 (28.9)      | 58.1 (14)        |
| CD45RA+CD4+/CD45RO+CD4+ (%) | 0.9 (0.5)        | 1 (0.6)         | 0.6 (0.1)        | 0.25 (0.2)       |
| CD45RACD8+CD45RO+CD8+ (%) | 2 (1.7)          | 1.3 (0.8)       | 0.6 (0.6)        | 0.5 (0.4)        |
| Living cells (%) | 67.3 (8.3)       | 72.2 (6.6)      | 55.8 (26)        | 68.4 (12.8)      |

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tion of the existence of a locoregional Th2 profile in control patients that switches toward a locoregional Th1 profile after rhIL-12 injection.

**DISCUSSION**

We studied various immunological effects of the i.t. administration of rhIL-12 in previously untreated HNSCC patients before surgery. The same dose and schedule was used as in our previous study, i.e., 100 or 300 ng/kg i.t., given once weekly in which pretreated HNSCC patients with a local recurrence or metastases after prior surgery and radiotherapy were treated (24). To our surprise, remarkable toxicity was observed in the present study, i.e., at the 100 ng/kg dose level grade 3 liver toxicity and at the 300 ng/kg dose level grade 3 liver toxicity, grade 3 fatigue, and grade 3 metabolic acidosis. The 300 ng/kg had dose-limiting toxicity. This toxicity was absent in the previous study of pretreated patients. The difference might be a reflection of the compromised immune status in the recurrent patients who had been pretreated with surgery and radiotherapy. Indications for this are the pretreatment number of lymphocytes (1767 versus 833/µl, respectively) and the higher and dose-

Fig. 2 The mRNA expression encoding interleukin (IL)-12p35 T-bet/c-Maf ratio in peripheral blood mononuclear cells in peripheral blood after the first IL-12 injection in 6 patients.

Fig. 3 The mRNA expression encoding IFN-γ and T-bet and the T-bet/c-Maf ratio in peripheral blood mononuclear cells in lymph nodes in 6 interleukin (IL)-12-treated (●, number 2–10) and 6 control patients (□, c1–c6). The IFN-γ mRNA expression was higher in the IL-12-treated patients (P < 0.002). The T-bet expression was comparable except in 1 patient. The T-bet/c-Maf ratio was higher in most patients (nonsignificant).
dependent IFN-γ production in this study compared with the earlier study. Another difference was the injection site: the primary tumor versus lymph node metastases or local recurrence. Triggering might be more efficient with an intact locoregional lymph node system.

Instead of a lower toxicity after i.t. administration compared with i.v. or s.c. administration, dose-limiting toxicity was observed after i.t. injection at the 300 ng/kg once-weekly dose level, compared with a maximal tolerated dose of 500 ng/kg i.v. twice weekly (19) and >2400 ng/kg s.c. once weekly. The liver toxicity in our present study was extremely high: 3 of 10 patients had grade 2 (n = 1) or 3 (n = 2) liver toxicity. Besides the different routes of administration, these studies differ in patient characteristics. Probably part of the observed toxicity in this study reflects highly efficient immune triggering via the primary tumor and in the regional lymph nodes after the relatively high concentration of rhIL-12 locally.

In this study, we compared 10 IL-12-treated patients with 20 control patients, who were eligible but preferred not to receive rhIL-12 injections. The patient characteristics and the duration of the follow-up of these control patients are similar to those of the IL-12-treated patients. We realize that this is not identical to a randomized approach, but such an approach was not considered feasible in this setting. After the limited number of two or three injections, we were not able to detect any clinical activity in the tumor. However, in 2 tested patients, an enlargement of the lymph nodes was seen 24 h after the first injection. To test if the IL-12-treated patients were doing equally well as control patients, overall and disease-free survival were analyzed. The IL-12-treated patients have a trend toward better overall survival, but it had not reached significance at the most recent follow-up.

