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Ablation of TNF or lymphotoxin signaling and the frequency of spontaneous tumors in p53-deficient mice

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Abstract

TNF plays diverse and contrasting roles in cancer, promoting skin carcinogenesis and metastasis, but also possessing potent antitumor effects in mice. TNF via TNFR1 axis induces NFκB, and may contribute to inflammation-facilitated neoplasia. On the other hand, lymphomas are cited as rare complications of anti-TNF therapy in humans. In order to address possible modulating role of TNF and of a related cytokine, LTα, in spontaneous tumorigenesis, we compared mice with p53-TNF, p53-LTα, p53-TNFR1 and p53-TNF-LT combined deficiencies. Unexpectedly, neither of these mice showed significant modulation of their survival or shift in the spectrum of emerging tumors, as compared to p53-deficient mice, arguing against direct link between TNF blockade and lymphoma development.

Keywords: Cytokine and chemokine biology; p53/mdm2; Tumor immunology; Knockout mice

1. Introduction

TNF was discovered due to its striking tumor-necrotizing activity in mice [1] in which case its primary target were cells of vascular endothelium [2–4]. Later, studies employing TNF gene deficient mice revealed unexpected function of TNF in development and maintenance of lymphoid tissue architecture and resolution of inflammatory reactions [5–8].

Whether TNF is pro- or anti-tumorigenic in vivo constitutes an important public health issue, since over a million of autoimmune patients worldwide are placed on continuous pharmacological TNF

Abbreviations: TNF, tumor necrosis factor; LT, lymphotoxin; TNFR, TNF receptor; NFκB, nuclear factor kappa B; KO, knockout.

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blockade [9,10]. Lymphomas are cited as rare complication of the therapy, although no clear mechanistic link to TNF ablation was established. If TNF is overall pro-tumorigenic or pro-metastatic, then some of these patients may actually benefit from its blockade. In mouse cancer studies TNF was found both tumor-promoting, as in skin carcinogenesis model [11,12], and participating in immune surveillance [13].

In order to evaluate the net effect of these possible opposite functions of TNF signaling in vivo, we took advantage of mice deficient in tumor suppressor p53 [14]. Spontaneous tumor development both in homozygous and heterozygous states of p53 deficiency has been widely utilized to evaluate the activity of candidate modifier genes. In this study we addressed possible modifying role of TNF and of a closely related cytokine, LTα.

2. Results and discussion

2.1. Genetic ablation of TNF or LTα does not result in significant changes in survival of p53-null mice

In agreement with published reports [15,16], p53-KO mice developed tumors by 3–5 months with a median survival around 20 weeks of age (Fig. 1A). Mice with double TNF-p53 deficiency showed only marginal increase in survival (Fig. 1A), arguing against any major role for TNF in tumor protection. On the other hand, in spite of established pro-carcinogenic TNF function in chemically induced skin tumors [11,12] we found no evidence for the modifying role of TNF in the development of spontaneous lymphomas in p53-null mice.

We then addressed in a similar experiment a possible contribution of LTα in spontaneous tumor development. LTα is a TNF-like cytokine which can engage TNF receptors, but in combination with LTβ it predominantly signals in vivo through a separate receptor, LTβR [17]. The net result of the LTα ablation in this model was difficult to predict. LTα is involved in NK cell differentiation, so its ablation may affect primary tumor growth and metastasis [18,19]. Additionally, local expression of LTα by tumor cells was shown to inhibit tumor growth [20]. On the other hand, in murine fibrosarcoma model the LTα/β → LTβR signaling axis was shown to play a pro-tumorigenic role [21].

LTα-deficient mice have defective development of lymphoid tissues [22,23]. Tumors, in particular lymphomas, are known to emerge from the thymus [24] and peripheral lymphoid organs, including mesenteric lymph nodes and Peyer's patches [25]. Should there be any significant modulation of tumor-free survival, the interpretation of the data would not be straightforward without additional experiments, as the LTα mice have anatomical defects

