IL-17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment

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Abstract

The aggressiveness of invasive ductal carcinoma (IDC) of the breast is associated with increased IL-17 levels. Studying the role of IL-17 in invasive breast tumor pathogenesis, we found that metastatic primary tumor-infiltrating T lymphocytes produced elevated levels of IL-17, whereas IL-17 neutralization inhibited tumor growth and prevented the migration of neutrophils and tumor cells to secondary disease sites. Tumorigenic neutrophils promote disease progression, producing CXCL1, MMP9, VEGF and TNFα, and their depletion suppressed tumor growth. IL-17A also induced IL-6 and CCL20 production in metastatic tumor cells, favoring the recruitment and differentiation of Th17. In addition, IL-17A changed the gene expression profile and the behavior of non-metastatic tumor cells, causing tumor growth in vivo, confirming the pro-tumor role of IL-17. Furthermore, high IL-17 expression was associated with lower disease-free survival (DFS) and worse prognosis in IDC patients. Thus, IL-17 blockade represents an attractive approach for the control of invasive breast tumors.

Introduction

Breast cancer continues to be one of the leading causes of cancer-associated deaths among women worldwide (1). The initiation of breast cancer is likely caused by a combination of oncogenic mutations that promote genetic instability and accelerated cellular proliferation (2). Cancer develops in a complex host-tissue microenvironment that comprises immune cells, fibroblasts and blood and lymphatic vessels (3). In the tumor microenvironment, inflammatory mediators, such as the cytokines IL-1, IL-6 and TGF-β, stimulate cancer cell proliferation and invasion and contribute to disease progression (4).
IL-17 is the major effector cytokine produced by Th17 cells, a subtype of T helper cell. Th17 differentiation is mediated by a combination of signaling via IL-6, IL-21, IL-1β, IL-23 and TGF-β through the intracellular signaling molecule STAT3, which induces expression of the transcription factor Rorγt (5). Th17 plays active roles in inflammation and autoimmune disease and appears to be important in pulmonary bacterial immunity as well as protection against infection by certain protozoa (6,7). Although IL-17 has been detected in several tumors, such as prostate, gastric, lung and colon cancers and lymphoma (8-13), whether the role of IL-17 is pro- or anti-tumoral remains controversial, and its function seems to depend on the cancer type (14). IL-17 acts in several cell types and leads to the production of GM-CSF, IL-1, IL-6, TNF-α, chemokines, activation of NOS2 and metalloproteinases, and leukocyte recruitment (6,15). Therefore, we hypothesized that IL-17 is a key cytokine in tumor pathogenesis and modulates tumor progression. In fact, we demonstrated that IL-17A production is increased in patients with invasive ductal carcinoma (IDC) of the breast and that tumor-infiltrating IL-17A-producing cells are positively correlated with the presence of regulatory T (Tregs) cells in the tumor microenvironment, as well as with tumor aggressiveness and a poor disease prognosis (16). These data prompted us to investigate how IL-17 affects invasive progression tumor progression. Herein, using a metastatic (4T-1) and non-metastatic (67NR) murine mammary tumor model, we demonstrated that IL-17A was produced by tumor-infiltrating CD4+ T lymphocytes during metastatic tumor progression and that the neutralization of IL-17 inhibited tumor growth and prevented the migration of tumorigenic neutrophils and metastasis. Moreover, IL-17 had a direct effect on 4T-1 tumor cells by stimulating the release of IL-6 and CCL20.
In addition, IL-17 altered the gene expression profile in 67NR tumor cells associated with tumor growth. High expression of IL-17 was also associated with lower disease-free survival (DFS) and worse prognosis in patients with IDC. Thus, our study uncovers a previously unknown, key role for IL-17 in tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophil recruitment.

Materials and Methods

Human subjects and study populations

Tumor biopsies and peripheral blood samples were obtained from 23 patients with IDC of the breast who received care at the Department of Gynecology and Obstetrics, Breast Disease Division, Ribeirão Preto School of Medicine, University of São Paulo, from 2008-2010. The inclusion criteria included patients with IDC of the breast who had not received prior therapy for cancer. Control skin biopsy samples were obtained from women undergoing cosmetic breast and abdominoplasty surgeries. All subjects signed an informed consent form releasing the use of their specimens before participating in the study. This study was approved by the Ethics Committee of the Ribeirão Preto Medical School Hospital.

Murine model of mammary carcinoma

Specific pathogen-free, 8- to 10-week-old female BALB/c mice were obtained from a local breeding facility at the University of São Paulo, Brazil. Mice were injected with 50 μL of a single-cell suspension containing 5×10⁴ of a 4T1 mouse metastatic mammary tumor cell line (purchased from the American Type
Culture Collection – ATCC, USA) or 67NR non-metastatic mammary tumor cell line provided by Jing Yang (University of California, San Diego) orthotopically into the fourth mammary fat pad, as described previously (17). The sizes of the primary tumors were assessed morphometrically using electronic calipers. Tumor volumes were calculated according to the following formula: tumor volume (mm$^3$) = $L \times W^2 / 2$, where $L$ represents the major axis (largest cross-sectional diameter) of the tumor, and $W$ represents the minor axis. The data are presented as the mean ± standard error (mean ± SEM). For the treatment with goat anti-mouse IL-17, antigen affinity-purified polyclonal antibody (AF-421-NA; R&D Systems), the mice received 5 doses of 10 µg of anti-IL-17 antibody by intraperitoneal (i.p.) injection, beginning 6 days after tumor-cell inoculation (dpi) and every 3 days thereafter. To deplete neutrophils, the mice received 5 doses of 30 µg of rat anti-mouse Ly6G (1A8; Biolegend) antibody by i.p injection, from 18 dpi and every 3 days thereafter. For the treatment with rat anti-mouse IL-6 antibody (MP5-20F3; BioXcell), the mice received 5 doses of 20 µg of anti-IL-6 antibody by i.p injection, and treatment was initiated at 1 dpi and every 4 days thereafter. Lung and tumor tissue specimens were routinely embedded in paraffin and stained with hematoxylin and eosin (H&E) to assess the presence of metastatic colonies. In total, 4-µm H&E-stained sections from at least three different levels were examined. All procedures were performed in accordance with the International Guidelines for the Use of Animals and by the local Ethics Committee at the University of São Paulo, Brazil.

