Jackson ImmunoResearch LABORATORIES INC.

ANTIBODIES FOR SEROLOGY AND DIAGNOSTICS

NEW

Anti-Human IgE Antibodies

For high-specificity immunoassay design and performance



Board of Regents, The University of Texas System EX2003
Microbiotica, Limited v. Board of Regents, The University of Texas System
PGR2023-00026

DOI: 10.1002/eji.201445052 Eur. J. Immunol. 2015. 45: 922–931

European Journal of Immunology

Interleukin-17 and interleukin-22 promote tumor progression in human nonmelanoma skin cancer

Lavinia Nardinocchi[1], Giulio Sonego[2], Francesca Passarelli[3], Simona Avitabile[1], Claudia Scarponi[1], Cristina Maria Failla[1], Stefano Simoni[2], Cristina Albanesi[1] and Andrea Cavani[1]

[1] Laboratory of Experimental Immunology, Istituto Dermopatico dell'Immacolata, Rome, Italy
[2] Day Surgery Unit, Istituto Dermopatico dell'Immacolata, Rome, Italy
[3] Department of Dermatopathology, Istituto Dermopatico dell'Immacolata, Rome, Italy

Interleukin-17 (IL-17) and IL-22 have been reported to play critical roles in autoimmunity and inflammation but information about their role in cancer is limited. In this study, we investigated the role of IL-17 and IL-22 in the progression of human skin basal-cell carcinoma (BCC) and squamous-cell carcinoma (SCC). We found that both tumor types are infiltrated with an high number of IL-17$^+$ and IL-22$^+$ T lymphocytes, as demonstrated by immunohistochemistry and by FACS analysis performed on peritumoral T-cell lines isolated from skin biopsies. In vitro studies demonstrated that proliferation and migration of the BCC- and SCC-cell lines M77015 and CAL27 were increased by IL-17 and IL-22. Moreover, IL-17, alone or in combination with TNF-α, was able to induce the production of two cytokines important for tumor progression, IL-6 and IL-8, in CAL27. We also showed that IL-17 upregulated NF-κB signaling, while IL-22 activated the STAT3 pathway and the antiapoptotic AKT protein in M77015 and CAL27. Finally, in vivo experiments demonstrated that IL-17 and IL-22 enhanced tumor growth in nude mice injected with CAL27. Altogether, our findings indicate that high levels of IL-22 and IL-17 in the BCC and SCC microenvironment promote tumor progression.

Keywords: cancer · dermatology · IL-17 · IL-22 · T cells


See accompanying Commentary by McAllister and Kolls.


Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Nonmelanoma skin cancer (NMSC) represents one of the most common malignancies in humans and keratinocyte-derived basal-cell carcinomas (BCCs) and squamous-cell carcinomas (SCCs) are responsible approximately for the 80 and 20% NMSC cases, respectively [1, 2]. Both types of NMSCs share important risk factors, such as fair skin and high degree of sun exposure [3]. Nevertheless, BCCs and SCCs have distinct metastatic attitude, being rare in BCC and much more common in SCC [4]. Tumor-infiltrating lymphocytes (TILs) are frequently found in malignant tumors, being suggestive of a host antitumoral immune response. Several independent studies have demonstrated that the presence of TILs correlated with increased survival and represents a predictive as well as prognostic biomarker in patients with breast cancer, colorectal cancer, glioma, and gastric cancer [5–7]. However, dissimilar data revealed that TILs functionally promoted epithelial carcinogenesis [8], and that TIL-secreted chemokines contributed to tumor angiogenesis and growth [9]. Although few studies have described the inflammatory infiltrate of BCCs and SCCs [10], the role of infiltrating T lymphocytes and cytokines secreted in BCC and SCC microenvironment in cancer progression has not yet been investigated. ILs carry out important functions

Correspondence: Dr. Andrea Cavani
e-mail: cavani@idi.it

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

in cell differentiation and inflammation [11–13]. IL-17, produced by activated memory T lymphocytes, in particular Th17 cells, regulates both innate immunity and host defense by inducing the expression of nuclear factor-kappa B (NF-κB); chemokines CXCL8, CXCL6, and CXCL1; growth factors such as G-CSF and GM-CSF; IL-6; and adhesion molecules (ICAM-1) leading to augmented neutrophil accumulation, granulopoeisis, and inflammatory responses [14]. Although important information about the role of IL-17 and Th17 cells in inflammation have accumulated, their involvement in tumor progression remains undefined [15, 16], and whether Th17 cells suppress or promote tumor progression is still a matter of debate [17]. In particular, the presence and the function of IL-17 in BCC and SCC microenvironment have not yet been studied. IL-22 belongs to the IL-10 cytokine family and plays several roles in host defense at mucosal surfaces as well as in tissue repair [18, 19]. IL-22 is a unique cytokine produced by immune cells, including Th-cell subsets and innate lymphocytes, that acts only on nonhematopoietic stromal cells, in particular epithelial cells, keratinocytes, and hepatocytes [20–22]. IL-22 mediates cellular inflammatory responses by activating intracellular kinases – including JAK1, Tyk2, and MAP kinases – and transcription factors, such as signal transducer and activator of transcription (STAT3) [23, 24]. Furthermore, IL-22 exhibits antiapoptotic and tumorigenic functions. Recent data show that its overexpression protects lung cancer cell lines from apoptosis via activation of STAT3 and its downstream antiapoptotic proteins Bcl-2 and Bcl-xL and inactivation of MAP kinases [25]. Moreover, it has been demonstrated that augmented IL-22 in colon cancer and ulcerative colitis microenvironment leads to tumor growth, inhibition of apoptosis, and promotion of metastasis by STAT3 activation [26]. Furthermore IL-22 activates the phosphorylation of STAT3 in oral SCC cell lines, leading to the upregulation of the antiapoptotic and mitogenic genes [27]. In this context, the aim of our study was to investigate the role of IL-17 and IL-22 in the growth and invasiveness of NMSC.

Results

IL-17$^+$ and IL-22$^+$ cells are present in the BCC and SCC inflammatory infiltrate

To investigate the distribution and localization of IL-17$^+$ and IL-22$^+$ cells in the inflammatory infiltrate, specimens from ten cases of BCC and ten cases of SCC were analyzed by immunohistochemistry (IHC). As shown in Figure 1, numerous IL-17$^+$ cells were detected in the peritumoral area of BCC (A) and SCC (C), paralleled by the high number of IL-22$^+$ cells (Fig. 1B and D). The inflammatory infiltrate was then isolated from ten BCC and six SCC biopsies and the resulting cells immediately analyzed by FACS. TILs from both BCC and SCC were enriched in CD4$^+$ and deprived in CD8$^+$ cells (Fig. 1E and F) when compared to PBMCs isolated from the same patients. Additionally, the average percentage of TILs positive for IL-17 (14.7% ± 6.1), IL-22 (8.7% ± 0.5), and TNF-α (70.5% ± 13.1) was significantly higher compared to circulating T cells. In the tumor infiltrate of BCCs and SCCs, the percentage of NK cells (CD3$^-$/CD56$^+$) was 9.2% ± 1.2. A minor and variable percentage of infiltrating NK cells release IL-17, but not IL-22 (data not shown). In contrast, average number of IFN-γ$^+$ cells (18.3% ± 7.1) infiltrating SCCs, but not of those infiltrating BCCs, was lower compared to circulating T cells (Fig. 1G–J). In Supporting Information Figure 1, one representative case of surface Ag staining (CD4 and CD8) and cytokine production (IL-17, IL-22, TNF-α, and IFN-γ) of BCCs, SCCs, and PBMCs is shown.