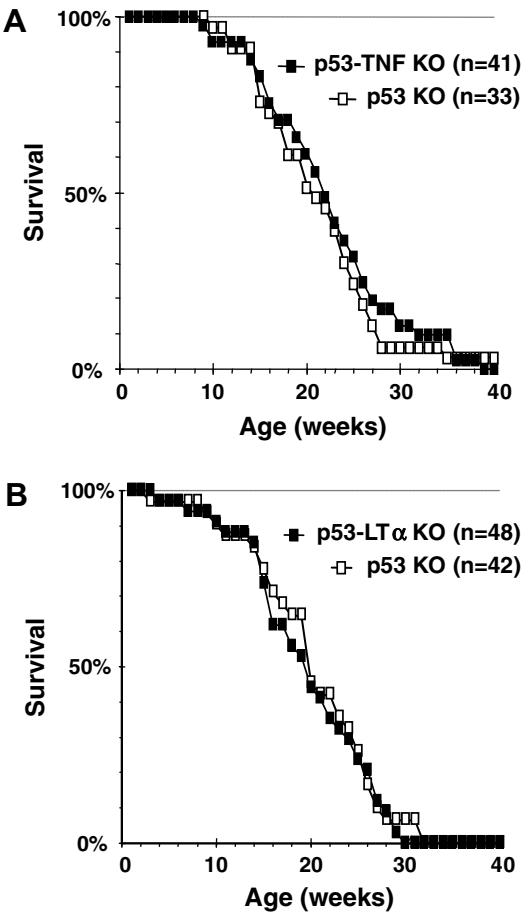

Fig. 1. Spontaneous tumorigenesis in p53-TNF, p53-LTα double deficient mice. (A) p53-TNF versus p53-null. (B) p53-LTα versus p53-null.

which may be related to spread of tumors in mice: they completely lack lymph nodes and Peyer's patches [22,23], i.e. sites where lymphomas may arise. Yet, the insensitivity of tumor-free survival of p53-deficient mice to LTα inactivation (Fig. 1B) not only suggested that LTα signaling is not important for tumorigenesis on this cancer-prone background, but also argued against the role of lymph nodes, Peyer's patches and correct microarchitecture of the spleen for tumor development and spread, at least in this model of cancer.

2.2. Genetic ablation of TNFR1 or combined ablation of TNF and LT does not significantly influences the disease onset or the spectrum of tumors in either $p53^{-/-}$ or $p53^{+/-}$ mice

The negative result with single TNF or LTα deficiencies did not formally exclude a redundant pro-tumorigenic or protective function mediated by these two related cytokines. Indeed, TNF and LTα can signal through the same

receptors, TNFR1 (p55) and TNFR2 (p75). TNFR1 is one of the main activators of the classical NFκB pathways in vivo [26,27]. Chronic inflammation and activation of NFκB may lead to cancer, especially when combined with inactivation of various modifier genes such as Mdr2 [8] or APC [28].

However, the tumor-free survival of double homozygous mice with TNFR1 and p53 deficiencies (Fig. 2A) suggested no pro- or anti-tumorigenic effects of TNFR1 ablation and perhaps revealed only a slight tendency to delay tumor-related mortality. Another model allowing to address the redundancy of TNF and LTα signaling in vivo, is TNF/LTβ/LTα triple-deficient mice [29]. While TNFR1 deficiency only affects TNF → TNFR1 and LTα → TNFR1 signaling, mice with triple cytokine deficiency also lack LTβ → LTβR, TNF → TNFR2 and LTα → TNFR2 cascades, which all potentially might modulate tumorigenesis.

Our results with the quadruple KO mice on C57BL/6 genetic background (see supporting information online) are in agreement with p53-TNFR1 data and suggested no significant effects of these multiple deficiencies in cytokine signaling on tumor (in particular, lymphoma) development.

Finally, $p53^{+/-}$ mice represent a model for spontaneous tumor growth which mimics the loss of only one allele of p53 tumor suppressor gene and is better related to hereditary human cancers, such as Li-Fraumeni syndrome. Therefore, the contribution of TNFR1 ablation was also evaluated in $p53^{+/-}$ mice (Fig. 2B). In this case the median survival was extended to more than 60 weeks, and the tumor-free survival curves were overlapping for the earlier time points with a tendency to slightly delayed tumor-related death in $p53^{+/-}$, $TNFR1^{-/-}$ mice. Thus, neither genetic ablation of TNFR1 nor combined ablation of TNF and LT does not grossly affect tumor-free survival of p53-null mice.

