

Inhibition of Soluble Tumor Necrosis Factor Prevents Chemically Induced Carcinogenesis in Mice

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Abstract

TNF is a potent promoter of carcinogenesis and potentially important target for cancer prevention. TNF is produced as functionally distinct transmembrane and soluble molecules (tmTNF and sTNF, respectively), but their individual roles in carcinogenesis are unexplored. We investigated the participation of tmTNF and sTNF in chemically induced carcinogenesis in mice. We found that injection of XPro1595, a dominant-negative TNF biologic (DN-TNF) and specific antagonist of sTNF, decreased tumor incidence and growth, and prolonged survival of 3-methylcholanthrene (MCA)-injected mice. Similar results were obtained following the exclusion of both TNF forms by either TNF-receptor 2-Fc fusion protein (TNFR2-Fc) treatment or *TNF* gene deletion. In addition, gene deletion of TNFR1, which is preferentially triggered by sTNF, was temporarily blocked, whereas gene deletion of TNFR2, which is preferentially triggered by tmTNF,

enhanced MCA-induced carcinogenesis. Concomitantly with carcinogenesis induction, MCA increased circulating IL1 α , accumulation of myeloid-derived suppressor cells (MDSC), STAT3 phosphorylation, and immunosuppression in the spleen. In sharp contrast, DN-TNF treatment dramatically decreased IL1 α and increased the essential immunoregulatory cytokines IL1 β , IL12p70, and IL17 in the peripheral blood of MCA-injected mice. In addition, MDSC accumulation, STAT3 phosphorylation, and immunosuppression in MCA-injected mice were prevented by DN-TNF treatment, TNFR2-Fc treatment, and/or gene deletion of TNF or TNFR1, but not deletion of TNFR2. These findings reveal that sTNF is both an essential promoter of carcinogenesis and a pivotal regulator of MDSCs, and indicate that sTNF could be a significant target for cancer prevention and therapy. *Cancer Immunol Res*; 4(5); 441–51. ©2016 AACR.

Introduction

Causal links of chronic inflammation and carcinogenesis have been increasingly documented (1, 2). The first indications were provided by epidemiologic findings that virtually all cancers arise in tissues undergoing chronic inflammation (2). The increased secretion of proinflammatory cytokines associated with gene polymorphisms was also found to be linked to increased risks to various inflammatory diseases and cancers (3, 4).

More pertinent evidence has been obtained in mouse models that have been modified to have either activated or suppressed NF- κ B in specific tissues (5). Specifically, *Mdr2*-knock-out mice spontaneously develop cholestatic hepatitis, a form

of chronic inflammation that triggers via TNF NF- κ B signaling in hepatocytes and leads to hepatocellular carcinoma. In contrast, these mice fail to develop cancer if activation of NF- κ B is suppressed by neutralization of TNF or when hepatocyte-specific inducible *I κ B*-super-repressor transgene is introduced (6). Similarly, mice lacking NF- κ B-activator *IKK β* in intestinal epithelial or myeloid cells show decreased colon-tumor incidence or size, respectively, after being fed with azoxymethane (AOM)/dextran-sulfate-sodium (DSS), a colitis-associated cancer inducer (7).

An inflammation-carcinogenesis link has been demonstrated in mice having overexpressed or deleted genes encoding proinflammatory cytokines or their receptors. Gastric-mucosa overexpression of *IL1 β* leads to local inflammation, recruitment of myeloid-derived suppressor cells (MDSC), and cancer development (8). In contrast, mice lacking the *TNF* gene are resistant to chemical carcinogenesis in the skin (9, 10). Similarly, *TNFR1* gene-deficient mice show reduced AOM/DSS-caused colon inflammation and tumor incidence (11). Extending these findings and supporting the central role for TNF in inflammation, carcinogenesis, and targeted cancer prevention, TNF neutralization by TNFR2-Fc (ENBREL) leads to reduction of carcinogen-induced inflammation and tumorigenesis (10).

The mechanism of TNF involvement in carcinogenesis has been partially elucidated. The combination of TNF, IFN γ , and IL1 β activates inducible nitric oxide synthase, leading to excessive production of nitric oxide, which causes DNA damage and inhibits DNA-repair proteins (12). More importantly, TNF directly induces transformation of carcinogen-initiated fibroblasts (13),

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inhibits carcinogen-induced cell death, and promotes cell proliferation via NF- κ B activation (14).

TNF has both transmembrane (tmTNF) and soluble (sTNF) forms (15). Transmembrane TNF is expressed on cell membrane as functional homotrimers of 26-kDa type-II protomers. The soluble form is also a homotrimer, but consists of the 17-kDa extracellular domain of tmTNF that was cleaved by TNF α -converting enzyme. Discovered as an endotoxin-induced serum factor causing hemorrhagic necrosis of tumors, and applied with limited use in treatment of limb sarcoma, TNF has been subsequently defined as a pleiotropic cytokine that mediates a number of vital functions, including structural organization of secondary lymphoid organs, apoptosis, cell proliferation/survival, inhibition of intracellular pathogens, immunoregulation, and inflammation (15). In addition, TNF deregulation plays important roles in pathogenesis of granuloma formation, autoimmune diseases, acute-phase reaction, septic shock, fever, and cachexia. These diverse functions are induced via cognate interactions of the two forms of TNF with two transmembrane receptors, TNFR1 and TNFR2.

Although TNF is mainly made by activated macrophages, TNFR1 is ubiquitously expressed by almost all mammalian cells, and TNFR2 is only expressed by immune and endothelial cells (15). Different immune cells express different levels of the two receptors. Both TNF receptors bind both TNF forms, but sTNF and tmTNF preferentially trigger TNFR1 and TNFR2, respectively (15, 16). The receptors for TNF have structurally distinct intracellular domains and transduce different signaling pathways. However, although only TNFR1 is associated with the signaling molecules that transduce apoptosis, both TNF receptors can bind molecules that transduce inflammation and signal cell survival. Due to the shared signaling molecules, both TNF receptors can crosstransduce signals to one another (15, 16). Because of this complexity, the specificity of sTNF and tmTNF stimulation of their preferred receptors is variable.

Recent research has begun to elucidate the individual roles of tmTNF and sTNF in immunity and inflammation. Mouse and human dendritic cell (DC)/natural killer (NK)-cell cross-talk, a central immunoregulatory mechanism that induces skewing and increases Th1-type cytokines and antitumor immunity, is a non-redundant function of tmTNF–TNFR2 interaction, not involving the sTNF–TNFR1 axis (17–19). In contrast, inflammation-driven autoimmune diseases can be prevented by selective sequestration of sTNF, or genetic deletion or antibody blockade of TNFR1, showing that inflammation is mediated preferentially by sTNF–TNFR1 interaction (20, 21). However, the individual roles of sTNF and tmTNF in carcinogenesis are still unexplored.

In the present study, we demonstrate a pivotal role of sTNF in expansion of MDSCs, development of immunosuppression, and promotion of carcinogenesis and tumorigenesis in a murine model of chemically induced carcinogenesis. These findings suggest that sTNF could be an important target for cancer prevention.

Materials and Methods

Mice

Eight-week-old wild-type C57BL/6 (B6), T-cell/B-cell-deficient SCID (B6.129S7-*Rag1*^{tm1mom}/J), TNF-deficient (TNFko, B6.129S-*Tnf*^{tm1Gkl}/J), TNFR1-deficient (TNFR1ko, B6.129S2-*Tnfrsf1a*^{tm1Mak}/J), and TNFR2-deficient (TNFR2ko, B6.129S2-*Tnfrsf1b*^{tm1Mwm}/J) female mice were purchased from The

Jackson Laboratory. All mice were homozygous and bred by standard brother–sister mating at The Jackson Laboratory. Mice were housed at the University of Pittsburgh Cancer Institute's Association for Assessment and Accreditation of Laboratory Animal Care internationally accredited animal facility. The animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents

The following reagents were used in the present study: human-recombinant IL2 (Chiron Corp.); TLR4 ligand, *Escherichia coli* lipopolysaccharide (LPS; Lonza); human TNFR2–Fc fusion protein (Amgen); clinical-grade pegylated DN-TNF XPro1595 biologic (20; Xencor); FITC-conjugated anti-CD3, anti-CD4, anti-CD11b, and anti-CD45R mAbs; phycoerythrin (PE)-conjugated anti-CD3, anti-Gr1, anti-CD25, anti-NK1.1, anti-CD11c, and anti-F4/80 mAbs; allophycocyanin-conjugated anti-Ly6C, anti-FoxP3, and anti-CD8 mAbs (all anti-mouse CDs); corresponding fluorochrome-conjugated isotype-control mAbs (eBioscience); mouse Quantikine IFN- γ ELISA kit (R&D Systems); and the Millipore mouse 32-plex cytokine Kit.

