Interleukin-22 Is Frequently Expressed in Small- and Large-Cell Lung Cancer and Promotes Growth in Chemotherapy-Resistant Cancer Cells

Sebastian Kobold, MD, Stefanie Völk,* Till Clauditz, MD,† Natascha Jennifer Küpper, MSc,* Sarah Minner, MD,† Amanda Tufman, MD,‡ Peter Düwell, PhD,§ Michael Lindner, MD,‖ Ina Koch, PhD,‖ Simon Heidegger, MD,* Simon Rothenfußer, MD,* Max Schnurr, MD,§ Rudolf Maria Huber, MD,‡ Waldemar Wilczak, MD,† and Stefan Endres, MD**

Introduction: In lung cancer, interleukin-22 (IL-22) expression within primary tissue has been demonstrated, but the frequency and the functional consequence of IL-22 signaling have not been addressed. This study aims at analyzing the cellular effects of IL-22 on lung carcinoma cell lines and the prognostic impact of IL-22 tissue expression in lung cancer patients.

Methods: Biological effects of IL-22 signaling were investigated in seven lung cancer cell lines by Western blot, flow cytometry, real-time polymerase chain reaction, and proliferation assays. Tumor tissue specimens of two cohorts with a total of 2300 lung cancer patients were tested for IL-22 expression by immunohistochemistry. IL-22 serum concentrations were analyzed in 103 additional patients by enzyme-linked immunosorbent assay.

Results: We found the IL-22 receptor 1 (IL-22-R1) to be expressed in six of seven lung cancer cell lines. However IL-22 signaling was functional in only four cell lines, where IL-22 induced signal transducer activator of transcription 3 phosphorylation and increased cell proliferation. Furthermore, IL-22 induced the expression of anti-apoptotic B-cell lymphoma 2, but did not rescue tumor cells from carboplatin-induced apoptosis. Cisplatin-resistant cell lines showed a significant up-regulation of IL-22-R1 along with a stronger proliferative response to IL-22 stimulation. IL-22 was preferentially expressed in small- and large-cell lung carcinoma (58% and 46% of cases, respectively). However, no correlation between IL-22 expression by immunohistochemistry and prognosis was observed.

Conclusion: IL-22 is frequently expressed in lung cancer tissue. Enhanced IL-22-R1 expression and signaling in chemotherapy-refractory cell lines are indicative of a protumorigenic function of IL-22 and may contribute to a more aggressive phenotype.

Key Words: Lung cancer, Small-cell lung cancer, Large-cell lung cancer, Interleukin-22, Interleukin-22-receptor 1.

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Interleukin-22 (IL-22) is a unique cytokine of the IL-10 family acting exclusively on interleukin-22-receptor 1 (IL-22-R1) positive epithelial cells.[1] IL-22 induces antimicrobial proteins such as defensins in epithelial cells, protects against damage, and promotes regeneration in injured tissues.[2] These functions, including up-regulation of antiapoptotic pathways and increased proliferation, are protective in acute inflammatory conditions and in tissue injury,[3,4] but may be deleterious in chronic states where proliferation or antiapoptosis promote disease, as demonstrated in psoriasis.[5] Several signal transducers downstream of the IL-22-R1 have been described, including signal transducer activator of transcription (STAT)1 STAT5, extracellular-signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38.[6] In cancer cells, however, the main signaling pathway downstream of the IL-22-R1 is STAT3, which mediates most IL-22–induced effects.[2,7,8] In the lung, IL-22 has been found to be expressed by alveolar epithelial cells, macrophages, and T cells.[9,10] Both exogenous IL-22 and IL-22 produced by immune cells protect lung tissue from acute injury induced by ventilation trauma or viral infection.[3,11] Thus, the lung is an organ in which epithelial cells can react to IL-22 and resident cell populations are capable of producing IL-22. Interestingly, many cancer cell lines, including those of lung origin, express the IL-22-R1.[12] Recent work by Zhang et al.[7] suggests that IL-22 is an autocrine mediator in non–small-cell lung cancer (NSCLC). In the current study, we analyzed 2300 lung cancer samples for IL-22 expression by immunohistochemistry and also

*Department of Internal Medicine IV, Division of Clinical Pharmacology and Center of Integrated Protein Science, Ludwig-Maximilians Universität München, Member of the German Center for Lung Research, Munich, Germany; †Institute of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; Departments of ‡Internal Medicine V, Thoracic Oncology Center, §Internal Medicine IV, Munich, Ludwig-Maximilians Universität München, Munich, Germany, Member of the German Center for Lung Research; and ‖Center for Thoracic Surgery, Asklepios Biobank for Lung Diseases, Asklepios Clinic München-Gauting, Comprehensive Pneumology Center Munich, München-Gauting, Germany, Member of the German Center for Lung Research.

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Address for correspondence: Sebastian Kobold, MD, Division of Clinical Pharmacology, Ludwig-Maximilians Universität München, Ziemssenstraße 1 80336, München, Germany. E-mail: Sebastian.kobold@med.uni-muenchen.de

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studied IL-22 serum levels in lung cancer patients. Further, we investigated IL-22-R1 expression, signal transduction, and proliferative response to IL-22 in lung cancer cell lines.

PATIENTS AND METHODS

Patients and Patient-Derived Samples

Two tissue microarrays (TMAs) were established from a total of 203 (TMA1) and 2097 (TMA2) lung cancer specimens of archived tissue samples at the Institute of Pathology at the University Medical Center Hamburg-Eppendorf, the University Hospital Freiburg, and the Kantonspital Basel (collected between 1994 and 2006), respectively. All tissues were obtained from patients who were undergoing lobectomy or pneumectomy. Tissue samples were formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded, and used for TMA construction, as previously described.[13] Hematoxylin-eosin–stained sections were used to define representative tumor regions. Tissue cylinders (0.6 mm in diameter) were then punched from that region of the block with the use of a self-developed semiautomated tissue arrayer.[14] Survival data were available for 1724 patients. The mean follow-up period was 29.1 months (range, 3–200 months). Survival data were either obtained from the cancer registry, or collected from the patients' attending physicians. All tissues included in these TMAs had been reviewed by an expert pathologist determining histological type and histological grade. The pathologically determined tumor stage (pT), nodal (pN), and metastasis (pM) status were obtained from the primary reports of the department of pathology. The collection and TMA-based screenings of human tumor samples were in compliance with the ethical principles for medical research established by the World Medical Association's Declaration of Helsinki in its current version. An overview of the patient cohort is given in Table 1.

Serum samples from 103 patients with early-stage lung cancer were obtained from the Asklepios Biobank of Lung Diseases. All patients gave their informed consent.

Serum samples from age- and sex-matched healthy donors (n = 70) were provided by the Bavarian Red Cross (Blutspendedienst des Bayerischen Roten Kreuzes gemeinnützige GmbH).

