THE ROLE OF TNF-TNFR2 SIGNAL IN IMMUNOSUPPRESSIVE CELLS AND ITS THERAPEUTIC IMPLICATIONS

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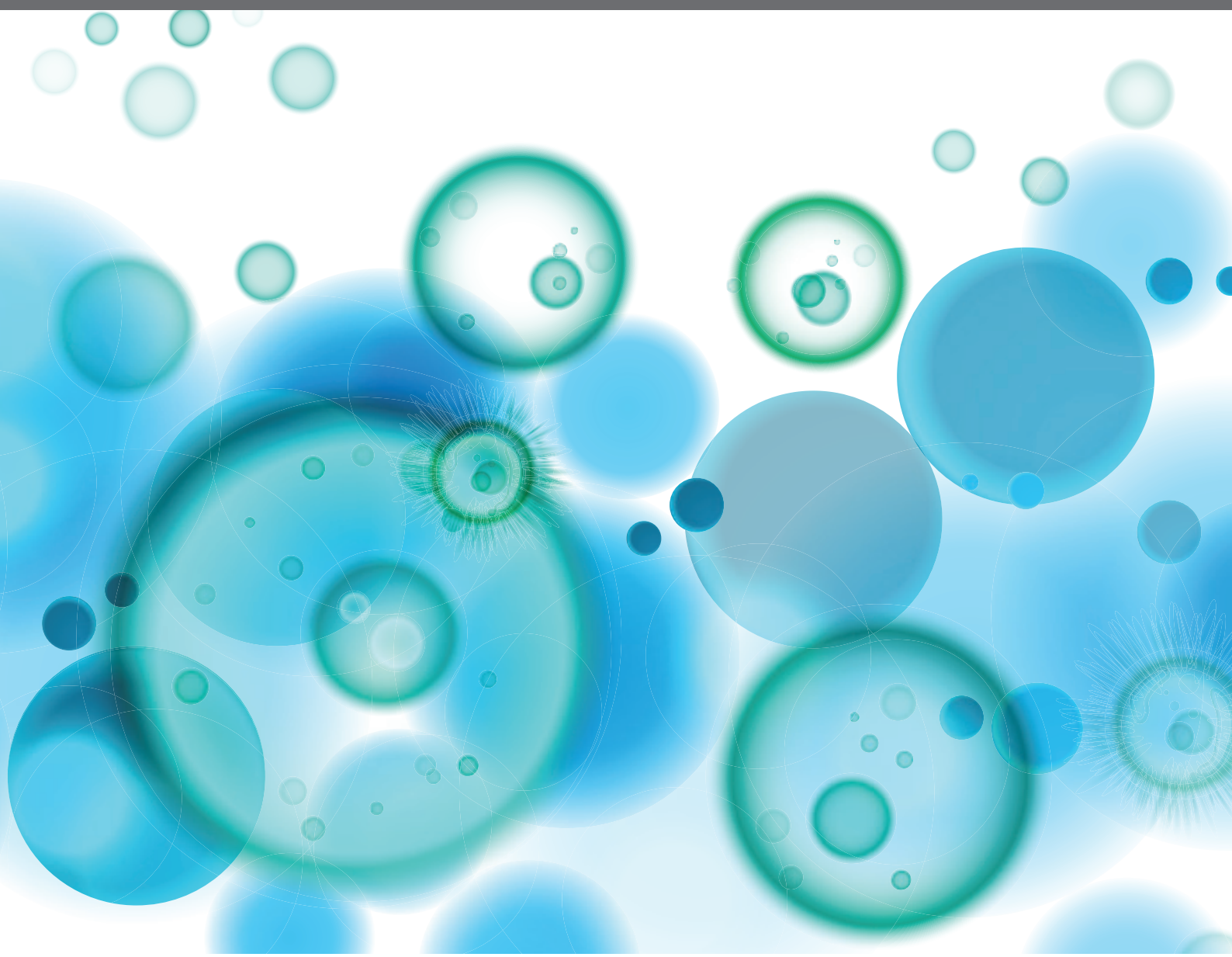

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THE ROLE OF TNF-TNFR2 SIGNAL IN IMMUNOSUPPRESSIVE CELLS AND ITS THERAPEUTIC IMPLICATIONS

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$CD4^+FoxP3^+$ regulatory T cells (Tregs) play an indispensable role in the maintenance of immune homeostasis and prevention of autoimmune diseases, and represent a major cellular mechanism of tumor immune evasion. Targeting of Tregs has great potential in the treatment of some major human diseases, including autoimmunity, transplant rejection, GvHD, and cancer, and are critical controllers of immunity to infectious pathogens. It is expected they will also be central to the control of allergic and inflammatory diseases. Understanding the biological pathways crucial for the regulation of Treg activity is a prerequisite for harnessing the immense therapeutic potential of Tregs. TNF is generally believed to be a master pro-inflammatory cytokine, and anti-TNF therapy has become a mainstay treatment for some autoimmune diseases. However, experimental evidence indicates that TNF preferentially activates Tregs, resulting in the expansive proliferation, phenotypic stability, and enhanced suppressive capacity of these immune suppressors. This effect of TNF is mediated by TNFR2, which is preferentially expressed by human and mouse Tregs. Furthermore, expression of TNFR2 is able to identify the most suppressive subset of Tregs. Although counterintuitive and contradictory to earlier reports, these findings have been supported by increasing experimental evidence from both human and mouse studies. These recent studies revealing the Treg-promoting effect of TNF not only leads to the redefinition of the immunological biology of this pleiotropic cytokine, they are also helpful in designing novel therapies in the treatment of cancer, autoimmune diseases, and GvHD, as well as enhancing current vaccines and immunomodulators. In this article collection, current knowledge on the cellular and molecular aspects of the Treg-stimulatory effect of the TNF-TNFR2 pathway will be discussed.

