The Tumor-Promoting Effect of TNF-α Involves the Induction of *Secretory Leukocyte Protease Inhibitor*[1]

Nick Devoogdt,[2] Hilde Revets, Anne Kindt, Yuan Qing Liu, Patrick De Baetselier, and Gholamreza Hassanzadeh Ghassabeh

According to the cancer immunoediting concept, inflammatory mediators play not only a critical role in promoting host protection against cancer but also contribute to cancer cell growth and survival. TNF-α is a critical factor in this network. However, the mechanisms underlying the tumor-promoting effect of TNF-α have not been fully elucidated yet. We previously reported that in vitro culture of Lewis lung carcinoma 3LL cells with TNF-α-producing macrophages resulted in enhanced resistance toward TNF-α-mediated lysis and increased malignancy of the 3LL cells. In this study, we analyzed the effects of endogenous TNF-α on TNF-α resistance and malignant behavior in vivo of low-malignant/TNF-α-sensitive 3LL-S cells and cancer cells derived from 3LL-S tumors that developed in wild-type or TNF-$\alpha^{-/-}$ mice. Interestingly, 3LL-S cells acquired a malignant phenotype in vivo depending on the presence of host TNF-α, whereas acquisition of TNF-α resistance was TNF-α-independent. This result suggested that malignancy-promoting characteristics of 3LL-S cells other than TNF-α resistance are influenced in vivo by TNF-α. We previously identified the malignancy-promoting genes, *secretory leukocyte protease inhibitor* (*SLPI*) and *S100A4*, as being up-regulated in 3LL-S cells upon their s.c. growth in wild-type mice. In this study, we show that *SLPI*, but not *S100A4*, was induced in 3LL-S cells both in vitro and in vivo by TNF-α, and that silencing of in vivo induced 3LL-S *SLPI* expression using RNA interference abrogated in vivo progression but did not influence TNF-α resistance. These data indicate that *SLPI* induction may be one mechanism whereby TNF-α acts as an endogenous tumor promoter. *The Journal of Immunology,* **2006, 177: 8046–8052.**

It is now well established that the immune system plays a critical role in suppressing tumor development. However, there is growing evidence that the interactions between the immune system and neoplastic cells can also result in the promotion of tumor progression (1, 2). Based on these dual effects of the immune system on developing tumors, Dunn et al. (3) have proposed the cancer immunoediting hypothesis that states that, besides its antitumor activity, the immune system can facilitate tumor progression, at least in part, by sculpting the immunogenic phenotype of tumors as they develop.

The microenvironment in and around tumors that is largely orchestrated by inflammatory cells that secrete proinflammatory cytokines and chemokines, as well as growth factors and matrix-degrading enzymes, plays an indispensable role in the neoplastic process by enhancing cell proliferation, survival, migration, and angiogenesis (4). The proinflammatory cytokine TNF-α, produced by immune cells such as macrophages, T- and B cells, is a pleiotropic cytokine with a paradoxal role in neoplastic disease. Under certain circumstances, TNF-α can selectively destroy tumor vasculature, inducing apoptosis and necrosis of cancer cells, and may serve as an effector molecule for immune-mediated cancer cell destruction (5–7).

In contrast, TNF-α may enhance tumor development and spread by contributing to the generation of an optimal tumor microenvironment, for instance via its influence on the expression of angiogenic and growth factors, cytokines, adhesion receptors, and proteases in both cancer and stromal cells (8). Hence, TNF-α, locally produced within the tumor microenvironment, may contribute to the immunoediting process by selection for and/or generation of cancer cell variants with increased resistance to TNF-α-mediated cytotoxicity and enhanced malignancy.

We previously reported that in vitro culture of 3LL cancer cells in the presence of TNF-α-producing macrophages leads to increased resistance to TNF-α-mediated cytotoxicity and enhanced malignancy of the tumor cells (9). Using this tumor model, we now aimed to analyze the in vivo effects of TNF-α on TNF-α resistance and malignancy of cancer cells, and the interrelationship between these two characteristics.

In this study, we demonstrated that in vivo endogenous TNF-α enhanced the malignancy of cancer cells. The increase in malignancy was shown to correlate with the induction of the promalignant gene *secretory leukocyte protease inhibitor* (*SLPI*) by TNF-α but not with increased resistance of cancer cells to this cytokine. Taken together, these data and our previous finding that *SLPI* is a malignancy-promoting gene (10), we established a new link between TNF-α and cancer cells, whereby host TNF-α contributed to tumor progression and metastasis via induction of *SLPI* expression in cancer cells.

Materials and Methods

Mice

Six- to 8-wk-old female wild-type (WT)[3] C57BL/6 (Harlan) and TNF-$\alpha^{-/-}$ mice (11) (provided by Dr. K. Sekikawa, Department of Immunology, National Institute of Animal Health, Tsukuba City, Japan) were

Department of Molecular and Cellular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Brussels, Belgium

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[2] Address correspondence and reprint requests to Dr. Nick Devoogdt, Department of Molecular and Cellular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Gebouw E, niveau 8, Pleinlaan 2, 1050 Brussels, Belgium. E-mail address: ndevoogd@vub.ac.be

[3] Abbreviations used in this paper: WT, wild type; shRNA, short-harpin RNA; d.p.i., days postinjection; DMBA, dimethylbenz(*a*)anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AU, arbitrary units.