IL-17 and IL-22 directly promote proliferation and migration of BCC- and SCC-cell lines

To determine whether Th17 or Th22 cells and their product IL-17 or IL-22 could directly affect tumor cells, in vitro experiments were performed with the SCC-cell line CAL27 and the BCC-cell line M77015, both expressing the IL-17 and IL-22 receptors (data not shown). As shown in Figure 2, recombinant IL-17 or IL-22 significantly enhanced BCC (Fig. 2A) and SCC (Fig. 2B) proliferation, as compared to untreated cells. Conversely, normal human keratinocytes showed a limited but significant proliferation response to IL-22, but no response to IL-17 (Fig. 2C). No effect on cell proliferation was present in the SCC12 SCC-cell line that did not express IL-17 or IL-22 receptor (data not shown).

Accordingly, the supernatant of activated TIL-derived T-cell clones producing either IL-17 or IL-22 (Supplementary Table 1), but not that of the Th1-cell clone 6.1, was able to induce proliferation of both M77015 (Fig. 2D and E) and CAL27 cells (Fig. 2F and G). To demonstrate that tumor-cell growth induced by supernatant of activated TIL-derived cell clones was dependent on IL-17 and IL-22, we performed blocking experiments with anti-IL-17 and anti-IL-22 Abs. As shown in Figure 2, results clearly indicate the capacity of both IL-17 and IL-22 blocking antibodies to reduce M77015 and CAL27 proliferation induced by supernatant of TIL-derived cell clones (Fig. 2D–G). Furthermore, IL-17 and IL-22 were able to induce CAL27- and M7715-cell migration in an in vitro scratch assay (Fig. 3), indicating that the cytokines promote local invasiveness of tumor cells.

IL-6 and IL-8 are induced in SCC cells by IL-17 treatment

The next step was to investigate whether IL-17 and IL-22 alone or in combination with other Th17- and/or Th22-derived cytokines could induce the secretion of cytokines and/or chemokines, known to influence tumor survival and progression, in tumor cells. Indeed, our previous data showed that in normal human keratinocytes [28, 29], IL-17, alone or in combination with TNF-α, induced the secretion of IL-8. To verify whether IL-17 had similar effects on BCC- and SCC-cell lines, we exposed CAL27 and M77015 cells to recombinant human IL-17, IL-22, with or without TNF-α, and cytokine release was investigated by ELISA. In particular, we decided to analyze IL-6 and IL-8 release, as it is known that both

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

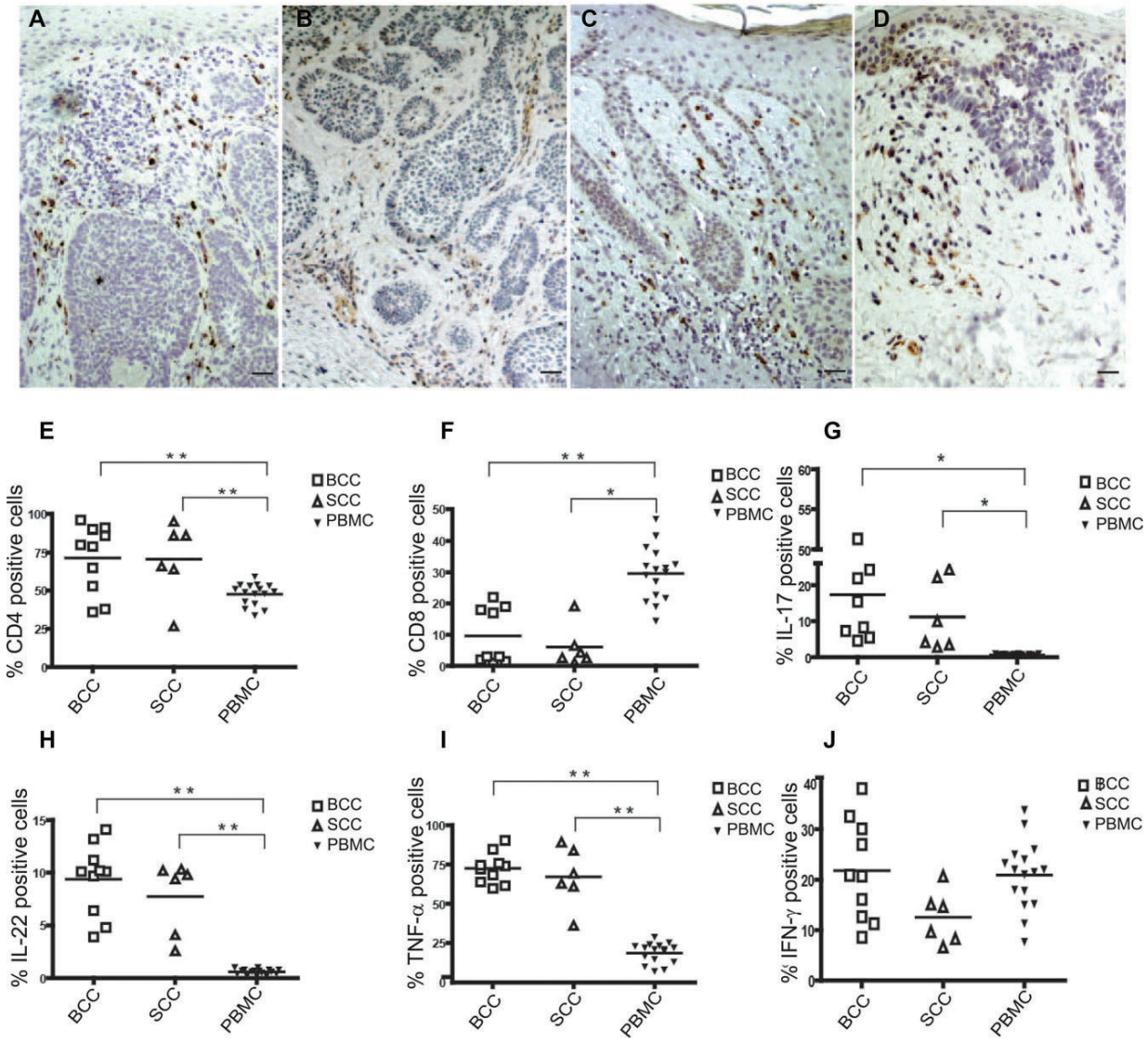


Figure 1. IL-17 and IL-22 are highly expressed in peritumoral infiltrate of human BCCs and SCCs. (A–D) Four micrometer histological sections were stained with mAb anti-IL-17 or anti IL-22. (A and C) IL-17 staining (red) in BCC (A) and SCC (C). (B and D) IL-22 staining (red) in BCC (B) and SCC (D), original magnification 100×, scale bars 35 μm. Representative images are shown: 10 BCC or SCC patients were investigated. (E–J) T-lymphocytes isolated from BCCs and SCCs; and PBMCs from the same patients were analyzed for (E) CD4 and (F) CD8 expression; and (G–J) expression of (G) IL-17, (H) IL-22, (I) TNF-α, and (J) IFN-γ by flow cytometry. Symbols represent individual patients, vertical bars show the mean. Ten patients with BCC and six patients with SCC were investigated. $^{*}p < 0.05$; $^{**}p < 0.01$, Student's *t*-test.