An insight of the physiological and pathological roles of such effects of TNF in an inflammatory reaction and immune response will be provided. The seemingly contradictory Treg-promoting effect of TNF and immunosuppressive effect of anti-TNF therapy will be analyzed. Recent efforts to translate such discoveries into therapeutic benefits will be introduced. The novel strategies in the treatment of cancer and GvHD, by down- or up-regulation of Treg activity through targeting TNFR2, will be highlighted. In addition to Tregs, TNFR2 has also been found to play a key role in the accumulation and immunosuppressive function of myeloid-derived suppressive cells (MDSCs) and Mesenchymal stem cells (MSCs). Therefore, the current understanding of the role of TNF-TNFR2 signal in other type of immunosuppressive cells, as well as its clinical and therapeutic implications, have also been considered.

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EDITORIAL
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Editorial: The Role of TNF-TNFR2 Signal in Immunosuppressive Cells and Its Therapeutic Implications

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Keywords: TNF, TNFR2, CD4$^+$Foxp3$^+$ regulatory T cells, myeloid-derived suppressive cells, mesenchymal stem cells, inflammation, cancer, autoimmune diseases

Editorial on the Research Topic

The Role of TNF-TNFR2 Signal in Immunosuppressive Cells and Its Therapeutic Implications

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TNF is generally believed to be a master pro-inflammatory cytokine, and anti-TNF therapy has become the mainstream treatment for some autoimmune diseases. However, experimental evidence indicates that TNF preferentially activates Tregs, resulting in their proliferation, and enhancing their phenotypic stability and suppressive capacity. This effect of TNF is mediated by the cell surface receptor TNFR2, which is highly expressed by both human and mouse Tregs (1–4). Furthermore, within Tregs, expression of TNFR2 identifies the most suppressive fraction of cells (5). This Research Topic summarizes the latest knowledge on the critical role that TNF-TNFR2 signaling play in modulating the biology of Tregs, as well as other immune-suppressive cell types. Moreover, it analyzes its implications in controlling harmful inflammatory responses, as well as the potential targeting of this key signaling pathway to develop new immunotherapies against cancer.

MECHANISM OF TNFR2 SIGNALING IN THE ACTIVATION AND EXPANSION OF TREGS

Although there is compelling evidence that TNF signaling through the TNFR2 receptor preferentially stimulates Tregs, the molecular mechanism had remained largely unknown. The study of human Tregs by Urbano et al. from Radboud University sheds some new light on this important topic. The results from their *in vitro* studies indicate that, through epigenetic regulatory pathways, the autocrine TNF-TNFR2 feedback loop promotes the stability of a highly suppressive phenotype for Tregs, namely one that expresses high levels of TIGIT, FOXP3, Helios, and EZH2. The study by He et al. from the University of Macau in turn provides both *in vitro* and *in vivo* experimental evidence that P38 MAPK is a component of the signaling pathway, which leads to TNFR2 mediated expansion of Tregs in response to TNF stimulation, as demonstrated by using small molecule inhibitors of known TNFR2 signaling pathways in T cells. The implications of the existence of such signaling pathways in disease are discussed by Yang et al. from Sun Yat-sen

University, summarizing prior knowledge on the signaling pathways pertaining to TNF-TNFR1 and TNF-TNFR2 interactions in the context of autoimmune diseases and tissue regeneration. The main conclusion was that targeting TNFR1 or TNFR2 signaling pathways has significant therapeutic implications.

ROLE OF TNFR2-EXPRESSING TREGS IN CANCER IMMUNOLOGY

The tumor microenvironment is often rich in both TNF, as well as in TNFR2-expressing immune cells, specifically immunosuppressive Tregs or myeloid-derived suppressor cells (MDSCs) (6, 7). High expression of TNFR2 is a characteristic of tumor associated Tregs that potently inhibit anti-tumor immune responses in many different types of cancer (5, 8–10). This idea is further substantiated by a study on patients with advanced epithelial ovarian cancer, by Kampan et al. from Monash University. Furthermore, this study found that IL-6 present in malignant ovarian cancer ascites is responsible for the up-regulation of TNFR2 expression and the expansion of highly suppressive Treg subsets. This finding may help to devise novel immunotherapies aiming to eliminate indirectly tumor-associated immune-suppressive Treg activity by inhibiting IL-6. Salomon et al. from Centre d'Immunologie et des Maladies Infectieuses discussed both historic and current understanding of the puzzling role of TNF as well as anti-TNF therapy in immune and inflammatory responses, leading up to our current understanding of the TNF-TNFR2 pathway and its decisive role in controlling the activation of Tregs. Through the analysis of recent reports on the therapeutic use of agonistic TNFR2-targeting agents in graft vs. host disease (GvHD), this review article raises the possibility of targeting of TNFR2-expressing Tregs using such agents, as potentially a safe and efficient approach to enhance anti-tumor immunity. In addition to its expression on immunosuppressive Tregs, TNFR2 can also be expressed by some tumor cells. Sheng et al. from Zhengzhou University summarized recent research regarding the role of TNFR2 in the promotion of carcinogenesis, cancer immune evasion and tumor growth. They concluded that TNFR2 was an ideal candidate for targeted tumor therapy. Moreover, this article clearly explains bi-directional signaling via TNFR2, given membrane-bound TNF (mTNF) preferentially binds to and activates TNFR2, and in addition to the forward signaling of mTNF→TNFR2, reverse signaling (e.g., TNFR2→mTNF) can also occur. In this case, mTNF acts as a receptor that can transduce activating intracellular signaling when interacting with either sTNFR2 or membrane-bound TNFR2 on the surface of cells. Qu et al. from Tianjin Medical University further takes up this discussion into the possible role of forward and reverse crosstalk between mTNF and TNFR2 in an immunosuppressive tumor microenvironment. Indeed, these reviews leave open the question of whether reverse signaling by TNFR2 expressing Tregs may substantially affect mTNF-expressing immune cells and tumor cells.