Induction, treatment, and measurement of carcinogenesis *in vivo*

Wild-type and TNF-, TNFR1-, and TNFR2-deficient B6 mice were injected s.c. with 0.1 mg MCA dissolved in 0.1 mL sesame oil (Sigma). Wild-type mice were then randomly divided into groups of 10 to 15 mice. PBS and its XPro1595-DN-TNF and ENBREL–TNFR2–Fc solutions (200 μ g/0.5 mL/mouse) were injected i.p. twice a week, for 12 weeks, beginning on the day of MCA injection. Appearance of 2-mm diameter tumors was detected by palpation twice weekly. Tumor growth was determined by measuring two perpendicular tumor diameters with calipers twice weekly. Data are presented as multiplications of two tumor diameters of individual tumors and their mean values. Survival was scored daily.

In vitro suppression of DC/NK-cell cross-talk

Endogenous DC/NK-cell cross-talk was assessed as follows. Splenocytes (2×10^6 /mL) of untreated and treated wild-type mice were resuspended in complete cell-culture medium (CM: RPMI-1640 medium, 0.1 mmol/L nonessential amino acids, 2 mmol/L sodium pyruvate, 1 mmol/L L-glutamine, 100 μ g/mL streptomycin, 100 U/mL penicillin, 10% FCS, Life Technologies; 50 μ mol/L 2-mercaptoethanol, Bio-Rad) supplemented with 1 μ g/mL LPS and 6,000 IU/mL IL2. The cell suspensions were seeded in 96-well round-bottom plates (BD-Biosciences) and incubated at 37°C for 24 hours.

Cell-mediated suppression of DC/NK-cell cross-talk was assessed as follows. SCID-mouse bulk splenocytes (composed of 50% NK1.1⁺CD3⁻ NK cells, 30% CD11c⁺ iDCs, and 20% F4/80⁺ monocytes/macrophages) served as NK-cell/DC cross-talk responders (22). Bulk splenocytes of untreated or MCA-injected (MCA/PBS-, MCA/DN-TNF-, or MCA/ENBREL-treated) wild-type mice served as stimulators/suppressors of SCID-mouse DC/NK-cell responders. The responders and stimulators/suppressors were suspended in CM, supplemented with LPS (1 μ g/mL) and IL2 (6,000 IU/mL), seeded either alone or mixed in 1:1 and 1:3 cell-to-cell ratios in 96-well round-bottom plates, and incubated at 37°C for 24 hours.

The assays were performed in triplicate. Following the incubations, cell-free supernatants were examined for the presence of IFN γ using ELISA.

Flow cytometry

Standard cell-surface flow cytometry with fluorochrome-conjugated antibodies was performed as described (17). B cells (CD45R⁺F4/80⁻), macrophages (CD45R⁻F4/80⁺), NK cells (CD3⁻NK1.1⁺), DCs (CD11b⁺CD11c⁺), CD4 (CD3⁺CD4⁺CD8⁻) and CD8 (CD3⁺CD4⁻CD8⁺) T cells, and monocytic (CD11b⁺Gr1^{lo/-}Ly6C^{+/hi}) and granulocytic (CD11b⁺Gr1^{hi}Ly6C⁺) MDSCs were examined using direct multicolor cell-surface staining. Regulatory T cells (Treg; CD4⁺Foxp3⁺CD25^{hi}) were examined as per the manufacturer's protocol (Biolegend). Phosphorylated STAT3 was investigated using PE-conjugated antiphosphorylated STAT3-tyrosine-705 (pSTAT3y705), Alexa488-conjugated anti-pSTAT3-serine-727 (pSTAT3s727), and corresponding isotype-control mouse-IgG2a mAbs (BD Biosciences), as described (23). Cells were analyzed on Beckman-Coulter CyAN ADP cytometer. Data analyses were performed using Summit 4.3 software (Beckman-Coulter).

Quantification of cytokines

Serum cytokines were quantified using the Millipore-multiplex mouse-cytokine kit, as recommended by the company. IFN γ was measured in the cell-culture-conditioned media using the mouse-IFN γ Quantikine ELISA Kit (R&D).

Statistical analysis

Data were statistically evaluated using 10.0-SPSS (SPSS Inc.) and 3.0.2.-R (www.R-project.org) software. Data are reported as mean \pm SD. Statistical significance was assessed using the Student *t* test. In addition, cancer appearance (tumor-development cumulative hazard) and survival time were analyzed using the Kaplan–Meier method. Exact log-rank tests were used to compare cancer-appearance and survival-time curves of experimental and control groups. $P \leq 0.05$ was considered statistically significant.

Results

Soluble TNF sequestration prevents MCA-induced carcinogenesis

DN-TNF selectively sequesters sTNF without affecting tmTNF and inhibits inflammations without affecting major immune mechanisms controlling intracellular pathogens and cancer (20, 21). Differently, TNFR2-Fc neutralizes both sTNF and tmTNF and inhibits both inflammations and major immune mechanisms (17–20). We initially examined the impact of XPro1595-DN-TNF and ENBRELEL-TNFR2-Fc on MCA-induced carcinogenesis in wild-type B6 mice (Fig. 1A). We found that 4-mm² tumors appear in all mice regardless of treatment on week 4 after MCA injection. The incipient tumors were more frequent in PBS-treated than in ENBRELEL-treated and, especially, DN-TNF-treated mice. The differences in tumor frequencies between PBS-treated and ENBRELEL-treated, PBS-treated and DN-TNF-treated, or ENBRELEL-treated and DN-TNF-treated mice were significant ($P = 0.009$, $P < 0.00005$, and $P = 0.027$, respectively).

To confirm the initial findings and evaluate in more detail the roles of sTNF and tmTNF in carcinogenesis, we compared the effects of PBS- and DN-TNF treatment (sTNF sequestration) in

wild-type mice with the effects of *TNF*, *TNFR1*, and *TNFR2* deletion (exclusions: sTNF and tmTNF, sTNF receptor and tmTNF receptor, respectively) in the gene-deficient mice on MCA-induced carcinogenesis. Tumor incidence (Fig. 1B), tumor size (Fig. 1C), and survival (Fig. 1D) were monitored. The time and incidence of tumor occurrence varied between the experimental groups (Fig. 1B). In PBS-treated wild-type and *TNFR2*-deficient mice, 4-mm² tumors appeared early and in the majority of animals (week 4: 6/10 and 7/10 mice, respectively). In these mouse groups, tumor incidence reached maximum (8/10 mice) on weeks 6 and 10, respectively. In sharp contrast, DN-TNF sequestration of sTNF, exclusion of sTNF activity by knocking out *TNFR1*, or exclusion of both sTNF and tmTNF by knocking out *TNF* led to a delayed (3 to 4 weeks) and infrequent (1/10, 2/10, and 2/10 mice, respectively) occurrence of tumors. Although the low tumor incidence remained unchanged in *TNF*-deficient and DN-TNF-treated mice, it notably increased in *TNFR1*-deficient mice (from 2/10 to 6/10 mice) at week 9 after MCA injection. Tumor frequency differences of PBS-treated versus DN-TNF-treated, *TNF*-deficient, and *TNFR1*-deficient mice were significant ($P = 0.0007$, $P = 0.0016$, and $P = 0.031$, respectively). Similarly, the differences in tumor frequencies of *TNFR2*-deficient versus DN-TNF-treated, *TNF*-deficient, and *TNFR1*-deficient mice were also significant ($P = 0.0013$, $P = 0.0026$, and $P = 0.029$, respectively).