We are the first to demonstrate that the observed IL-12-induced lymphopenia in humans is caused by a redistribution of peripheral blood lymphocytes to extravascular sites, e.g., lymph nodes. In the neck dissection specimen of the IL-12-treated patients, larger and a higher number of lymph nodes were found. This lymphadenopathy is in agreement with the observation that normal mice receiving IL-12 develop a marked splenomegaly (29) and that in a murine tumor model i.t. injection of IL-12 plasmid DNA resulted in lymphadenopathy and splenomegaly (30). In the lymph nodes of the IL-12-treated patients, a higher percentage of NK cells and a lower percentage of T cells and Th cells were measured. Surprisingly, the percentage of B cells was slightly higher (nonsignificant); Tc cells were the same. The same trends were seen in the primary tumors. The percentages in the lymph nodes and primary tumor correspond with the observation of the extreme decrease of NK cells, the late recurrence of B cells in the peripheral blood, and the moderate decline of Th cells after rhIL-12 injection. However, Tc cells decreased in the peripheral blood, but no increase was detected in the lymph nodes. Our results of the differential redistributions of the lymphocyte subsets are in agreement with those of Gately et al. (29), who observed, after administration of IL-12 to normal mice, an increase in the numbers of NK cells and B cells in the spleen and NK cells and Tc cells in the liver, whereas the number of Th cells in these sites remained essentially unchanged. Our observation in the human lymph nodes is exactly the same as this observation in the spleen of mice. It is imaginable that the disappearing Tc cells in the peripheral blood in our study accumulate in the liver together with NK cells, as seen in the mouse model. The measured redistribution of activated immune cells from the peripheral blood to the lymph nodes (and primary tumor), clearly show that monitoring the immune effects solely in the peripheral blood, after IL-12 or other immune therapy, can be misleading.

Remarkably, in the peripheral blood, lymph nodes, and the primary tumor, a higher percentage of the CD3+CD8+ cells were positive for CD28 and CD62L. This observation cannot be simply explained by the hypothesis that the CD28+CD8+ and CD62L+CD8+ extravasate preferentially, leaving the CD28+ and CD62L+ behind. The CD28+CD8+ and CD62L+CD8+ are also present in a higher percentage in the lymph nodes.

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Because of the higher percentage at both locations (blood and lymph nodes), it appears that a true up-regulation of CD28 and CD62L on CD8+ cells occurs. CD28 up-regulation on CD8+ cells after IL-12 administration is not previously described. CD28 plays an important role in the initiation and expansion of the T-cell response. It has been described that CD28 expression is lost in a subset of CD8+ cells, which correlates with the inability to produce IL-2 and proliferate after activation by antigen-presenting cells. These functions could be restored by reexpressing CD28 in vitro (31). Moreover, CD28+CD8+ cells produce more IFN-γ after IL-12 stimulation than CD28-CD8+ cells in vitro (32). Thus, these data suggest that after IL-12 treatment CD8+ cells are activated in vivo. CD62L (L-selectin) is a lymphocyte homing receptor and plays a key role in the migration of naïve T cells through the lymph nodes. CD62L expression on Th1 cells can be maintained by IL-12 and thereby facilitate the traffic of Th1 cells (33). Moreover, it was shown that CD8+ cells expanded in vitro in the presence of exogenous IL-12 acquired CD62L (34). So, this study confirms the in vitro observations that the CD62L is up-regulated on CD8+ cells after IL-12 treatment.