The spectrum of emerging tumors (Table 1) did not significantly differ between p53 KO and LTα/TNF/LTβ/p53 KO mice or between p53 KO and TNFR1/p53 KO mice, with lymphomas constituting about half of the tumors. In spite of the fact that $p53^{+/-}$ mice remained tumor-free much longer than p53-null mice (Fig. 2B), lymphomas clearly remained the dominant type of tumors.

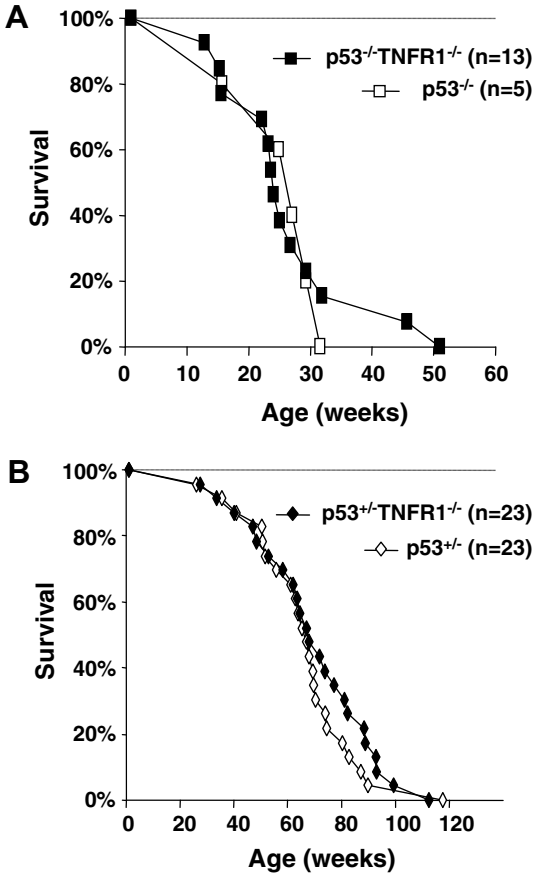

Fig. 2. Spontaneous tumorigenesis in p53-TNFR1 double deficient mice. (A) $p53^{-/-}TNFR1^{-/-}$ versus p53-null. (B) $p53^{+/-}TNFR1^{-/-}$ versus $p53^{+/-}$.

3. Concluding remarks

Recent advances in understanding the molecular mechanisms linking chronic inflammation and cancer resulted in a growing interest in the role of TNF as one of the key inflammatory mediators of cancer development [30]. LT has received much less attention, even though LTβR agonists were suggested as potential anti-cancer therapeutics [31] (but see also [21]). Strong LTα activation (most likely, in infiltrating T-cells) via alternative NFκB pathway was reported in a mouse model of prostate cancer [32], however, it remained unclear whether LT played any causative role in this system. The involvement of TNF-mediated inflammation in mouse models was demonstrated for a number of tumor types which are frequently associated with inflammation in humans. Examples include mice bearing orthotopically growing pancreatic tumors [33], melanoma cells [34] and a number of others. However, owing to complexity of TNFR signaling, the possible role of TNF in cancer development appears more complex than just one of inflammatory mediators. For example, TNF has recently been implicated in rejection of transplantable MCA-induced sarcomas [35] and in immune surveillance against spontaneous pancreatic tumors [13]. The pro-inflammatory role of TNF has been demonstrated in several mouse models of gastrointestinal cancer, with inflammation

Table 1
Tumor types in various mice with p53 and TNF/TNFR1 deficiencies

Histologic findings	Experiment 1		Experiment 2		Experiment 3	
	$p53^{-/-}$	$p53^{-/-}$ TNF/$LT^{-/-}$	$p53^{-/-}$	$p53^{-/-}$ $TNFR1^{-/-}$	$p53^{+/-}$	$p53^{+/-}$ $TNFR1^{-/-}$
Number of lesions						
Lymphoma	22	17	3	8	14	12
Sarcoma	11	11	1	1	5	4
Carcinoma	3	1		2	3	2
Others	8	2	1	2	1	5
Total	44	30	5	13	23	23

In Experiment 1, the actual number of mice analyzed for tumor types was 24 for $p53^{-/-}$TNF/$LT^{-/-}$ and 32 for $p53^{-/-}$ mice. Four mice had double and 1 mouse had triple lesions in $p53^{-/-}$TNF/$LT^{-/-}$ mice, and 8 mice had double and 2 mice had triple lesions in $p53^{-/-}$ mice.

induced either by chemical treatment [36] or with transgenic expression of inflammatory mediators [37]. However, no evidence was found for the role of TNF in spontaneous tumor development in a classical model of intestinal cancer based on mutations in APC tumor suppressor gene [38].