cytokines induce proliferation and invasion of many tumor cells [30–32]. As shown in Figure 4, IL-17, but not IL-22 alone, augmented both IL-6 (Fig. 4A) and IL-8 (Fig. 4B) secretion by CAL27 cells. Moreover, IL-17 showed a slightly additive effect on TNF-α treatment. In contrast, basal levels of IL-6 and IL-8 in M77015 were below detection levels. Secretion was increased by TNF-α treatment, but not by IL-17 or IL-22 (data not shown). These data indicate that IL-17 has a role in SCC progression not only directly by inducing tumor-cell proliferation and migration, but also indirectly by promoting the production of cytokines such as IL-6 and IL-8.

BCC- and SCC-cell lines upregulate ERK1/2 and STAT3/NFκB pathway in response to IL-22 and IL-17

In order to evaluate BCC- and SCC-cell responsiveness to IL-22 and IL-17 in terms of activation of known intracellular molecular cascades active in healthy keratinocytes, we analyzed IL-22 and IL-17 molecular signaling in time-course experiments. STAT3 was phosphorylated in normal human keratinocytes after 5 and 15 min of IL-22 treatment (2.5 ± 0.18-fold induction over time 0) and then returned to basal levels (Fig. 5A) [24]. Differently, STAT3 was constitutively activated in M77015 and CAL27 cells (Fig. 5A).

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

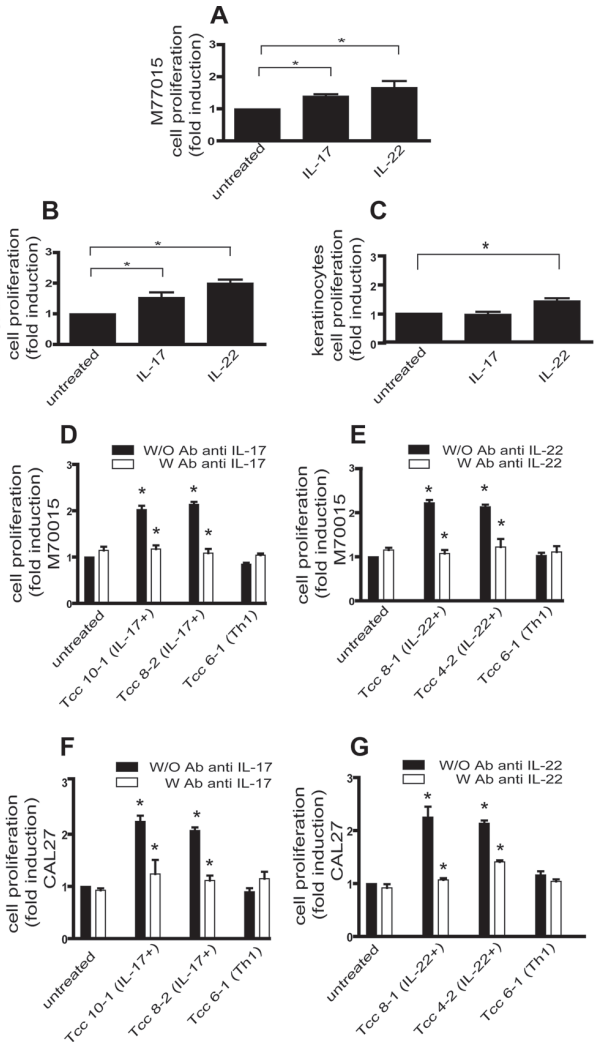


Figure 2. IL-17, IL-22, Th17, and Th22 TILs promote proliferation of a BCC and SCC cell line. (A–C) M77015 cells (A), CAL27 cells (B), and healthy human keratinocytes (C) were treated with 50 μM of IL-17 or IL-22. (D–G) M77015 cells (D and E) and CAL27 cells (F and G) were treated with the supernatant of TIL-derived T-cell clones from two BCC biopsies (9-1, 10-1, 11-1, 4-2, 8-2) in presence of 10 μg/mL IL-17 and 10 μg/mL IL-22 blocking antibodies. Proliferation was evaluated by crystal violet incorporation. Data are shown as mean densitometric values + SEM of two samples pooled from three experiments performed, and expressed as fold increase over the values of proliferation in untreated cells assumed as 1. $^*p < 0.05$, Student's t-test.

Upon IL-22 treatment, M77015 cells showed STAT3 phosphorylation levels similar to those of normal human keratinocytes only after 1 h of treatment (2.6 ± 0.21-fold induction over time 0), but residual phosphorylated STAT3 was still present after 5 h. On the other hand, in CAL27 cells treated with IL-22, STAT3 showed different phosphorylation kinetics than in M77015 cells and normal human keratinocytes, with a peak of activation already at 15 min (2.9 ± 0.17-fold induction), maintained up to 5 h. We previously demonstrated [24] that IL-22 induces ERK1/2 phosphorylation in normal keratinocytes with the first peak of activation at 5 min followed by a slight reduction and by a second peak of activation at 1 h that lasted up to 6 h (Fig. 5A). IL-22-treated M77015 cells showed kinetics of ERK1/2 activation similar to those of normal cells, although the levels of phosphorylated ERK1/2 were higher, especially at the second peak of activation (2.1 ± 0.11- vs. 1.7 ± 0.22-fold induction). Surprisingly, CAL27 cells showed a substantial decrease of ERK1/2 phosphorylation after IL-22 treatment (Fig. 5A).

Since IL-22 can exert antiapoptotic effects on cancer cells [25] and it is able to induce AKT phosphorylation in human keratinocytes and fibroblasts such as synoviocytes [33], we tested whether BCC and SCC cells showed peculiar AKT activation. Indeed, we found that while CAL27 and normal keratinocytes had similar levels of phosphorylated AKT in response to IL-22, M77015 expressed very low level of basal AKT and IL-22 slightly induced its phosphorylation (Fig. 5B).

IL-17 promotes NF-κB activation in different tumor-cell lines [34]. P65 subunit of NF-κB, when phosphorylated in Ser536 and released from the IκB inhibitory complex, induces the transcription of target genes. We observed that phosphorylated p65 was constitutively present in both M77015 and CAL27 cells and rapidly upregulated after IL-17 treatment. Phosphorylated p65 amounts and induction kinetics were similar in normal keratinocytes and M77015 cells, whereas CAL27 cells showed a higher and earlier NF-κB activation, with a peak of induction at 5 min (Fig. 5C). As expected, IkB-α degradation paralleled p65 phosphorylation in both keratinocyte and tumor cells.