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Table I. *Description of 3LL variants used in this study*

Cell Line	Description	Reference
3LL-S	Subclone of 3LL	(9)
3LL-R	Subclone of 3LL obtained after coculture with inflammatory macrophages	(9)
3LL-S-sc^{WT}	Cancer cells derived from s.c. 3LL-S tumor grown in WT mice	This study
3LL-S-sc^{KO}	Cancer cells derived from s.c. 3LL-S tumor grown in TNF-$\alpha^{-/-}$ mice	This study
2B4, 2C2, and 2C6	3LL-S subclones transfected with pRNAU6.1/Neo plasmid containing SLPI shRNA	This study
5A1 and 5B3	3LL-S subclones transfected with pRNAU6.1/Neo plasmid containing scrambled control shRNA	This study

used in all experiments. Mice were kept at our animal facility in accordance with national legislation and approval of the Animal Experimental Committee of the Vrije Universiteit Brussel.

Cell lines and culture conditions

Different cell lines are described in Table I. Briefly, the 3LL-R variant was generated by coculturing 3LL cells in vitro with inflammatory macrophages and subsequent cloning in soft agar (9). The 3LL-S variant is a subclone from the parental 3LL cells (9). The 3LL-S-sc^{WT} and 3LL-S-sc^{KO} cell lines were obtained by s.c. inoculation of 2×10^6 3LL-S cells in WT and TNF-$\alpha^{-/-}$ mice, respectively, homogenization of the resulting tumor tissue, and in vitro propagation of cancer cells for at least 15 days to eliminate contaminating host cells. All cell lines were maintained in RPMI 1640 supplemented with 0.3 mg/ml L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated FCS (all obtained from Invitrogen Life Technologies).

General molecular techniques

Unless otherwise noted, nucleic acids were handled according to standard protocols. Total RNA was prepared using TRIzol reagent, and reverse transcription was performed using Superscript II Reverse Transcriptase (both obtained from Invitrogen Life Technologies) as recommended by the suppliers. Northern blots were exposed to a phosphorimager screen (Bio-Rad), and gene-specific signals were quantified using the Molecular Analyst software (Bio-Rad).

Analysis of SLPI *and* S100A4 *expression by real-time PCR*

Real-time PCR was performed exactly as described in Ref. 12. Gene expression was normalized using *mouse ribosomal protein S12* as a housekeeping gene, for which primer sequences are described (12). Other primer pairs used are 5′-CACATATACCCTCACAGCAC-3′ and 5′-CTATCAAAATCGGAGCCTGC-3′ for *SLPI*, and 5′-GTACTGTGTCTTCCTGTCC-3′ and 5′-GCTCAGCACTGTGCACATG-3′ for *S100A4*.

Characterization of s.c. 3LL-S tumors in WT and TNF-$\alpha^{-/-}$ mice

s.c. 3LL-S tumors were harvested and frozen in OCT compound (Sakura). Immunohistochemistry was conducted on acetone-fixed frozen sections cut at 8 μm. Specimens were treated with 10% normal rabbit or donkey serum before incubation with appropriately diluted anti-mouse F4/80 (Serotec), CD11b, CD4, CD8, CD19 Abs, and isotype-matched controls (BD Pharmingen) or anti-mouse SLPI Ab (v-17) and isotype-matched control (Santa Cruz Biotechnology). Following an extensive rinse in PBS, appropriately diluted biotinylated secondary Abs (Vector Laboratories) were applied. Positive cells were stained using the sequential application of the Vectastain Elite ABC kit and the diaminobenzidine substrate kit (Vector Laboratories). Specimens were briefly counterstained with hematoxylin before microscopic analysis. Pictures were taken at ×200 (see Fig. 2, *B–I*) or ×100 (see Fig. 3*G*) magnifications.

TNF-α *expression in s.c. 3LL-S tumors*

3LL-S tumors from WT or TNF-$\alpha^{-/-}$ mice were grinded in a mortar using liquid N_2. Total RNA from both in vitro-cultured cells and tumor tissue was isolated, and RT-PCR was performed using Superscript II RT and HotStar *Taq* polymerase (Invitrogen Life Technologies). We used primer pairs 5′-CCTTCACAGAGCAATGACTC-3′ and 5′-GTCTACTCCCAGGTTCTCTTC-3′ for *TNF-α* and primer pairs 5′-TCAACGTCACACTTCATGATG-3′ and 5′-ACACTGTGCCCATCTACGAG-3′ for *β-actin.*

Western blot analysis

Cells were lysed in modified radioimmunoprecipitation assay buffer containing complete protease inhibitor mixture (Roche Diagnostics), sonicated, and equal amounts of total protein were run on a 12% SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane. Membranes were immunoblotted with anti-SLPI (v-17; Santa Cruz Biotechnology) or anti-β-actin (AC-15; Abcam) Abs at 1/1000 or 1/5000 dilutions, respectively, and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Evaluation of tumorigenicity

A total of 2×10^6 cells was injected s.c. in the flank of WT or TNF-$\alpha^{-/-}$ mice, and tumor length (L) and width (W) were measured at different time points using a caliper. The tumor volume (V) was calculated as follows: $V = W \times W \times L \times 0.4$.

Evaluation of experimental metastatic potential

A total of 2×10^6 cells was injected i.v. via the tail vein in WT mice. Experimental metastatic potential was measured by survival of the mice.

In vitro induction of SLPI, S100A4, *and* TNF-α *mRNA in 3LL-S cells by TNF-α*

A total of 5×10^6 3LL-S cells in complete medium was treated for 4 h at 37°C with 0, 0.5, 2, 5, and 20 ng/ml recombinant mouse TNF-α (Innogenetics). Viability of the treated cells was >95% as measured by the trypan blue exclusion test. Gene expression levels were evaluated by real-time PCR.

