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A short course of neoadjuvant IRX-2 induces changes in peripheral blood lymphocyte subsets of patients with head and neck squamous cell carcinoma

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Abstract

Objective—IRX-2, a primary cell-derived biologic with pleotropic immune activity, was shown to induce increased lymphocyte infiltrations into the tumor of patients with head and neck squamous cell cancer (HNSCC) after 10 days of neoadjuvant therapy (Berinstein et al. 2011). In the same patients enrolled in the Phase II study, peripheral blood lymphocyte subsets were monitored pre- and post-IRX-2 therapy to evaluate changes induced by IRX-2.

Methods—Absolute lymphocyte numbers were determined in whole blood using the TetraONE System. Lymphocytes were further separated on Ficoll—Hypaque gradients and evaluated by multiparameter flow cytometry. Lymphocyte numbers, including regulatory T cells (Treg) and naïve, memory and effector T cells, were compared in pre- and post-therapy specimens.

Results—Total lymphocyte numbers remained unchanged after IRX-2 therapy. Significant changes occurred in numbers of circulating B cells and NKT cells, which decreased following IRX-2 therapy. The frequency of circulating Treg ($CD4^+CD25^{high}$) remained unaltered (e.g., 6.7 ±

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0.6% vs. 7.5 ± 0.8%; means ± SEM) as was the $CD8^+$/Treg ratio (6.6 before and 6.7 after IRX-2 therapy). The mean absolute number of $CD3^+CD45RA^+CCR7^+$ (naïve) T cells was decreased after IRX-2 therapy but numbers of total memory (i.e., central and peripheral) and terminally differentiated T cells were unchanged.

Conclusions—IRX-2-mediated reductions in B and NKT cell numbers in the blood suggest a redistribution of these cells to tissues. A decrease in naïve T cells implies their up-regulated differentiation to memory T cells. Unchanged Treg numbers after IRX-2 therapy indicate that IRX-2 does not expand this compartment, potentially benefiting anti-tumor immune responses.

Keywords

Cytokines; Lymphocytes; Cancer; Neoadjuvant IRX-2; HNSCC

Introduction

Patients with head and neck squamous cell carcinoma (HNSCC), especially those with advanced disease, have profoundly dysfunctional anti-tumor immunity, which likely results from tumor-induced immune suppression [1]. Therefore, immunotherapy with a goal of restoring the compromised anti-tumor immune responses and thus improving outcome is a rational strategy for treatment of HNSCC [1]. Nevertheless, immunotherapy, including antibody-based interventions or anti-tumor vaccines, has not been successful in patients with HNSCC, and to date has had little impact on disease control [2, 3]. Interestingly, only small proportions of patients with HNSCC are responsive to immune interventions such as, e.g., cetuximab therapy [2]. It has been suggested that a spectrum of immune escape mechanisms which promote tumor progression are responsible for the resistance of HNSCC to immunotherapy [4, 5].

Early studies with recombinant interleukin-2 (rIL-2) delivered peritumorally or systemically to patients with advanced HNSCC indicated that cytokine therapy, while able to up-regulate anti-tumor immune responses, had little impact on clinical outcome or patient survival [6, 7]. On the other hand, a natural mix of cytokines (NCM) rich in IL-2 when administered perilymphatically to tumor-involved lymph nodes induced complete or partial, although transient, clinical immune responses in patients with HNSCC treated by Cortesina and colleagues [8, 9]. These studies provided first evidence that the outcome of patients with HNSCC could be improved by cytokine-based therapy [10]. These results provided a rationale for the next series of cytokine-based immune therapies in this patient group. In investigator-initiated studies using a NCM some encouraging but anecdotal clinical responses to this form of locoregional therapy were observed in patients with HNSCC [11, 12]. Based on these results, IRX Therapeutics prepared a similar product that was manufactured under cGMP conditions and was designated as “IRX-2.” This product was evaluated for safety and activity in FDA-approved multicenter Phase I and Phase II clinical trials for patients with HNSCC [13, 14]. The therapy consisted of a multicomponent biologic (IRX-2) produced by phytohemagglutinin (PHA)-activated human peripheral blood mononuclear cells (PBMC) and delivered perilymphatically in combination with a regimen of low-dose cyclophosphamide and a cyclooxygenase inhibitor, indomethacin, as well as zinc in a multivitamin oral formulation [13, 14]. This regimen of drugs was expected to reduce or eliminate tumor-induced immune suppression mediated by regulatory T cells (Treg) and/or prostaglandin E_2 (PGE_2) and to provide a supplement of zinc, which is usually deficient in patients with HNSCC [15–17].

