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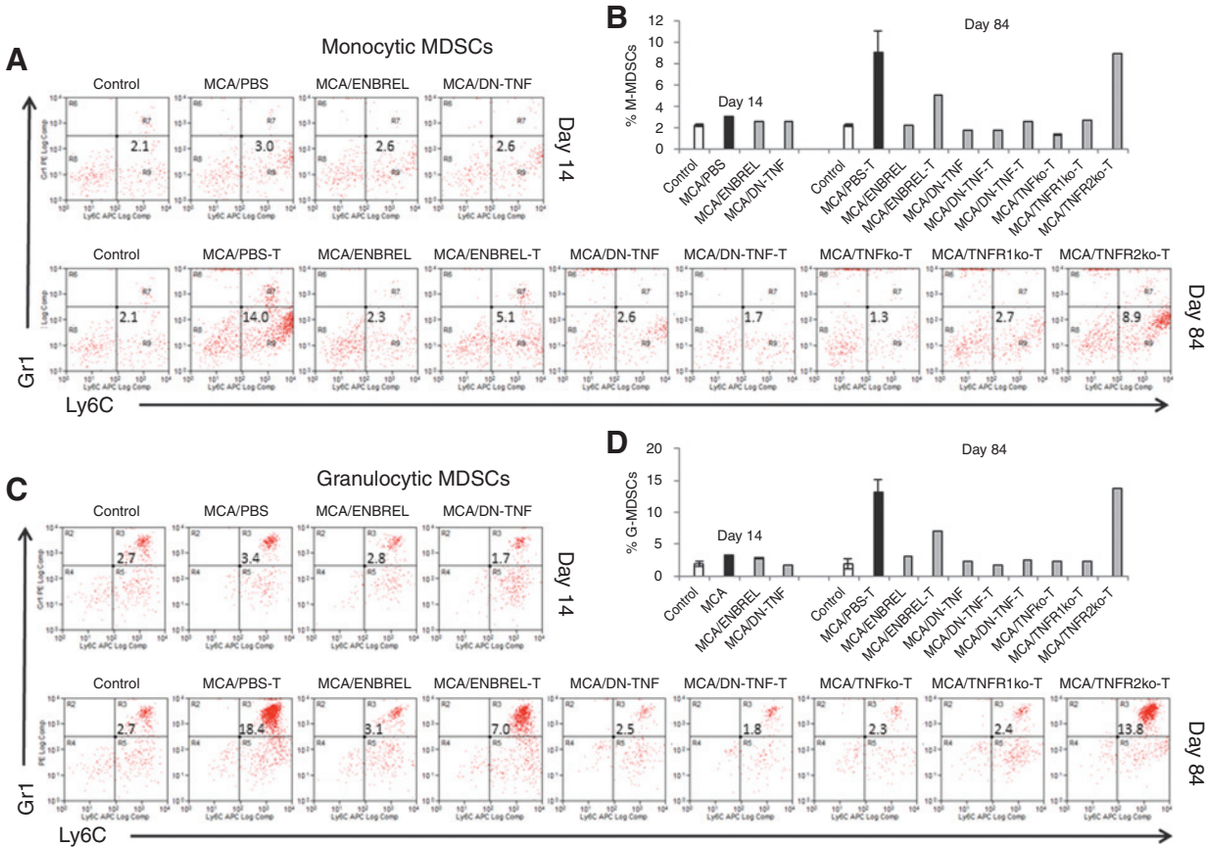

Figure 3.

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 ± 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