TABLE 1. Clinical and Pathologic Characteristics of 2097 Patients (TMA2)[a]

Characteristics	Study Cohort on TMA No. of Patients
Histology	
Squamous cell carcinoma	980 (47%)
Adenocarcinoma	569 (27%)
Bronchioalveolar carcinoma	92 (4%)
Large-cell carcinoma	379 (18%)
Small-cell carcinoma	63 (3%)
Adenosquamous carcinoma	14 (1%)
Follow-up	
Overall survival (mo)	
Median	23.0
Mean	35.2
Sex	
Male	1265 (77%)
Female	377 (23%)
Age (yr)	
≤60	702 (40%)
>60	1070 (60%)
Mean age (yr)	63 (11–92)
pT category	
pT1	453 (22%)
pT2	1167 (56%)
pT3	300 (14%)
pT4	154 (7%)
pN category	
pN0	1027 (51%)
pN1	489 (24%)
pN2	442 (22%)
pN3	58 (3%)
pM category	
pM0	1958 (93.8%)
pM1	130 (6.2%)

[a]Numbers do not add up to 2097 in the different categories because of cases with lack of data.

pT, pathologically determined tumor stage; pN, pathological nodal; pM, pathological metastasis; TMA, two tissue microarray.

Cell Lines

Lung cancer cell lines H187, H1339, LOU-NH91, HCC827, and A549 were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkultur (DSMZ), Braunschweig, Germany). The cisplatin-resistant cell lines H1339-R and HCC827-R have been described.[15] De novo cisplatin-resistant cell lines HCC827-R2 and A549-R were generated, as previously described.[15]

Scratch Assay

Scratch assay was performed, as described.[16] Cells were stimulated for 4 hours in 1% fetal calf serum (FCS) medium (Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) supplemented with 1% penicillin-streptomycin [PS] of 10,000 U/ml and 10,000 µg/ml stock solution; respectively) and 1% L-glutamine (of 200 mm stock solution) with or without IL-22 (Peprotech, Hamburg, Germany) or gardiquimod (InvivoGen, Toulouse, France) at the indicated concentrations. Cell-free area was measured and calculated using Image J software (freeware established by R. Stallmann and available for download under http://rsbweb.nih.gov/ij/download.html). Mitomycin C was used at 10 µg/ml.

Western Blot

Western blot was performed, as previously described.[17] Antibodies used included polyclonal antibody against IL-22-R1 (Sigma, St. Louis, MO), monoclonal antibody against pSTAT3 (Tyr705, clone B-7; Santa Cruz, Heidelberg, Germany), monoclonal antibody against STAT3 (clone F-2; Santa Cruz), polyclonal antibody against BCL-2 (N19, sc492; Santa

Cruz), monoclonal antibody against β-actin (clone C4; Santa Cruz), polyclonal antibody against mouse immunoglobulin (IgG), horseradish peroxidase (HRP)-labeled (Cell Signaling, Frankfurt, Germany), and polyclonal antibody against rabbit IgG, HRP-labeled (Santa Cruz).

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked immunosorbent assay for IL-22 detection was obtained from Research & Development, (R & D) Abington, United Kingdom. Absorption was then measured at 450 nm using a Mithras LB940 multilabel plate reader (Berthold Technologies, Bad Wildbad, Germany).

Real-Time Polymerase Chain Reaction

Quantitative RT-PCR was performed as previously described.[17] The following primers were designed using the online Roche assay design center: IL-22-R1-fwd: 5′-TTA TTT TTC CAT TGT CTC TGT CCA -3′, IL-22-R1-rev: 5′-TGG CAG CAC ATT TCC TTG TAA CT – 3′, HPRT-fwd: 5′ - CGA GCA AGA CGT TCA GTC CT - 3′, and HPRT-rev: 5′ - TGA CCT TGA TTT ATT TTG CAT ACC–3′, together with the probes 28 and 73 of the Roche Universal Probe Library (Roche, Mannheim, Germany). Primer efficiency was determined experimentally by serial dilutions of cDNA at 2.13 for the IL-22-R1 primer pair and at 1.95 for the HPRT pair. Expression of IL-22-R1 relative to HPRT was calculated using the light cycler relative quantification software based on the formula: relative expression = NT × ETCpT/NR × ERCpR where NT is the number of target molecules at detection threshold Cp, NR is the number of reference molecules at detection threshold Cp, ET is the efficiency of target amplification, and ER is the efficiency of reference amplification.

Vitality and Proliferation Assay

For assessing cell vitality, cell titer blue assay was performed in accordance with the protocol of the manufacturer (Promega, Mannheim, Germany). Stimulation by or neutralization of IL-22 was performed in serum-free medium (DMEM or RPMI, 1% PS and 1% L-Glutamine). Positive controls for vitality or proliferation were assessed in culture medium supplemented as above including, in addition, 10% FCS. For IL-22 neutralization, polyclonal antibody against IL-22-R (R & D) and monoclonal antibody against IL-22 (clone 142928; R & D) were added to the culture. For STAT3 inhibition, the specific inhibitor STATTIC (Sigma, Taufkirchen, Germany) was used at a concentration of 25 μM.

Apoptosis Assay by Annexin V-Propidium Iodide Staining

Cells were treated either with carboplatin (Ratiopharm, Ulm, Germany) at the indicated concentrations: STATTIC (10 μM) or IL-22 (100 ng/ml) for 48 hours in supplemented culture medium (DMEM or RPMI with 10% FCS, 1% PS and 1% L-Glutamine). Afterward, cells were stained with propidium iodide nucleic acid stain (Invitrogen, Paisley, United Kingdom) and annexin V allophycocyanin (APC)-conjugated antibody (ImmunoTools, Friesoythe, Germany). Cells were studied by flow cytometry using a FACS Canto II (Becton Dickinson, Heidelberg, Germany), and data were analyzed with Flow Jo software (Version 7.6.5; Tree Star Inc., Ashland, Oregon).

IL-22 Immunohistochemistry

Freshly cut 3-μm thick TMA sections were analyzed on 1 day in a single experiment. IL-22 expression was detected with a polyclonal rabbit antibody (ab18944; abcam) in a dilution of 1:150 after peroxidase blocking with H_2O_2 (DAKO S2023; DAKO, Hamburg, Germany) for 10 minutes. Slides were pretreated at high-temperature in an autoclave (121°C) with citrate buffer, pH 6 for 5 minutes. The Envision system (DAKO 5007) was used to visualize immunostaining. The IL-22 antibody was pretested on a separate TMA containing biopsies of normal tissue (colon; $n = 3$; endometrium, $n = 4$, lung, $n = 4$, epididymidis, $n = 2$) as negative controls (no IL-22 expression) and biopsies with malignant tissue (colon cancer; $n = 10$), where IL-22 expression has been previously described (positive control)[18].