This study presents results of immune monitoring of lymphocytes in the peripheral blood of 25 patients with resectable HNSCC who were treated with IRX-2 in a single-arm open-label Phase II trial of neoadjuvant IRX-2 delivered perilymphatically prior to surgery. Because

IRX-2 contains a combination of immunostimulatory cytokines, including IL-2, the expectation was that its delivery in the vicinity of tumor-involved lymph nodes might alter activation or differentiation of lymphocytes in the lymph nodes and promote lymphocyte migration to the tumor, which would be reflected in the patients' peripheral blood as well.

Materials and methods

Phase II clinical study

Subjects with newly diagnosed, biopsy-confirmed HNSCC were enrolled in an FDA-approved clinical protocol of IRX-2 (IRX-2 2005-A). This was a multicenter study. The study design and the time points for delivery of all drugs and blood draws are presented in Fig. 1. All enrolled subjects received perilymphatic IRX-2 daily for 10 days prior to surgery. Cyclophosphamide was given at a dose of 300 mg/m^2 on day 1. IRX2 was administered daily for 10 days subcutaneously and bilaterally at doses of 1 ml/ injection (115 IU IL-2/ injection) into the draining cervical lymph nodes. Indomethacin was given orally at 250 mg 3× a day, and zinc (24 mg) was given orally daily throughout the 21-day period. Peripheral venous blood specimens were collected at time points indicated in Fig. 1.

Demographics, clinical and pathologic data for patients enrolled in this study are provided in the accompanying paper.[1]

Collection and processing of blood specimens

Peripheral blood specimens were harvested into green-top vacutainer tubes containing sodium heparin, which were placed in temperature-controlled shipping kits and immediately shipped for the next-day delivery to the Immunologic Monitoring Laboratory (IML) at the University of Pittsburgh. Samples were logged into the IML data base and then processed. A fraction of each whole blood specimen was used to determine absolute numbers of immune cells based on their surface phenotype using the TetraOne System (Beckman Coulter, Miami FL), and the remainder was centrifuged on Ficoll—Hypaque gradients to isolate PBMC. The PBMC were recovered, washed, counted and cryopreserved using a Cryo-Med (ThermoForma, Marietta, OH). Cells were stored in 2-ml vials in the vapor phase of liquid N_2 storage units until thawed and tested.

Antibodies and reagents

The following anti-human monoclonal antibodies were used for flow cytometry: anti-CD3, anti-CD8, anti-CD4, anti-CD20, anti-CD16, anti-56, anti-CD45RA, anti-CCR7, anti-CD27, and anti-CD25. All Abs and their respective isotypes used as negative controls were purchased from Beckman Coulter, except for anti-CCR7 Abs, which were from BD Biosciences, San Jose, CA. Ab dilutions were determined by titrations using freshly isolated or activated PBMC obtained from normal controls (NC).

Immunophenotyping and flow cytometry

Thawed and washed PMBC were stained for cell surface markers as previously described [15]. The phenotypic markers were assessed in batched specimens using paired cryopreserved/thawed PBMC from each subject that were obtained on days 1 and 21. Briefly, pre-titered volumes of each Ab was added to 100 μl of the cell suspension and incubated for 20 min at RT in the dark, washed twice with staining buffer (0.1% w/v BSA and 0.1% w/v NaN_3 in PBS) and finally fixed in 1% (v/v) paraformaldehyde in PBS. Four-color flow cytometry was performed using a Beckman Coulter FC500 flow cytometer

[1]Berinstein et al. [26].

equipped with CXP software. Lymphocytes were gated based on forward/ side scatter and excluding debris, monocytes and granulocytes. At least 10^5 cells were acquired for analysis. The data were analyzed using Coulter CXP software.