TNF blockers are widely used drugs to treat autoimmune disease, such as rheumatoid arthritis, Crohn's disease, psoriatic arthritis and psoriasis [39]. If endogenous TNF plays a role in protection against cancer as suggested by recent reports [13,35], then its continuous blockade may facilitate neoplasia in some of immunocompromised patients. On the other hand, if pro-carcinogenic activities of TNF observed in mice translate to humans [40], then continuous blockade of TNF signaling may have some beneficial effects for patients. Additional benefits may be due to inhibition of reported pro-metastatic role of TNF [41,42].

Our findings do not support the hypothesis on the protective role of TNF (or LTα) in spontaneous tumorigenesis in p53-deficient mice. On the other hand, – within the limitations of the experimental system used – we found that tumor-promoting role of TNF is minor.

4. Materials and methods

4.1. Mice

p53-null mice [43] were from Jackson lab or from Bomholtgaard Breeding Facilities, Denmark. TNF-deficient mice and LTα-deficient mice were described previously [7,23]. All mice were housed in SPF conditions. Experiments using animals were done under properly approved animal protocols for S.A.N. (animal facility NCI Frederick, MD, USA) and T.B. (animal facility at the Max-Delbrück-Center, Berlin-Buch, Germany). Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). Since not all mice deficient in TNF/LT ligands were fully backcrossed to C57BL6 background by the beginning of our experiments, matched experimental groups ($p53^{-/-}$ and double or multiple deficiency) were generated by first obtaining double heterozygotes ($p53^{+/-}$, $TNF^{+/-}$ or $p53^{+/-}$, $LT\alpha^{+/-}$ and so on) and then intercrossing the offspring. In order to maximize the yield of experimental animals, the breeding was then performed as $p53^{+/-} \times p53^{+/-}$ on either wild type or TNF/LT single or multiple deficient background. All progenies were genotyped for p53 locus, and $p53^{-/-}$ mice or $p53^{+/-}$ were monitored for tumor formation and survival. Genotyping was performed by PCR using DNA from tail biopsies and the following primers: p53X7 5′-CAC ATG TAC TTG TAG TGG ATG G; p53X65 5′-ACA GCG TGG TGG TAC CTT AT; p53neo19 5′-CTA TCA GGA CAT AGC GTT GG (for p53 locus); KO41 5′-TGA GTC TGT CTT AAC TAA CC; KO42 5′-CCC TTC ATT CTC AAG GCA CA; KO49 5′-CTC TTA AGA CCC ACT TGC TC (for TNF locus); Pri1lta 5′-CTT GTG TCT GTC TTG CGT; Pri2lta 5′-GTC TCT CGG CAG TTA AGC; pri7lta 5′-ATA ACT GTG ACT TGA ACC (for LTα locus).

TNFR1-deficient were described previously [44]. The p53 and TNFR1 double knockout mice were generated by breeding a single $p53^{-/-}$ male with several $TNFR1^{-/-}$ female mice. The offsprings were then intercrossed to obtain $p53^{-/-}$ $TNFR1^{-/-}$, $p53^{-/-}$ $TNFR1^{+/-}$, $p53^{+/-}$ $TNFR1^{-/-}$ and $p53^{+/-}$ $TNFR1^{+/-}$ mice. Genomic typing of mice was performed by PCR using the following primers: 5′-CTC TCT TGT GAT CAG CAC TG; 5′-CTG GAA GTG TGT CTC AC for TNFR1 locus and 5′-CTG GAA GTG TGT CTC AC; 5′-CCA AGC GAA ACA TCG CAT CGA GCG A for neo^R gene.

4.2. Pathology

A necropsy was performed on each mouse when moribund. Tumor and other tissues were fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin/eosin. Only mice that succumbed to a confirmed tumor were included in the survival analysis.

4.3. Statistical analysis

Survival data sets were compared using Wilcoxon two sample test (http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html) and no significant difference was found in any of the experiments shown ($p > 0.05$).

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