THE ROLE OF TNFR2-EXPRESSING TREGS IN OTHER DISEASES

The significant role that TNFR2-expressing Tregs can play in the pathogenesis and treatment of other types of diseases are also included in this Research Topic. Pegoretti et al. from University of Groningen discussed the possibility of selectively modulating the individual TNF receptors, TNFR2 or TNFR1, for the treatment of multiple sclerosis (MS), a neurodegenerative autoimmune disease which is currently resistant to anti-TNF treatment. Mancusi et al. from the University of Perugia discussed the need to evaluate whether Treg activation via TNFR2 could be used to practically enhance the yield, purity, and/or efficacy of Tregs used for cell therapy. Furthermore, they propose that such an approach has the potential for quick clinical translation in HSCT trials, since it is reported that Tregs have the capacity to prevent GvHD and promote immune reconstitution. Ahmad et al. from Universiti Sains Malaysia analyzed the role of TNF-TNFR2 interactions in immune tolerance to allergens and concluded that targeting TNF-TNFR2 interactions may represent a novel strategy for the treatment of allergic inflammatory responses. Based on the idea that genetic variation in the promoter of the TNFRSF1B gene could have a major impact on disease susceptibility, as well as potential responsiveness to TNFR2-targeting therapies, Li and Anderson from National Cancer Institute at Frederick proposed a key transcription factor binding site that may have significant effects on TNFRSF1B promoter activity, and suggested that it should be considered in future studies.

THE ROLE TNFR2 SIGNALING IN OTHER TYPE OF IMMUNOSUPPRESSIVE CELLS

In addition to conventional $CD4^+$ Tregs, TNFR2 also plays a key role in modulating the activity of other type of immunosuppressive cells, such as myeloid-derived suppressive cells (MDSCs), Mesenchymal stem cells (MSCs) and $CD8^+$ Treg cells. In this Research Topic, Chavez-Galan et al. from University of Geneva studied the role that mTNF may play a role in promoting accumulation and enhancing function of MDSC in the pleural cavity during an acute mycobacterial infection. They found that the interaction of mTNF expressed by MDSCs and TNFR2 expressed by CD4 T cells is required for protection against the lethal inflammatory responses, which are sometimes associated with pleural mycobacterial infection. However, Schmid et al. from the University of Regensburg did not observe significant effects on MDSCs after TNFR2 agonist treatment *in vivo*. In addition to CD4 Tregs, some TNFR2-expressing CD8 T cells also have suppressive capacity. Ye et al. from Huazhong University of Science and Technology analyzed the functional consequences of TNFR2 expression by $CD8^+Foxp3^+$ Tregs and by $CD8^+$ Teffs, and concluded that a TNF-TNFR2 mediated coordinated complex events ultimately result in strong $CD8^+$ T cell-mediated immune responses. Mesenchymal stem cells

(MSCs) have immunosuppressive properties which may be therapeutically harnessed in the treatment of autoimmune diseases. Yan et al. from the University of Macau discusses the role of TNF signaling through TNFR1 and TNFR2 on the biology of MSCs. The effect of TNF in MSC-based therapy for autoimmune and inflammatory diseases is also discussed.

EFFECT OF TNFR2-TARGETING PHARMACOLOGICAL AGENTS ON TREG ACTIVITY

Synthetic glycine coated 50 nm polystyrene nanoparticles (PS50G) are able to inhibit allergic airway inflammation. Mohamud et al. from Monash University reported that PS50G treatment preferentially stimulates the expansion of highly suppressive TNFR2$^+$ Tregs, which express high levels of Ki-67, LAP, and CTLA-4. This is likely caused by the induction of CD103$^+$ DCs in mice treated with PS50G. This property of engineered nanoparticles may prove to be useful in the treatment of inflammatory human diseases. Urbano et al. from Radboud University reported that the combination treatment with rapamycin and a TNFR2 agonist antibody was able enhance hypo-methylation of the FOXP3 gene, and consequently promote the stability of Tregs. Clear therapeutic potential has spurred the development of agonistic or antagonistic TNFR2-targeting biological agents in the recent years. Zou et al. from the University of Macau provides an overview regarding the latest progress in the study of TNFR2-targeting pharmacological agents and their therapeutic potential through the up- or down-regulation of Treg activity. In addition to protein drugs, this review suggests that small molecule inhibitors of TNFR2 may also have therapeutic value. Shaikh et al. from University of Macau performed a virtual screening of 400,000 naturally occurring small molecule compounds against TNF-binding sites of TNFR2. Their results indicate that a number of compounds could block the ligand-binding site of TNFR2. *In vitro* and *in vivo* studies are now needed to verify the results of this virtual study. Progranulin (PGRN) is a protein with immunosuppressive properties, which purportedly inhibits TNF-induced TNFR1/2 signaling by directly binding to TNFR1 and TNFR2. However, Lang et al. from University Hospital of Würzburg didn't observe a direct interaction between PGRN and TNFR1/2 in cellular binding studies.

Taken together, 19 primary research reports and review articles in this Frontiers Topic further support and substantiate the decisive role of TNF-TNFR2 interactions in promoting the activation, expansion and phenotypic stability of Tregs, as well as other immunosuppressive cells which may further include CD8 Tregs, MDSCs and MSCs. The critical role of TNFR2 signaling in helping maintain immune homeostasis, in promoting an immunosuppressive tumor microenvironment and in dampening autoimmune or allergic responses, is highlighted across these articles, as is an exploration of the molecular mechanisms that underlie this interaction. Emerging trends in the development of TNFR2-targeting therapeutics are further highlighted. We thus believe this article collection will be helpful for investigators performing fundamental research, as well clinical researchers. Given the substantial potential that targeting TNF-TNFR2 signaling offers for treatment of multiple diseases, we hope this collection of articles will spur further research, and eventually lead to new useful treatments.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Transmembrane Tumor Necrosis Factor Controls Myeloid-Derived Suppressor Cell Activity *via* TNF Receptor 2 and Protects from Excessive Inflammation during BCG-Induced Pleurisy