**Gene expression measurement by quantitative real-time PCR (qPCR)**
Total RNA isolation and real quantitative real-time PCR (qPCR) analyses were conducted as detailed in Supplementary data. The primers are listed in Supplementary Table S1. The analyses were performed using the cycle threshold (Ct) method, which allows for quantitative analysis of the expression of a factor using the formula $2^{-\Delta\Delta\text{Ct}}$, in which $\Delta\text{Ct} = \text{Ct target gene} - \text{Ct of the housekeeping gene GAPDH (glyceraldehyde phosphate dehydrogenase) or } \beta$-actin, and $\Delta\Delta\text{Ct} = \Delta\text{Ct sample} - \Delta\text{Ct}$.

**Microarray analysis**

To assess the gene expression profiles of the biopsies of patients with breast tumors and 67NR and 4T-1 tumor cell lines, RNA was isolated and microarray analyses were conducted as detailed in Supplementary data.

**Immunohistochemistry analysis**

Frozen breast tumor tissue sections (5 µm) were prepared and subjected to immunohistochemistry analysis using the antibodies described in Supplementary data.

**Isolation of leukocytes and cell culture**

To characterize the inflammatory infiltrate in the tumors, lung and spleen, cells were isolated tissue as described in Supplementary data. Mononuclear cells isolated from murine mammary tumors were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) (Sigma) for 4 hours in 48-well plates. Lung cells were stimulated in vitro with ultrapure LPS (100 ng/ml) (Invivogen) for 6 hours in 24-well plates. For all conditions, brefeldin A (BD Biosciences) was added.
during the last 2 hours for the determination of intracellular cytokines by flow cytometry. Splenocytes from mice were stimulated with anti-CD3 (2 µg/ml) plus anti-CD28 antibody (1 µg/ml) (BD Biosciences) for 72 hours to measure the IL-17 and IFN-γ levels in the supernatants by ELISA. The murine mammary tumor cell lines 4T1 and 67NR were treated with recombinant murine IL-17A (rIL-17) (400 ng/ml) (Biosource) in 24-well plates. After 48 hours, IL-6 and CCL20 levels were measured in the supernatants by ELISA, the cells were collected, and the mRNA was extracted to evaluate the gene expression by qPCR.

**Flow cytometry assay**

For T cell analysis, antibodies to the following mouse proteins were obtained from BD Bioscience: CD3 (145-2C11), CD4 (RM4-5), CD8, (53-6.7), TCR γδ (UC7-13D5), IL-17A (TC11-18H10) and IFN-γ (XMG1.2) or their respective isotype controls (BD Biosciences). For neutrophil analysis, antibodies to the following mouse proteins were obtained from Biolegend: CD11b (M1/70), F4/80 (BM8), MHCII (M5/114.15.2), Ly6G (1A8) and TNF-α (MP6-XT22). Intracellular cytokine staining was performed according to the manufacturer’s instructions (BD Biosciences). Data acquisition was performed using the FACSCanto II and analyzed according to size based on forward-scatter and granulosity by side-scatter dot plots using the FlowJo software.

**FACS-based cell sorting**

For fluorescence-activated cell sorting (FACS), single-cell suspensions from spleens and tumors obtained from 4T-1 tumor-bearing mice were isolated and stained, and the Ly6G+F4/80− cells were identified by gating on CD11bMHCII−
cells. Sorting was performed with an Aria FACS (BD Biosciences), and the pelleted cells were added directly to Trizol reagent for gene expression analysis.

**Air pouch model**

Air pouches were created in the dorsal side of the back of BALB/c. PBS, CCL20 (400 ng), and 4T1 or 67NR supernatant were injected into the air pouch; 20 h later, the cell infiltrates were harvested by pouch lavage and stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) (Sigma) for 4 h, and brefeldin A (BD Biosciences) was added during the last 2 h to determine the intracellular IL-17 by flow cytometry.

**Statistical analysis**

The statistical analysis was performed using an unpaired t-test or ANOVA followed by Bonferroni’s multiple comparison tests. Kaplan-Meier curves were used to assess the influence of immune parameters on DFS. The significance of these parameters was calculated using the log-rank test (5.0 GraphPad Software). All values were considered significantly different at p < 0.05.