Absolute lymphocyte counts

A standard single-platform technique, TetraCHROME System (Beckman Coulter, Miami, FL), based on four-color flow cytometry in the presence of counting beads was used. The identification of lymphocytes by expression of bright CD45 and low side scatter signals was followed by the identification of T cell subsets based on the expression of CD3, CD4, and CD8, as previously described [18]. Briefly, 100 μl aliquots of heparinized anticoagulated blood were incubated with 10 μl of tetraCHROME reagent containing labeled anti-CD45, anti-CD3, anti-CD20, and anti-CD56 Abs. Specimens were then lysed with the ImmunoPrep Reagent System at the Coulter TQ-Prep Workstation. Leukocyte morphology and cell surface integrity were maintained by a gentle, no-wash erythrocyte lysing method. Immediately before analysis, 100 μl of Flow count Fluorospheres (Beckman Coulter) were added to the stained cells, and the beads were counted along with cells. The sample acquisition and analysis were performed on the FC500 flow cytometer with a fully automated software-reagent combination. The number of cells (or cell subsets) per microliter was obtained by calculating the number of cells counted × concentration of beads/ number of beads counted. In each experiment, blood obtained from patients was evaluated together with blood from at least one NC.

Statistical analysis

Flow cytometry results (percentages or absolute numbers of lymphocytes) obtained prior to and after therapy were compared using Wilcoxon's signed rank sum test. The two-sided P values were considered significant at $P < 0.05$.

Results

Peripheral blood specimens obtained from 25 patients with HNSCC were tested at baseline (day 1) prior to the start of IRX-2 regimen and also on day 21 after IRX-2 delivery was completed before surgery (Fig. 1). The total lymphocyte counts (means ± SEM) were 1,612 ± 111 on day 1 and 1,507 ± 128 on day 21. There were no significant changes in these values between day 1 and day 21. Absolute numbers and percentages of various lymphocyte subsets in the peripheral blood were determined at both time points and are presented in Table 1. The only significant pre- to post-treatment differences were observed in the B lymphocyte number, which was reduced ($P < 0.005$) and the NKT cell number which was also reduced ($P < 0.03$) following therapy with IRX-2. The absolute numbers of the other lymphocyte subsets in the peripheral circulation remained unchanged (Table 1). Notably, absolute numbers and the frequency of circulating Treg ($CD4^+CD25^{high}$) remained unaltered after IRX-2 therapy (e.g., 6.7 ± 0.6% vs. 7.5 ± 0.8%; means ± SEM). The ratio of $CD8^+$/ Treg cells was 6.6 before IRX-2 therapy and 6.7 afterward. As we previously reported [17], the percentage of Treg in the peripheral circulation of our patients with HNSCC was increased relative to that in normal controls (12.3 ± 3% vs. 2 ± 1.5%; means ± SEM). This frequency was unchanged following IRX-2 therapy.

Although no changes in total T lymphocytes were observed after IRX-2 therapy, subtyping for naïve, memory or differentiated T cells revealed some post-therapy effects. Figure 2 shows that the mean number of naïve ($CD3^+CD45RA^+$ $CCR7^+$) T cells in the blood was decreased in the patients' peripheral circulation after IRX-2 therapy ($P < 0.04$) as was the mean number of central memory T cells. However, no significant differences were detected in numbers of total memory T cells (i.e., central and peripheral) in pre- versus post-therapy

samples possibly because the mean number of central memory ($CD3^+CD45RA^-CCR7^+CD27^+$) T cells was decreased while that of peripheral effector memory ($CD3^+CD45RA^-CCR7^-CD27^-$) T cells was somewhat increased after IRX-2 therapy (Fig. 2). Although, the number of terminally differentiated ($CD3^+CD45RA^+CCR7^-CD27^-$) T cells was slightly lower in post-therapy samples, this difference did not reach statistical significance.

Discussion

IRX-2 is a primary cell-derived biologic with pleotropic immune modifying activity. When administered to patients in the Phase 2 trial, IRX-2 was safe and increased lymphocyte infiltration into the tumor and T cell activation in situ as compared to biopsy prior to treatment [26]. As observed in tumor samples procured from surgery performed after the IRX-2 regimen was completed, these in situ changes significantly correlated with disease outcome, as the OS was significantly prolonged in patients whose tumor samples were strongly infiltrated with $CD3^+$ T cells [26]. In the study presented here, IRX-2 was shown to induce relatively minor changes in absolute numbers of circulating lymphocyte subsets when delivered to the patients as the neoadjuvant perilymphatic regimen prior to surgery.