IL-17 and IL-22 promote tumor growth in vivo

To evaluate whether IL-17 and IL-22 could have an effect on tumor growth in vivo, we generated tumor xenografts in athymic nude mice by injecting CAL27 cells subcutaneously in the animal back. Experiments were conducted only with CAL27 cells as M77015 cells were not tumorigenic in vivo. Mice treated with IL-17 or IL-22 over the course of 2 weeks displayed increased tumor volumes compared with those treated with vehicle alone, reaching a volume of 1.1 ± 0.15 cm^3 for IL-17 group and 1.2 ± 0.19 cm^3 for IL-22 group compared to a volume of 0.75 ± 0.10 cm^3 in the control group after 14 days (Fig. 6A). At day 14, mice were sacrificed and tumors harvested and processed for histological and IHC analysis. Interestingly, H&E staining did not show significant differences among the three conditions (Fig. 6B). It is known that IL-17 could induce massive myeloid-cell infiltration. To investigate the contribution of myeloid-cell infiltration to the augmented tumor size observed after IL-17 and IL-22 injection in nude mice, tumor specimens were stained with anti-CD11c. Results indicate the increased infiltration of $CD11c^+$ cells in the peritumoral area of IL-17 injected mice, but not in tumoral area (Fig. 6B).

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

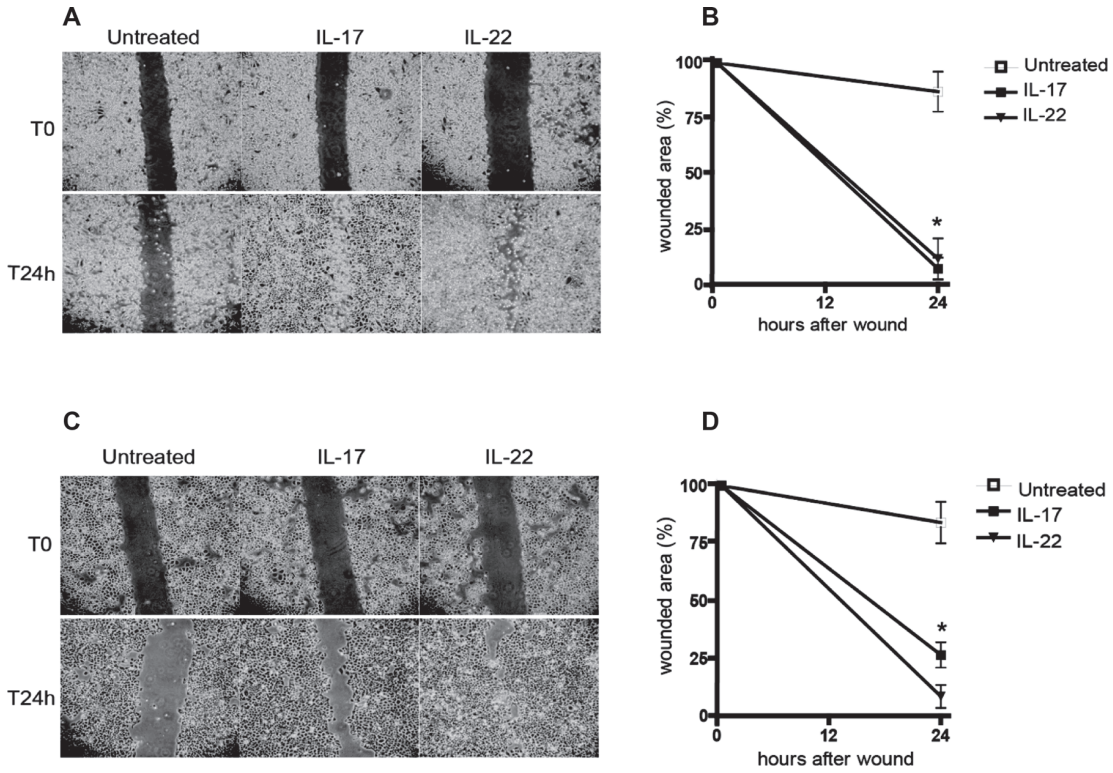


Figure 3. IL-17 and IL-22 induce BCC- and SCC-cell migration. (A–D) Confluent cell layers of (A, B) M77015 and (C, D) CAL27 were scratched and afterward treated with 50 ng/mL IL-17 and IL-22 for 24 h and microscopy pictures were taken 24 h after the scratch with a digital camera. (A, C) Representative microscopy images are shown (magnification 200×). (B, D) The residual gap between migrating cells was determined by a computer-assisted image analysis system (Axiovision, Zeiss) and expressed as percentage of initial scratched area. Data are shown as mean ± SEM of single replicates pooled from at least three experiments . *$p < 0.05$, Student's *t*-test.

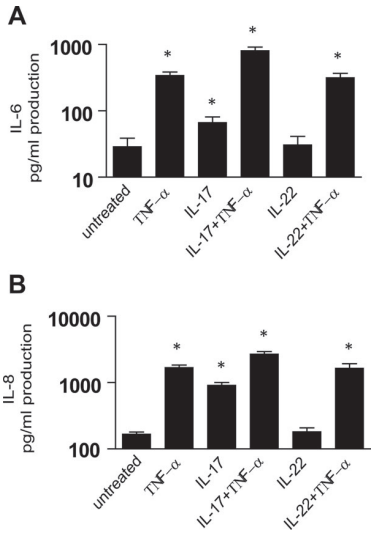


Figure 4. IL-17 enhances the release of IL-6 and IL-8 from CAL27 cells. CAL27 cells were treated with 50 ng/mL TNF-α and 50 ng/mL IL-17 or IL-22, alone or in combination. After 48 h, (A) IL-6 and (B) IL-8 levels in the supernatants were measured by ELISA. Data are shown as mean ± SEM of duplicate samples from three experiments.*$p < 0.05$, Student's *t*-test.

Discussion

Tumorigenesis is a multistage process initiated by mutations that activate oncogenes or inhibit tumor suppressor genes [11]. However, neoplastic cells often require additional factors from the microenvironment to support their survival, favor their growth, and induce metastases to distant organs. In particular the microenvironment can exert inhibitory effects on malignant cells, but during their progression, tumors circumvent these inhibitory signals and instead use these surrounding cells to their own ends, causing the inappropriate growth, invasion, and, ultimately, metastasis [35].

Clinical data and experimental mouse models have provided a definitive link between inflammation and cancer [36, 37], and cytokines are important mediators of communication between tumor cells and the inflammatory microenvironment. Although in different types of cancer the role of the TIL and its relationship to prognosis has been extensively studied, in NMSC the functions of particular T-lymphocyte subsets have not received much attention.