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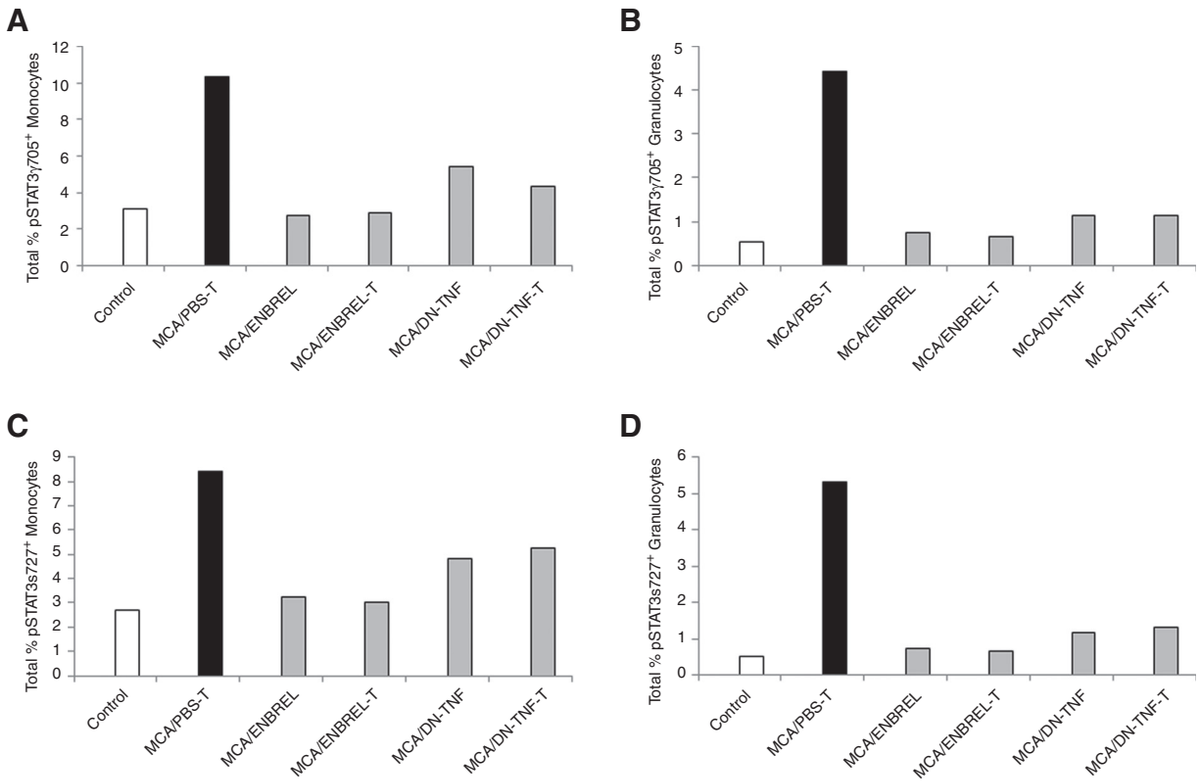

Figure 4.
sTNF sequestration prevents MCA-induced activation of STAT3 in myeloid cells. Splenocytes of healthy/untreated control (Control) and MCA-injected/PBS-treated (tumor-bearing: MCA-T), ENBREL-treated (tumor-free: MCA/ENBREL; and tumor-bearing: MCA/ENBREL-T), and XPro1595-treated (tumor-free: MCA/DN-TNF; 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 IFNγ secretion (17, 19). IL2/LPS stimulation induced enhanced NK-cell/DC cross-talk and IFNγ 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 IFNγ (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 IFNγ 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