For each tissue spot, IL-22 staining was scored by cytosplasmatic staining intensity (0, 1+, 2+, or 3+) and by estimation of the fraction of positive tumor cells. A composite score was built from these two parameters according to the following scheme: Low expression was defined as a staining intensity of 0 and 1+ in less than 70% of tumor cells or a staining intensity of 2+ in less than 30% of tumor cells. Positive expression was defined as a staining intensity of 1+ in more than 70% of tumor cells, a staining intensity of 2+ in more than 30% of tumor cells, or a staining intensity of 3+.

Statistical Analyses

For statistics JMP 7.0 software (SAS institute Inc., Cary, NC) or GraphPad Prism Software, version 5.0b (GraphPad Software, LaJolla, CA) were used. All Student's *t* tests were two-sided and *p* values less than 0.05 were considered significant. Differences between experimental conditions were analyzed using the unpaired Student's *t* test. Regression analysis was used to compare variances. To study the relationship between IL-22 expression and clinical–pathological parameters, contingency table analysis and χ^2 test (likelihood) was used. Overall survival was analyzed by the Kaplan–Meier method and was compared using log-rank test.

RESULTS

Lung Cancer Cell Lines Express IL-22-R1 but React Heterogeneously to IL-22 Stimulation

Seven lung cancer cell lines were studied for the biological effects of IL-22 signaling: A549, HCC827, H1339, H187, LOU-NH91, HCC827-R, and H1339-R (the latter 2 cisplatin-resistant). Six of seven cell lines (85%) were found positive for IL-22-R1 expression by Western blot and RT-PCR (Fig. 1*A* and data not shown). None of the cell lines were found to express IL-22 (data not shown). To test for IL-22-R1 functionality, cell lines were stimulated with recombinant IL-22, and subsequent STAT3 phosphorylation was analyzed by Western blot (Fig. 1*B*). Four of the six cell lines (66%) expressing IL-22-R1 showed IL-22-induced STAT3 phosphorylation (see Fig. 1*B*, *C* for A549 and HCC827-R, data not shown for H1339-R

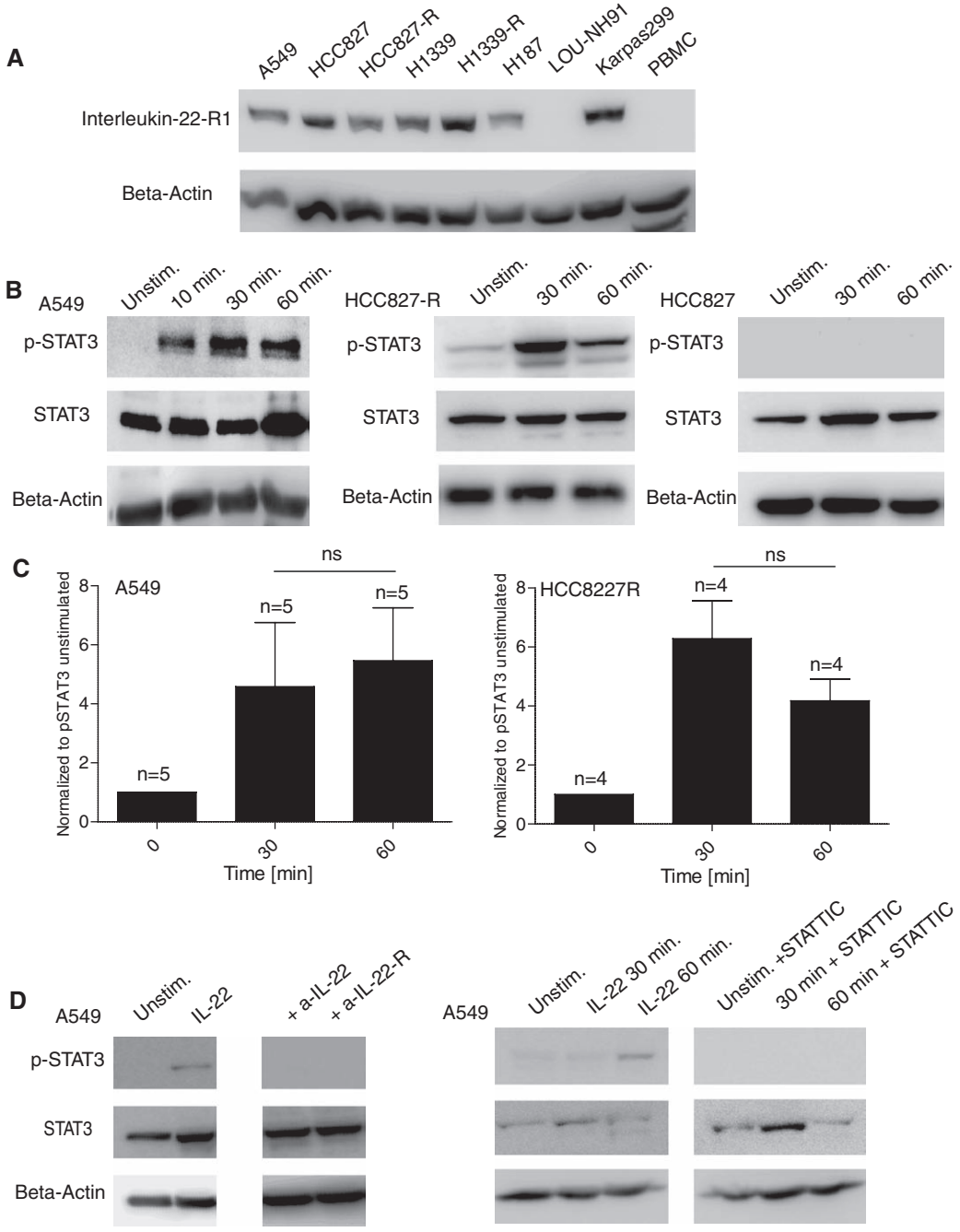

FIGURE 1. IL-22-R1–positive lung carcinoma cell lines react to IL-22 in a specific manner. *A*, Expression analysis of IL-22-R1 in the lung cancer cell lines A549, HCC827, HCC827-R, H1339, H1339-R, H187, and LOU-NH91 by Western blot. The cell line Karpas 299 and human PBMC served as positive and negative controls, respectively. β-actin served as loading control. *B*, Detection of STAT3 phosphorylation on IL-22 stimulation in the cell lines A549, HCC827-R and HCC827. Lysates were made 10, 30, and 60 minutes after stimulation. STAT3 and β-actin served as loading controls. *C*, Densitometric analysis of pSTAT3 of five, respectively four independent stimulations of cell lines A549 and HCC827R with IL-22 (100 ng/ml) for 30 and 60 minutes. *D*, Stimulation of cell line A549 with IL-22 in the presence or absence of IL-22 or IL-22-R1 blocking antibodies. IL-22–induced STAT3 phosphorylation was detected after 30 minutes of stimulation with IL-22. A549 was also stimulated with IL-22 in the presence or absence of the specific STAT3 inhibitor STATTIC (25 μM). STAT3 phosphorylation is shown after 30 and 60 minutes, respectively. For all Western blots, the results of one of three representative experiments are shown. PBMC, peripheral blood mononuclear cells; IL-22, interleukin-22.