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Pleural tuberculosis (TB) is a form of extra-pulmonary TB observed in patients infected with *Mycobacterium tuberculosis.* Accumulation of myeloid-derived suppressor cells (MDSC) has been observed in animal models of TB and in human patients but their role remains to be fully elucidated. In this study, we analyzed the role of transmembrane TNF (tmTNF) in the accumulation and function of MDSC in the pleural cavity during an acute mycobacterial infection. *Mycobacterium bovis* BCG-induced pleurisy was resolved in mice expressing tmTNF, but lethal in the absence of tumor necrosis factor. Pleural infection induced MDSC accumulation in the pleural cavity and functional MDSC required tmTNF to suppress T cells as did pleural wild-type MDSC. Interaction of MDSC expressing tmTNF with CD4 T cells bearing TNF receptor 2 (TNFR2), but not TNFR1, was required for MDSC suppressive activity on CD4 T cells. Expression of tmTNF attenuated Th1 cell-mediated inflammatory responses generated by the acute pleural mycobacterial infection in association with effective MDSC expressing tmTNF and interacting with CD4 T cells expressing TNFR2. In conclusion, this study provides new insights into the crucial role played by the tmTNF/TNFR2 pathway in MDSC suppressive activity required during acute pleural infection to attenuate excessive inflammation generated by the infection.

Keywords: transmembrane tumor necrosis factor, TNF receptor 2, myeloid-derived suppressor cells, BCG infection, BCG-induced pleurisy

AUTHOR SUMMARY

Tumor necrosis factor (TNF) is an essential cytokine for host protection and control of tuberculosis (TB) infection that remains one of the leading causes of morbidity and mortality worldwide. Pleural TB is a frequent form of extra-pulmonary TB observed during a primary TB infection or after reactivation. Accumulation of myeloid-derived suppressor cells (MDSC) limiting T cell responses has been previously observed in TB patients. We have evaluated the role of TNF in MDSC function

during acute infection in a murine model of BCG-induced pleurisy. We observed that transmembrane TNF (tmTNF) is crucial for the activity of MDSC and that tmTNF expressed on MDSC interacts with CD4 T cells expressing TNF receptor 2 (TNFR2) for suppressive activity that regulates the inflammatory process associated with pleural mycobacterial infection. This work highlights the essential role of tmTNF during acute mycobacterial pleurisy that is required to attenuate the excessive inflammatory response associated with pleural mycobacterial infection.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that remains a major health problem worldwide causing high morbidity and mortality. The pulmonary form is the most common form of TB infection but extra-pulmonary TB accounts for about one-third of reported TB cases (1). Generally, host immunity to a primary TB infection is able to mount an effective immune response inducing Th1-type cytokines, but in a minority of infected individuals, immunity appears inefficient resulting in an active TB (2). Pleural TB is considered as a form of extra-pulmonary TB which is a frequent clinical problem consisting in the accumulation of fluid and pleural cells in the pleural cavity subsequent to *Mycobacterium tuberculosis* infection (3, 4). Pleural TB has been reported as a primary TB pleurisy consequent to the rupture of pulmonary subpleural caseous lesions into the pleural space (5). Pleural TB can also be observed in patients with reactivation of latent TB and, in certain cases, associated with the use of corticosteroid and anti-TNF treatments or presence of comorbidities as HIV/AIDS and diabetes (6).

During acute pleural mycobacterial infection, the activity of inflammatory cells can be controlled by tolerogenic cells that attenuate the inflammatory process associated with the infection. Among these, MDSC are a heterogeneous population of innate cells that expand during cancer, inflammation, and infection, and play different roles depending on pathological processes (7). MDSC have been described as natural suppressor cells inhibiting the proliferative response of T-helper lymphocytes. MDSC have been distinguished as two distinct phenotypes: polymorphonuclear $Ly6G^{+}GR\text{-}1^{high}$ and mononuclear $Ly6G^{-}GR\text{-}1^{dim}$ MDSC (8). High frequencies of MDSC in blood and lung of patients with TB have been reported (9–11). In BCG vaccination studies in mice, MDSC were shown to restrain T cell priming by NO-dependent mechanisms (12). In a murine model of TB, MDSC have been shown to accumulate in the lung and other organs during progressive TB (13, 14). A study has reported that during chronic TB infection, there was excessive MDSC accumulation in the lung of sensitive mice and their depletion ameliorated disease outcome (15). The studies reported so far on MDSC activity during mycobacterial infection have been performed during chronic TB infection and results have shown that expansion of MDSC is associated with severity of the infection as MDSC prevent immune responses against mycobacteria (16).

Tumor necrosis factor is an important cytokine involved in the pathogenesis of several human inflammatory diseases and host defense mechanisms against many pathogens. TNF is first synthesized as a precursor or transmembrane form (tmTNF) and then cleaved by the TNF-α converting enzyme (TACE) under any stimuli which induce TNF producing soluble TNF (solTNF) (17). Using genetic mouse models expressing a mutated transmembrane form of TNF that cannot be cleaved by TACE (tmTNF KI mice), it has been shown that tmTNF mediates host protection against *Mycobacterium bovis* BCG and acute *M. tuberculosis* infections (18–21). We have also shown that inhibition of solTNF, by dominant-negative TNF biologics that do not block tmTNF, preserved immunity during acute BCG and *M. tuberculosis* infections and this treatment was efficient in preventing acute liver injury (22, 23). Anti-TNF therapies neutralizing soluble and tmTNF have shown their efficacy for the treatment of autoimmune inflammatory diseases; however, the mechanisms by which TNF can control immune tolerance during infection and how this can be disrupted by TNF inhibition remains unclear.

Recent studies on MDSC in the context of chronic inflammation have shown that TNF can block differentiation of MDSC and increase their intrinsic suppressive function (24). Inhibition of TNF during chronic inflammation decreased MDSC suppressive activity and enhanced maturation toward macrophages and dendritic cells restoring *in vivo* immune functions (24). More recently, using a model of sterile inflammation, it has been shown that membrane expression of TNFR2 on MDSC was required for differentiation and functionality (25). TNF signaling through TNFR2 promoted survival of MDSC helping tumor evasion (26). In mouse models of carcinogenesis, neutralization of TNF resulted in reduced MDSC accumulation and delayed the tumor growth (27). Together, these data show that the TNF pathway plays a critical role in the regulation of MDSC function. However, at present, whether TNF is required for MDSC accumulation and activity during acute mycobacterial infection and whether the TNF interaction with either TNFR1 or TNFR2 is required for MDSC suppressive function is unclear.