Tumors grew slowly or remained dormant in all groups of mice until week 10 after MCA injection. Afterward, in the test groups with frequent tumors (PBS-treated wild-type and *TNFR1*- and *TNFR2*-deficient mice), the tumors grew with a variable velocity and differed widely in size within each group, at all time points of their measurements. In sharp contrast, the infrequent tumors in DN-TNF-treated and *TNF*-deficient mice remained small (4–9 mm²) and dormant until week 12 after MCA injection. Thus, between weeks 10 and 14 after MCA injection, tumors were significantly larger in PBS-treated, *TNFR1*-deficient, and *TNFR2*-deficient mice than in DN-TNF-treated and *TNF*-deficient mice (vs. DN-TNF: $P = 0.017$, $P = 0.023$, and $P = 0.029$; vs. *TNF*-knockout: $P = 0.027$, $P = 0.031$, and $P = 0.036$, respectively; Fig. 1C).

The lower tumor frequency and slower tumor growth in DN-TNF-treated and *TNF*-deficient mice than in PBS-treated, *TNFR1*-deficient, and *TNFR2*-deficient mice resulted in significant prolongation of mouse survival. At the experiment's endpoint, day 112 (week 16) after MCA injection, 9 of 10 DN-TNF-treated and 10 of 10 *TNF*-deficient mice were alive. In sharp contrast, 5 of 10 *TNFR1*-deficient (vs. DN-TNF, TNFko: $P = 0.001$ and $P = 0.003$, respectively), 4 of 10 PBS-treated (vs. DN-TNF and TNFko: $P = 0.0026$ and $P = 0.0037$, respectively), and 1 of 10 *TNFR2*-deficient (vs. DN-TNF and TNFko: $P = 0.00033$ and $P = 0.00012$, respectively) mice were alive (Fig. 1D).

These findings demonstrate that sTNF is critical, whereas tmTNF is dispensable, for MCA-induced carcinogenesis. They also suggest that tmTNF, in contrast to sTNF, could have a protective role in carcinogenesis.

Sequestration of sTNF modulates immunoregulatory cytokines

MCA-induced cancers are immunogenic (24). Therefore, sTNF exclusion-induced increased resistance to MCA-induced carcinogenesis could be caused by a modified and/or enhanced antitumor immune response. Type and quantity of secreted cytokines define type and effectiveness of antitumor immune responses. We next examined cytokines in the sera of healthy/untreated

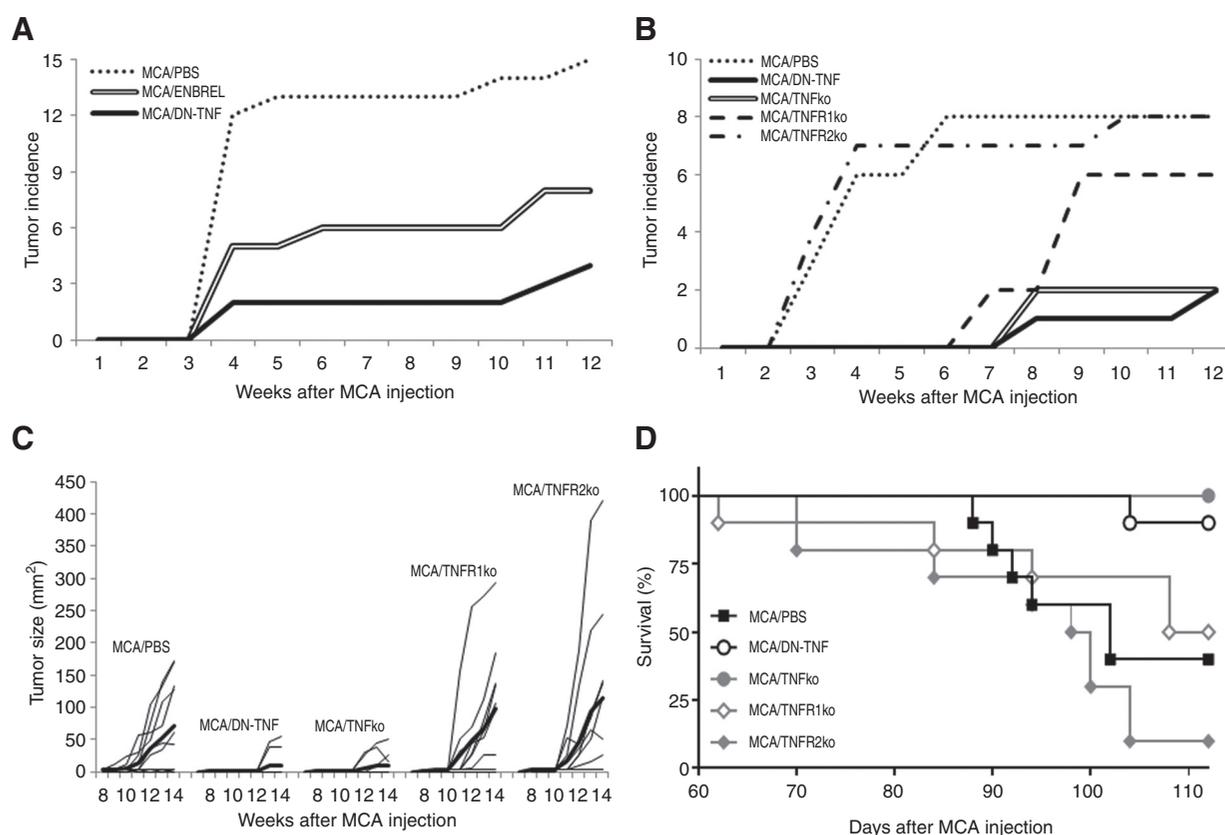


Figure 1.

Sequestration of sTNF or deletion of *TNF* or *TNFR1* genes protects mice from MCA-induced carcinogenesis. A, wild-type B6 mice were injected s.c. with MCA as described in Materials and Methods. The MCA-injected mice were randomized into three groups of 15 mice each. The control-group mice were injected i.p. with PBS (0.5 mL). The two other groups of mice were injected i.p. with either ENBREL or XPro1595 (DN-TNF; 200 μ g/0.5 mL PBS/mouse), respectively. The injections of PBS, ENBREL, and DN-TNF were performed twice a week, for 12 weeks, starting on the day of MCA injection. B–D, 20 wild-type, 10 *TNF*-deficient (TNFko), 10 *TNFR1*-deficient (TNFR1ko), and 10 *TNFR2*-deficient (TNFR2ko) B6 mice were injected with MCA, as described above. Ten of the MCA-injected wild-type mice were also injected i.p. with PBS, whereas the other 10 mice were injected with XPro1595 biologic. The MCA-injected gene-deficient mice were left untreated. MCA injections and treatments were performed as described in A. A and B present the cumulative tumor incidence. C presents the individual tumor sizes and their mean (thick lines). D, cumulative survival. The analyses were performed as described in Materials and Methods. In A, the differences of MCA/PBS versus MCA/ENBREL or MCA/DN-TNF, and MCA/ENBREL versus MCA/DN-TNF are significant ($P = 0.009$, $P < 0.00005$, and $P = 0.027$, respectively). In B, the differences of MCA/PBS versus MCA/DN-TNF, MCA/TNFko and MCA/TNFR1ko; and MCA/TNFR2ko versus MCA/DN-TNF, MCA/TNFko and MCA/TNFR1ko are significant ($P = 0.0007$, $P = 0.0016$, and $P = 0.031$; $P = 0.0013$, $P = 0.0026$, and $P = 0.029$, respectively). In C, the differences of MCA/PBS versus MCA/DN-TNF and MCA/TNFko; MCA/TNFR1ko versus MCA/DN-TNF and MCA/TNFko; and MCA/TNFR2ko versus MCA/DN-TNF and MCA/TNFko are statistically significant ($P = 0.017$, $P = 0.027$; $P = 0.023$, $P = 0.031$; $P = 0.029$ and $P = 0.036$, respectively). In D, the differences of MCA/DN-TNF versus MCA/TNFR1ko, MCA/PBS and MCA/TNFR2ko; MCA/TNFko versus MCA/TNFR1ko, MCA/PBS and MCA/TNFR2ko; and MCA/TNFR2ko versus MCA/PBS and MCA/TNFR1ko are statistically significant ($P < 0.0001$, $P < 0.0003$, $P < 0.0001$; $P < 0.0001$, $P < 0.0001$, $P < 0.0001$; $P = 0.037$ and $P = 0.029$, respectively).