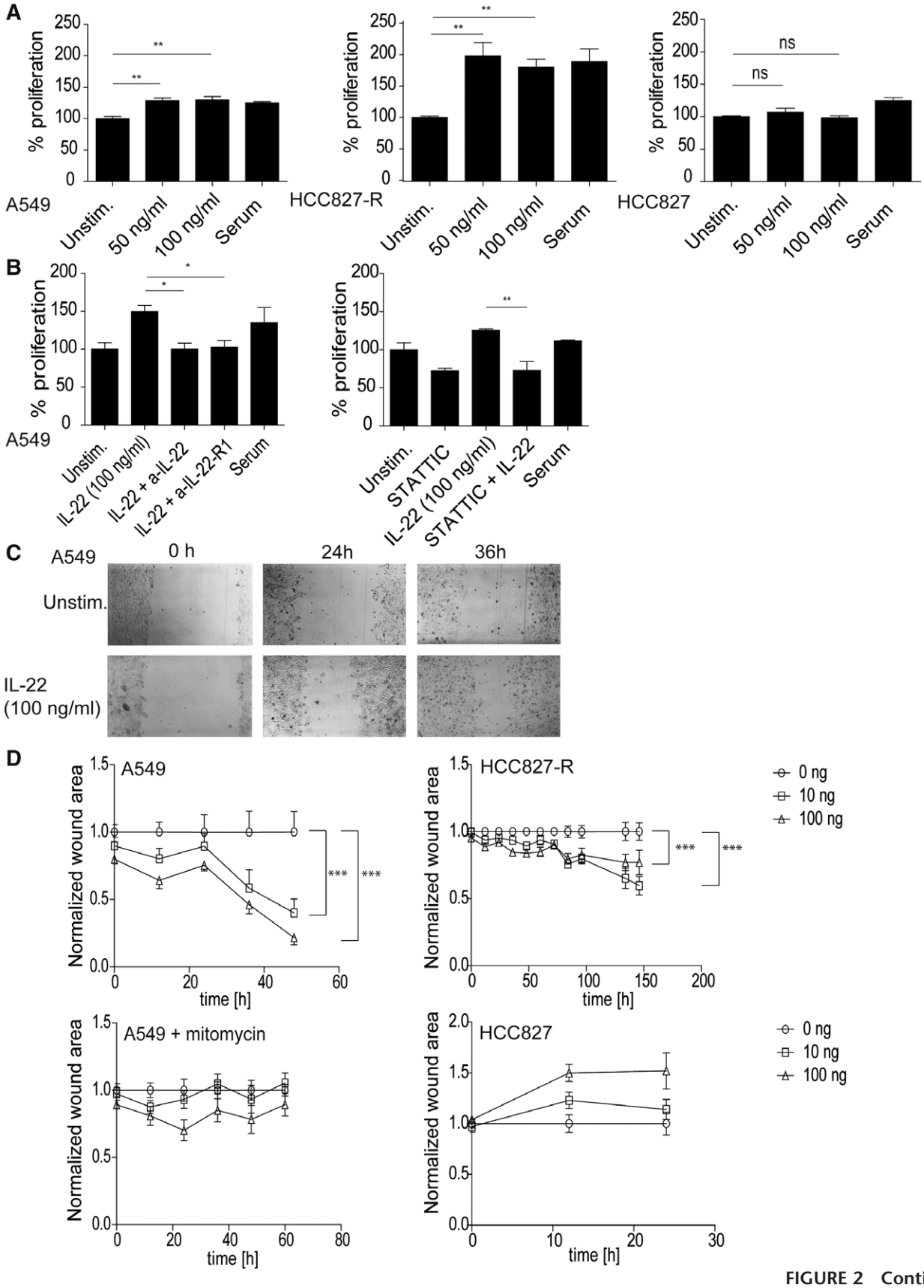

FIGURE 2 Continued

and H187). The cell lines H1339, LOU-NH91, and HCC827 did not respond by STAT3 phosphorylation (see Fig. 1*B* for HCC827, data not shown for H1339 and LOU-NH91). Specificity of the signaling was demonstrated using IL-22 neutralizing and IL-22-R1 blocking antibodies and a specific STAT3 phosphorylation inhibitor (Fig. 1*D*).

IL-22 Induces Proliferation in IL-22 Responsive Lung Cancer Cell Lines

Lung cancer cell lines that are reactive to IL-22 as evidenced by STAT3 phosphorylation were tested for IL-22–induced proliferation. IL-22 stimulation resulted in an increase in proliferation as compared with unstimulated cells (Fig. 2*A*). The observed effect was dependent on IL-22 signaling through STAT3 because IL-22–blocking antibodies and STAT3 inhibition completely suppressed IL-22–induced cell proliferation (Fig. 2*B*). Subsequently, we investigated the impact of IL-22 on re-epithelialization in a standardized scratch assay. IL-22 induced faster re-epithelialization of the scratch as compared with the unstimulated control (Fig. 2*C*). Because IL-22 stimulation showed no dose dependency between 100 and 50 ng/ml in proliferation assays, we chose to test the effect of IL-22 in scratch assay using 100 ng/ml and 10 ng/ml (Fig. 2*C*). A trend toward a dose dependency was observed. Again, the faster re-epithelialization was only observed in IL-22-sensitive cell lines (Fig. 2*D*). Suppression of IL-22-mediated re-epithelialization by mitomycin C treatment, which blocks proliferation but not migration, indicates that replenishment of the scratch area is mediated predominantly by tumor cell proliferation (Fig. 2*D*).