In this study, we have investigated the role of tmTNF in MDSC generation and suppressive activity in a model of acute pleural mycobacterial infection. Our data show that tmTNF on MDSC interact with CD4 T cells expressing TNFR2 but not TNFR1. TmTNF-TNFR2 interaction plays a critical role for MDSC suppressive activity on T cells which allows attenuation of the inflammation within the pleural cavity. Our data indicate that MDSC exert a beneficial function limiting inflammation during acute mycobacterial infection and favoring disease resolution.

MATERIALS AND METHODS

Animals

C57BL/6 wild-type (WT), deficient for TNF (TNF KO) (28), and transmembrane form TNF knockin ($tmTNF^{\Delta 1-9,K11E}$, deletion of amino acids 1–9 and substitution at position 11) (29) TNFR1 KO mice (30) and double TNFR1/TNFR2 (Jackson laboratory) (31) were used. CD4cre/TNFR2fl/fl mice that do not have TNFR2 on the surface of T cells were obtained by crossing C57BL/6NTac-Tg(CD4-cre*)* (32) (from Taconic farms) with TNFR2fl/fl mice (from EUCOMM *via* Institut Clinique de la souris, France from Prof Daniela Mannel, University of Regensburg, Germany). For experiments, adult mice (8–12 week old) were housed in animal facility of the Medical Faculty, University of Geneva

(Geneva, Switzerland). All animal experiments were carried out in accordance with institutional guidelines and were approved by the academic ethical committee on animal experimentation and the cantonal veterinary office from Geneva (authorization No. GE167/14).

M. bovis BCG

Mycobacterium bovis BCG Pasteur strain 1173 P2 was grown in Middlebrook 7H9 broth containing ADC (Difco), and middle-log phase bacilli were washed and frozen aliquots kept frozen at −80°C until use.

M. bovis BCG Infection

BCG-induced pleurisy infection was generated by intrapleural cavity injection of 10^6 CFU of *M. bovis*. BCG in 100 μL of saline as previously reported (33). Mice were monitored twice a week for body weight and sacrificed at day 14 post-infection or followed for survival studies. Groups of naïve littermates or uninfected mice were analyzed in the same way as infected animals.

Pleural Cell and Fluid Preparation

Thoracic cavities from naïve and infected mice were washed twice with 1 mL of 2 mmol/L EDTA-phosphate-buffered saline (PBS), samples were centrifuged, and supernatants containing pleural fluid were frozen at −80°C for cytokine evaluation. Pleural cells were suspended in PBS-1% bovine serum albumin, counted, and used for the different techniques such as Flow cytometry, enrichment of MDSC, and cytospin followed by May-Grünwald-Giemsa and Ziehl-Neelsen (ZN) staining as reported (33).

Multiparametric Flow Cytometry Analysis

The frequency of immunological cellular subpopulations in pleural cells was analyzed by flow cytometry. Briefly, cells were stained for 30 min at 4°C with different combinations of the following fluorochrome-conjugated mAb: GR-1 (Clone RB6-8C5), F4/80 (Clone BM8), Ly6C (Clone HK1.4), CD3 (Clone 145-2c11), and CD4 (Clone GK1.5) (BioLegend), and CD11c (Clone HL3) (BD Bioscience), and iNOS (Clone CXNFT) (Cell Signaling technology, eBioscience). After antibody incubation, cells were washed with PBA (phosphate buffered saline containing 0.1% Sodium Azide and 0.2% Albumin bovine). Data were collected using a FACs CyAn flow cytometer (Beckman Coulter, Inc.) and analyzed with FlowJo (Tree Star) software. 100,000 events were acquired per sample.

Enrichment of MDSC

Single-cell suspensions were obtained from the pleural cavity of infected mice. MDSC were enriched by using magnetic microbeads kit (MDSC isolation kit; Miltenyi Biotec) and AutoMACS Pro Separator (Miltenyi Biotec). First, polymorphonuclear $Ly6G^+GR\text{-}1^{high}$ MDSC (PMN-MDSC) subpopulation were indirectly magnetically labeled with anti-Ly-6G and retained. The unlabeled cell fraction (depleted of $Ly\text{-}6G^+Gr\text{-}1^{high}$) was indirectly magnetically labeled with anti-GR1 and the mononuclear $Ly6G^-GR\text{-}1^{dim}$ MDSC (MO-MDSC) subpopulation isolated by positive selection. The purity of MDSC subpopulations was evaluated by flow cytometry (surface marker) and by cytospin (May-Grünwald-Giemsa stain) to identify morphology (PMN- vs MO- MDSC) and intracellular bacilli with ZN staining.

In Vitro Proliferation and Suppression Assay of T Cells

Spleen cells from WT, TNFR1-KO, and TNFR2-CD4 KO mice were prepared as described previously (18). Bulk splenocytes were stimulated with plate-immobilized anti-CD3 (Clone 145-2C11) plus soluble anti-CD28 (Clone 37.51) (eBioscience), both antibodies were used at concentration of 1 μg/mL. Splenocytes were co-incubated with varying ratios of MDSC (1:1, 1:2, 1:4), after 48 h of co-culture at 37°C, supernatants were collected for cytokine measurements and cells for proliferation assay using KI-67 (Clone 16A8) (Biolegend). Briefly, cells were harvested, washed with PBA, and then surface molecules (CD3, CD4) were stained as described above. Subsequently, cell pellet was suspended in Fixation/permeabilization solution (eBioscience) at 4°C, washed with permeabilization buffer (eBioscience), and then stained with KI-67 for 30 min at 4°C. Cells were washed and analyzed by flow cytometry. Splenocytes with polyclonal stimuli were considered as 100% proliferation (Positive control).