(control) and MCA-injected/PBS-, ENBREL- or DN-TNF-treated mice, 2 weeks after MCA injection (Fig. 2). In MCA-injected/PBS-treated mice, the central proinflammatory cytokines IL1 β , IL12p40/p70, and IL17 were either unchanged (IL1 β , IL17; Fig. 2A and C) or slightly decreased (IL12p40/p70; Fig. 2B) relative to control mice. In contrast, the levels of these cytokines increased significantly in MCA-injected/DN-TNF-treated mice (IL1 β : $P = 0.026$; IL12p40/70: $P = 0.05$; IL17: $P = 0.039$). The cytokines did not change in MCA-injected/ENBREL-treated mice. In contrast, IL1 α levels were high in control mice, notably increased in MCA-injected/PBS-treated mice, but strongly decreased below control levels by ENBREL ($P = 0.012$) or DN-TNF ($P = 0.023$) treatment. These findings indicate that sTNF suppresses and sTNF sequestration enables inflammasome (IL1 β), Th1 (IL12), and Th17 (IL17) responses in MCA-injected

mice. Because these responses did not differ in control and MCA-injected/ENBREL-treated mice (neutralized sTNF and tmTNF), the increased responses after sTNF-sequestration could be mediated by tmTNF. In addition, the strong suppression of IL1 α by ENBREL or DN-TNF indicates that sTNF upregulates IL1 α . Intriguingly, at this early stage of carcinogenesis, sTNF was not detectable in the sera of MCA-injected mice. However, sTNF could be initially present in biologically significant quantities in tumor tissues (1).

Sequestration of sTNF averts MDSC accumulation

Both carcinogens and cancer induce strong immunosuppression, which enables cancer development and growth (25–27). MDSCs are a heterogeneous population of immature myeloid cells that strongly suppress immune functions (28). Although MDSCs are rare in healthy organisms, their frequency and activity

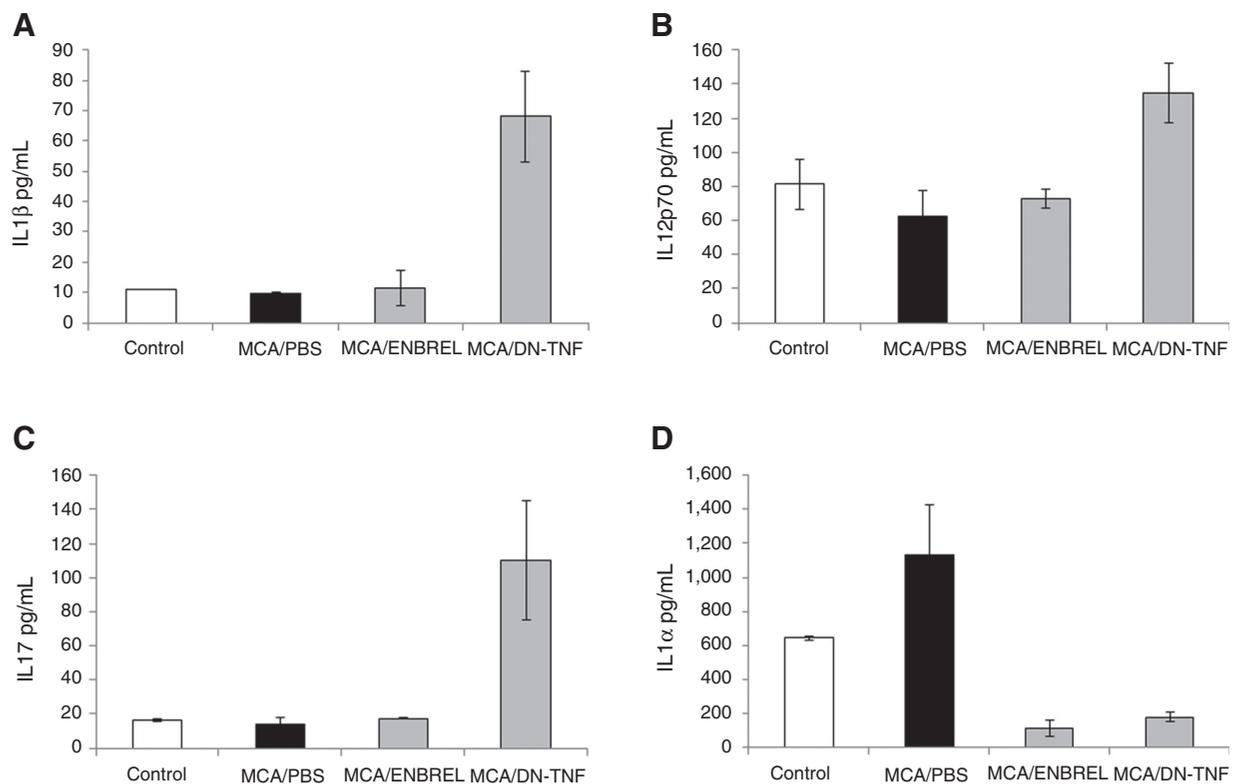


Figure 2.

Sequestration of sTNF by DN-TNF and neutralization of both sTNF and tmTNF by TNFR2-Fc affect secretion of immunoregulatory cytokines in MCA-injected mice. Healthy/untreated (control), MCA-injected/PBS-treated (MCA/PBS), MCA-injected/ENBREL-treated (MCA/ENBREL), and MCA-injected/XPro1595-treated (MCA/DN-TNF) wild-type mice (3 mice per each group) were sacrificed, and their sera obtained 14 days after MCA injection. Sera were examined for the presence of IL1 β (A), IL12p40/70 (B), IL17 (C), and IL1 α (D) using the LUMINEX assay. The data represent mean values of triplicates \pm SE. In A, B, and C, the increases of cytokine levels in the sera of MCA/DN-TNF-treated mice are statistically significant relative to that of control and MCA/PBS-treated mice ($P = 0.026$, $P = 0.05$, and $P = 0.039$, respectively). In D, the decreases of IL1 α levels in the sera of MCA/ENBREL- and DN-TNF-treated mice versus control and MCA/PBS-treated mice are statistically significant ($P = 0.012$ and $P = 0.023$, respectively).

greatly increase in cancer-host bone marrow, peripheral lymphoid tissue, and tumor. It is believed that MDSCs play significant roles in tumor immune escape and failure of immunotherapy. However, MDSC presence and function in chemically induced carcinogenesis are underexplored (28). We examined changes in frequency of MDSCs (Supplementary Fig. S1; Fig. 3A–D) and macrophages (Supplementary Fig. S2) following MCA injection and/or exclusion of sTNF or tmTNF. MCA induced increases of spleen monocytic (CD11b⁺Gr1^{lo/-}Ly6C^{+/hi}; Fig. 3A and B) and granulocytic (CD11b⁺Gr1^{hi}Ly6C⁺; Fig. 3C and D) MDSC frequency in wild-type mice. Increased frequencies of MDSCs were notable at the cancer initiation (day 14: 1.4- and 1.3-fold, respectively), and highly augmented in the tumor-established stage (day 84: 6.7- and 6.8-fold, respectively) of carcinogenesis. In contrast, MCA-induced carcinogenesis did not change splenic F4/80⁺ macrophage frequency (Supplementary Fig. S2A and S2B). However, ENBREL neutralization of both TNF forms decreased frequencies of monocytic and granulocytic MDSCs and macrophages in both tumor-free and tumor-bearing MCA-injected mice. Sequestration of sTNF by XPro1595-DN-TNF invariably and dramatically decreased frequencies of monocytic MDSCs, granulocytic MDSCs, and macrophages to or below their control levels, in MCA-injected both tumor-free and tumor-bearing mice. Similar decreases of MDSC and macrophage frequencies were

observed in MCA-injected tumor-bearing *TNF*- and *TNFR1*-deficient mice. In contrast with MCA-injected/DN-TNF- or ENBREL-treated wild-type and *TNF*- or *TNFR1*-deficient mice, and similar to MCA-injected/PBS-treated wild-type mice, MCA-injected/*TNFR2*-deficient mice lacking preferential tmTNF receptor possessed highly increased frequencies of both monocytic and granulocytic MDSCs, and unchanged frequencies of macrophages, relative to control mice. FoxP3⁺CD4⁺CD25⁺ Treg frequencies were found unchanged in all MCA-injected groups of mice (Supplementary Fig. S3). These data show that MCA induces increased MDSC accumulation during carcinogenesis. They also provide evidence that sTNF, but not tmTNF, is required for the MCA-induced accumulation of MDSCs and maintenance of the macrophage population, and that treatment with the selective sTNF inhibitor XPro1595-DN-TNF efficiently prevents MDSC accumulation.