IL-22 Does Not Protect Lung Cancer Cell Lines from Chemotherapy-Induced Apoptosis

IL-22 has been described to protect epithelial cells from apoptosis through up-regulation of antiapoptotic proteins such as BCL-2.[19] We used the chemotherapeutic drug carboplatin, which induces apoptosis in cancer cells by cross-linking of DNA strands. Treatment with IL-22 had no impact on cell viability or on the rate of apoptosis in carboplatin-exposed A549 or HCC827-R cells (Fig. 3*A*, *B*). Similar observations were made for the chemotherapeutic drug 5-FU (Supplementary Figure 1A, Supplemental Digital Content 1, http://links.lww.com/JTO/A434). IL-22 induced a weak BCL-2 up-regulation in these cell lines (Fig. 3*C*). Gardiquimod served as a positive control for Bcl-2 induction (Supplementary Figure 1C, Supplemental Digital Content 1, http://links.lww.com/JTO/A434).[20]

Chemotherapy-Resistant Cell Lines Up-regulate IL-22-R1 Expression and Signaling

Because resistance to standard chemotherapeutic agents such as cisplatin is a common event in lung cancer treatment, we attempted to determine whether drug-resistant cells have an altered response to IL-22. Cell lines A549 and HCC827 were repeatedly exposed to cisplatin, which resulted in resistance to both cisplatin and cisplatin-induced apoptosis (Fig. 4*A* and Supplementary Figure 1B, Supplemental Digital Content 1, http://links.lww.com/JTO/A434). These cell lines were designated A549-R and HCC827-R2, respectively. Interestingly, A549-R and HCC827-R2 up-regulated IL-22-R1 expression as assessed by RT-PCR and Western blot (Fig. 4*B*). Up-regulation of IL-22-R1 expression correlated with a stronger IL-22-induced proliferative response as compared with passage-matched non-resistant controls (Fig. 4*C*). These data indicate that cisplatin-resistant cell lines are more sensitive to IL-22 signaling and this may contribute to the aggressive phenotype of these cells.

IL-22 Is Preferentially Expressed in Primary Large- and Small-Cell Lung Cancer

To further investigate how the above-described in vitro effects impact on human lung cancer disease, we first tested whether IL-22 is expressed in primary lung cancer tissue, by staining 203 samples from patients with NSCLC and small-cell lung cancer (SCLC) (TMA1; Table 2) for IL-22 expression. IL-22 was expressed in all histological subtypes with the highest positive proportion in SCLC (85%; Fig. 5*A*, *B*). Adenocarcinoma (ADCA), bronchoalveolar carcinoma, and large-cell carcinoma exhibited similar expression rates with 54%, 45%, and 44%, respectively. Morphologically, the tumor tissue, that is, the cancer cells themselves mainly stained positive for IL-22 expression. Squamous cell carcinoma (SQCC) showed the least frequent expression of IL-22 (23%). To confirm these findings, we next analyzed a second cohort containing 2097 samples from patients with NSCLC (n = 2034; 97%) or SCLC (n = 63; 3%) for IL-22 expression (Table 3). Of 2097 TMA tissue spots stained for IL-22 expression, 1815 spots (86%) were evaluable for IL-22 expression analysis.

FIGURE 2. Continued IL-22 induces STAT3-mediated cell proliferation in lung carcinoma cell lines. *A*, Cell proliferation measurement by cell titer blue assay on IL-22 (either 50 or 100 ng/ml as indicated) stimulation for the cell lines A549, HCC827-R, and HCC827. Ten percent serum served as positive control and serum-free medium as baseline control. Values were normalized to the mean value of unstimulated controls. The results of one of three representative experiments are shown. The error bars represent the standard error of the mean of the replicates (n = 6). *B*, Cell proliferation measurement by cell titer blue assay on IL-22 stimulation (100 ng/ml) for the cell line A549 in the presence or absence of IL-22 or IL-22-R1 blocking antibodies or STATTIC. Values were normalized to the mean value of unstimulated controls. The results of one of three representative experiments are shown. The error bars represent the standard of mean of the replicates (n = 3). *C*, Light microscopy image of representative scratches applied to cell line A549 in the presence or absence of IL-22. Results are shown for the time points 0, 24, and 36 hours. The results of one of three representative experiments are shown. *D*, Measurement of cell-free surface in a scratch assay in the presence or absence of IL-22 (10 ng/ml or 100 ng/ml, respectively) for the cell lines A549, HCC827-R, and HCC827. Extent of re-epithelialization was normalized to the unstimulated controls. The cell line A549 was treated, in addition, with mitomycin C in the presence of IL-22. The results of one of three representative experiments are shown. Error bars represent the standard error of the mean of the replicates (n = 12). For all experiments, *indicates a p value less than 0.05, **indicates a p value less than 0.01, and ***indicates a p value less than 0.001, and a nonsignificant difference. IL-22, interleukin-22; ns, nonsignificant.