PCR Analysis for Genotyping CD4-cre TNFR2 Mice

DNA extracted from the tail was used for PCR to detect homozygous mice with TNFR2 deletion. Primers were CD4-cre F: 5′-CCCAACCAACAAGAGCTC-3′, and CD4-cre R: 5′-CCCAGAAATGCCAGATTAGG-3′. Amplifications were performed using the following program: preheating stage at 95°C for 3 min, 35 cycles at 95°C for 45 s, 56,7°C for 30 s, 72°Cfor 45 s, and extension at 72°C for 5 min.

Flow-Sorting of CD4 T Cells from the Spleen

Spleen from 14-day infected mice was dissociated and CD4 T cells flow-sorted using magnetic microbeads kit (mouse CD4 T cells isolation kit; Miltenyi Biotec) and AutoMACS Pro Separator (Miltenyi Biotec).

Cytokine Evaluation Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine levels in the pleural fluid and cell supernatants were assessed by ELISA. IL-2, IFN-α, IFN-γ, IL-12p70, IL-6, IL-10, and the chemokine CCL2 (MCP-1) were quantified in the pleura fluid and in cellular supernatants in accordance with the manufacturer's instructions.

Western Blot

Enriched MO-MDSC and PMN-MDSC were lysed and subjected to SDS-PAGE and transferred to membranes. The primary antibodies used were polyclonal Arginase 1 and iNOS (Cell Signaling Tech) and secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit. Blots were developed with chemiluminescence substrate ECL. The density of the bands was analyzed using the online ImageJ 1.39c software. Actin was used as loading control as reported (34).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad Soft., La Jolla, CA, USA). *P*-value <0.05 was considered as statistically significant. Experiments with two groups were analyzed with an unpaired Student's *t*-test and one-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons.

RESULTS

Transmembrane TNF is Sufficient to Rescue Mice from BCG-Induced Pleurisy and to Prevent Excessive Accumulation of Neutrophils in the Pleural Cavity

We have previously reported that a mouse model of pleural infection shows that TNF or its receptors are crucial to control *M. bovis* BCG-induced pleurisy (33). To further evaluate whether transmembrane TNF (tmTNF) or soluble-TNF form is required for protection during pleural mycobacterial infection, we analyzed mice that express a mutated form of TNF (tmTNF$^{\Delta 1-9,K11E}$ or tmTNF KI) that cannot be cleaved by TACE and do not produce solTNF (29). As previously reported after an i.v. BCG infection, we observed that tmTNF KI mice were able to control BCG-induced pleurisy as they survived for more than 14 weeks post-infection as is the case for WT mice (**Figure 1A**). By contrast, TNF KO mice did not resist pleural BCG infection and died at 7–9 weeks after infection (**Figure 1A**). Pleural cavity cytokine profiles in WT and tmTNF KI mice exhibited no differences at 14 weeks post-infection, suggesting that tmTNF KI resolved the infection as observed in WT mice (Figure S1 in Supplementary Material). Evaluation of cells accumulated in the pleural cavity at day 14 post-infection showed higher cell numbers in tmTNF KI mice than in WT mice but TNF KO mice had twofold higher cell numbers compared with WT mice (**Figure 1B**). Pleural BCG infection in TNF KO, but not tmTNF KI mice resulted in expansion of multinucleated giant cells containing numerous vacuoles and many bacilli (**Figure 1C**). TNF KO cells were previously shown to be deficient in iNOS expression and unable to eliminate bacteria which led to a miliary TB (33). Accumulated cells were mainly myeloid CD11b$^+$ cells and the total number was not affected by the absence of TNF (**Figure 1D**). Nevertheless, the number of GR1$^+$ cells significantly increased in TNF KO but not in tmTNF KI mice suggesting that tmTNF controls neutrophil recruitment (**Figure 1E**). However, both solTNF and tmTNF regulated the recruitment of CD3 lymphocytes in BCG-infected mouse pleural cavity (**Figure 1F**). Our data show that tmTNF is mandatory for the control of cell recruitment and protection against BCG-induced pleurisy in mice.

BCG-Induced Pleurisy Activates Expansion of Monocytic and Granulocytic MDSC

Mycobacterium tuberculosis infection in human and in mouse has been associated with the accumulation and expansion of MDSC which may contribute to aggravation and impaired control during chronic infection. We evaluated the presence of MDSC in the pleural cavity following BCG-induced pleurisy. Two types of analyses have been performed according to previous publications on MDSC during mycobacterial infection (13, 15). A gate on CD11b$^+$ F4/80$^+$ myeloid cell population showed that naïve TNF KO and tmTNF KI mice presented similar cell numbers compared to WT mice, but BCG infection induced a 6-fold increase at day 14 post-infection in all groups of mice (**Figure 2A**). Co-expression of Ly6C$^+$ and GR1$^+$ was evaluated on CD11b$^+$ F4/80$^+$ subset which may contain cells with a phenotype of MDSC (**Figure 2B**). The CD11b$^+$ F4/80$^+$ Ly6C$^+$ GR1$^+$ subpopulation expanded during infection and an increased frequency was found in TNF KO mice, but not in tmTNF KI mice, suggesting that tmTNF, but not solTNF regulates their expansion in the pleural cavity of infected mice (**Figures 2C,D**). To further confirm the expansion of cells with a phenotype of MDSC, a second analysis of pleural cells was done by evaluating cells co-expressing CD11b$^+$ GR1$^+$ as previously described (14). The analysis confirmed that this subset expanded during the infection and its frequency was higher in TNF KO mice compared with WT and tmTNF KI mice (Figure S2 in Supplementary Material).