IRX-2-induced changes in the peripheral blood were not expected to be significant given a short (10-day) course of the locoregional treatment. We did observe a statistically significant decrease in circulating B cells following IRX-2 therapy. Although the patients were pre-treated with a non-chemotherapeutic dose of cyclophosphamide (an agent known to decrease lymphocyte levels when used at high doses), it is unlikely that at the dose used, there was a specific impact on B cell and not on T cells. Seen by itself, this decrease in absolute B cell numbers in the blood from pre- to post-therapy might not deserve attention. However, the presence of increased numbers of B cells in the tumor after IRX-2 therapy was concurrently seen in the same group of patients as described by Berinstein et al. [26]. This suggests that IRX-2 enhances migration of B cells from the blood to tumor sites and tumor-draining lymph nodes. In previous clinical studies with a natural biologic similar to IRX, we also observed an increase of B cells and plasma cells in lymph nodes, which significantly correlated with the degree of B cell infiltration into the tumor [12]. Thus, we hypothesize that IRX-2-driven mobilization of B cells to lymph nodes and the tumor is responsible for the observed decrease of B cell numbers in the circulation. Although B cell numbers and functions in HNSCC have not been extensively studied, at least one study reports that the presence of $CD20^+$ B cells and of $CD8^+$ T cells in metastatic lymph nodes of patients with HNSCC is associated with favorable clinical outcome [19]. Differentiation of IRX-2-activated B cells into plasma cells could result in the production of anti-tumor antibodies with potential therapeutic effects. Perhaps an extended regimen of IRX-2 allowing for a longer period of B cell activation and differentiation could be even more beneficial for patients with HNSCC, and in the upcoming FDA-approved randomized trial, confirmation of the IRX-2-specific effect on B cells will be one of the primary assessments.

The second aspect of IRX-2 delivery concerns the differentiation of circulating T cells. We observed that the numbers of naïve T cells are significantly decreased in the circulation after IRX-2 therapy, while the numbers of total memory and of terminally differentiated T cells remain unchanged. This suggests that IRX-2 promotes the differentiation of naïve T cells, increasing their transition from the naïve to memory phenotype. At the same time, IRX-2 did not enlarge the peripheral compartment of terminally differentiated effector T cells, which in patients with HNSCC is expanded relative to NC and contains T cells sensitive to apoptosis [20]. This suggests that during IRX-2 therapy, transiting of maturing effector T cells from the memory to terminally differentiated T cell compartment is less active and instead, the memory compartment serves as a source of mature effector T cells moving into

tissues. We have previously reported that a rapid turnover of differentiating T cells in the peripheral circulation may be in part responsible for a paucity of anti-tumor effector cells in patients with HNSCC [21]. The observed effects of IRX-2 after a short course of therapy on lymphocyte differentiation in the periphery suggest that IRX-2 can alter and perhaps normalize lymphocyte homeostasis in patients with HNSCC.

Since IRX-2 contains IL-2, it was possible that its delivery to patients with HNSCC would lead to expansion of Treg, as seen in clinical trials with IL-2 that was used at significantly higher (100–1,000 fold) levels [22]. As previously reported, patients with HNSCC have an increased frequency of Treg in the circulation relative to NC [23], i.e., 5 ± 3% versus 2 ± 1.5% of total $CD4^+$ T cells (means ± SD). Treg accumulate at the tumor site in HNSCC [24]. Treg are dependent on IL-2 for their survival and function [25], and IRX-2 delivery could further increase the Treg pool resulting in undesirable suppression of anti-tumor immune responses. Importantly, the IRX-2 regimen did not increase the frequency or absolute numbers of Treg in this Phase II trial, and the $CD8^+$/Treg ratios remained unchanged after IRX-2 therapy. This further suggests that immunotherapy with IRX-2 could be especially effective in up-regulating effector T cell activation and their functions without concomitantly increasing Treg numbers.

The reported results of IRX-2 effects on peripheral blood lymphocytes of patients with HNSCC provide an encouragement for the future use of this pleotropic biologic in cancer therapies. Administered locoregionally for a short time period prior to surgery, it had significant effects on absolute numbers of lymphocyte subsets, confirming its potential utility as a modulator of anti-tumor effector cells in vivo. Together with increased lymphocyte infiltrations into tumors of the HNSCC patients treated with IRX-2 as reported by Berinstein et al. [26], our results indicate that IRX-2 deserves to be evaluated in randomized clinical trials to confirm its beneficial effects on the restoration of anti-tumor immunity in patients with cancer.