The present study investigated the role of two inflammatory cytokines IL-17 and IL-22 in human skin BCCs and SCCs. Our data demonstrate that IL-22$^+$ and IL-17$^+$ T cells are enriched in the

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

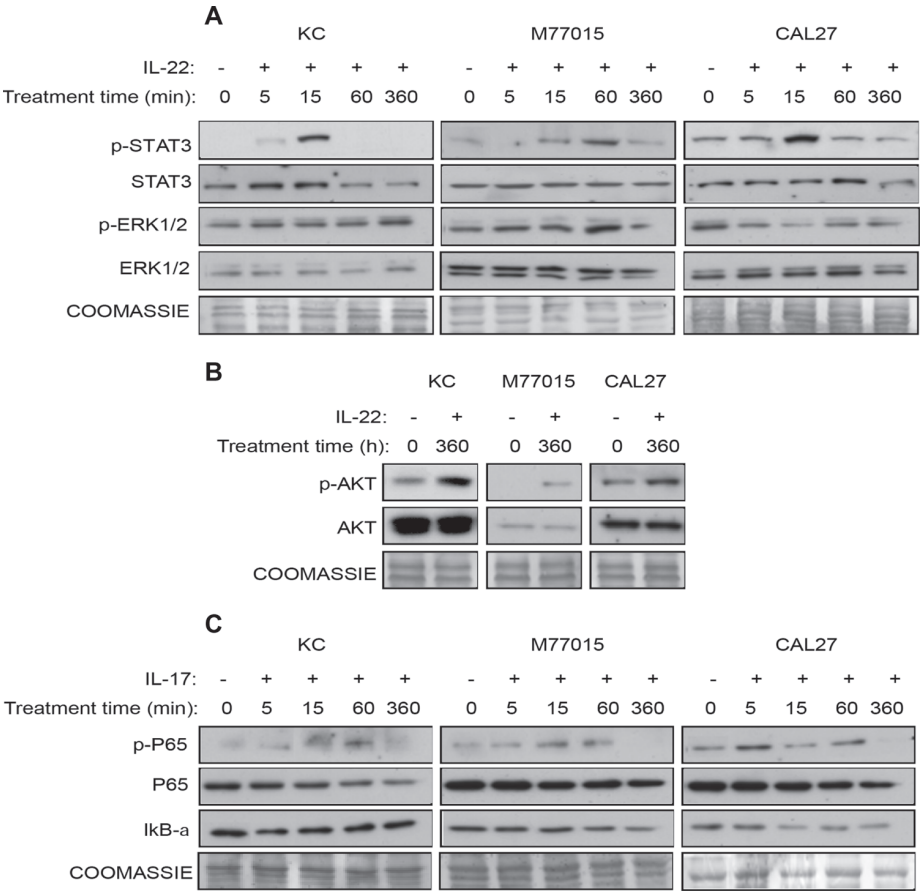


Figure 5. IL-17- and IL-22-dependent molecular signals in BCC- and SCC-cell lines. (A) Healthy human keratinocytes (KC) and M77015 and CAL27 cells were treated with 50 ng IL-22 for 5, 15, 60, and 360 min. STAT3 phosphorylation in Y705 (p-STAT3), total STAT3, phosphorylated ERK1/2 (p-ERK1/2), and total ERK was detected by Western blot. Coomassie staining was used as loading control. (B) Healthy human keratinocytes (KC) and M77015 and CAL27 cells were treated with 50 ng IL-22 for 360 min. AKT phosphorylation in S473 (p-AKT) and total AKT were determined by Western blot. Coomassie staining was used as loading control. (C) Healthy keratinocytes (KC) and M77015 and CAL27cells were treated with 50 ng IL-17 for 5, 15, 60, and 360 min. P65 phosphorylation in S536 (p-P65), total P65, and IκBα were determined by Western blot. Coomassie staining was used as loading control. Representative images of three independent experiments are shown. Densitometric analysis is reported within the text.

tumor microenvironment of BCCs and SCCs. Our result is consistent with studies that indicate the elevated IL-17 and IL-22 expression in several human tumors, such as ovarian cancer, prostate cancer, breast cancer, hepatocellular carcinoma, esophageal cancer, and gastric cancer [26, 38–48].

Further, IL-17 and IL-22 promote tumor-cell proliferation and migration in vitro, and tumor-cell growth in vivo. In SCCs, this effect is partly mediated by the release of IL-8 and IL-6 induced by IL-17 alone or in combination with TNF-α. These data may have important consequence on tumor progression as previously reported for different tumor types [30, 32, 38].

Our data also showed that in SCC and BCC inflammatory infiltrates, the percentage of IFN-γ^+ T lymphocytes and $CD8^+$ TILs is lower compared with the respective PBMCs. This finding supports the notion that in tumor microenvironment the Th1- and CD8-mediated cytotoxic responses are inhibited.

Recent evidence has shown that the activity of most of the inflammatory cytokines converges on NF-κB and STAT3 [34, 37]. STAT3 is constitutively phosphorylated in SCCs and, to a minor extent, in BCC-cell lines, and it is strongly upregulated starting from 15 min after IL-22 exposure. In contrast, in normal keratinocytes STAT3 phosphorilation is not constitutive, but rapidly induced by IL-22 treatment [24]. The constitutive activation of STAT3 in CAL27 and M77015 may reflect the high proliferation rate and migration capability of cancer cells compared with healthy keratinocytes. In addition, the cell line CAL27 showed a unique response to IL-22, which downregulates p-ERK1/2 in the SCC line, in contrast with healthy keratinocytes and the BCC line M77015. This result indicates that in SCC-cell line the only mediator of IL-22 signaling is STAT3.

As demonstrated in other cancer models, IL-22 can have also antiapoptotic effects [25]. Here we demonstrated that in CAL27 p-Akt is upregulated after 6 h upon IL-22 treatment. Thus, exposure to IL-22 stimulates CAL27 proliferation and contrasts tumor-cell apoptosis, while M77015 lacked basal AKT activation and IL-22 only slightly induced its phosphorylation. These data