**Results**

**Metastatic primary tumor-infiltrating lymphocytes produce IL-17A**

To investigate the role of IL-17A in the pathogenesis of invasive breast cancer, we injected 4T1 metastatic mouse mammary tumor cells into the mammary fat pads of BALB/c mice. The 4T1 cells line grows rapidly at the primary site and forms metastasis in the lung, liver, bone and brain, which makes this cell line an excellent model for studying the progression of breast cancer (17). To better
characterize IL-17-associated responses in the 4T1 tumor model, we evaluated the kinetics of IL-17A and IFN-γ production by tumor-infiltrating lymphocytes isolated from mice with metastatic (4T-1) and non-metastatic (67NR) tumors. We found that, as early as 15 days post-inoculation (dpi) with tumor cells, 4T-1 tumor-infiltrating lymphocytes produced high levels of IL-17A compared with 67NR tumor-infiltrating lymphocytes (Fig. 1A), whereas both produced similar levels of IFN-γ. To assess the kinetics of IL-17A and IFN-γ production during the metastatic tumor development, the cytokine levels in the supernatants of total splenocytes were evaluated. We found that the maximum peak of IL-17A production occurred at 15 and 25 dpi, whereas the production of IFN-γ peaked at 25 dpi (Fig. 1B). In addition, the levels of IL-17A were 15-fold higher after tumor induction. To determine the cellular source of these cytokines, leukocytes were isolated from the tumor microenvironment and stimulated with PMA and ionomycin. We found that CD3+CD4+, CD3+CD8+ and CD3+γδ+ T cells produced IL-17A and IFN-γ at all assessed points (Fig. 1C and D). In particular, we observed an increase in the number of IL-17A-producing CD3+CD4+ and TCRγδ+ T cells at 25 and 35 dpi, while the number of IL-17+CD8+ T cells was only slightly elevated at 25 dpi (Fig. 1C). However, we found increased numbers of IFN-γ-producing CD3+CD8+ and TCRγδ+ T cells at 35 dpi, while the numbers of CD3+CD4+ IFN-γ+ T cells were maintained throughout the entire period (Fig. 1D). Together, these data reveal that IL-17A is produced by CD3+CD4+ (Th17) and TCRγδ+ T cells during metastatic primary tumor growth.

**IL-17 promotes tumor growth and controls neutrophil recruitment**
To further investigate whether IL-17A is involved in the pathogenesis of invasive breast cancer, we treated 4T-1-inoculated BALB/c mice with anti-IL-17-neutralizing antibody and analyzed tumor progression. 4T-1-inoculated mice treated with anti-IL-17A antibodies showed a significant reduction in tumor volume in comparison to mice treated with an IgG control (Fig. 2A). The tumor volumes in the 4T-1-inoculated and anti-IL-17-treated mice were similar to those of mice injected with 67NR tumors (Fig. 2A). Similarly, TC-1 bearing-C57BL/6 mice treated with anti-IL-17 antibodies showed a significant reduction of tumor volume (Supplementary Fig. S1). Additionally, anti-IL-17 treatment led to 100% of survival (as assessed on day 50 dpi) in mice inoculated with a metastatic mammary tumor, in comparison with 80% of mortality of the 4T-1 tumor-inoculated BALB/c mice (Fig. 2B). All animals that survived up to 50 dpi were sacrificed due to the large size of their primary tumor. Moreover, the administration of anti-IL-17A-neutralizing antibodies significantly reduced tumor mass (Fig. 2C) and size in tumor-bearing mice (Fig. 2D), that was accompanied by a decreased polymorphonuclear cells migration compared with the control mice (Fig. 2E). To assess whether IL-17 favors disease progression, we analyzed the histopathological changes at 35 dpi in the lungs of BALB/c mice with metastatic mammary tumors that were treated with anti-IL-17 or IgG control antibodies. Control antibody-treated mice showed a complete loss of lung architecture and presented numerous colonies of tumor cells and a predominance of polymorphonuclear cells in the lungs. On the contrary, anti-IL-17-treated mice exhibited preserved lung architecture, reduced numbers of tumor cells and polymorphonuclear cells (Fig. 3A). These results were further confirmed by flow cytometry analysis of lung-extracted leukocytes, which showed a significant
reduction in the number of neutrophils in the lungs of anti-IL-17-treated mice compared with those of control antibody-treated mice (Fig. 3B). We then evaluated the expression of the pro-tumorigenic neutrophil markers TNF-\(\alpha\) and MMP-9. As expected, the number of TNF-\(\alpha\)-producing CD11b\(^+\)Ly6G\(^+\) neutrophils was significantly elevated in the lungs of animals with a mammary tumor and treated with a control antibody but was massively reduced in the lungs of anti-IL-17A-treated mice (Fig. 3C). qPCR analysis of total lung tissue showed a significant reduction in the relative expression of MMP-9 in animals treated with anti-IL-17 in comparison to those treated with control antibody (Figure 3D). On the other hand, the expression of IFN-\(\gamma\) was similar in both groups (Supplementary Fig. S2). Together, these results indicate that IL-17 participates in tumor progression, possibly by recruiting neutrophils to secondary sites affected by the tumor, thus favoring disease progression.