Generation of 3LL-S SLPI and scrambled control short-hairpin RNA (shRNA) transfectants

shRNA constructs were generated in the pRNAU6.1/Neo plasmid, as described by the provider (GenScript). The sequence 5′-AGGCAAGATGTATGATGCTTA-3′ was selected as *SLPI*-targeting sequence, and the scrambled sequence 5′-TCTAATTGTAGCAAGGATAGG-3′ was used as control. SLPI and scrambled control shRNA constructs were electroporated in 3LL-S cells, and cells were subcloned and selected in complete medium containing 1.75 mg/ml G418 (Invitrogen Life Technologies). *SLPI* expression levels in transfectants were evaluated by real-time PCR.

Evaluation of TNF-α-mediated cytotoxicity

TNF-α-induced cancer cell cytotoxicity was measured through uptake of the membrane impermeable dye propidium iodide (Calbiochem) as described previously (13). Briefly, 5×10^4 cells were incubated for 24 h in the presence 0, 0.5, 5, 50, or 500 ng/ml TNF-α. After addition of propidium iodide (10 μg/ml), cellular fluorescence was measured in a fluorescence-activated cell sorting analyzer (FACSVantage; BD Biosciences). Living and dead cells were quantified using the CellQuest program (BD Biosciences). Percentage of lysis is determined as [percentage of dead cells/(percentage of living + percentage of dead cells)] × 100.

Statistical analysis

Statistical analyses were performed by the two-tailed unpaired Student's *t* test.

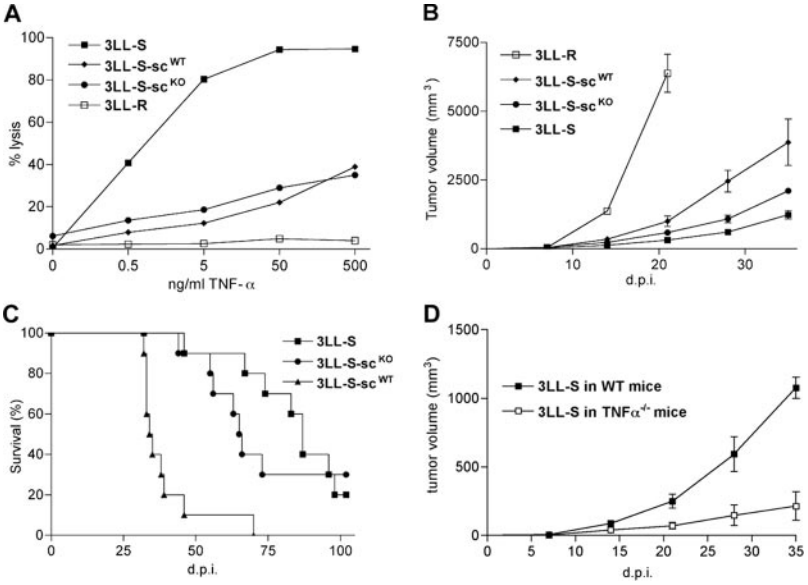

FIGURE 1. Sensitivity to TNF-α-mediated cytotoxicity and malignancy in WT or TNF-$\alpha^{-/-}$ mice of 3LL-S, 3LL-R, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells. *A*, Lysis of 3LL-S, 3LL-R, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells by increasing concentrations of recombinant mouse TNF-α. *B*, Tumorigenicity of 3LL-S, 3LL-R, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells injected s.c. in WT mice. *C*, Survival of WT mice injected i.v. with 3LL-S, 3LL-S-sc^{WT}, or 3LL-S-sc^{KO} cells. *D*, Tumorigenicity of 3LL-S cells injected s.c. in WT or TNF-$\alpha^{-/-}$ mice. All data shown are representatives of at least three independent experiments. Resistance of 3LL-S-sc^{WT} and 3LL-S-sc^{KO} cells to TNF-α-mediated lysis was comparable with several similarly obtained ex vivo variants.

Results

s.c. growth of 3LL-S cells enhances their malignancy and resistance to TNF-α-mediated cytotoxicity

Previous experiments have shown that coculture of 3LL cells in vitro with TNF-α-producing macrophages resulted in the generation of a variant (3LL-R) with increased resistance to TNF-α-mediated cytotoxicity (Fig. 1*A*) and enhanced malignancy (Fig. 1*B*; $p = 0.005$ at 21 days postinjection (d.p.i.)) as compared with 3LL-S cells that were not cocultured with these macrophages (9). To investigate the effect of TNF-α on these characteristics of cancer cells in vivo, low-malignant/TNF-α-sensitive 3LL-S cells were injected s.c. in WT C57BL/6 mice, and tumor-derived cancer cells (hereafter referred to as 3LL-S-sc^{WT}) were analyzed for their malignancy and resistance to TNF-α-mediated cytotoxicity.

Upon s.c. growth of 3LL-S cells in WT mice, these cancer cells became more malignant, as evidenced by the higher tumorigenicity of 3LL-S-sc^{WT} cells as compared with 3LL-S cells (Fig. 1*B*; $p = 0.0009$ at 35 d.p.i.). The increase in malignancy of 3LL-S-sc^{WT} cells was also reflected by a shorter survival time of mice that were inoculated i.v. with these cells, as compared with mice receiving 3LL-S cells (Fig. 1*C*; $p < 0.0001$). Concomitantly, local growth of 3LL-S cells also increased their resistance to TNF-α-mediated cytotoxicity (Fig. 1*A*).