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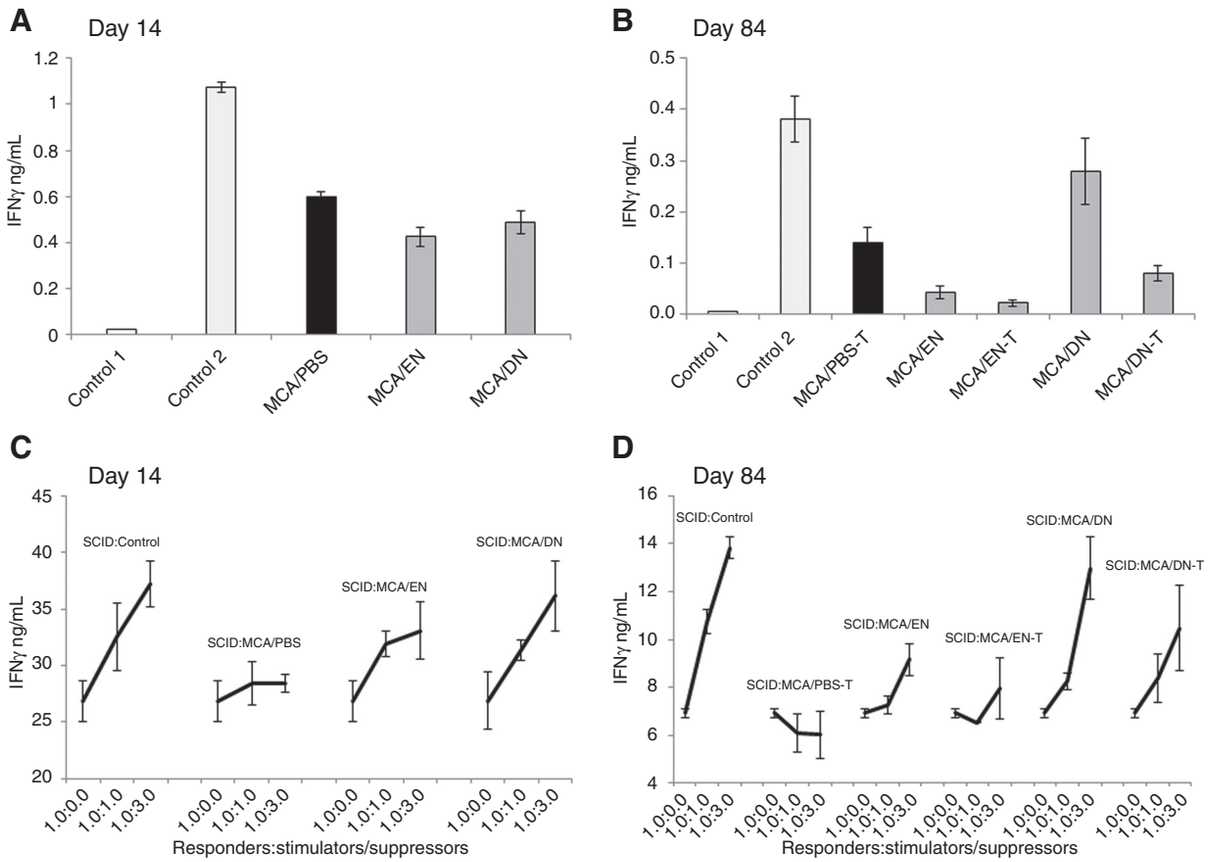

Figure 5.

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 μg/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 IFNγ 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 ± SD of IFNγ 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 (1.0:0.0) 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 IFNγ 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.

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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 LTα and possibly LTα2β1 (35), partially prevented carcinogenesis. These findings indicate that besides tmTNF, LTα or LTα2β1 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, IL1β, 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 IL1α (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.

IL1α and IL1β bind to the same receptors (39). Both cytokines are produced as 31-kDa precursors, which are processed by the proteases calpain and IL1β converting enzyme, respectively, generating 17-kDa soluble forms that are released outside of cells. Soluble IL1β is immunologically active. In contrast, soluble IL1α is seemingly immunologically inactive in spite of its ability to bind to IL1 receptors. However, soluble IL1α becomes immunologically active after binding to a cell-membrane mannose-like receptor. The calpain-mediated cleavage of IL1α precursor and release of 17-kDa IL1α also lead to the generation of the functional 14-kDa IL1α N-terminal propiece. The IL1α propiece functions in cells as a transcription factor, activating oncogenes and inhibiting tumor-suppressor genes, and is considered as an "endokine." Complementarily, secreted 17-kDa IL1α can function as a growth factor for malignant cells. Moreover, immunologically inactive soluble IL1α, capable of binding IL1 receptors, could block and prevent IL1 receptor interaction with the immunologically active forms of IL1, and thus function as a potent negative regulator of immune responses.

As IL1α is overexpressed in cells exposed to chemical carcinogens (39), our findings that the soluble IL1α level is elevated in MCA-injected and strikingly decreased in DN-TNF–treated mice indicate that sTNF can enhance generation of propiece and soluble IL1α. As a result, the two upregulated forms of IL1α 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-κB 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 IL1α, 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 IL1α, VEGF, and GM-CSF (28, 39).

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

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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), TGFβ, IL10, arginase, nitric oxide, and reactive oxygen species (28, 43, 44). MDSC-derived immunosuppressive molecules, especially PGE2, TGFβ, 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 potently 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 immunoregulatory 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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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Sobo-Vujanovic, L. Vujanovic, F. Concha-Benavente, Y. Lin, N.L. Vujanovic
Writing, review, and/or revision of the manuscript: A. Sobo-Vujanovic, L. Vujanovic, A.B. DeLeo, R.L. Ferris, N.L. Vujanovic
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Sobo-Vujanovic, L. Vujanovic, N.L. Vujanovic
Study supervision: N.L. Vujanovic

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