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References

1. Whiteside TL. Immunobiology of head and neck cancer. Cancer Metastasis Rev. 2005; 24:95–105. [PubMed: 15785875]
2. Specenier P, Vermorken JB. Cetuximab in the treatment of squmaous cell carcinoma of the head and neck. Exp Rev Anti-cancer Ther. 2011; 11:511–524.
3. Janke M, Peeters B, Zhao H, deLeeuw O, Moorman R, Arnold A, Ziouta Y, Fournier P, Schirrmacher V. Activation of human T cells by a tumor vaccine infected with recombinant newcastle disease virus producing IL-2. Int J Oncol. 2008; 33:823–832. [PubMed: 18813797]
4. Ferris RL, Whiteside TL, Ferrone S. Immune escape associated with functional defects in antigen-processing machinery in head and neck cancer. Clin Cancer Res. 2006; 12:3890–3895. [PubMed: 16818683]
5. Young RM. Protective mechanisms of head and neck squamous cell carcinomas from immune assult. Head Neck. 2006; 28:462–470. [PubMed: 16284974]
6. Hadden JW. The immunology of head and neck cancer: prospects for immunotherapy. Clin Immunother. 1995; 3:362–385.
7. Whiteside TL, Letessier E, Hirabayashi H, Vitolo D, Bryant J, Barnes L, Snyderman C, Johnson JT, Myers E, Herberman RB, et al. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. Cancer Res. 1993; 53:5654–5662. [PubMed: 8242620]

8. Cortesina G, De Stefani A, Giovarelli M, Barioglio MG, Cavallo GP, Jemma C, Forni G. Treatment of recurrent squamous cell carcinoma of the head and neck with low doses of interleukin-2 injected perilymphatically. Cancer. 1988; 62:2482–2485. [PubMed: 3263896]
9. Cortesina G, De Stefani A, Galeazzi E, Cavallo GP, Badellino F, Margarino G, Jemma C, Forni G. Temporary regression of recurrent squamous cell carcinoma of the head and neck is achieved with a low but not with a high dose of recombinant interleukin-2 injected perilymphatically. Br J Cancer. 1994; 69:572–576. [PubMed: 8123489]
10. De Stefani A, Valente G, Forni G, Lerda W, Ragona R, Cortesina G. Treatment of oral cavity and oropharynx squamous cell carcinoma with perilymphatic interleukin-2: Clinical and pathologic correlations. J Immunother. 1996; 19:125–133.
11. Hadden JW, Endicott J, Baekey P, Skipper P, Hadden EM. Interleukins and contrasuppression induce immune regression of head and neck cancer. Arch Otolaryngol Head Neck Surg. 1994; 120:395–403. [PubMed: 8166968]
12. Meneses A, Verastegui E, Barrera JL, Zinser J, de la Garza J, Hadden JW. Histologic findings in subjects with head and neck squamous cell carcinoma receiving perilymphatic natural cytokine mixture prior to surgery. Arch Pathol Lab Med. 1998; 122:447–454. [PubMed: 9593347]
13. Freeman SM, Franco JL, Kenady DE, Baltzer L, Roth Z, Brandwein HJ, Hadden JW. A Phase I safety study of an IRX-2 regimen in subjects with squamous cell carcinoma of the head and neck. Am J Clin Oncol. 2011; 34:173–178. [PubMed: 20539208]
14. Wolf GT, Fee WE, Dolan RW, Moyer JS, Kaplan MJ, Spring PM, Suen J, Kenady DE, Newman JG, Carroll WR, et al. Novel neoadjuvant immunotherapy regimen safety and survival in head and neck squamous cell cancer. Head Neck. 201110.1002/hed.21660
15. Berlinger NT. Deficient immunity in head and neck cancer due to excessive monocyte production of prostaglandins. Laryngoscope. 1984; 94:1407–1411. [PubMed: 6593559]
16. Hadden J. The treatment of zinc deficiency as an immunotherapy. Int J Immunopharmac. 1995; 17:697.
17. Strauss L, Bergmann C, Gooding W, Johnson JT, Whiteside TL. The frequency and suppressor function of CD4+ $CD25^{high}$Foxp3+ T cells in the peripheral circulation of patients with squamous cell carcinoma of the head and neck. Clin Cancer Res. 2007; 13:6301–6311. [PubMed: 17975141]
18. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. Clin Cancer Res. 2004; 10:3755–3762. [PubMed: 15173082]
19. Pretscher D, Distel LV, Grabenbauer GG, Wittlinger M, Buettner M, Niedobitek G. Distribution of immune cells in head and neck cancer: CD8+ T-cells and CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro- and hypopharyngeal carcinoma. BMC Cancer. 2009; 9:292–301. [PubMed: 19698134]
20. Hoffmann TK, Donnenberg AD, Finkelstein SD, Donnenberg VS, Friebe-Hoffman U, Myers EN, Appella E, DeLeo AB, Whiteside TL. Frequencies of $tetramer^{+}$ T cells specific for the wild-type sequence $p53_{264–272}$ peptide in the circulations of patients with head and neck cancer. Cancer Res. 2002; 62:3521–3529. [PubMed: 12067999]
21. Kim J-W, Ferris RL, Whiteside TL. Chemokine receptor 7 (CCR7) expression and protection of circulating CD8+ T lymphocytes from apoptosis. Clin Cancer Res. 2005; 11:7901–7910. [PubMed: 16278415]
22. Berntsen A, Brimnes MK, thor Straten P, Svane IM. Increase of circulating $CD4+CD25^{high}$FOXP3+ regulatory T cells in patients with metastatic renal cell carcinoma during treatment with dendritic cell vaccination and low-dose interleukin-2. J Immunother. 2010; 33:425–434. [PubMed: 20386464]
23. Bergmann C, Strauss L, Wang Y, Szczepanski MJ, Lang S, Johnson JT, Whiteside TL. T regulatory type 1 cells (Tr1) in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease. Clin Cancer Res. 2008; 14:3706–3715. [PubMed: 18559587]
24. Bergmann C, Strauss L, Zeidler R, Lang S, Whiteside TL. Expansion of human T regulatory type 1 cells in the microenvironment of COX-2 overexpressing head and neck squamous cell carcinoma. Cancer Res. 2007; 67:8865–8873. [PubMed: 17875728]