Hence, upon local in vivo growth in WT mice, 3LL-S cells simultaneously acquired an increased malignant phenotype and enhanced resistance to TNF-α-mediated cytotoxicity.

Endogenous TNF-α contributes to the in vivo progression of 3LL-S cells but not to increased TNF-α resistance

To further address the role of host TNF-α in the in vivo progression of 3LL-S cells, s.c. growth of these cells was compared between WT and TNF-$\alpha^{-/-}$ mice. As shown in Fig. 1*D*, 3LL-S tumors grew significantly faster in the flank of mice expressing endogenous TNF-α than in TNF-$\alpha^{-/-}$ mice ($p = 0.0006$ at 35 d.p.i.), demonstrating that endogenous TNF-α exerted a tumor growth-promoting effect in this model.

Next, cancer cells derived from s.c. 3LL-S tumors grown in TNF-$\alpha^{-/-}$ mice (hereafter referred to as 3LL-S-sc^{KO} cells) were compared with 3LL-S-sc^{WT} and 3LL-S cells in function of their capacity to form s.c. tumors and to metastasize in WT mice, and in respect to their sensitivity to TNF-α-mediated cytotoxicity. 3LL-S-sc^{KO} cells were significantly less malignant than 3LL-S-sc^{WT} cells as evidenced by their reduced tumorigenicity (Fig. 1*B*; $p = 0.008$ at 35 d.p.i.) and prolonged survival time of mice upon i.v. inoculation (Fig. 1*C*; $p = 0.0004$) in WT mice. Surprisingly, 3LL-S cells grown s.c. in the absence of endogenous TNF-α also acquired enhanced resistance to TNF-α-mediated cytotoxicity. Indeed, 3LL-S-sc^{KO} cells were as resistant to lysis by TNF-α as 3LL-S-sc^{WT} cells (Fig. 1*A*). Therefore, in vivo TNF-α enhanced the malignancy of 3LL-S cells but not their resistance to TNF-α-mediated cytotoxicity.

To investigate whether TNF-α was locally produced within the tumors, RT-PCR was performed on 3LL-S total tumor tissues. These experiments revealed *TNF-α* expression in tumor tissues grown in WT mice. Under these conditions, we did not detect *TNF-α* mRNA in 3LL-S tumors that developed in TNF-$\alpha^{-/-}$ recipients, nor in in vitro-cultured 3LL-S cells (Fig. 2*A*).

Next, the infiltrate of 3LL-S tumors grown s.c. in TNF-$\alpha^{-/-}$ mice was studied by immunohistochemistry and compared with that of tumors grown in WT animals. Both in WT and TNF-$\alpha^{-/-}$ mice, staining for F4/80 (Fig. 2, *B* and *C*) and CD11b markers (data not shown) revealed massive infiltration of macrophages in 3LL-S tumors, especially at tumor edges. 3LL-S tumors were also infiltrated by $CD4^+$ (Fig. 2, *D* and *E*) and $CD8^+$ T lymphocytes (Fig. 2, *F* and *G*), but not by B lymphocytes, as evaluated by staining with an anti-CD19 Ab (data not shown). Moreover, these studies revealed that overall the infiltration of 3LL-S tumors by macrophages, $CD4^+$ and $CD8^+$ T lymphocytes, and B lymphocytes was not affected by endogenous TNF-α (Fig. 2, *B–G*).

Taken together, the RT-PCR and immunohistochemical analyses suggested that *TNF-α* mRNA detected in tumor tissue in WT mice was produced by local tumor-infiltrating cells. However, these data do not exclude the possibility that part of this *TNF-α* mRNA was produced by 3LL-S cells themselves in response to host TNF-α. Yet, as measured by quantitative real-time PCR, in vitro stimulation of 3LL-S cells with 0.5, 2, 5, and 20 ng/ml recombinant TNF-α did not induce *TNF-α* mRNA expression in these cells (data not shown).

Collectively, these data showed that during 3LL-S tumor formation, locally produced TNF-α contributed to increased tumor growth and malignancy of cancer cells but not to their resistance to TNF-α-mediated cytotoxicity. Hence, host TNF-α does not seem