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

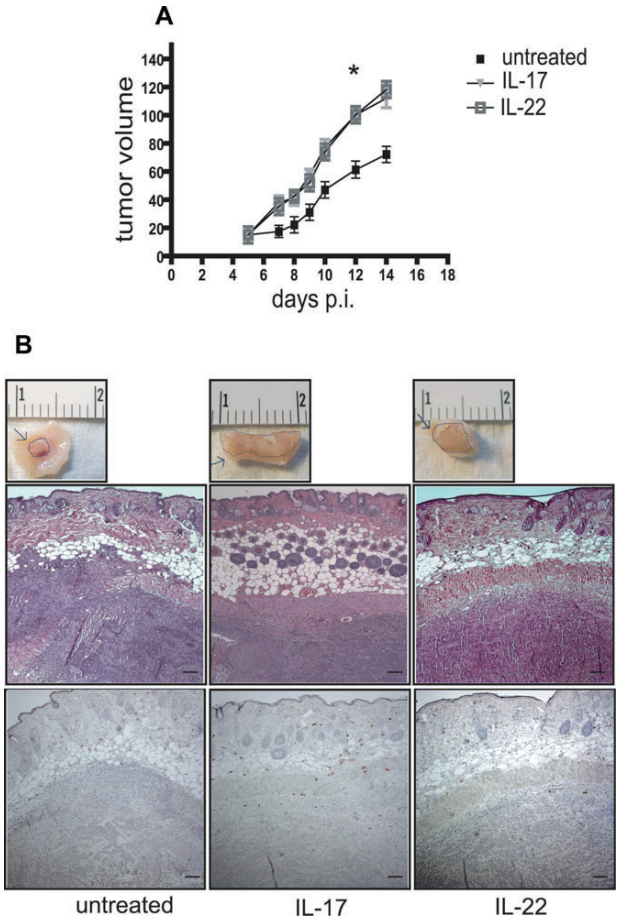


Figure 6. IL-17 and IL-22 support tumor growth in vivo. (A) CAL27-cell xenografts were established in nude mice. Tumors were treated with IL-17 or IL-22 (0.5 μg for each mouse) at day 5 and then daily for additional 8 days. Tumor volumes were measured every day. *Y*-axis, tumor volume; *X*-axis, calibration time after CAL27 injection (p.i.= post-injection). Data are reported as average tumor volume ± SEM of the five animals of a single experiment, representative of three experiments. $^{*}p < 0.05$, Student's *t*-test. (B) Representative images of xenografts of each group of untreated or IL-17- or IL-22-treated mice are shown (top). Corresponding H&E staining (middle). Staining with mAb against CD11c (red staining, bottom). Original magnification 50×. Scale bars: 100 μm.

indicate that the SCC-cell line is more responsive to antiapoptotic stimuli, also because the CAL27-cell line has a mutation in p53 gene that confers an intrinsic resistance to apoptosis by cancer cells.

A number of reports have indicated that the activation of NF-κB is a major mechanism by which IL-17 regulates target gene expression, especially in different tumor-cell lines [34]. Accordingly, our data demonstrated that NF-κB/p65 phosphorylation is induced within 15 min after IL-17 treatment as in SCC and BCC tumor cell lines, as well as in healthy keratinocytes. NF-κB/p65 phosphorylation was paralleled by the down modulation of IκBα, a negative regulator of NF-κB in both tumor-cell lines.

Finally, in vivo experiments confirmed the role of IL-17 and IL-22 in tumor growth. Both IL-17 and IL-22 administration at the site of CAL27-cell xenografts in athymic nude mice progressively increased the tumor size as compared to PBS-treated mice. These data are coherent with previous reports demonstrating the slower tumor progression in IL-17$^{-/-}$ or IL-17 receptor$^{-/-}$ mice [16].

Altogether, our study demonstrates a previously unknown protumorigenic role of IL-22 and IL-17 in human SCCs and BCCs and indicates that treatment with anti-IL-22 or anti-IL-17 antibodies may be a valuable therapeutic approach in selected cases. Additionally, our data encourage the administration of anti-IL-17 or anti-IL-22 biological drugs in patients affected by psoriasis and other autoimmune diseases at risk for the development of skin cancers.

Material and methods

Tumor samples

Tumor biopsies were obtained from 20 patients with BCC and 16 patients with SCC. Patients were enrolled in the study after written informed consent. The study was performed according to the Declaration of Helsinki with regard to scientific use, and approved by the ethical committee of Istituto Dermopatico dell'Immacolata, IDI-IRCCS, Rome, Italy.

Cell isolation from skin biopsies

Fresh tumor tissues were washed in RPMI 1640 (Lonza, Basel, Switzerland). Fatty, connective, and necrotic tissue parts were removed. Tissues were minced into 1–2 mm pieces with a scalpel, and placed in culture in complete RPMI 1640 with 5% human serum (complete medium) and 60 U/mL recombinant human (rh) IL-2 (Novartis, Varese, Italy). After 4–7 days, cells emigrated from tissue samples were collected and placed in starvation with minimal IL-2 supply before phenotypic characterization, functional assays, and T-cell cloning by limiting dilution (0.6 cells/well) in the presence of irradiated allogeneic feeder cells plus 1% Phytohemaglutinin-M (Roche, Mannheim, Germany)

Cell lines and culture condition

PBMCs were isolated from healthy volunteers by Ficoll-Paque PLUS (Lonza) density-gradient centrifugation. M77015-08- and SCC12-cell lines from the American Tissue Culture Collection and CAL27-cell line – kindly provided by Cedric Gaggioli, INSERM U634, Nice, France – were grown in DMEM (Biocrhom AG, Cambridge, UK), 10% Fetalclone II serum (HyClone Laboratories). Normal human keratinocytes were obtained from skin biopsies of healthy volunteers, as previously described [28], cultured in serum-free medium, keratinocyte growth medium (KGM, Clonetics, Walkersville, MD), and used at 60–80% confluence.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Flow cytometric analysis

For intracytoplasmic detection of cytokines, lymphocytes were incubated with PMA (10 ng/mL), ionomycine (1 μg/mL), and monensin (BD Biosciences, Milan, Italy) for 6 h at 37°C and 5% CO_2. After 2-h incubation, brefeldin (BD Biosciences) was also added for the last 4 h. Cells were collected, fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained for 20 min with the anticytokine mAb in the presence of Perm/Wash solution (BD Biosciences). Acquisition and analysis were done using a FACSAria equipped with Diva software (BD Biosciences). The mAbs used for intracytoplasmic detection of cytokines and surface Ag were as follows: TNF-α-FITC, IFN-γ-PB, CD4-FITC, CD25-PE, CD56-allophycocyanin, CD8-PB, CD3-allophycocyaninCy7 (BD Biosciences); IL-17-PE and IL-17RA-PE (EBiosciences, Frankfurt, Germany); IL-22-allophycocyanin and IL-22Ra1-allophycocyanin (R&D Systems, Abingdon, UK).

IHC and H&E staining

Tumor tissues were harvested and fixed with 4% paraformaldehyde. Paraffin-embedded 5-μm sections were dewaxed and rehydrated. After quenching endogenous peroxidase, achieving Ag retrieval, and blocking nonspecific binding sites, sections were incubated with primary mAbs for 1 h at room temperature. Staining kits were purchased from ScyTek (ScyTek Laboratories, West Logan, UT). Single staining was developed using 3-amino-9-ethylcarbazole (DAKO, Glostrum, Denmark) followed by counterstaining with hematoxylin. The mAbs used for the study were the IL-17 (R&D Systems) and IL-22 (Novus Biologicals, Cambridge, UK), CD11c (Novus Biologicals).

Western blotting

Protein extracts were prepared by solubilizing cells in RIPA buffer containing a mixture of protease and phosphatase inhibitors (Roche, Mannheim, Germany). Proteins were resolved on a 10% SDS-PAGE, transferred to PVDF filters, and probed with primary mAbs. Filters were developed using the ECL-plus detection system (Amersham Biosciences, Buckinghamshire, UK) or the SuperSignal West Femto kit (Pierce, Rockford, IL, USA). Equal loading of proteins was assessed by performing the Coomassie staining of the filters. The mAbs used for the study were as follows: phospho-AKT (Ser473) and phospho-p65 (Ser536; Cell Signaling, Danvers, MA, USA); AKT1/2/3, IkBα, p65, p-ERK1/2, and ERK1 (Santa Cruz Biotechnology, CA, USA); phospho-STAT3 (Tyr 705, Cell Signaling); STAT3 and (Santa Cruz).