To explore if these subpopulations contain functional MDSC, pleural cells at day 14 post-infection were fractionated into two subpopulations according to the presence of GR1 using MDSC-isolation kits as described (13). A first fraction was defined as granulocytic or polymorphonuclear MDSC cells (PMN-MDSC) and characterized by flow cytometry as CD11b$^+$F4/80$^+$Ly6G$^+$GR1^{high}Ly6C^{int} (**Figure 3A**). Further examination by light microscopy confirmed a polymorphonuclear phenotype as expected (**Figure 3A**). Flow cytometry analysis of iNOS expression showed that tmTNF was sufficient to maintain the expression of iNOS in the MDSC population; however, a small fraction of PMN-MDSC from TNF KO produced iNOS (**Figures 3A,B**). The second isolated fraction was characterized as CD11b$^+$F4/80$^+$Ly6G$^-$GR1^{dim}Ly6C^{high} and the phenotype defined as mononuclear MDSC cells (MO-MDSC). WT and tmTNF KI MO-MDSC showed iNOS expression that was lower for TNF KO cells (**Figures 3D,E**). The poor ability of TNF KO MDSC to produce iNOS was confirmed using CD11b$^+$ GR1$^+$ as main MDSC markers after flow-sorting (Figures S3A,B in Supplementary Material). The capacity of MDSC to contain intracellular mycobacteria was evaluated. Analyses of cells containing bacilli by ZN staining of MDSC sorted preparations revealed that phagocytic MDSC is a very rare event for pleural MDSC. We could observe few PMN-MDSC and MO-MDSC containing one or two bacilli in TNF KO cells (**Figures 3C,F**). The frequency of MDSC containing bacilli in WT and tmTNF KI cells was very low. Our data show that BCG-induced pleurisy induces accumulation of MDSC in the pleural cavity and tmTNF regulates their accumulation.

Transmembrane TNF Restores Mononuclear MO-MDSC and Polymorphonuclear PMN-MDSC Suppressive Functions on CD4 T Cells

Considering the phenotypic similarity of the two defined myeloid fractions with reported MDSC, we assessed their functional