Soluble TNF-dependent STAT3 activation in myeloid cells

The transcription factor STAT3 plays an essential role in regulation of MDSC development and function (28, 29). Its active, phosphorylated form, pSTAT3, is considered a hallmark of MDSCs. To validate the carcinogen-induced sTNF-dependent expansion of MDSCs, we investigated whether MCA induces sTNF-dependent pSTAT3 in myeloid cells. Splenocytes of

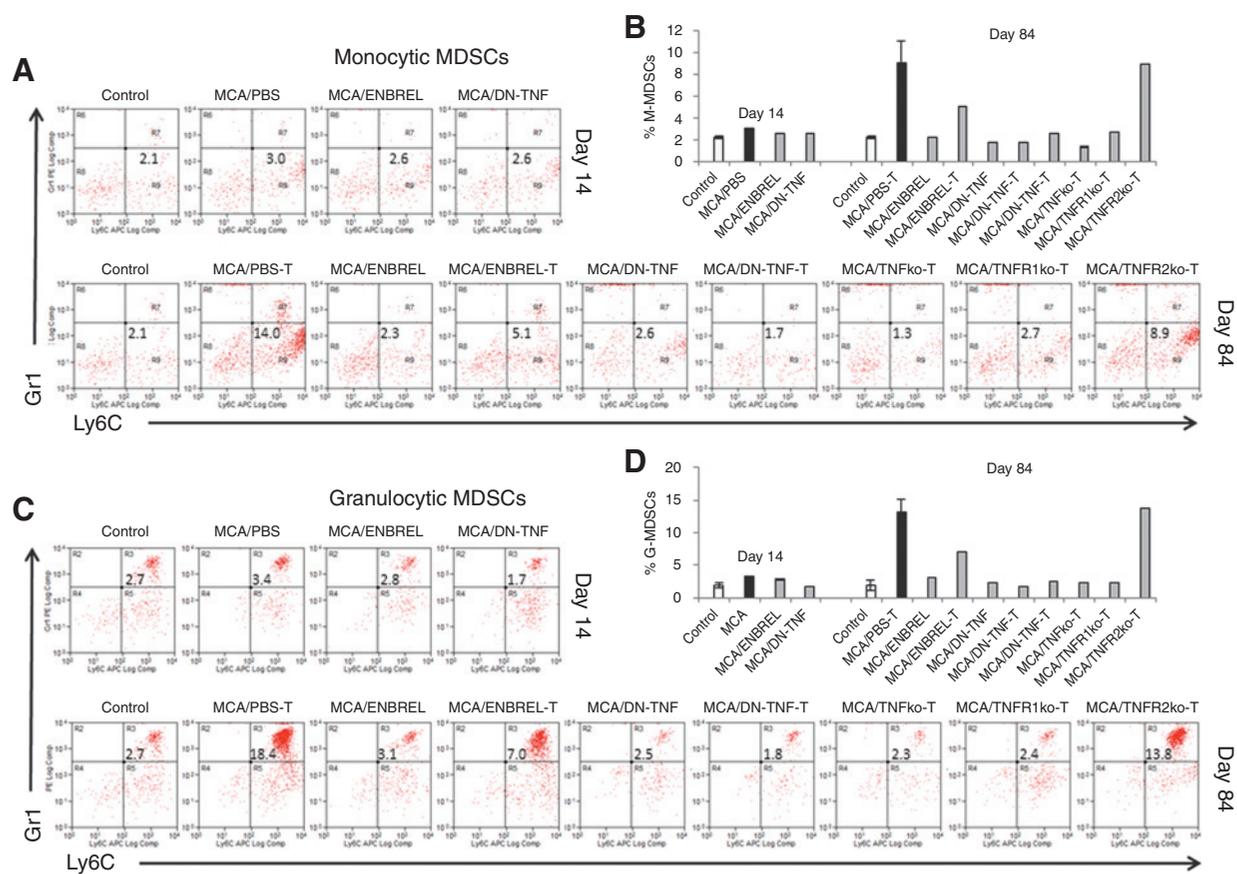


Figure 3. sTNF exclusion averts MCA-induced MDSC accumulation. Similar experimental groups were set up as described in the Fig. 1 legend. Both tumor-free and tumor-bearing (T) mice were examined: healthy/untreated control (Control); MCA-injected/PBS-treated (tumor-free:tumor-bearing: MCA/PBST), ENBREL-treated (tumor-free: MCA/ENBREL; tumor-bearing: MCA/ENBREL-T), XPro1595-treated (tumor-free: MCA/DN-TNF; tumor-bearing: MCA/DN-TNF-T); and/or tumor-bearing MCA-injected/TNFko (MCA/TNFko-T), TNFR1ko (MCA/TNFR1ko-T), and TNFR2ko-T (MCA/TNFR2ko-T) mice. Spleens were obtained from the listed groups of mice on days 14 (A, B) and/or 84 (C, D) following MCA injection, and stained with fluorochrome-conjugated antibodies to CD11b, Gr1, and Ly6C. Stained splenocytes were analyzed by three-color flow cytometry. The gating/analysis strategy is presented in Supplementary Fig. S1. CD11b⁺Gr1^{lo/-}Ly6C⁺/hi monocytic MDSCs (A, B) and CD11b⁺Gr1^{hi}Ly6C⁺ granulocytic MDSCs (C, D) were scored. Dot-plot data present nongranular Gr1⁻Ly6C⁺CD11b⁺ (A) and granular Gr1⁺Ly6C⁺CD11b⁺ (C) cells. Histograms show individual and mean (2–3 replicates \pm SD; control, MCA/PBS-T, and DN-TNF-T) percentages of monocytic (B) and granulocytic (D) MDSCs.

healthy/untreated-control and MCA-injected/PBS-, ENBREL-, or DN-TNF-treated wild-type mice were examined by flow cytometry for the presence of pSTAT3y705 and pSTAT3s727 residues at day 84 after MCA injection (Fig. 4). Monocytes and granulocytes were defined by forward-scatter (size) and side-scatter (granularity), as nongranular/large- and granular/medium-size cells, respectively (Supplementary Fig. S4). The identities of cell populations were confirmed using their labeling with fluorochrome-conjugated antibodies to Gr1, CD11b, Ly6C, and F4/80, and back-gating (Supplementary Figs. S1 and S2, data not shown). In controls, the frequencies of splenic monocytes and granulocytes expressing pSTAT3 were low (Fig. 4). In contrast, frequencies of STAT3y705⁺ (Fig. 4A and B) and pSTAT3s727⁺ (Fig. 4C and D) monocytes (Fig. 4A and C) and granulocytes (Fig. 4B and D) were highly increased in MCA-injected/PBS-treated mice. The MCA-induced increases in pSTAT3⁺ cell frequencies were prevented in both tumor-free and tumor-bearing mice by ENBREL or DN-TNF treatment. These data show that MCA and/or MCA-induced tumors activate the essential MDSC transcription factor STAT3 in myeloid cells, which parallels and correlates with MDSC

accumulation. They also show that sTNF is not only important for MCA-induced accumulation of MDSCs but also for MCA-induced activation of STAT3 in myeloid cells.