**Pro-tumorigenic neutrophils control tumor growth in metastatic mammary tumors**

The findings reported above led us to assess the contribution of neutrophils to disease progression in our tumor model. We first analyzed the numbers of neutrophils in the spleens of metastatic and non-metastatic tumor-bearing mice. The numbers of Ly6G\(^+\)F4/80\(^-\) neutrophils (CD11b\(^+\)MHCII\(^-\)) were significantly increased in 4T-1 tumor-bearing mice in comparison to 67NR tumor-bearing hosts and naive mice (Fig. 4A). Furthermore, we found high numbers of tumor infiltrating-neutrophils at 25 and 35 dpi within the 4T-1 tumors, as assessed by flow cytometry (Fig. 4B) and histological analysis (Fig. 4C). In line with the finding that pro-tumorigenic neutrophil contributes to angiogenesis, invasion,
metastasis and immunosuppression (18), we also found that neutrophils isolated from the spleens and tumors of 4T-1 tumor-bearing mice presented a high expression of genes related to pro-tumorigenic neutrophils, including Cxcl1, Tnf-α, Mmp-9 and Vegf (Fig. 4 D). To further test the role of tumor-associated neutrophils, we treated BALB/c mice undergoing metastatic tumor (4T-1) induction with anti-Ly6G-neutralizing antibody and analyzed tumor progression. The depletion of neutrophils in vivo did not alter an IL-17A production in the tumor (Supplementary Fig. S3A and B); however, promoted a significant reduction of the volume (Fig. 4 E-F) and weight of the tumor mass (Fig. 4 G) in tumor-bearing mice. Thus, the recruitment of pro-tumorigenic neutrophils contributes to tumor progression.

IL-17A induces the production of IL-6 and CCL20 in murine metastatic mammary carcinoma cells

To determine the effects of IL-17A on tumor cells, the 4T-1 and 67NR cells lines were cultured for 48 hours in the presence or absence of recombinant IL-17A (rIL-17). The 4T-1 cells exhibited high CCL20 production that was increased with IL-17 treatment (Fig. 5 B). However, we found a low level of IL-6 expression in the 4T-1 cells, which was increased with IL-17 treatment (Fig. 5 A-B). Therefore, IL-6 production is dependent IL-17 on 4T-1 cells. By contrast, non-metastatic 67NR cells demonstrated low expression of IL-6 and CCL20 independent of IL-17 treatment (Fig. 5 A-B). Thus, metastatic and non-metastatic tumor cells display different responses to IL-17A in vitro.

IL-6 is required for Th17 differentiation and is involved in the inflammation associated with tumorigenesis (19,20). To test the relevance of IL-6 in tumor
progression and its contribution to the responses of Th17 cells. BALB/c mice undergoing 4T-1 tumor induction were treated with anti-IL-6-neutralizing antibody, and tumor progression was evaluated. The depletion of IL-6 in vivo significantly lowered the tumor volume in comparison with control mice (treated with IgG control) (Fig. 5C). Additionally, our data showed a decrease in the numbers of CD4^+IL-17A^+ T cells (Fig. 5D) and neutrophils (Ly6G^+F4/80^-) gated on CD11b^hiMHCII^- cells in the spleens of animals treated with anti-IL-6 (Fig. 5E) compared to the control group. Similarly, we found a reduction of IL-17A (Supplementary Fig. S3A and D) and polymorphonuclear cells (Fig. 5F) in tumor of mice treated with anti-IL-6 mAb compared with the controls.

To further evaluate whether the Th17 response in the tumor is dependent on factors produced by metastatic tumor cells in vivo, we generated an air pouch in the dorsal side of BALB/c mice and locally injected 4T-1 and 67NR cell supernatant, CCL20 as an inflammatory stimulus or PBS as a control. Twenty hours later, we found that the numbers of IL-17-producing CD3^+ cells recruited into the air pouch were significantly higher in the 4T-1 supernatant-treated group than in the PBS control group and comparable to the CCL20–treated mice (Fig. 5 G). In response to the 67NR supernatant, we observed that similar numbers of IL-17^+CD3^+ cells were recruited into the air pouch compared with the control group (treated with PBS). Therefore, the 4T-1 cell supernatants induce the migration of pre-existing T cells producing IL-17. IL-17 promotes the production of IL-6 and CCL20 initiates a feedback loop to foster the amplification of the Th17 response.
IL-17A directly affects non-metastatic mammary tumor cell behavior in vivo

To assess whether IL-17 affects the growth of non-metastatic primary tumor, we pre-treated 67NR cells with rIL-17 before injecting them into BALB/c mice and analyzed the tumor progression. The IL-17-pre-treated 67NR cells promoted a significantly increased tumor volume compared with that of mice injected with untreated 67NR cells and comparable to that of 4T-1 tumor-bearing mice (Fig. 6A). Moreover, the number of neutrophils in the spleens (Fig. 6B) and tumor (Fig. 6C) of mice inoculated with IL-17-pre-treated 67NR cells was increased compared with those that received untreated 67NR cells and it was similar to that of 4T-1 tumor-bearing mice (Fig. 6B).

To understand how IL-17 induces an increase in non-metastatic tumor growth, we analyzed the gene expression profile of the 67NR cells lines that were either treated or not treated with IL-17A, using a microarray analysis. Strikingly, using a threshold of at least twofold, we identified 1742 up-regulated and 2592 down-regulated genes in the IL-17-treated cells compared with the untreated cells (Fig. 6D). In addition, we found an increased expression of genes related to cellular adhesion (Itga1, Itgb1, Itgb3, Cadm4, Icam1, Spam1, Ceacam13 and Ceacam19); cellular growth and survival (Cdc20b, Cdc25b, Erg1, Egfr, Ciapin1, Bcl2l14 and Il24); chemokine and chemokine receptors (Ccr1, Ccr2, Cxcr2, Cxcr3, Cxcr4, Cxcr5, Ccl3, Cxcl2, Cxcl11); and cytokines, receptors and molecules associated with angiogenesis and metastasis (Tgfb1i1, Il1a, Il18r1, Il18rap, Tnfsf12, Ptger3, Pdcd11, Hmox1, Twist1, Vegfa) (Fig. 6D). A complete list of the differentially expressed genes is presented in Supplementary Table S2-3.
To confirm whether IL-17 actually promotes more aggressive tumor cells, we compared the gene expression profile of 4T-1 cells with IL-17-treated 67NR cells. Interestingly, we found 279 up-regulated genes in common between the IL-17-treated 67NR cells and 4T-1 cells compared with the untreated 67NR cells (Fig. 6E). Among the differentially expressed genes, we identified Itga1, Icam-1, Egfr, Cxcr3, Cxcr4, Ccl3, Il1a, LTB4r2 and Twist1 related to the cellular adhesion, growth and migration of tumor cells. Taken together, these results indicate that IL-17 has a direct effect on tumor cells, altering the gene expression profile and making the cells more aggressive, favoring tumor growth in vivo.