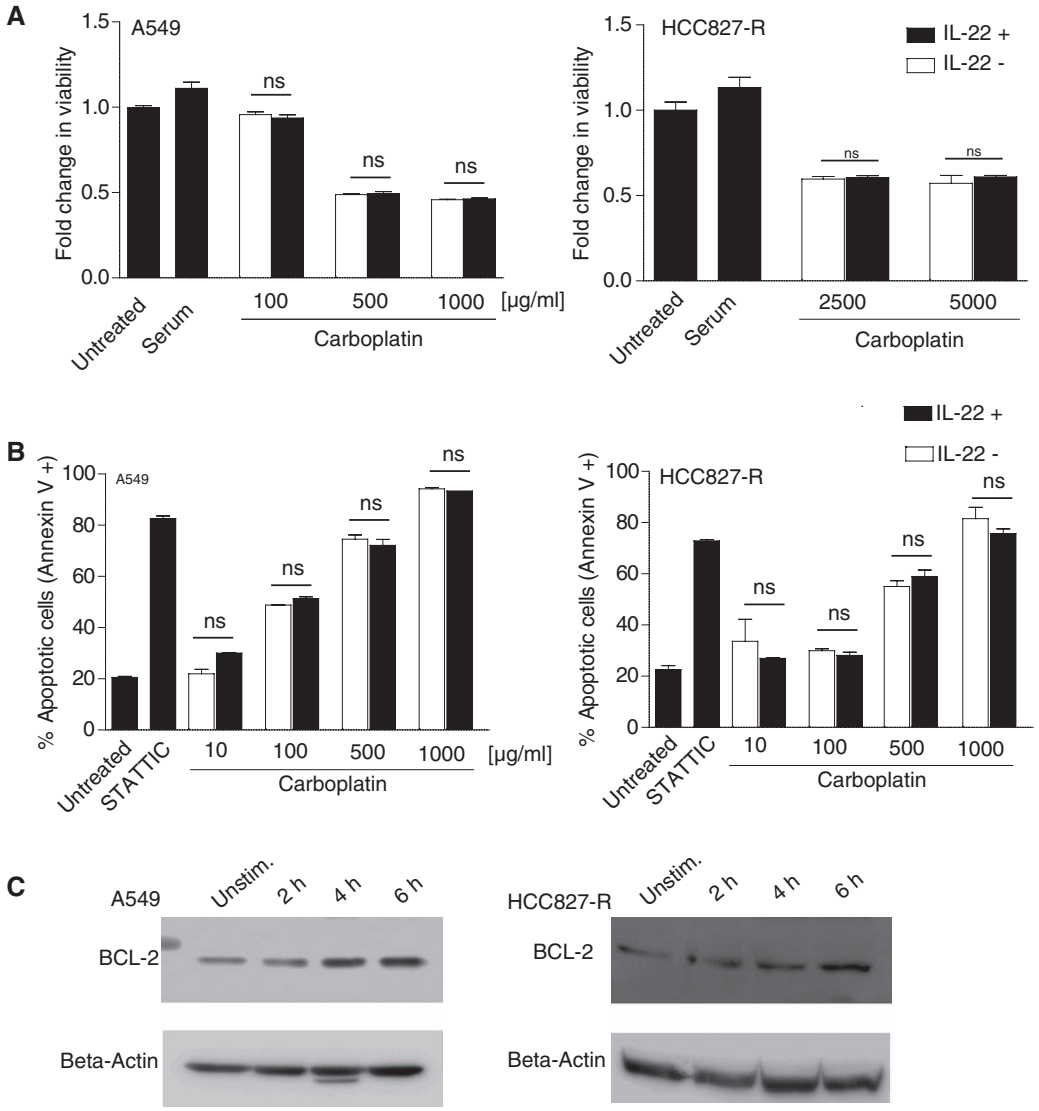

FIGURE 3. IL-22 does not protect lung carcinoma cell lines from chemotherapy-induced apoptosis. *A,* IL-22 responsive cells were treated with increasing doses of carboplatin for 48 hours (100, 500, and 1000 µg/ml). Cell viability was measured by cell titer blue assay. Values were normalized to the untreated controls. The results of one of three representative experiments are shown. The error bars represent the standard error of the mean of the replicates ($n = 4$). *B,* IL-22 responsive cells A549 and HCC827R were treated with increasing doses of carboplatin (10, 100, 500, and 1000 µg/ml) for 48 hours and were analyzed for the induction of apoptosis by flow cytometry. Total apoptotic cells are calculated by adding annexin V+ PI– and annexin V– PI+ cells. STATTIC (10 µM) served as a positive control. No significant differences were found between IL-22-treated and untreated cells. The results of one of three representative experiments are shown. The error bars represent the standard error of the mean of the replicates ($n = 12$). *C,* Cell lines A549 and HCC827R were treated for 2, 4, or 6 hours with recombinant IL-22 (100 ng/ml) and were analyzed for BCL-2 induction by Western blot. β-actin served as loading control. The results of one of three representative experiments are shown. IL-22, interleukin-22; V Pi, propidium iodide; ns, nonsignificant; BCL-2, B-cell lymphoma 2.

The remaining 282 samples were noninformative because of absence of tissue on the TMA or a lack of unequivocal tumor cells in the arrayed samples. Expression of IL-22 was again found in a majority of SCLC samples (58%; Fig. 1*C*). Large-cell carcinoma had the second highest proportion of IL-22 expression (46%; Fig. 1*C*), whereas, ADCA, bronchoalveolar carcinoma, and SQCC expressed IL-22 only in smaller proportions of samples (33%, 24%, and 28%, respectively). Contingency table analysis confirmed a higher expression in SCLC and large-cell carcinoma compared with the other histological subtypes ($p < 0.0001$; Table 3). Expression of IL-22 by large-cell carcinoma is supported by the analysis of serum IL-22 levels in a small cohort ($n = 103$) of patients undergoing surgery for ADCA, SQCC, or large-cell carcinoma. Two of the three patients in this cohort with large-cell lung carcinoma had higher IL-22 concentrations in their serum (998 and 964 pg/ml) compared with matched healthy controls (mean 53 pg/ml), with ADCA (mean 28 pg/ml), or with SQCC (mean