25. Czystowska M, Strauss L, Bergmann C, Szajnik M, Rabinowich H, Whiteside TL. Reciprocal granzyme/perforin-mediated death of human regulatory and responder T cells is regulated by interleukin-2 (IL-2). J Mol Med. 2010; 88:577–588. [PubMed: 20225066]

26. Berinstein NL, Wolf GT, Naylor PH, Baltzer L, Egan JE, Brandwein HJ, Whiteside TL, Goldstein L, El-Naggar A, Badoual C, Fridman W-H, White JM, Hadden JW. Increased lymphocyte infiltration in patients with head and neck cancer treated with the IRX-2 immunotherapy regimen. Cancer Immunol Immunother. 2011 (in press).

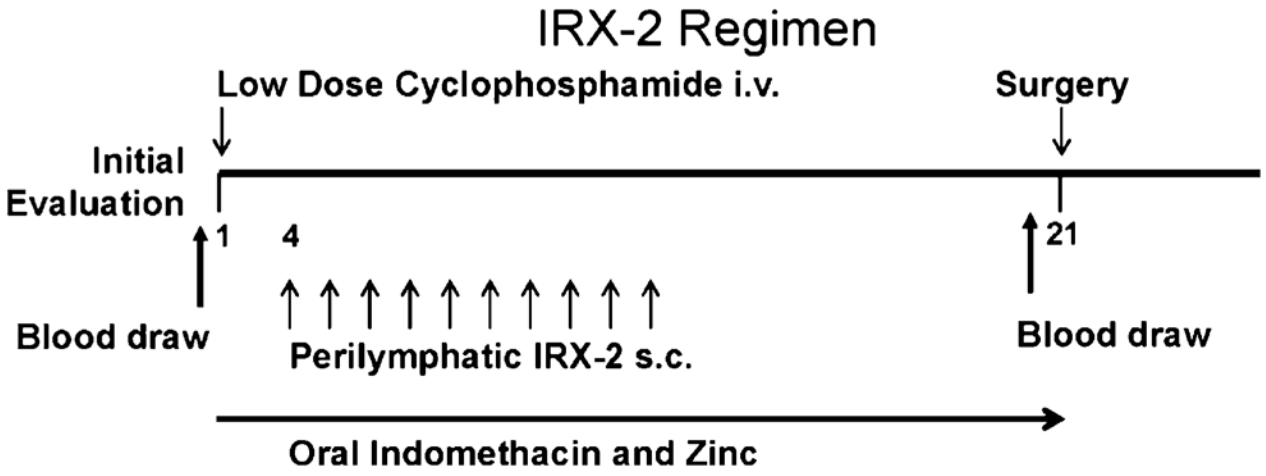

Fig. 1.
A schema showing the IRX-2 regimen used in the Phase II protocol and time points for *blood* draws