**IL-17A expression predicts clinical outcome in human invasive breast cancer**

The results described above, along with our recent observation that the presence of IL-17-producing T cells in the tumoral microenvironment is associated with disease progression in patients with breast cancer (16), led us to inquire if the tumor microenvironment of patients with IDC could, in fact, promote Th17 differentiation. We first assessed whether cytokines that drive Th17 differentiation could be promptly measured in such tumors. A qPCR analysis revealed that the expression of IL-6 (p = 0.003) and CCL20 (p = 0.003) mRNA was significantly increased in breast tumor tissue when compared to control tissue (Fig. 7A). Interestingly, IL-6 expression was up regulated in IL-17 hi human breast tumor samples, however the expression of CCL20 was similar in IL-17 hi and IL-17 lo samples (Supplementary Fig. S4). An immunohistochemistry analysis confirmed that IL-6 and CCL20 protein was selectively expressed in
tumor tissues but not in control-tissue biopsies (Fig. 7B). These data indicate that invasive human breast tumors provide a favorable environment for the development, maintenance and migration of Th17 cells.

To further investigate how IL-17 production could directly affect the biology of tumors in humans, we grouped breast tumor samples according to their expression of IL-17A and then compared their gene expression profiles using microarray analysis. Strikingly, principal component analysis revealed that low and high IL-17 producers showed distinct global gene expression profiles (Fig. 7C). Using a threshold of at least twofold and a p-value < 0.01, we identified 74 up-regulated (red) and 168 down-regulated (blue) genes in tumor samples from patients with IL-17hi in comparison to IL-17lo tumors (Fig. 7 C-D, Supplementary Table S4-5). In addition, a substantial proportion of the differentially expressed genes in these samples were related to tumor progression, and their expression was confirmed by qPCR (Supplementary Fig. S4). Genes that were up-regulated in IL-17hi tumor samples included USP28, a ubiquitin-specific protease involved in the proliferation of tumor cells (21), BCL-2 (B-cell lymphoma 2), a dominant repressor of cell death that promotes cell survival (22), and FAIM (Fas apoptosis inhibitory molecule), an anti-apoptotic molecule (21). By contrast, the expression of GAS-7 (growth arrest-specific protein 7), a gene related to cell growth/arrest (22), was reduced compared to the IL-17lo tumor samples.

To further investigate the clinical significance of IL-17A in human breast cancer, the clinicopathological factors of breast cancer, such as tumor size, lymph node status (positive and negative axilla) and the survival of patients, were analyzed relative to the intratumoral expression of IL-17A. The clinical characteristics of
the patients studied are reported in Supplementary Table S5. Interestingly, we found that 75% of the patients with IL-17\textsuperscript{hi} expression exhibited tumors larger than 5 cm (T3); 92% of these patients were lymph node positive, and 42% showed recurrent disease. In contrast, 64% of patients with IL-17\textsuperscript{lo} expression presented tumors up to 5 cm and were classified as T2, 73% of them presented negative axilla, and 100% of the patients showed no recurrence of disease (Supplementary Table S6). In contrast, patients with low expression of IL-17A demonstrated improved DFS rates compared to patients with high expression of this gene (Fig. 7E; p = 0.047). Together, the results indicate that IL-17A promotes the development of aggressive tumors through mechanisms involving direct effects on tumor cells and the immune system.

Discussion

In this study, we uncovered the mechanism by which the pro-inflammatory cytokine IL-17A promotes metastatic mammary primary tumor progression. We showed that the presence of IL-17A in tumor tissues isolated from IDC patients correlates with poor prognosis and a high expression of genes that promote the proliferation and survival of these tumors. Confirming the data from humans, in our murine mammary tumor model, IL-17 changes the behavior of non-metastatic cells, promoting tumor growth. Thus, we propose that inhibition of IL-17 could have broad clinical applications in invasive breast cancer. Recently, we demonstrated that the enrichment of Treg cells in IDC patients correlates with up-regulation of IL-17A expression and invasiveness of the tumor (16). Consistent with these findings, tumor-infiltrating Th17 cells were shown to correlate with worse prognosis in patients with colorectal and lung cancer.
Despite the potential therapeutic implications of these observations, especially in the context of the link between inflammation and tumor growth, the mechanisms underlying the modulation of tumor progression by IL-17 are not yet known.