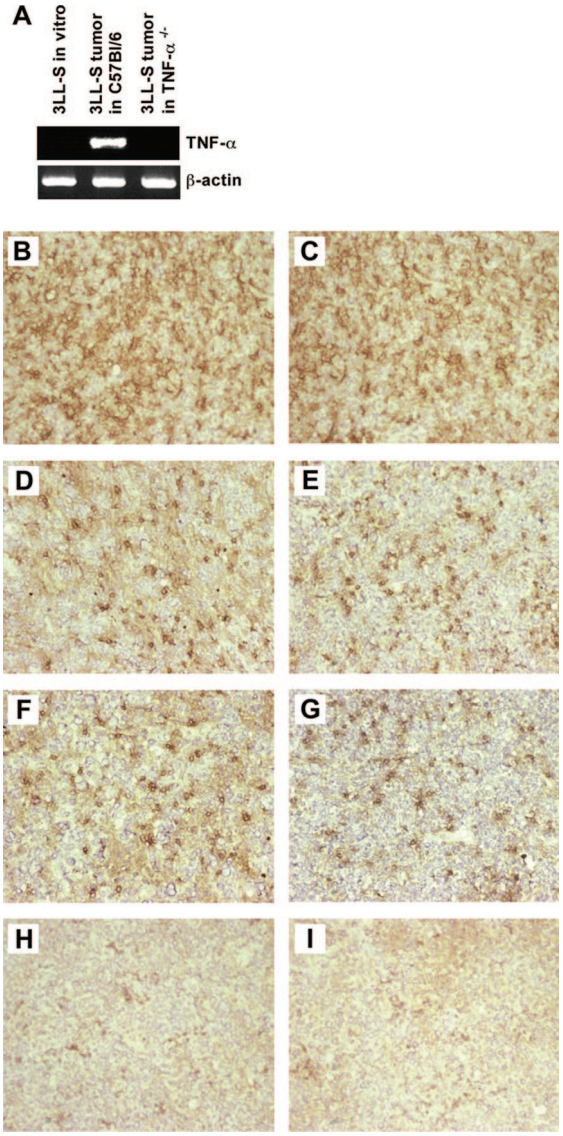

FIGURE 2. Local TNF-α expression and immune cell infiltration of s.c. 3LL-S tumors grown in WT or TNF-$\alpha^{-/-}$ mice. *A*, RT-PCR for *TNF-α* and *β-actin* on 3LL-S cells and s.c. 3LL-S tumor tissue grown in WT or TNF-$\alpha^{-/-}$ mice. Two independent experiments were performed. *B–I*, Immunohistochemical analysis of sections from s.c. 3LL-S tumors grown in WT (*B*, *D*, *F*, and *H*) or TNF-$\alpha^{-/-}$ mice (*C*, *E*, *G*, and *I*). Sections were immunostained for the macrophage marker F4/80 (*B* and *C*) and T cell markers CD4 (*D* and *E*) and CD8 (*F* and *G*). Immunostaining with the control Ab is shown in *H* and *I*. All original magnifications were ×200. Two independent immunostainings were performed on sections from four different tumors isolated from WT or TNF-$\alpha^{-/-}$ mice. Representative pictures are shown.

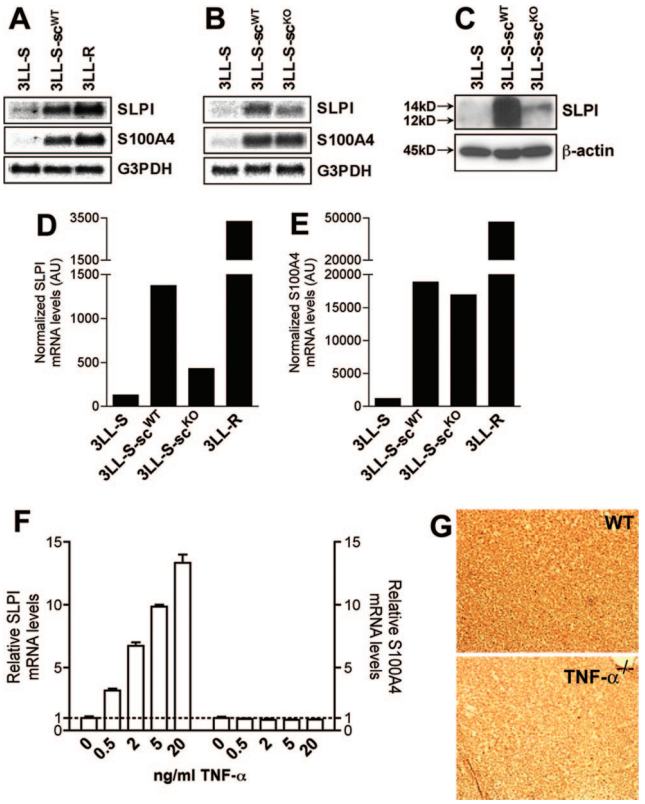

FIGURE 3. *SLPI* and *S100A4* expression in 3LL-S, 3LL-R, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells. *A* and *B*, Northern blot analysis of *SLPI*, *S100A4*, and *G3PDH* in in vitro-cultured 3LL-S, 3LL-R, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells. Representative pictures of at least five experiments are shown. *C*, Western blot analysis of SLPI and β-actin in lysates of in vitro-cultured 3LL-S, 3LL-S-sc^{WT}, and 3LL-S-sc^{KO} cells. Representative pictures of three experiments are shown. *D* and *E*, Expression levels of *SLPI* (*D*) and *S100A4* (*E*) after normalization of Northern blot signals against the housekeeping gene *G3PDH*. *F*, Real-time PCR analysis for *SLPI* (*left*) and *S100A4* (*right*) mRNA expression in 3LL-S cells treated for 4 h with increasing concentrations of recombinant mouse TNF-α. Representative data of at least three experiments are shown. *G*, Immunohistochemical analysis of SLPI expression in sections of 3LL-S s.c. tumors isolated from WT (*top panel*) or TNF-$\alpha^{-/-}$ (*bottom panel*) mice. All original magnifications were ×100. Representative pictures of immunostainings of sections from four different tumors are shown.

to promote 3LL-S tumor progression via an immunoediting process involving TNF-α-mediated cytotoxicity, suggesting that the tumor-promoting effect of TNF-α is not likely mediated by selection for and/or generation of cancer cell variants with increased TNF-α resistance.