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected, filtered, and measured for their content of IFN-γ, IL-17, IL-22, TNF-α, and IL-6 by using ELISA duoset kit from R&D systems; CXCL8 was measured with OptEIA kits (BD PharMingen), as per the manufacturer's protocol.

Migration assay

Normal human keratinocytes and CAL27 and M77015 cells at 100% of confluence were starved overnight and then scratched with the tip of a p-200 pipette to create a uniform cell-free zone in each well. Cellular debris were removed by PBS washing, and wounded monolayers were incubated or not with 50 ng/mL IL-22 or 50 ng/mL IL-17 (R&D Systems). Microscopy pictures were taken at different time-points after treatment with a digital camera. The residual gap between migrating cells was measured with a computer-assisted image analysis system (Axiovision; Zeiss, Germany), and expressed as percentage of the initial scratched area.

Crystal violet assay

A total of 1×10^4 cells were seeded in 96-well plates in triplicate for each condition. The day after, cells were starved for 24 hours and then stimulated or not with 50 ng/mL IL-22 or 50 ng/mL IL-17 (R&D Systems), in presence or not of 10 μg/mL IL-17 and 10 μg/mL IL-22 blocking antibodies (R&D Systems). After 24–48 h of treatment, cells were stained with 0.5% crystal violet (145 mmol/L NaCl, 0.5% formal saline, 50% ethanol) for 40 min. Crystal violet was eluted from cells with 33% acetic acid and absorption of the supernatant from each sample was measured at 540 nm at an ELISA reader (model 3550 UV ELISA reader, Bio-Rad, Hercules, CA, USA).

Tumor growth in nude mice

Six-week-old male CD-1 nude (*nu/nu*) mice (Charles River Laboratories, Calco, Italy) were injected subcutaneously with 3×10^6 CAL27 cells and tumors were allowed to grow for 1 week. Mice were then randomized in three groups (five mice for each group) and treated with 0.5 μg IL-17 or IL-22 or 1× PBS once a day for 8 days injecting the compounds on the tumor boundaries. Tumor dimensions were measured every day and their volumes were calculated from caliper measurements of two orthogonal diameters (x and y, larger and smaller diameters, respectively) by using the formula volume $= xy^2/2$. The experiment was repeated twice with similar results. All mouse procedures were carried out in accordance with institutional standard guidelines. The experimental design has been authorized by the Italian Health Minister (number 226/13, investigator involved Cristina Maria Failla).

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Statistical analysis

Statistical analysis was done using a two-tailed Student´s *t*-test. Statistically significant differences were defined as $p < 0.05$ or $p < 0.001$.

Acknowledgments: This work was supported by grants from the Italian Minister of Health.

Conflict of interest: Andrea Cavani has served as a paid speaker for Novartis and Abbott. Simona Avitabile received a grant from Abbott.

References

1 **Ridky, T. W.**, Nonmelanoma skin cancer. *J. Am. Acad. Dermatol.* 2007. **57**: 484–501.

2 **Glogau, R. G.**, The risk of progression to invasive disease. *J. Am. Acad. Dermatol.* 2000. **42**: 23–24.

3 **Armstrong, B. K. and Kricker, A.**, The epidemiology of UV induced skin cancer. *J. Photochem. Photobiol. B* 2001. **63**: 8–18.

4 **Kim, R. H. and Armstrong, A. W.**, Nonmelanoma skin cancer. *Dermatol. Clin.* 2012. **30**: 125–39, ix.

5 **Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M. et al.**, Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006. **313**: 1960–1964.

6 **Leffers, N., Gooden, M. J., de Jong, R. A., Hoogeboom, B. N., ten Hoor, K. A., Hollema, H., Boezen, H. M. et al.**, Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. *Cancer Immunol. Immunother.* 2009. **58**: 449–459.

7 **Galon, J., Pages, F., Marincola, F. M., Thurin, M., Trinchieri, G., Fox, B. A., Gajewski, T. F. et al.**, The immune score as a new possible approach for the classification of cancer. *J. Transl. Med.* 2012. **10**: 1.

8 **Daniel, D., Meyer-Morse, N., Bergsland, E. K., Dehne, K., Coussens, L. M. and Hanahan, D.**, Immune enhancement of skin carcinogenesis by $CD4^+$ T cells. *J. Exp. Med.* 2003. **197**: 1017–1028.

9 **Numasaki, M., Fukushi, J., Ono, M., Narula, S. K., Zavodny, P. J., Kudo, T., Robbins, P. D. et al.**, Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003. **101**: 2620–2627.

10 **Moussai, D., Mitsui, H., Pettersen, J. S., Pierson, K. C., Shah, K. R., Suarez-Farinas, M., Cardinale, I. R. et al.**, The human cutaneous squamous cell carcinoma microenvironment is characterized by increased lymphatic density and enhanced expression of macrophage-derived VEGF-C. *J. Invest. Dermatol.* 2011. **131**: 229–236.

11 **Vendramini-Costa, D. B. and Carvalho, J. E.**, Molecular link mechanisms between inflammation and cancer. *Curr. Pharm. Des.* 2012. **18**: 3831–3852.

12 **Kolls, J. K. and Linden, A.**, Interleukin-17 family members and inflammation. *Immunity* 2004. **21**: 467–476.

13 **Ouyang, W., Kolls, J. K. and Zheng, Y.**, The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 2008. **28**: 454–467.

14 **Spolski, R. and Leonard, W. J.**, Cytokine mediators of Th17 function. *Eur. J. Immunol.* 2009. **39**: 658–661.

15 **Martin-Orozco, N. and Dong, C.**, The IL-17/IL-23 axis of inflammation in cancer: friend or foe? *Curr. Opin. Investig. Drugs* 2009. **10**: 543–549.

16 **Wilke, C. M., Kryczek, I., Wei, S., Zhao, E., Wu, K., Wang, G. and Zou, W.**, Th17 cells in cancer: help or hindrance? *Carcinogenesis* 2011. **32**: 643–649.

17 **Maniati, E., Soper, R. and Hagemann, T.**, Up for Mischief? IL-17/Th17 in the tumour microenvironment. *Oncogene* 2010. **29**: 5653–5662.

18 **Zenewicz, L. A. and Flavell, R. A.**, Recent advances in IL-22 biology. *Int. Immunol.* 2011. **23**: 159–163.

19 **Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H. A. et al.**, STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J. Exp. Med.* 2009. **206**: 1465–1472.

20 **Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F. et al.**, Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J. Clin. Invest.* 2009. **119**: 3573–3585.

21 **Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. and Sallusto, F.**, Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat. Immunol.* 2009. **10**: 857–863.

22 **Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M., Murphy, A. J., Karow, M. and Flavell, R. A.**, Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* 2007. **27**: 647–659.

23 **Boniface, K., Bernard, F. X., Garcia, M., Gurney, A. L., Lecron, J. C. and Morel, F.**, IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J. Immunol.* 2005. **174**: 3695–3702.