To study the role of IL-17 in tumor progression, we took advantage of the previously established 4T-1 metastatic breast cancer model in BALB/c mice, which is an excellent model for studying the progression of breast cancer in humans. This model has been used to study antitumor immune mechanisms that counteract tumor growth and metastasis (17). Using this model, we showed that IL-17A is produced in the metastatic primary tumor microenvironment by CD3+CD4+ and CD3+γδ+ T cells. And IL-17 blockade significantly inhibited tumor growth, indicating the role of IL-17 in mammary tumor growth. In TC-1 tumor model, we also showed that the tumor-infiltrating lymphocytes produce IL-17A, and that its blockage reduces the volume tumor. Another models as B16 melanoma, tumor growth was inhibited in IL-17−/− mice and in IFN-γ/IL-17 double-knockout mice (24). On the contrary, in the ovarian carcinoma model, tumor growth was increased in IL-17−/− mice because the absence of IL-17 caused decreased numbers of IFN-γ–producing NK and T cells (25). Although it was suggested that IFN-γ plays a role in the regulation of antitumor immune responses (26), our results indicate that IFN-γ does not play a direct role in controlling tumor development in the 4T1 model. In addition, and in contrast with IL-17, the IFN-γ production was neither altered with the disease progression nor when IL-17 was blocked. However, we cannot exclude the possibility of an indirect role of IFN-γ in this process via regulation of IL-17.
production (27-29). Thus, it is conceivable that the downstream effects of the IL-17 blockade in regulating tumor growth is tumor model-specific.

The mechanism by which IL-17 promotes the growth of metastatic primary mammary tumors is neutrophil-dependent. Once the IL-17 blockade-induced reduction in mammary tumor size was accompanied by decreased numbers of neutrophils in the tumor microenvironment and tumor cell colonies at secondary sites of tumor formation. This beneficial effect of anti-IL-17A antibody therapy was also associated with decreased MMP-9 expression and reduced numbers of TNF-α-producing neutrophils, indicating that the main role of IL-17 in this model is to promote the recruitment of pro-tumorigenic neutrophils. Tumor-associated neutrophils differentiate in response to tumor-produced factors, such as TGF-β, that is produced by tumor and inflammatory cells (14) and contribute to angiogenesis, metastasis and immunosuppression in tumor-bearing hosts (30).

In a lung cancer model, it has been shown that neutrophils invading the lungs exert pro-tumorigenic activity through the release of MMP-9, which favors the survival and establishment of tumor cells (31). Furthermore, neutrophils also promote the release of pro-inflammatory factors, such as TNF-α, in a melanoma model (32) and human tumors, including hepatocellular and cervical carcinomas (33), which, in turn, favor angiogenesis and metastasis. To facilitate invasion, neutrophils might directly degrade the extracellular matrix via the release of several enzymes, such as neutrophil elastase, cathepsin G, proteinase-3, MMP-8 and MMP-9 (34). In our studies, neutrophils isolated from tumor sites and the spleen produced high levels of CXCL1, TNFα, MMP-9 and VEGF, all of which are associated with disease progression (35). In line with this finding, despite
the depletion of neutrophils \textit{in vivo} did not change the IL-17A production into the tumor, it promoted a inhibition of 4T-1 tumor growth, confirming the pro-tumorigenic role of neutrophils in this model.

In addition to the recruitment of neutrophils, IL-17 also induces IL-6 and CCL20 production in metastatic tumor cells, which in turn promote the recruitment and differentiation of Th17 cells. The initial recruitment of IL-17-producing T cells creates a feed-forward loop that sustains the inflammatory environment at the tumor site with high production of IL-6 and CCL20, maintaining the profile of the Th17 response. Indeed, the depletion of IL-6 \textit{in vivo} promotes the inhibition of tumor growth and decreases the Th17 response and neutrophil migration, suggesting that IL-17-mediated tumor progression is dependent on IL-6.

Regardless, CCL20 is an essential factor affecting the prevalence of the Th17 response in a tumor. Interestingly, the direct effects of IL-17 in inducing the production of inflammatory cytokines was specific to metastatic (4T-1) tumor cells, as the non-metastatic tumor cell line (67NR) was refractory to the effects of IL-17, although both tumor cell lines expressed the receptor for IL-17. Nonetheless, these results explain why the Th17 response is increased in metastatic tumor-bearing mice compared with non-metastatic tumor-bearing mice.

In line with this finding, tumor tissues from patients with IDC showed higher expression of IL-6 and CCL20, which would also provide a favorable environment for the migration and maintenance of Th17 cells. Based on these observations and the previously established tight association between TGF-β and IL-6 with tumor incidence and progression in patients with cancer, it appears likely that CD4$^+$ T cells entering an established tumor are more likely to
be exposed to conditions that induce the differentiation of Th17 compared with other Th subsets (20,36,37). Thus, the feed-forward loop we are describing for the murine 4T1 models might very well be present in human breast tumors. Interestingly, the presence of IL-17 in patients with IDC was associated with a worse prognosis and a high expression of genes related to the proliferation and survival of these tumors. This effect occurs because IL-17 determines the aggressiveness of the tumor cells. IL-17 is able to change the behavior of non-metastatic 67NR cells, inducing the expression of various genes associated with the cellular adhesion, proliferation and migration of tumor cells. For example, breast tumor cells with high ICAM-1 (38), CXCR3 (39) and Twist (40) expression exhibit metastatic potential. These alterations generated in 67NR cells after treatment with IL-17 induced increased tumor growth and levels of neutrophils; this finding might explain why IL-17 induces increased expression of mediators such as CXCL11 and Ltb4r2 (leukotriene B4 receptor 2) in 67NR tumor cells, which are genes related to neutrophil chemotaxis (41).