Endogenous TNF-α induces the expression of SLPI, *but not* S100A4, *in 3LL-S cells*

We previously reported the increased expression of the malignancy-promoting genes *SLPI* and *S100A4* during 3LL-S tumor progression in WT mice (10). In this study, we investigated whether TNF-α affected *SLPI* and *S100A4* expression in 3LL cells. To this end, *SLPI* and *S100A4* expression was compared between 3LL-R cells (i.e., cells obtained by in vitro coculture of 3LL cells with TNF-α-producing macrophages) and control 3LL-S cells. Northern blot analyses (Fig. 3*A*) revealed that *SLPI* and *S100A4* expression was 26- (Fig. 3*D*) and 41-fold (Fig. 3*E*) higher in 3LL-R than in 3LL-S cells, respectively. In addition, 3LL-S cells were treated in vitro for 4 h with increasing concentrations of TNF-α, and *SLPI* and *S100A4* expression was measured via real-time PCR. TNF-α induced the expression of *SLPI* in a dose-dependent manner: 0.5, 2, 5, and 20 ng/ml TNF-α resulted in 3-, 7-, 10-, and 14-fold induction, respectively, of *SLPI* in 3LL-S cells (Fig. 3*F*, *left*; $p < 0.01$ for all concentrations). In contrast, *S100A4* expression was not affected under these conditions (Fig. 3*F*, *right*; p = NS).

Next, to evaluate the gene-inducing activity of endogenous TNF-α during 3LL-S tumor progression, *SLPI* and *S100A4* expression was compared between 3LL-S-sc^{WT} and 3LL-S-sc^{KO} cells. As shown in Fig. 3, *B* and *C*, endogenous TNF-α clearly contributed to increased *SLPI* mRNA and protein expression upon s.c. growth of 3LL-S cells. Indeed, the *SLPI* mRNA level in 3LL-S-sc^{KO} cells was only 3-fold higher than that in 3LL-S cells, as

compared with an 11-fold induction in 3LL-S-sc^{WT} cells (Fig. 3*D*). In contrast, no significant differences in *S100A4* expression levels were observed between 3LL-S-sc^{WT} and 3LL-S-sc^{KO} cells (Fig. 3, *B* and *E*).

Finally, to confirm that SLPI protein production depends on endogenous TNF-α at the tumor site, immunohistochemistry was performed with an anti-mouse SLPI Ab on sections of s.c. 3LL-S tumors that developed either in WT or in TNF-$\alpha^{-/-}$ mice. Detection of SLPI was uniform and diffuse with some cellular immunostaining, as expected for a secreted protein (Fig. 3*G*). Ag specificity was confirmed by immunohistochemistry with an isotype-control Ab (data not shown). Importantly, SLPI protein detection was reproducibly more prominent in sections of 3LL-S tumors that developed in WT mice as compared with tumors derived from TNF-$\alpha^{-/-}$ mice, demonstrating that endogenous TNF-α contributes strongly to SLPI protein production at the tumor site (Fig. 3*G*).

SLPI *is required for 3LL-S tumor progression but does not influence TNF-α sensitivity*

We previously reported that *SLPI* overexpression in 3LL-S cells enhanced their malignant behavior (10). In this study, to investigate whether *SLPI* induction by local environmental factors including TNF-α plays a major role in tumor progression of 3LL-S cells, cells were stably transfected with a *SLPI*-down-regulating shRNA (SLPI shRNA) construct and subsequently analyzed for their capacity to form s.c. tumors in WT or TNF-$\alpha^{-/-}$ animals. A scrambled SLPI shRNA sequence served as negative control in these experiments. Three 3LL-S SLPI shRNA transfectants (2B4, 2C2, and 2C6) with ~90% reduction in their *SLPI* mRNA levels ($p < 0.01$) as compared with 3LL-S cells and two control shRNA transfectants (5A1 and 5B3) were generated (Fig. 4*A*; *SLPI* mRNA levels in control transfectants and 3LL-S cells were not significantly different). In agreement with our previously published overexpression studies (10), down-regulation of *SLPI* expression in 3LL-S cells dramatically reduced their capacity to form s.c. tumors in WT mice. Indeed, whereas control transfectants were tumorigenic in WT mice, SLPI shRNA transfectants completely regressed (Fig. 4*B*; $p < 0.02$ when each SLPI shRNA transfectant was compared with each control transfectant at 28 d.p.i.). Thus, counteracting in vivo-induced *SLPI* expression in 3LL-S cells by RNA interference abrogated tumor progression. As expected, tumors of 3LL-S SLPI shRNA transfectants invariably regressed also in TNF-$\alpha^{-/-}$ (data not shown; p = NS when comparing tumor volume between WT and TNF$\alpha^{-/-}$ mice for each SLPI shRNA transfectant at 28 d.p.i.). Furthermore, corroborating with the discordant growth of 3LL-S cells in WT and TNF-$\alpha^{-/-}$ mice (Fig. 1*D*), control transfectants grew faster in the presence than in the absence of endogenous TNF-α (Fig. 4*C*; $p = 0.01$ and 0.08 when comparing tumor volume between WT and TNF-$\alpha^{-/-}$ mice at 28 d.p.i. of 5B3 and 5A1, respectively).

Serine protease inhibitors have been reported to inhibit cytotoxicity mediated by TNF-α (14, 15). However, the fact that 3LL-S-sc^{KO} cells were as resistant to the cytotoxic activity of TNF-α as 3LL-S-sc^{WT} cells (Fig. 1*A*), although their *SLPI* expression levels were significantly different (Fig. 3, *B–D*), did not favor a protective role for the serine protease inhibitor SLPI against the cytotoxic action of TNF-α on 3LL-S cells. Yet, to completely exclude such a role, 3LL-S *SLPI* and control shRNA transfectants were tested for their sensitivity to TNF-α-mediated cytotoxicity. These experiments revealed that *SLPI* did not affect TNF-α-mediated cytotoxicity of 3LL-S cells. Indeed, both 3LL-S SLPI and control shRNA transfectants were as sensitive to lysis by TNF-α as 3LL-S cells (Figs. 1*A* and 4*D*). Our previously reported (10) *SLPI*-overexpressing and mock-transfected 3LL-S cells also did not differ in their TNF-α sensitivity (data not shown).