MCA induces and sTNF inhibition averts cell-mediated suppression of NK-cell/DC cross-talk

NK-cell/DC cross-talk is a central immunoregulatory mechanism that defines type and extent of innate and adaptive immune responses (30–32). We have previously determined that the cross-talk leading to a high Th1 response, which efficiently controls cancer growth, is mainly mediated via cell-to-cell contact and tmTNF (17–19, 33). Carcinogens and tumors suppress NK cells and/or DCs (26, 27, 34) that may disable NK-cell/DC cross-talk and allow carcinogenesis and tumor growth. Because MCA concomitantly induced sTNF-dependent carcinogenesis, suppression of central immunoregulatory cytokines that are upregulated in NK-cell/DC cross-talk, and accumulation of MDSCs, we examined whether MDSC-mediated suppression of NK-cell/DC cross-talk is an MCA-induced immunosuppression mechanism (Fig. 5). We initially assessed the endogenous NK-cell/DC cross-talk

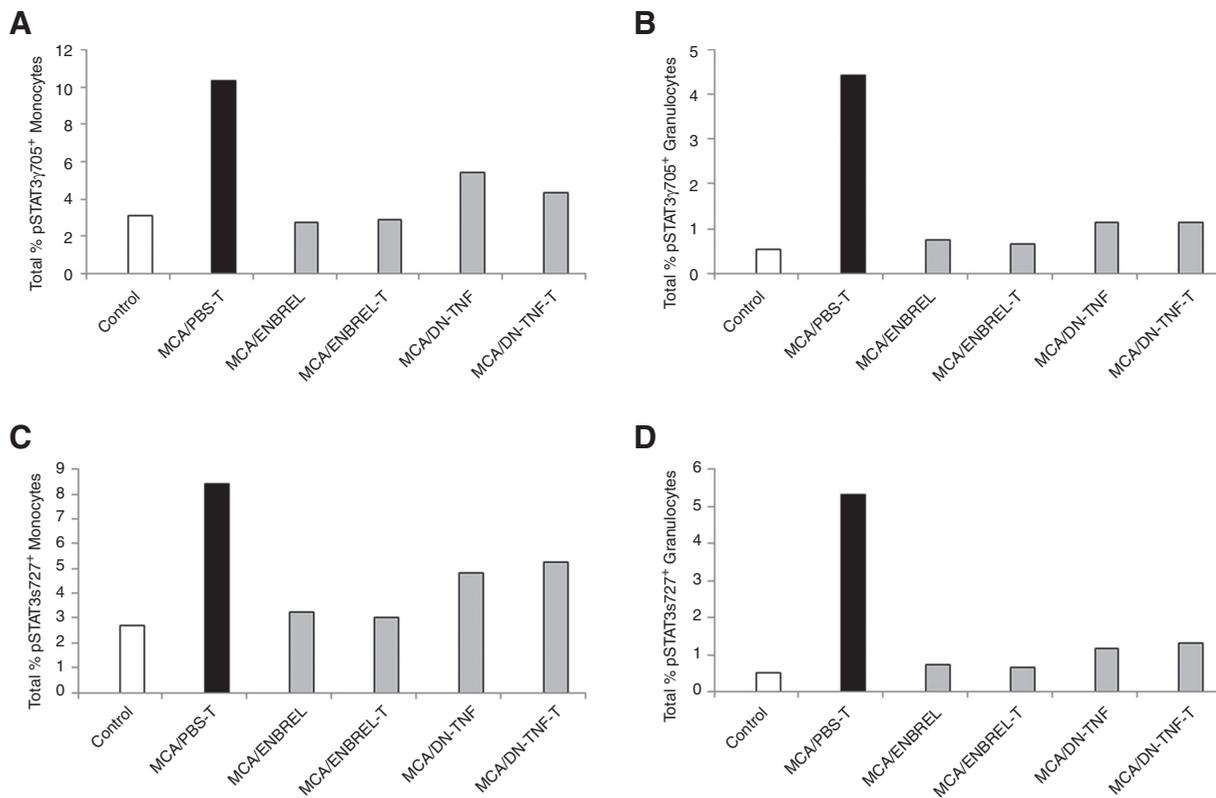


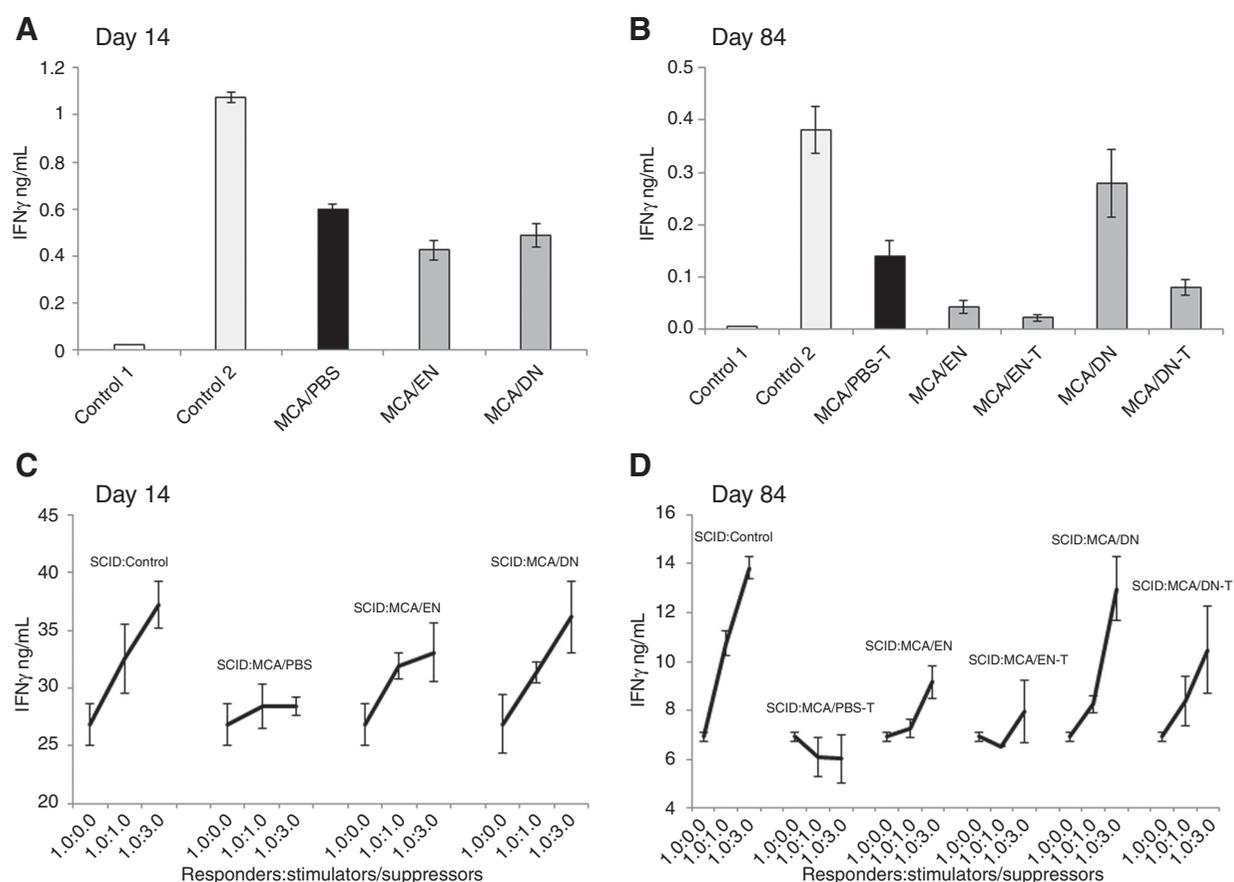
Figure 4.

sTNF sequestration prevents MCA-induced activation of STAT3 in myeloid cells. Splenocytes of healthy/untreated control (Control) and MCA-injected/PBS-treated (tumor-bearing: MCA-T), ENBREL-treated (tumor-free: MCA/ENBREL; and tumor-bearing: MCA/ENBREL-T), and XPro1595-treated (tumor-free: MCA/DN-TNF; and tumor-bearing MCA/DN-TNF-T) wild-type mice were obtained on day 84 after MCA injection and examined by flow cytometry for intracellular presence of the phosphorylated STAT3y705 (A, B) and STAT3s727 (C, D). The analyses of gated large nongranular mononuclear cells (monocytes; A, C) and middle-sized granular (granulocytes; B, D) were performed as shown in Supplementary Fig. S3. Data represent total percentages of stained monocytes and granulocytes.

among splenocytes of healthy/untreated-control and MCA-injected/PBS-, ENBREL-, and DN-TNF-treated wild-type mice (Fig. 5A and B). Splenocytes were stimulated with IL2 and LPS, to activate NK cells and DCs, respectively, and promote their cross-talk and IFN γ secretion (17, 19). IL2/LPS stimulation induced enhanced NK-cell/DC cross-talk and IFN γ secretion in healthy untreated mouse splenocytes (control 2) relative to their unstimulated counterpart (control 1). Both 14 and 84 days after carcinogen injection, the IL2/LPS-stimulated splenocytes of all MCA-injected mice regardless of treatment, except of DN-TNF-treated tumor-free mice 84 days after MCA injection, showed significant decreases of Th1 response (Fig. 5A and B). The decreases of activity were particularly pronounced in splenocytes of ENBREL-treated mice. These findings show that NK-cell/DC cross-talk is suppressed throughout MCA-induced carcinogenesis. The enhanced suppression of NK-cell/DC cross-talk in all ENBREL-treated mice and lack of suppression in DN-TNF-treated tumor-free mice at day 84 after MCA confirm that endogenous NK-cell/DC cross-talk is primarily mediated by tmTNF (17–19).