24 **Sestito, R., Madonna, S., Scarponi, C., Cianfarani, F., Failla, C. M., Cavani, A., Girolomoni, G. et al.**, STAT3-dependent effects of IL-22 in human keratinocytes are counterregulated by sirtuin 1 through a direct inhibition of STAT3 acetylation. *FASEB J.* 2011. **25**: 916–927.

25 **Zhang, W., Chen, Y., Wei, H., Zheng, C., Sun, R., Zhang, J. and Tian, Z.**, Antiapoptotic activity of autocrine interleukin-22 and therapeutic effects of interleukin-22-small interfering RNA on human lung cancer xenografts. *Clin. Cancer. Res.* 2008. **14**: 6432–6439.

26 **Jiang, R., Wang, H., Deng, L., Hou, J., Shi, R., Yao, M., Gao, Y.** et al, IL-22 is related to development of human colon cancer by activation of STAT3. *BMC Cancer* 2013. **13**: 59.

27 **Naher, L., Kiyoshima, T., Kobayashi, I., Wada, H., Nagata, K., Fujiwara, H., Ookuma, Y. F. et al.**, STAT3 signal transduction through interleukin-22 in oral squamous cell carcinoma. *Int. J. Oncol.* 2012. **41**: 1577–1586.

28 **Albanesi, C., Cavani, A. and Girolomoni, G.**, IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-gamma and TNF-alpha. *J. Immunol.* 1999. **162**: 494–502.

29 **Pennino, D., Eyerich, K., Scarponi, C., Carbone, T., Eyerich, S., Nasorri, F., Garcovich, S. et al.**, IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific Th1 cells to kill autologous keratinocytes. *J. Immunol.* 2010. **184**: 4880–4888.

30 **Hartman, Z. C., Poage, G. M., den Hollander, P., Tsimelzon, A., Hill, J., Panupinthu, N., Zhang, Y. et al.**, Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer. Res.* 2013. **73**: 3470–3480.

31 **Gu, F. M., Li, Q. L., Gao, Q., Jiang, J. H., Zhu, K., Huang, X. Y., Pan, J. F. et al.**, IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

tumor progression in hepatocellular carcinoma. *Mol. Cancer*. 2011. **10**: 150.

32 **Desai, S.**, **Laskar, S. and Pandey, B. N.**, Autocrine IL-8 and VEGF mediate epithelial-mesenchymal transition and invasiveness via p38/JNK-ATF-2 signalling in A549 lung cancer cells. *Cell. Signal*. 2013. **25**: 1780–1791.

33 **Mitra, A.**, **Raychaudhuri, S. K. and Raychaudhuri, S. P.**, IL-22 induced cell proliferation is regulated by PI3K/Akt/mTOR signaling cascade. *Cytokine* 2012. **60**: 38–42.

34 **Karin, M. and Greten, F. R.**, NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol*. 2005. **5**: 749–759.

35 **Joyce, J. A. and Pollard, J. W.**, Microenvironmental regulation of metastasis. *Nat. Rev. Cancer*. 2009. **9**: 239–252.

36 **Mantovani, A.**, **Allavena, P.**, **Sica, A. and Balkwill, F.**, Cancer-related inflammation. *Nature* 2008. **454**: 436–444.

37 **Yu, H.**, **Kortylewski, M. and Pardoll, D.**, Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat. Rev. Immunol*. 2007. **7**: 41–51.

38 **Gu, F. M.**, **Li, Q. L.**, **Gao, Q.**, **Jiang, J. H.**, **Zhu, K.**, **Huang, X. Y.**, **Pan, J. F. et al.**, IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Mol. Cancer*. 2011. **10**: 150.

39 **Jiang, R.**, **Tan, Z.**, **Deng, L.**, **Chen, Y.**, **Xia, Y.**, **Gao, Y.**, **Wang, X. et al.**, Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. *Hepatology* 2011. **54**: 900–909.

40 **Steiner, G. E.**, **Newman, M. E.**, **Paikl, D.**, **Stix, U.**, **Memaran-Dagda, N.**, **Lee, C. and Marberger, M. J.**, Expression and function of pro-inflammatory interleukin IL-17 and IL-17 receptor in normal, benign hyperplastic, and malignant prostate. *Prostate* 2003. **56**: 171–182.

41 **Miyahara, Y.**, **Odunsi, K.**, **Chen, W.**, **Peng, G.**, **Matsuzaki, J. and Wang, R. F.**, Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 15505–15510.

42 **Kuang, D. M.**, **Peng, C.**, **Zhao, Q.**, **Wu, Y.**, **Zhu, L. Y.**, **Wang, J.**, **Yin, X. Y. et al.**, Tumor-activated monocytes promote expansion of IL-17-producing $CD8^+$ T cells in hepatocellular carcinoma patients. *J. Immunol*. 2010. **185**: 1544–1549.

43 **Numasaki, M.**, **Fukushi, J.**, **Ono, M.**, **Narula, S. K.**, **Zavodny, P. J.**, **Kudo, T.**, **Robbins, P. D. et al.**, Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003. **101**: 2620–2627.

44 **Wang, L.**, **Yi, T.**, **Zhang, W.**, **Pardoll, D. M. and Yu, H.**, IL-17 enhances tumor development in carcinogen-induced skin cancer. *Cancer Res*. 2010. **70**: 10112–10120.

45 **Zhuang, Y.**, **Peng, L. S.**, **Zhao, Y. L.**, **Shi, Y.**, **Mao, X. H.**, **Chen, W.**, **Pang, K. C. et al.**, CD8(+) T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer. *Gastroenterology* 2012. **143**: 951–62.e8.

46 **Tartour, E.**, **Fossiez, F.**, **Joyeux, I.**, **Galinha, A.**, **Gey, A.**, **Claret, E.**, **Sastre-Garau, X. et al.**, Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. *Cancer Res*. 1999. **59**: 3698–704.

47 **Bian, G. and Zhao, W. Y.**, IL-17, an important prognostic factor and potential therapeutic target for breast cancer? *Eur. J. Immunol*. 2014. **44**: 604–605.

48 **Benevides, L.**, **Cardoso, C. R.**, **Tiezzi, D. G.**, **Marana, H. R.**, **Andrade, J. M. and Silva, J. S.**, Enrichment of regulatory T cells in invasive breast tumor correlates with the upregulation of IL-17A expression and invasiveness of the tumor. *Eur. J. Immunol*. 2013. **43**: 1518–1528.

Abbreviations: **BCC**: basal-cell carcinoma · **IHC**: immunohistochemistry · **NMSC**: Nonmelanoma skin cancer · **SCC**: squamous-cell carcinoma · **TILs**: tumor-infiltrating lymphocytes

Full correspondence: Dr. Andrea Cavani, Laboratory of Experimental Immunology, Istituto Dermopatico dell'Immacolata, IDI-IRCCS, via dei Monti di Creta 104, 00167 Rome, Italy
e-mail: cavani@idi.it

See accompanying Commentary:
http://dx.doi.org/10.1002/eji.201545456

Received: 23/7/2014
Revised: 28/10/2014
Accepted: 3/12/2014
Accepted article online: 9/12/2014

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

15214141, 2015, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.201445052, Wiley Online Library on [19/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License