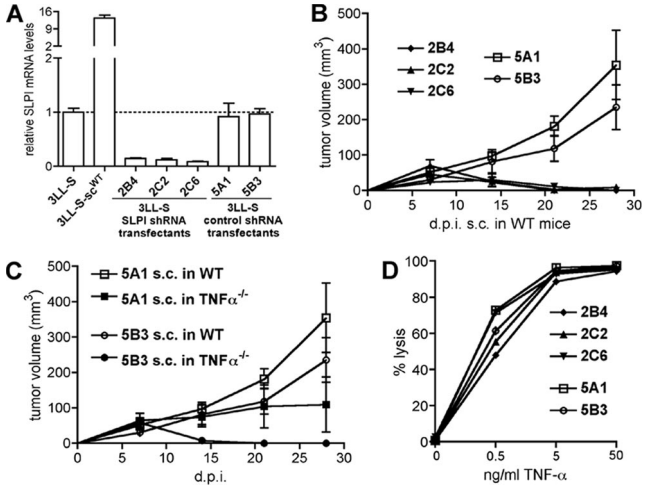

FIGURE 4. Effect of shRNA-mediated *SLPI*-silencing in 3LL-S cells on s.c. growth in WT or TNF-$\alpha^{-/-}$ mice and resistance to TNF-α-mediated cytotoxicity. *A*, Relative *SLPI* expression in 3LL-S, 3LL-S-sc^{WT}, 3LL-S SLPI shRNA-transfectants 2B4, 2C2, and 2C6, and 3LL-S scrambled control shRNA transfectants 5A1 and 5B3 as determined by real-time PCR analysis. Representative data from five independent experiments are shown. *B*, Tumorigenicity of 2B4, 2C2, 2C6, 5A1, and 5B3 injected s.c. in WT mice. *C*, Tumorigenicity of 5A1 and 5B3 injected s.c. in WT or TNF-$\alpha^{-/-}$ mice. Data depicted in *B* and *C* are representatives of two independent experiments. *D*, Cell lysis of 2B4, 2C2, 2C6, 5A1, and 5B3 by recombinant mouse TNF-α.

Collectively, these experiments establish TNF-α-inducible *SLPI* as a key tumor-promoting gene for 3LL-S cancer cells, independent of TNF-α resistance.

Discussion

Recent reports have expanded the appreciation of a role for the neoplastic microenvironment in tumor progression and led to the cancer immunoediting concept to describe more accurately the dual host-protecting and tumor-sculpting actions of the immune system. It has become apparent that the cross-talk between cancer cells and their close surrounding, including innate immune cells that secrete cytokines among other signaling molecules, can result in both tumor promotion and progression (3, 16). TNF-α is a cytotoxic immune-effector molecule for many types of cancer cells and may be regarded as a part of such an immunoediting process (5). Growing evidence from different animal cancer models indicates that the inflammatory mediator TNF-α may promote cancer development and spread (8).

In this study, we scrutinized a tumor model based upon s.c. growth of TNF-α-sensitive/low-malignant 3LL-S cells that allows us to investigate the dichotomic nature of TNF-α, i.e., tumor promotion vs cancer prevention. In this model, endogenous TNF-α exerted tumor-promoting activity: 3LL-S tumors developed faster in WT as compared with TNF-$\alpha^{-/-}$ mice, and cancer cells derived from s.c. 3LL-S tumors that formed in WT mice were more malignant than those derived from tumors that developed in TNF-$\alpha^{-/-}$ animals. Upon s.c. growth, TNF-α-sensitive 3LL-S cells acquired a TNF-α-resistant phenotype, even in the absence of endogenous TNF-α. However, there was no correlation between TNF-α resistance and malignancy, suggesting that acquisition of TNF-α resistance might be required but not sufficient to confer a full malignant phenotype upon these cells. Yet, we cannot exclude the possibility that TNF-α resistance, as measured by an in vitro

cytotoxicity assay, might not truly reflect the in vivo situation in which a complex interplay between various molecules might affect the extent to which these cells can be sensitive to different cytotoxic molecules, including TNF-α itself. Nevertheless, while probing the tumor-promoting role for TNF-α, we investigated whether *SLPI* and *S100A4*, which we formerly identified as malignancy-promoting genes during 3LL-S tumor progression (10), are involved in the TNF-α-driven tumorigenicity in this model. Our results showed that *SLPI* mRNA and protein expression was increased to a much higher extent when 3LL-S cells were grown in WT than in TNF-$\alpha^{-/-}$ mice. In addition, in vitro treatment of 3LL-S cells with TNF-α increased *SLPI* expression, whereas *S100A4* expression remained unaffected. Finally, shRNA-mediated *SLPI* silencing in 3LL-S cells completely abrogated the capacity of these cells to develop tumors. Collectively, these data demonstrated that 1) TNF-α stimulated tumor progression of 3LL-S cells; 2) the tumor-promoting activity of TNF-α was due, at least in part, to the induction of *SLPI* expression; and 3) TNF-α-inducible *SLPI* is required for full in vivo progression of 3LL-S cells.