Next, we directly assessed whether MCA-induced MDSCs could suppress NK-cell/DC cross-talk. In these experiments, the responder cells (responders) were bulk SCID-mouse splenocytes, composed of 50% NK cells, 30% DCs, and 20% monocytes/macrophages. The stimulator/suppressor cells

(stimulators/suppressors) were bulk splenocytes of healthy/untreated (control), MCA-injected/PBS-treated (tumor-free: MCA/PBS; tumor-bearing: MCA/PBS-T), MCA-injected/ENBREL-treated (tumor-free: MCA/EN; tumor-bearing: MCA/EN-T), and MCA-injected/DN-TNF-treated (tumor-free: MCA/DN; tumor-bearing: MCA/DN-T) wild-type mice, obtained on days 14 and 84 after MCA injection. Responders and stimulators/suppressors, either alone or mixed in 1:1 and 1:3 ratios, were incubated in cell-to-cell contact in the presence of IL2/LPS, for 24 hours. Very similar results were obtained with splenocytes of mice harvested on days 14 (Fig. 5C) and 84 (Fig. 5D) after MCA injection. At both time points, SCID NK cells and DCs interacted strongly and secreted large amounts of IFN γ (SCID:control, 1.0:0.0). The reaction was greatly increased in a dose-dependent manner by adding to SCID-mouse splenocytes healthy/untreated wild-type mouse splenocytes, which had low MDSC frequency (Fig. 3). Importantly, the observed increases in IFN γ secretion were 6- to 10-fold greater than the responses of the corresponding stimulators/suppressors alone (control 2, Fig. 5A and B), indicating a possible synergistic cooperation of SCID and healthy/untreated wild-type mouse splenocytes. In contrast, MCA/PBS-mouse splenocytes containing a large population of MDSCs (Fig. 3) strikingly decreased the SCID-mouse NK-cell/DC cross-talk to baseline, on day 14, or below baseline, on day 84. In sharp

**Figure 5.**

sTNF sequestration prevents MCA induction of cell-mediated suppression of NK-cell/DC cross-talk. As described in the Fig. 3 legend, splenocytes of healthy/untreated (control), MCA-injected/PBS-treated (tumor-free: MCA/PBS; and tumor-bearing: MCA/PBS-T), ENBREL-treated (tumor-free: MCA/EN; and tumor-bearing: MCA/EN-T), and XPro1595-treated (tumor-free: MCA/DN; and tumor-bearing: MCA/DN-T) wild-type mice were obtained on days 14 (A, C) and/or 84 (B, D) after MCA injection. These splenocytes, either alone (A, B) or mixed with SCID splenocytes (SCID) at 1:1 and 3:1 ratios (C, D), were stimulated with LPS (1 μ g/mL) and IL2 (6,000 IU/mL), for 24 hours. After this stimulation, the cell-culture-conditioned media were collected and examined for the presence of IFN γ using ELISA. In A and B, control 1 is unstimulated, and control 2 is IL2/LPS-stimulated splenocytes of healthy/untreated mice. Data are mean of triplicates \pm SD of IFN γ ng/mL. In A, MCA/PBS, MCA/EN, and MCA/DN are significantly lower than control 2 ($P = 0.00041$, $P = 0.00057$, and $P = 0.0016$, respectively). In B, MCA/PBS and MCA/EN are significantly lower than control 2 ($P = 0.0047$ and $P = 0.0016$, respectively). In C, SCID:control (1.0:1.0-1.0:3.0) and SCID:MCA/PBS (1.0:1.0-1.0:3.0) are significantly lower than SCID:control (1.0:1.0-1.0:3.0; $P = 0.026$ and $P = 0.001$, respectively); and SCID:MCA/EN (1.0:1.0-1.0:3.0) and SCID:MCA/DN (1.0:1.0-1.0:3.0) are significantly higher than SCID:MCA/PBS (1.0:1.0-1.0:3.0; $P = 0.019$ and $P = 0.001$, respectively). In D, SCID:Control (1.0:0.0) and SCID:MCA/PBS-T (1.0:1.0-1.0:3.0) are significantly lower than SCID:control (1.0:1.0-1.0:3.0; $P = 0.0034$ and $P = 0.0033$, respectively); and SCID:MCA/EN (1.0:3.0), SCID:MCA/DN (1.0:1.0-1.0:3.0), and SCID:MCA/DN-T (1.0:1.0-1.0:3.0) are significantly higher than SCID:MCA/PBS-T (1.0:1.0-1.0:3.0; $P = 0.027$, $P = 0.028$, and $P = 0.046$, respectively).

contrast, MCA/ENBREL-mouse splenocytes and, more prominently, MCA/DN-TNF-mouse splenocytes stimulated SCID NK-cell/DC cross-talk measured by IFN γ secretion. Both tumor-free and tumor-bearing MCA/DN-TNF-mouse splenocytes lacking MDSCs (Fig. 3) stimulated SCID-mouse NK-cell/DC cross-talk as strongly as wild-type control-mouse splenocytes. In contrast, MCA/ENBREL-treated mouse splenocytes having low to moderate frequency of MDSCs (Fig. 3) slightly (day 14) and moderately (day 84) inhibited SCID NK-cell/DC cross-talk relative to that of control- or MCA/DN-TNF-treated mouse splenocytes. These findings indicate that MCA and MCA-induced tumor strongly stimulate not only the expansion but also the immunosuppressive activity of MDSCs, which in turn inhibits NK-cell/DC cross-talk. They also suggest that the expansion and immunosuppressive activity of MDSCs are both dependent on sTNF and can be efficiently prevented by XPro1595-DN-TNF treatment.

Discussion

Previous studies showed that mice lacking either *TNF* or *TNFR1* genes are resistant to chemically induced carcinogenesis in the skin (9, 10) or colon (11), respectively. Similarly, wild-type mice that undergo chemically induced carcinogenesis and treatment with ENBREL show reduced tumorigenesis (10). These studies, however, did not consider possible distinct roles in carcinogenesis of two functionally different TNF forms. Here, we show that selective exclusion of sTNF by XPro1595-DN-TNF treatment or *TNFR1* deletion, or elimination of both sTNF and tmTNF by ENBREL treatment or *TNF* deletion, prevented carcinogenesis, decreased tumor growth, and prolonged survival of MCA-injected mice. In contrast, selective exclusion of tmTNF by *TNFR2* deletion enhanced MCA-induced carcinogenesis. We therefore have demonstrated that sTNF is essential in MCA-induced carcinogenesis.

The data also suggest that tmTNF, in opposition to sTNF, could have a protective role in carcinogenesis.

Notably, although DN-TNF treatment or TNF deletion, excluding sTNF or both TNF forms, almost completely prevented carcinogenesis, ENBREL treatment, neutralizing the two TNF forms and also LT α and possibly LT α 2 β 1 (35), partially prevented carcinogenesis. These findings indicate that besides tmTNF, LT α or LT α 2 β 1 could be protective in MCA-induced carcinogenesis.

A perplexing finding in our study was that exclusion of sTNF by *TNFR1* deletion only temporarily prevented carcinogenesis, and led to the late development of fast-growing tumors in the majority of MCA-injected mice. This may indicate that in addition to sTNF playing a major role, some other, less potent, factor(s) could also promote carcinogenesis by inducing late generation of TNFR1-expressing cancer cells. These cancer cells could be susceptible to and controlled by TNF-mediated cytotoxicity in wild-type mice (27, 36, 37). In contrast, in *TNFR1*-deficient mice, the MCA-induced cancer cells lack TNFR1 and may be resistant to TNF-mediated killing, and vigorously grow in the absence of the immune control.