The PK data of the injected rhIL-12 were comparable with the PK data of the earlier rhIL-12 i.t. study. The main difference is that in this study the area under the curve from 0–24 h after the second or third injection decreased compared with the first injection. This is in accordance with observations after s.c. and i.v. administration (18, 19). The produced IFN-γ was clearly higher, and the produced IL-10 was slightly higher in this study compared with the earlier i.t. study. Both IFN-γ and the IL-10 production decreased after the repeated injection compared with the first injection. The diminished production of IL-10 after repeated injections is in agreement with an earlier observation after s.c. administration (35) and in disagreement with another s.c. study (36). The IL-10 production observed after rhIL-12 administration has been suggested to be responsible for the down-regulation of clinical and immunological effects. However, our data show that despite the IL-10 production in the blood, a potent Th1 environment is present locoregionally in the lymph nodes of IL-12-treated patients. Moreover, recent data imply that IL-10 has a distinct role in the different phases of the immune response. Besides its potent capacity to down-regulate immunity during the effector phase, IL-10 might actually enhance immunity (e.g., IL-10 is known to enhance NK cell function) during the onset of an immune response (37). Therefore, it is doubtful that the observed IL-10 production after IL-12 administration can simply be regarded as a negative side effect.

In contrast to the peripheral blood in which no differences in IFN-γ expression were seen, we detected a 128-fold higher IFN-γ expression in the lymph nodes of the IL-12-treated patients than in control patients. Plasma IFN-γ protein was clearly produced after rhIL-12 injection, starting after 4 h and maximal after 24 h. IFN-γ mRNA levels were low in the PBMCs isolated 8 h after IL-12 injection. Possibly 8 h after treatment is too late to detect the peak of IFN-γ mRNA. In the earlier i.t. study, we detected a slight increase in expression after 4 h. In addition, our data are also in line with the finding that the IFN-γ-producing cells (i.e., NK, Tc, and Th1 cells) might extravasate and are no longer present in the circulation. Although slightly higher IL-10 and IL-12p35 levels were found in the blood after 24 and 8 h, respectively, no differences of these cytokines were seen in the lymph nodes.

The impressive decline in mRNA T-bet expression in the peripheral blood occurred to the same extent as in the former study. T-bet expression in the lymph nodes was higher in one IL-12-treated patient and approximately the same in the others. The resection of the primary tumor and the lymph nodes was performed 24 h after the last injection. Probably this was too late to measure a higher increase of T-bet in the lymph nodes. However, the T-bet/c-Maf ratio (Th1/Th2 ratio) decreased in the peripheral blood in all IL-12-treated patients and increased in 4 of 6 IL-12-treated patients. Remarkably, the 2 patients who did not show an increase in this ratio both had local recurrence of their disease after 6 months; the other 4 patients were disease free. Possibly the ability to up-regulate the T-bet/c-Maf ratio is a prognostic or predictive favorable sign. The observation that the IL-12-treated patients with the largest tumors had the lowest T-bet and IFN-γ expression in their lymph nodes supports this hypothesis.

The observation that the PBMCs isolated from the lymph nodes of the control patients exhibited a Th2 profile compared with the PBMCs of the peripheral blood is also interesting. In contrast, in the IL-12-treated patients, a clear Th1 profile in the lymph nodes was observed compared with the peripheral blood. The Th2 profile is probably an indication of locoregional immunosuppression, which switched to a Th1 profile after rhIL-12 treatment. Such a switch has been shown in mice in vivo with infectious diseases treated with IL-12 (38) but was not earlier shown in humans.

In summary, our data demonstrate that in previously untreated HNSCC patients, the i.t. administration of rhIL-12 is more toxic at doses of 100 and 300 ng/kg than in pretreated HNSCC patients. Its main biological effects locoregionally are a redistribution of lymphocytes from the peripheral blood to the enlarged lymph nodes in the neck, a significant higher percentage of NK cells and a lower percentage of Th cells in the lymph nodes and the primary tumor, and a huge increase in mRNA-encoding IFN-γ in the lymph nodes. A switch from the Th2 profile, present in control patients, to a Th1 profile in the lymph nodes after rhIL-12 administration occurred. The IL-12-treated patients, who showed no increase in the T-bet/c-Maf ratio in the lymph nodes, had the worst prognosis.

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Intratumoral Administration of Recombinant Human Interleukin 12 in Head and Neck Squamous Cell Carcinoma Patients Elicits a T-Helper 1 Profile in the Locoregional Lymph Nodes

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