It should be noted that, in a TNF-α-free 3LL-S tumor environment, *SLPI* expression was still induced, although to a level much lower than that in a TNF-α-containing environment (Fig. 3, *B* and *C*). Hence, besides TNF-α, other host factors can contribute to the induction of *SLPI* expression. In fact, we found that IL-1β, which was also detected in the 3LL-S tumor microenvironment, increased *SLPI* expression in 3LL-S cells in vitro to ~3-fold (data not shown).

Using the 3LL tumor model, we found TNF-α to be expressed in s.c. 3LL-S tumors that developed in WT mice, but not in tumors that developed in TNF-$\alpha^{-/-}$ animals. The observation that s.c. 3LL-S tumors were highly infiltrated by macrophages and T cells, suggested that either of these host cells might be the source of locally produced TNF-α.

Several indications prompted us to study whether *SLPI* expression in 3LL-S cells rendered them less susceptible to TNF-α-mediated cytotoxicity: 1) s.c. growth not only increased malignancy and *SLPI* expression of 3LL-S cells but also their resistance to TNF-α-mediated cytotoxicity; 2) coculture of 3LL cells with inflammatory macrophages in vitro increased both resistance to TNF-α-mediated cytotoxicity and *SLPI* expression levels of 3LL cells; and 3) serine protease inhibitors inhibit TNF-α-mediated cytotoxicity (14, 15). However, because *SLPI* expression levels in 3LL-S, ex vivo variants, 3LL-S SLPI overexpressors, and SLPI shRNA and control transfectants, did not correlate with the extent to which these cells were lysed by TNF-α, we concluded that *SLPI* expression and resistance to TNF-α lysis were not interrelated.

The finding that augmentation of cancer cell *SLPI* expression by host TNF-α might play a role in the malignancy-promoting activity of this cytokine is consistent with observations made in other mouse models of TNF-α-enhanced tumorigenesis. For instance, in a dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinogenesis model, carcinogen-induced skin cancer formation is severely impaired by treatment with antagonistic TNF-α Abs or in the absence of host TNF-α and TNF-α signaling (17–19). Most notably, the TPA-induced promotion phase of tumor formation, characterized by epigenetic alterations in gene expression, is most significantly affected by the absence of endogenous TNF-α, and *SLPI* was identified as one of the genes most prominently induced by TPA in the dorsal skin and with very high expression levels in advanced stages of DMBA/TPA-induced skin tumors (20). Similarly, in an inflammation-associated model of liver cancer, treatment with antagonistic TNF-α Abs resulted in apoptosis of transformed hepatocytes and failure to develop hepatocarcinomas (21). Both acute and chronic phases preceding carcinogenesis were associated with concomitantly increased *TNF-α* and *SLPI* expression levels (22). All of these data and our study are in line with the characteristics of *SLPI* as an acute phase inflammation-responsive gene inducible by cytokines such as TNF-α (23, 24) and promoting tumor formation. SLPI also exhibits anti-inflammatory properties, for instance, by inhibiting activation of the inflammatory mediator NF-κB (25). SLPI might therefore be expected to inhibit inflammation-induced tumor progression. Indeed, SLPI has been demonstrated to inhibit liver metastasis of 3LL cells by attenuating the host inflammatory response (26). It is worth mentioning that we did not observe any effect of *SLPI* expression on NF-κB activity in various 3LL variants used in this study, neither under constitutive conditions nor when these cells were treated with TNF-α (data not shown). Taken together (these data and ours), it can be suggested that the role of *SLPI* in tumor progression may depend on the type of target tissue and/or type of inflammatory stimuli. Further studies are needed to clarify which parameters determine whether *SLPI* exerts tumor-promoting or anti-inflammatory/cancer-preventing activities during inflammation-associated carcinogenesis.

The mechanism(s) by which *SLPI* promotes malignancy is not yet known, although we have demonstrated that the promalignant activity of *SLPI* is dependent on its protease inhibitory activity (10), independent of its ability to enhance cancer cell proliferation (10), and not due to a protective role against TNF-α-mediated cytotoxicity (this study). Several possible mechanisms for the promalignant activity of *SLPI* have been proposed (27). For instance, SLPI has been shown to stimulate fibroblasts to produce hepatocyte growth factor (28), a potent stimulator of tumor growth and progression (29). Also, SLPI was shown to bind to proepithelin, a pleiotropic, positive regulator of cancer cell malignancy (30), thereby protecting it from degradation by proteases (31). Additional studies should be performed to address these possibilities but are beyond the scope of the present work.

In conclusion, this study dissects three characteristics that are increased upon s.c. growth of 3LL-S cells and their contribution to malignant behavior: *SLPI* and *S100A4* expression, and resistance to TNF-α-mediated cytotoxicity. Although resistance to TNF-α may contribute to the malignant behavior of 3LL cells, in this study we showed that other factors, such as *SLPI* and *S100A4*, are causally involved. The expression of *SLPI* in 3LL-S cells was regulated at least in part by TNF-α, providing a plausible explanation for the tumor-promoting activity of endogenous TNF-α in our tumor model. In fact, our data indicate that the immunoediting oeuvre of TNF-α goes further than the induction of resistance of cancer cells to TNF-α-mediated cytotoxicity, but also includes malignancy sculpting via the induction of *SLPI*, independent of TNF-α resistance. Therefore, in addition to identifying a new mechanism linking TNF-α and cancer, our results identified a new type of sculpting feature by which TNF-α promotes neoplastic disease through induction of *SLPI* and in turn represents a new target for treatment of inflammation-associated cancer.

Disclosures

The authors have no financial conflict of interest.

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