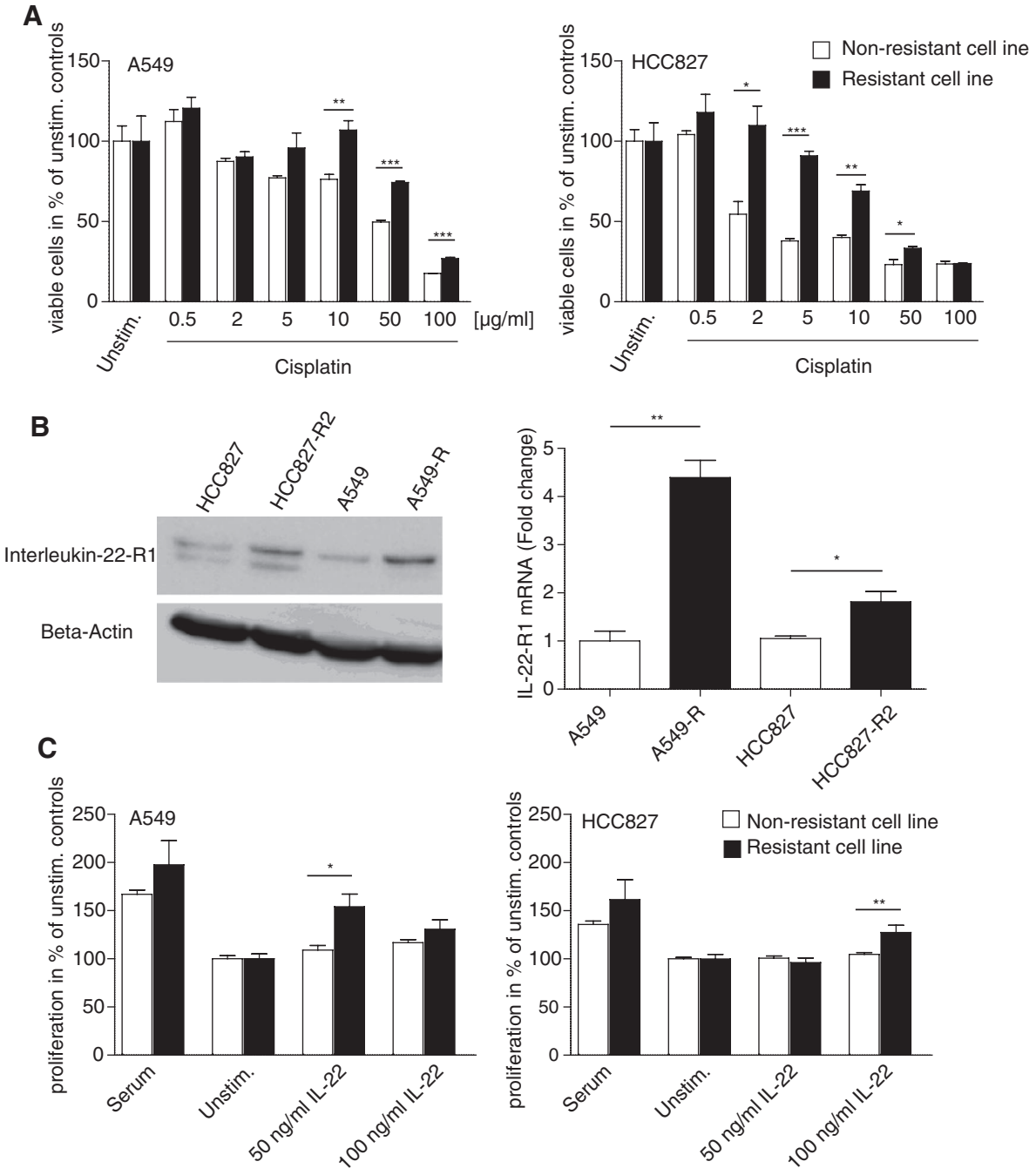

FIGURE 4. Cisplatin-resistant lung carcinoma cell lines are more sensitive to IL-22. *A*, Cell lines A549, A549-R, HCC827, and HCC827-R2 were treated for 4 hours with increasing doses of cisplatin (0.5, 2, 5, 10, 50, and 100 µg/ml) and vitality was measured by cell titer blue assay. The results of one of three representative experiments are shown. The error bars represent the standard error of mean of the replicates ($n = 3$). *B*, Expression of IL-22-R1 in the cell lines A549, A549-R, HCC827, and HCC827-R2 was evaluated both on protein (Western blot) and mRNA levels (RT-PCR). For RT-PCR data relative expression of IL-22 to hypoxanthin-phosphorybosyltransferase was normalized to the baseline level of the nonresistant control cells (A549 and HCC827, respectively). The results of one of three representative experiments are shown. The error bars represent the standard error of mean of the replicates ($n = 3$). *C*, Cell lines A549, A549-R, HCC827, and HCC827-R2 were stimulated with increasing concentrations of IL-22 (50 and 100 ng/ml). Ten percent serum served as positive control and cells in serum-free medium as baseline. Values were normalized to the mean value of unstimulated controls. The results of one of three representative experiments are shown. The error bars represent the standard error of mean of the replicates ($n = 6$). For all experiments, *indicates a p value less than 0.05, **a p value less than 0.01, ***indicates a p value less than 0.001 and a nonsignificant difference. RT-PCR, real-time polymerase chain reaction; IL-22, interleukin-22; IL-22-R 1,interleukin-22-receptor 1.

TABLE 2. Correlation of IL-22 Expression with Pathologic Characteristics of TMA1

Histology	IL-22 High (%)	IL-22 Low (%)
Adenocarcinoma	30 (45)	36 (55)
Bronchioalveolar carcinoma	18 (24)	56 (76)
Large-cell carcinoma	20 (44)	24 (56)
Small-cell carcinoma	11 (85)	v2 (15)
Squamous cell carcinoma	13 (23)	44 (77)
Unknown	6 (60)	4 (40)

TMA, two-tissue microarray.

88 pg/ml) patients (Supplementary Figure 1A, Supplemental Digital Content 1, http://links.lww.com/JTO/A434). Because of this differential expression of IL-22 among different lung cancer subtypes, we investigated a possible correlation of IL-22 expression with clinical–pathological parameters.

IL-22 Expression Does Not Correlate with Prognosis in Resected NSCLC and SCLC

We first tested the patient cohort of TMA2 for known prognostic parameters such as pathologically determined pT, pN, and pM. Advanced T stage (pT3 or pT4), positive nodal status (pN1–3), and pM1 all correlated significantly with reduced overall survival ($p < 0.0001$ for all comparisons, Supplementary Figure 2A, Supplemental Digital Content 2, http://links.lww.com/JTO/A435). Having established that the studied cohort is representative for standard prognostic factors, we analyzed the influence of IL-22 expression on prognosis in the complete cohort (Fig. 5*D*) and in the different histological subtypes separately (Supplementary Figure 2B, Supplemental Digital Content 2, http://links.lww.com/JTO/A435). IL-22 did not correlate with prognosis in any of the groups analyzed (Fig. 5*D*; Supplementary Figure 2B, Supplemental Digital Content 2, http://links.lww.com/JTO/A435). Moreover, we found no significant difference in IL-22 expression between different tumor stages (Table 3).

DISCUSSION

So far, a single study has reported IL-22 to be expressed in a small cohort of lung carcinoma samples.[7] The significance of this finding for the patients remained unclear. Our current study is the first to address the frequency of expression and the prognostic value of IL-22 expression in lung cancer. Analyzing two independent patient cohorts, we found SCLC and large-cell carcinoma to predominantly express IL-22 among all subtypes analyzed. In IL-22-responsive cell lines (4 of 7), IL-22 induced STAT3-mediated proliferation but not migration or protection from chemotherapy-induced apoptosis. We found

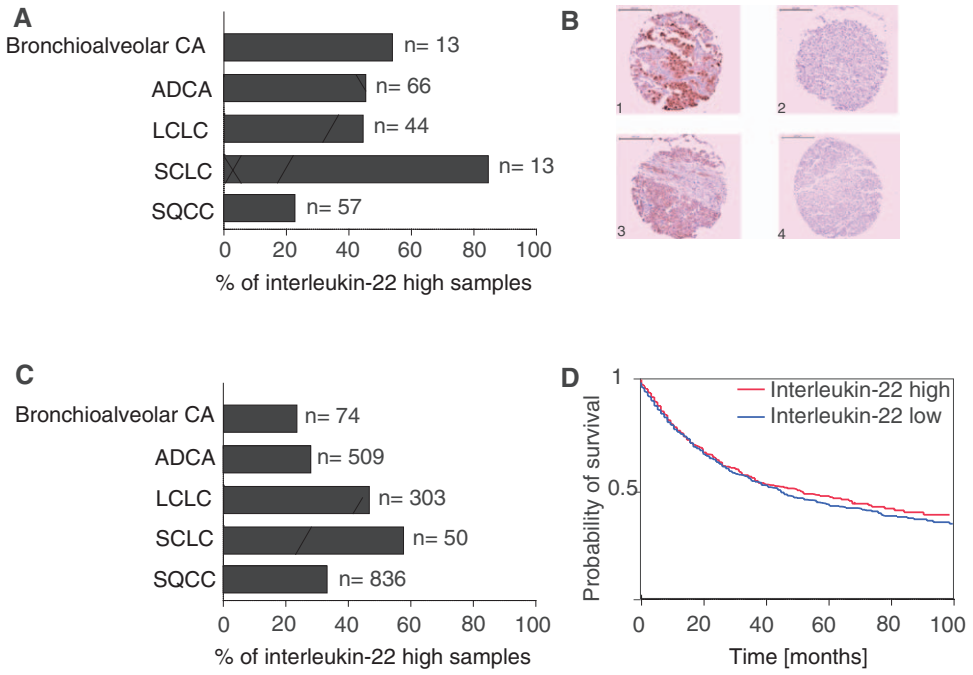

FIGURE 5. IL-22 is expressed in primary lung carcinoma tissue. *A*, IL-22 expression in the different lung cancer entities of TMA1 ($n = 215$). Tissue spots were rated either as low IL-22 expressors, or high IL-22 expressors. *B*, Exemplary staining of IL-22 high large-cell carcinoma,[1] IL-22 low large-cell carcinoma,[2] IL-22 high squamous cell carcinoma,[3] and IL-22 low squamous cell carcinoma[4] samples. *C*, IL-22 expression between the different lung cancer entities on TMA2 ($n = 1815$). Tissue spots were rated either as low IL-22 expressors, or high IL-22 expressors. *D*, Overall survival of all patients in TMA2 represented on a Kaplan–Meier survival curve ($n = 1724$). Survival is compared between the groups of IL-22 high expressors versus IL-22 low expressors. No significant difference in survival between the two groups was found. ADCA, adenocarcinoma; IL-22, interleukin-22; SCLC, small-cell lung cancer; LCLC, large-cell lung cancer; SQCC, squamous cell carcinoma; TMA, two-tissue microarray.