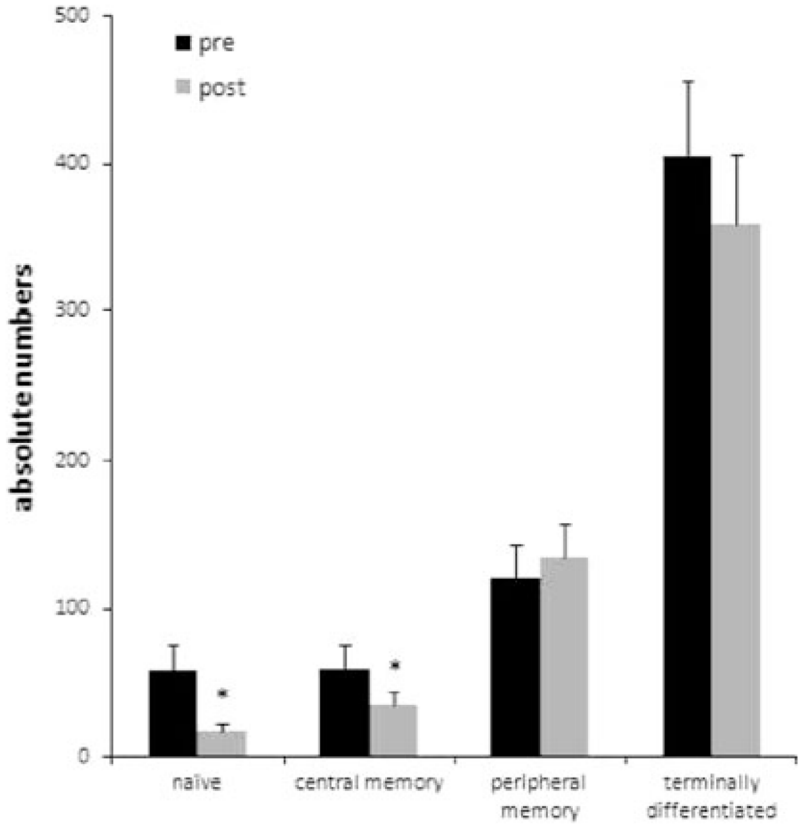

Fig. 2.

Absolute *numbers* of differentiated T lymphocytes in the peripheral blood defined by flow cytometry and based on differential expression of CD3, CD45RA/RO and CCR7 prior to and after IRX-2 therapy. The data are means ± SD. The *asterisks* indicate significant differences (pre- to post-therapy) at $P < 0.04$

Table 1

Absolute numbers of lymphocyte subsets in the peripheral blood of patients with HNSCC prior to and after IRX-2 treatment

Cell type	Surface markers	day 1 (pre)	day 21 (post)
Total lymphocytes		1,612 ± 111	1,507 ± 128
T lymphocytes	$CD3^+$	1,178 ± 90	1,121 ± 103
Helper	$CD4^+$	817 ± 68	762 ± 69
Cytotoxic	$CD8^+$	352 ± 40	350 ± 47
Activated helper	$CD4^+CD25^+$	192 ± 22	187 ± 19
Activated cytotoxic	$CD8^+CD25^+$	N/A	N/A
Regulatory	$CD4^+CC25^{high}$	53 ± 6	52 ± 7
B lymphocytes	$CD19^+CD3^-CD14^-$	282 ± 26	200 ± 16**
NK cells	$CD3^+CD16^+CD56^+$	143 ± 13	138 ± 19
NKT cells	$CD3^+CD16^+CD56^+$	46 ± 7	36 ± 5*

Lymphocyte subsets were evaluated by flow cytometry as described in section “Materials and methods”. Absolute numbers of all subsets were calculated based on the percent of positive cells present in the total lymphocyte fraction determined for every patient and every time point. The data are means ± SEM

The two asterisks indicate a significant pre- to post-IRX-2 difference at $P < 0.005$ and one asterisk at $P < 0.03$

N/A not available