Taken together, our data support a model in which pre-existing T cells producing IL-17 are recruited to the tumor site promote an inflammatory environment with a high IL-6 and CCL20 production that promotes their own differentiation in addition to promoting the recruitment of pro-tumorigenic neutrophils. Once at the tumor site, tumor-associated neutrophils secrete mediators, such as MMP-9, VEGF, TNF-α and CXCL1, which together promote tumor invasion and metastasis to secondary sites such as the lung. Notably, the induced tumor cell trigger determines the response profile that will be formed in the tumor microenvironment. The presence of IL-17 is crucial for disease aggressiveness. Therefore, our results support the idea that IL-17, and perhaps
expression of the IL-17 receptor, in tumor cells could serve as a biomarker for the prognosis of aggressive breast cancer. Further studies will be needed to determine whether these findings can be generalized to other types of invasive tumors. Nonetheless, our findings illustrate the therapeutic potential of IL-17 inhibition for specific breast cancer types, which represents an attractive approach for the treatment of invasive breast tumors.

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**References**

receptor in normal, benign hyperplastic, and malignant prostate. Prostate 2003;56:171-82.


Figure Legends

Figure 1. IL-17A is produced in murine metastatic mammary tumors. (A) Cytokine production by tumor-infiltrating cells isolated from BALB/c mice...
subjected to an induction protocol with non-metastatic (67NR) and metastatic (4T-1) mammary tumors on day 15 post-inoculation (pi). Cells were stimulated with PMA + ionomycin, and the expression of IL-17A and IFN-γ was analyzed in CD3⁺ cells using flow cytometry. (B) IL-17A and IFN-γ levels (measured by ELISA) in the supernatants of splenocytes isolated at day 0, 15, 25 and 35 pi with 4T-1 and stimulated with anti-CD3+anti-CD28 (closed bars) or unstimulated (open bars) for 72 hours. (C-D) Total numbers of PMA/ionomycin-stimulated, CD3-gated, CD4⁺, CD8⁺ and TCRγδ⁺ T cells producing IL-17A (C) and IFN-γ (D) in isolates from the tumor microenvironment (measured by flow cytometry). The data shown are representative of three independent experiments with similar results and are shown as the mean ± SEM of five mice (* p < 0.05, comparing periods of 15 days to 25 days; + p < 0.05, comparing periods of 25 days to 35 days; # p < 0.05, comparing periods of 15 days to 35 days).

Figure 2. Treatment with anti-IL-17 inhibits the growth of murine mammary tumors. BALB/c mice were subjected to an induction protocol with mammary tumor cells (4T1, squares) or treated with neutralizing anti-IL-17A (filled squares) or rat Immunoglobulin (IgG control) (open squares) antibodies or (67NR, asterisks). (A) The volume of the tumors was measured at 10 dpi. (B) The percent survival was assessed daily. (C) At 35 dpi, the weight of the tumors was measured. (D) Photographs show representative tumors in mice treated with the IgG control (left column) or anti-IL-17 antibodies (right column) at 35 dpi. (E) Immunohistochemical staining in tumor tissue of the mice treated with the IgG control (left column) or anti-IL-17 mAb (right column) at 35 dpi was used to detect Ly6G protein. Scale bars = 50 µm.

Figure 3. Blockade of IL-17 inhibits neutrophil recruitment to the lung in a metastatic mammary tumor model. BALB/c mice were subjected to an induction protocol with mammary tumors and treated with anti-IL-17A or IgG control antibodies. (A) Representative H&E-stained sections of lung tissue collected at 35 dpi; original magnification, 10 x (upper column) and 100 x (bottom column). Scale bars = 50 µm. (B) Flow cytometric analysis of the numbers of neutrophils (Ly6G⁺F4/80⁻) gated on CD11b⁺MHCII⁻ cells and (C) TNFα⁺ cells gated on Ly6G⁺MHCII⁺ cells after isolation from the lung and stimulation with 100 µg/ml of LPS for 6 hours. (D) mRNA was isolated from fragments of lung tissue from both groups at 35 dpi, and the relative expression of MMP-9 was evaluated by qPCR. The data shown are representative of two independent experiments with similar results and are shown as the mean ± SEM of five mice per group (*p < 0.05).

Figure 4. Increased numbers of neutrophils and expression of genes related to the tumorigenic neutrophils phenotype in metastatic murine mammary tumors. BALB/c mice subjected to an induction protocol with non-metastatic (67NR) and metastatic (4T-1) mammary tumor cells at day 0, 15, 25 and 35 post-inoculation (dpi). The numbers of neutrophils (Ly6G⁺F480⁻) gated on CD11b⁺MHCII⁻ cells were analyzed in the population of granulocytes isolated from the spleens (A) and tumors (B) by flow cytometry. (C) Representative, H&E-stained sections of the tumor microenvironment collected at 15, 25 and 35 dpi; original magnification, 10 x (left column) and 100 x (right column). Scale bars = 50 µm. (D) Ly6G⁺F4/80⁻ cells gated on CD11b⁻MHCII⁻ cells were sorted
from the spleens and tumors of 4T-1 tumor-bearing mice, and the expression of MMP-9, VEGF, TNF-α and CXCL1 was analyzed by qPCR. The data shown are normalized to B-actin expression. The data shown represent the means ± SEM compared to Ly6G+F4/80- cells isolated from the spleens of naive mice. (E) The volume of the tumors obtained from BALB/c mice subjected to an induction protocol with mammary tumor cells and treated with anti-Ly6G or rat Immunoglobulin (IgG control) antibodies was measured at 10 dpi. (F) Photographs are representative of tumors in mice treated with IgG control (left column) or anti-Ly6G (right column) antibodies. (G) At 35 dpi, the weight of the tumors was measured. Data are representative of two independent experiments with similar results and are shown as the mean ± SEM of four mice per group (*p < 0.05).