Our findings support the following scenario for the initiation of carcinogenesis. Carcinogens induce injury and cell necrosis in target tissues. Necrotic cells release damage-associated molecular pattern (DAMP) molecules, including heat-shock proteins, high-mobility group box 1 (HMGB1), DNA, RNA, S100 molecules, and purine metabolites (38). DAMPs induce activation of innate immunity effector cells, such as macrophages, DCs, and NK cells, which consequently release proinflammatory cytokines, such as sTNF. The proinflammatory cytokines induce procarcinogenic inflammation. In parallel, tissue protective/healing anti-inflammatory and immunosuppressive feedback mechanisms develop. In our study, IL1 β , IL12, and IL17, which are immunoregulatory cytokines produced by innate-immunity activated effectors and mediate the effective Th1 and Th17 anticancer immune mechanisms, are unchanged during the initiation of MCA-induced carcinogenesis, but enhanced after sTNF exclusion by DN-TNF. In the context of carcinogenesis, these findings indicate that, during carcinogenesis, the activation of anticancer immune mechanisms might be stimulated, but downregulated by sTNF-induced immunosuppression. In support of this possibility, potentially immunosuppressive soluble IL1 α (39) is notably secreted in healthy mice, significantly increased during the initiation of MCA-induced carcinogenesis, and strikingly decreased below its baseline levels by sTNF sequestration with DN-TNF.

IL1 α and IL1 β bind to the same receptors (39). Both cytokines are produced as 31-kDa precursors, which are processed by the proteases calpain and IL1 β converting enzyme, respectively, generating 17-kDa soluble forms that are released outside of cells. Soluble IL1 β is immunologically active. In contrast, soluble IL1 α is seemingly immunologically inactive in spite of its ability to bind to IL1 receptors. However, soluble IL1 α becomes immunologically active after binding to a cell-membrane mannose-like receptor. The calpain-mediated cleavage of IL1 α precursor and release of 17-kDa IL1 α also lead to the generation of the functional 14-kDa IL1 α N-terminal propeptide. The IL1 α propeptide functions in cells as a transcription factor, activating oncogenes and inhibiting tumor-suppressor genes, and is considered as an "endokine." Complementarily, secreted 17-kDa IL1 α can function as a growth factor for malignant cells. Moreover, immunologically inactive soluble IL1 α , capable of binding IL1 receptors, could block and prevent IL1 receptor interaction with the immu-

nologically active forms of IL1, and thus function as a potent negative regulator of immune responses.

As IL1 α is overexpressed in cells exposed to chemical carcinogens (39), our findings that the soluble IL1 α level is elevated in MCA-injected and strikingly decreased in DN-TNF-treated mice indicate that sTNF can enhance generation of propeptide and soluble IL1 α . As a result, the two upregulated forms of IL1 α could promote malignant transformation of target cells, mediate growth of newly generated malignant cells, and inhibit IL1-induced immune responses.

Soluble TNF and tmTNF preferentially trigger TNFR1 and TNFR2 signaling pathways, respectively. However, both TNF receptors are associated with TRAF2 adapter molecule, share TRAF1, have similar inflammatory/survival signaling pathways, and can cross-talk and cross-transduce signals one to the other (15, 16). Two complementary mechanisms of sTNF-dependent MDSC accumulation at the periphery have been defined (40, 41). The first one is dominant in chronic inflammation and is mediated by sTNF-induced S100A8/A9-RAGE signaling in MDSC progenitors leading to MDSC generation and differentiation arrest (40). As MDSC progenitors might lack TNFR2 and express only TNFR1, sTNF mediates this mechanism solely via TNFR1. The second mechanism is dominant in mice rapidly developing transplanted tumors and likely acute inflammation (41). This mechanism leads to protection from apoptosis and prolongation of survival of MDSCs by inducing NF- κ B activation, cFLIP upregulation, and caspase-8 inhibition. Paradoxically, the latter sTNF-mediated mechanism requires TNFR2. In this mechanism, sTNF could selectively trigger a TNFR1 cell-survival signal that could be either executed solely through the TNFR1 signaling pathway, if the expression of TNFR1 is prevalent, or the TNFR1-originated survival signal could be cross-transduced, amplified, and executed through the TNFR2 signaling pathway, if the expression of TNFR2 is prevalent. In agreement with these findings and considerations, we showed that MCA-induced chronic inflammation-based carcinogenesis caused in both tumor-free and tumor-bearing mice STAT3 phosphorylation in myeloid cells and accumulation of MDSCs, both of which were prevented by the exclusion of sTNF or *TNFR1*, but not *TNFR2*. These findings demonstrate that sTNF mediates MDSC accumulation during carcinogenesis and suggest that this mainly occurs by selectively triggering TNFR1 on MDSC progenitors, leading to MDSC generation and differentiation arrest.

During carcinogenesis initiation, low increases of MDSC frequency occur. In contrast, during tumorigenesis, high increases of MDSC frequency occur. This difference could be caused by the involvement of different MDSC-regulatory cytokines at different stages of carcinogenesis. sTNF appears to be involved throughout the carcinogenic process. Secreted IL1 α , which can also mediate growth and homing of myeloid cells (39), could be involved in the MDSC growth and regulation at least during carcinogenesis initiation. On the other hand, VEGF and GM-CSF, which are implicated in MDSC growth in tumor-bearing hosts, could be involved when tumors are established (28). Our data suggest that sTNF has a central regulatory role in MDSC accumulation during carcinogenesis, which could comprise their generation and differentiation arrest (40), survival extension (41), and growth stimulated by IL1 α , VEGF, and GM-CSF (28, 39).

Similar to MDSC accumulation, two TNF-dependent mechanisms are implicated in regulation of MDSC-immunosuppressive

functions. Thus, although sTNF mediates the acquisition and maintenance of MDSC-immunosuppressive functions (40), tmTNF highly expressed on *non-cleavable-TNF*-transduced tumor cells amplifies these functions (42). As activated immune cells of both innate and adaptive immunity, but not tumor cells, highly express tmTNF, the latter mechanism might be induced by activated immune cells interacting with MDSCs. In parallel with the sTNF-dependent accumulation of MDSCs in the spleen of MCA-injected mice, and in agreement with the published results and considerations, we observed potent sTNF-dependent MDSC-mediated immunosuppression of the central immunoregulatory NK-cell/DC cross-talk. The ultimate MDSC-immunosuppressive function could be a result of sTNF-dependent baseline suppressive activity and its tmTNF-mediated amplification induced by activated NK cells and DCs (17).

Similar to MDSC expansion, immunosuppressive activity was completely eliminated by sTNF exclusion with DN-TNF treatment in both tumor-free and tumor-bearing mice. As the frequency of other potential immunosuppressive cells, including CD4⁺CD25⁺Foxp3⁺ Treg and F4/80⁺ macrophages, did not change during MCA-induced tumorigenesis, it is conceivable that MDSCs are the main mediators of the splenocyte immunosuppression that evolves during chemical carcinogenesis. MDSCs strongly suppress T-cell responses by producing a number of immunosuppressive molecules, including prostaglandin E2 (PGE2), TGF β , IL10, arginase, nitric oxide, and reactive oxygen species (28, 43, 44). MDSC-derived immunosuppressive molecules, especially PGE2, TGF β , and IL10, are also known as potent suppressants and/or modulators of NK cells and DCs (45–47). Therefore, our findings could indicate that MDSCs potentially suppress NK cells and/or DCs, which leads to abrogation of their cross-talk in MCA-induced carcinogenesis. As NK-cell/DC cross-talk is the central immunoregulatory mechanism that defines the quality and extent of effective anticancer immune responses, MDSC-mediated immunosuppression could be a critically important mechanism of cancer immune escape in MCA-induced carcinogenesis.

In conclusion, this article reports on the novel findings that specific sTNF sequestration prevents chemically induced carcinogenesis, expansion of MDSCs, and suppression of Th1-type innate immunity in mice. The study reveals a pivotal role of sTNF in carcinogenesis and defines a sTNF–TNFR1-negative immunoreg-

ulatory axis of innate immunity. The newly defined negative immunoregulatory axis balances the positive tmTNF–TNFR2 immunoregulatory axis of innate immunity (17–9). It also complements the well-established immune checkpoints of adaptive immunity, through which targeting with antibodies leads to durable clinical responses in a large proportion of patients with advanced cancer (48–50). In this context, the sTNF–TNFR1 axis of innate immunity could be viewed as an additional immune checkpoint, targeting of which might be beneficial in both immunoprevention and immunotherapy of cancer.

Disclosure of Potential Conflicts of Interest

R.L. Ferris is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Celgene, Merck, and ONO Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

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