TABLE 3. Correlation of IL-22 Expression with the Clinical and Pathologic Characteristics of TMA2[a] (n = 1815)

Characteristics	IL-22 High (%)	IL-22 Low (%)	p
Histology			>0.0001
Adenocarcinoma	146 (29)	363 (71)	
Bronchioalveolar carcinoma	7 (54)	6 (46)	
Large-cell carcinoma	141 (47)	162 (53)	
Small-cell carcinoma	29 (58)	21 (42)	
Squamous cell carcinoma	283 (34)	553 (66)	
Sex			0.44
Male	400 (37)	693 (63)	
Female	112 (34)	215 (66)	
Age (yr)			0.49
≤60	207 (14)	385 (26)	
>60	333 (22)	574 (38)	
pT category			0.12
pT1	124 (36)	219 (64)	
pT2	355 (36)	641 (64)	
pT3	94 (36)	167 (64)	
pT4	32 (25)	94 (75)	
pN category			0.18
pN0	314 (37)	540 (63)	
pN1	136 (32)	293 (68)	
pN2	131 (34)	256 (66)	
pN3	22 (43)	29 (57)	
pM category			0.81
pM0	571 (35)	1063 (65)	
pM1	34 (36)	60 (64)	

[a]Numbers do not add up to 1815 in the different categories because of cases with lack of data.

pT, pathologically determined tumor stage; pN, pathological nodal; pM, pathological metastasis.

de novo–generated cisplatin-resistant cell lines to up-regulate IL-22-R1 and have a stronger proliferative response to IL-22 stimulation, indicating a possible role of IL-22 when a relapse after chemotherapy has occurred. However, in the large clinical cohort, IL-22 expression did not correlate with survival time in any of the histological subtypes.

Our data are in agreement with the data of Zhang et al.,[7] who have shown IL-22 expression in lung cancer samples both by immunohistochemistry and RT-PCR. Our data confirm the finding by Zhang et al. and other groups that lung cancer cells are responsive to IL-22.[3,9] Importantly, expression of IL-22-R1 has been found to be up-regulated in lung cancer samples.[21] However, because all lung cancer cell lines were found negative for IL-22 protein expression, expression of IL-22 in primary tissue may arise from nontumor cells or from the interaction between tumor and nontumor cells and stroma. The frequent expression we have found in large-cell lung correlates with the elevated serum concentrations we have found in large-cell lung cancer patients. No elevated serum concentrations were found in patients with the IL-22 low-expressing ADCA or SQCC. In contrast to the findings of Zhang et al.,[7] who suggested a chemotherapy-protective role of IL-22 through Bcl-2 up-regulation, IL-22 had no impact on chemotherapy-induced apoptosis in the current study, irrespective of the mechanism of apoptosis induction (adduct formation with DNA for carboplatin or blockade of thymidine synthesis by 5-fluorouracil), despite a slight Bcl-2 up-regulation. This discrepancy may be explained by the only modest impact on apoptosis described in the previous study.

The impact of cisplatin resistance on the expression of the IL-22-R1 may reflect a response to cell stress induced by chemotherapy. Although the exact mechanism and the regulation of this receptor remains unaddressed, up-regulation of IL-22-R1 has been reported to be induced by proinflammatory cytokines such as interferon-α and tumor necrosis factor-α.[22,23] The up-regulation of IL-22-R1 in a setting of acute stress may contribute to a rescue of these cells, if IL-22 is present.[23]

Not surprisingly, none of the analyzed tumor cell lines secreted notable levels of IL-22, congruent with previous reports.[7] This discrepancy versus the positive IL-22 expression in primary tumor tissue may be because of differences in expression between cell lines and primary tumors observed in other contexts.[24] However, because IL-22 expression has not been demonstrated in healthy epithelial cells, the source of IL-22 in the tumor environment may be the infiltrating immune cells. In fact, tumor infiltrating IL-22–positive T cells have been demonstrated in lung cancer, hepatocellular carcinoma, and gastric cancer.[25–27] Other potentially IL-22 producing cells (e.g., Th17-cells) have been detected in several cancer types, including lung cancer.[28] In addition, IL-22 can be produced by NK and myeloid cells, also found in the tumor microenvironment.[29,30]

It is surprising that despite IL-22 expression in a substantial number of the tumors, we were unable to find a correlation with prognosis, as has been suggested for IL-22 in gastric and hepatocellular carcinoma.[26,27] According to our data, there may be several explanations for this discrepancy: First, we have found lung cancer cell lines to respond heterogeneously to IL-22, which, if representative of the patients' situation, may indicate that not every cancer will respond to IL-22. Second, the impact of IL-22 on proliferation only and not on apoptosis may be insufficient to overcome the therapeutic effect of the operation performed in these patients. Third, IL-22-R1 expression may vary among tumor cells and because the receptor is a prerequisite for IL-22 reaction, IL-22 expression would need to be compared with IL-22-R1 expression, which according to our data directly impacts on IL-22 response. However, no loss or down-regulation of IL-22-R1 in cancer patients has been demonstrated so far. Most reports analyzing IL-22-R1 in lung cancer or other tumor types agree that IL-22-R1 is strongly expressed within all cancer tissues analyzed.[7,21,27] Accordingly, a receptor-based lack of effect of IL-22 may only be because of mutations in the coreceptor IL-10R2 or to downstream signaling defects, such as activating STAT3 mutations.[31,32] Because most patients will receive chemotherapy at some point during the course of their disease, it seems possible, based on our observation, that chemotherapy-resistant cells are more prone to IL-22 stimulation. In this case tumor tissue IL-22 expression prognostic at a later time

point in the disease course not covered by the tissue samples studied here. In conclusion, we found no evidence for IL-22 as a prognostic biomarker in early, potentially curable lung cancer. Further studies are needed to address the impact of IL-22 in advanced lung cancer, especially in SCLC and large-cell lung cancer.

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