Figure 5. IL-17A induces the expression of IL-6 and CCL20 in 4T-1 tumor cells lines. 67NR and 4T-1 cells were either treated or not treated with rIL-17. After 48 h, the mRNA was extracted and the culture supernatants were collected. (A) The relative expression of IL-6 and CCL20 was measured by qPCR. (B) The IL-6 and CCL20 levels were measured in the culture supernatants by ELISA. The data are representative of two independent experiments with similar results (*p < 0.05). (C) The volume of tumors obtained from BALB/c mice subjected to an induction protocol with (4T-1) metastatic mammary tumor cells and treated with anti-IL-6 or rat Immunoglobulin (IgG control) antibodies was measured at 10 dpi. At 35 dpi, the numbers of (D) Th17 cells (IL-17+CD4+CD45+ gated on CD3+ cells and (E) neutrophils (Ly6G+CD45+ gated on CD11b+MHCII+ cells isolated from the spleen were assessed by flow cytometry. (F) Immunohistochemical staining in tumor tissues of the mice treated with the IgG control (left column) or anti-IL-6 mAb (right column) at 35 dpi was used to detect Ly6G protein. The data are representative of two independent experiments with similar results and are shown as the mean ± SEM of four mice per group (*p < 0.05). BALB/c mice received PBS, 400 ng of CCL20 and/or a 4T-1 or 67NR supernatant injected into the air pouch, and the cell infiltrates were harvested at 20 h post-injection. (G) The total number of IL-17A-producing CD3+ cells harvested per pouch was analyzed by flow cytometry. The data are shown as the mean ± SEM of three mice per group (*p < 0.05).

Figure 6. IL-17A directly affects breast-cancer tumor cell behavior in vivo. 67NR cells were either treated or not treated with rIL-17 in vitro, and after 48 h, these cells were used to inoculate BALB/c mice to induce mammary tumors. (A) The volumes of the tumors were measured at 5 dpi and monitored daily. (B) At 35 dpi, the numbers of neutrophils (Ly6G+CD45+ gated on CD3+ cells were analyzed in the population of granulocytes isolated from the spleen by flow cytometry. (C) Immunohistochemical staining in tumor tissues of the mice receiving untreated 67NR cells (up column) or treated with rIL-17 (down column) at 35 dpi was used to detect Ly6G protein. The data are representative of two independent experiments with similar results and are shown as the mean ± SEM of four mice per group (*p < 0.05). 67NR cells were either treated or not treated with rIL-17. After 48 h, the mRNA was extracted, and the gene expression profiles were analyzed by a microarray. (D) Heat map displaying the hierarchical clustering of the gene expression profiles. The table presents
selected differentially expressed genes of interest. The complete list of the differentially expressed immune response is presented in Supplemental Table S2-3. (E) The Venn diagram shows the number of up-regulated genes in the 4T-1 cells and IL-17-pretreated 67NR cells in relation to the untreated 67NR cells. The intersection of the diagram indicates the number of up-regulated genes in common between the 4T-1 and IL-17-pretreated 67NR cells.

Figure 7. Expression of Th17-promoting cytokines in the tumor microenvironment of patients with IDC. RNA from breast tumor and healthy control tissue was extracted and analyzed by qPCR. (A) The relative expression levels of IL-6 and CCL20 are shown. Data are reported as the mean ± SEM of patients (N = 23) or healthy control subjects (N = 8) who were tested individually. The control values were significantly different compared to those of patients with IDC (*p < 0.05, t-test). (B) Immunohistochemical staining of healthy control (left column) and breast tumor tissue (right column) was used to detect IL-6 and CCL20 protein. Scale bars = 50 µm. RNA from breast tumor tissue was extracted, and the gene expression profiles of samples with low and high IL-17A expression were analyzed by microarray. (C) Principal component analysis of gene expression microarray 3D scatter plot showing the first three principal components; each patient sample is connected to a centroid for each group (IL-17-positive and IL-17-negative). (D) Heat map showing the hierarchical clustering of the gene expression profiles of samples with low and high IL-17A expression as analyzed by microarray. Values are expressed as the relative units of induction (positive values in red), repression (negative values in blue) and no modulation (shown in gray). (E) The bar graph shows the number of genes that were down- and up-regulated. (F) Kaplan-Meier curves illustrate the DFS of patients according to the expression of IL-17A. Log-rank p values for the DFS of patients with low and high gene expression were calculated. The data represent the mean ± SEM of 23 patients with IDC (*p < 0.05).
Figure A shows the expression levels of IL-17A and IFN-γ in 67NR and 4T-1 cells stained with anti-CD3, with the numbers indicating the mean fluorescence intensity (MFI) for each sample.

Figure B illustrates the IL-17 production in different days post-infection (p.i.) for the indicated groups. The graph shows a significant (*p < 0.05) increase in IL-17 production over time.

Figure C displays the numbers of CD3+CD4+ and CD3+CD8+ T-cells, as well as CD3+γδ+ cells, categorized by IL-17A expression levels over days post-infection. The graph indicates a peak in IL-17A expression at 15 days p.i. for CD3+CD4+ cells.

Figure D presents the IFN-γ expression levels for each of the CD3+CD4+, CD3+CD8+, and CD3+γδ+ cell populations over different days p.i. The IFN-γ expression shows a significant increase (#p < 0.05) in CD3+CD8+ cells by 25 days p.i. compared to earlier time points.
IL-17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment

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