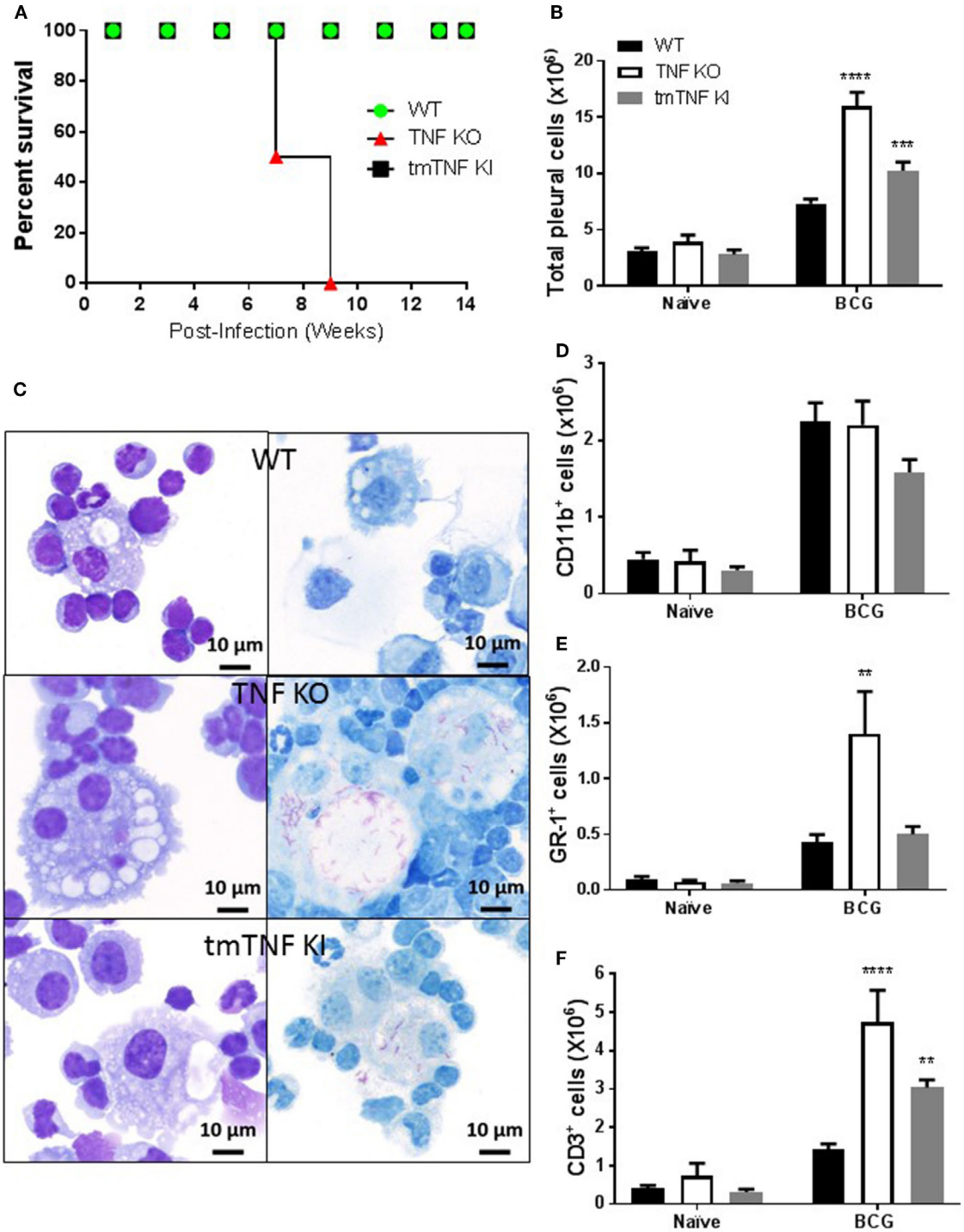

FIGURE 1 | Membrane bound tumor necrosis factor (TNF) is sufficient to activate an efficient immune response during BCG-induced pleurisy. **(A)** The survival of BCG-infected mice was monitored for 14 weeks post-infection (n = 7–9 from two experiments). **(B)** Total number of cells from the pleural cavity recovered in naïve littermate or uninfected and in mice infected with BCG for 14 days. (Bar graphs show means ± SEM of four experiments (n = 6, naïve and n = 20, infected mice/per group) wild-type (WT) and TNF KO and infected tmTNF KI mice). **(C)** Photomicrographs representative from cytospin preparation with cells isolated from the pleural cavity at day 14 post-infection and stained with May-Grunwald-Giemsa (MGG) (left) and Ziehl-Neelsen (ZN) staining (right) that shows intracellular bacilli (red) in macrophages in WT and tmTNF KI and in giant cells in TNF KO cells. **(D)** Quantification of the percentage of $CD11b^+$, **(E)** $GR\text{-}1^+$, and **(F)** $CD3^+$ cells was performed by flow cytometry analysis and absolute numbers were obtained considering the total cell number recovered from the pleural cavity in individual mouse (n = 4, naïve and n = 9, infected mice). Bar graphs show means ± SEM **(D–F)** of two experiments (**P < 0.001, ***P < 0.0001, and ****P < 0.00001 vs WT, ANOVA and Bonferroni *post hoc* test). Scale bars = 10 µm.

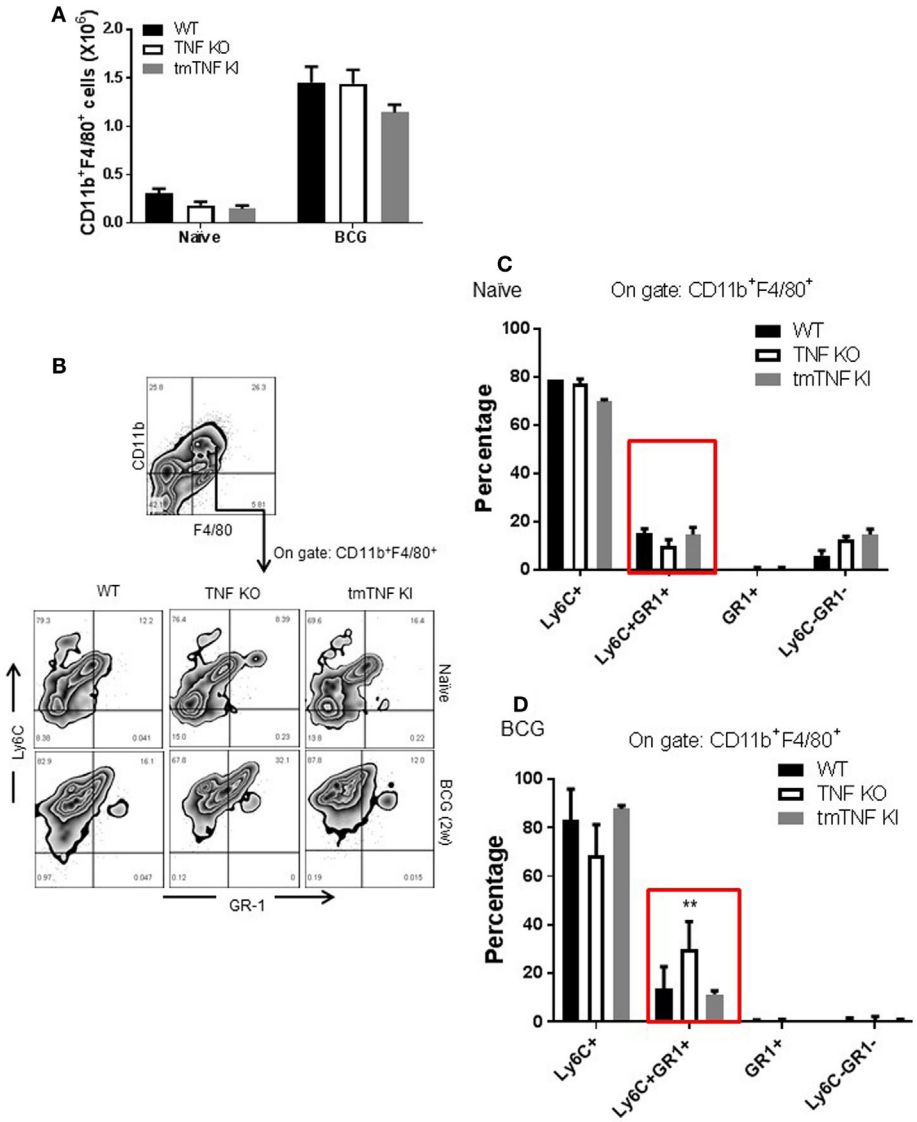

FIGURE 2 | Absence of tumor necrosis factor (TNF) induces an elevated expansion of cells with a phenotype of myeloid-derived suppressor cells (MDSC) phenotype in the pleural cavity of BCG-infected mice which is restored by tmTNF. **(A)** Absolute numbers of $CD11b^+$ $F4/80^+$ cells obtained by flow cytometry analysis considering the total cell number per pleural cavity in individual mouse. **(B)** Representative zebra plot corresponding to the strategy used to identify Ly6C and GR1 coexpression on gated $CD11b^+$ $F4/80^+$ subsets. **(C)** The frequency of $Ly6C^+$, $Ly6C^+GR1^+$, $GR1^+$, and $Ly6C^-GR1^-$ subpopulations on gated $CD11b^+$ $F4/80^+$ cells is shown in naïve mice, and **(D)** in BCG- infected mice at day 14 post-infection. Bar graphs show means ± SEM of two experiments ($n = 5$, naïve, $n = 9$ infected mice wild-type (WT) and TNF KO, and $n = 5$ tmTNF KI mice, $^{**}P < 0.001$ vs WT. ANOVA and Bonferroni *post hoc* test).

characteristic in terms of suppression of T cell proliferation by flow cytometry (Figure S4 in Supplementary Material). Co-culture experiments of pleural MO-MDSC ($Ly6G^-GR\text{-}1^{dim}$) from WT mice with stimulated splenocytes from naïve mice revealed a partial inhibition of CD4 T cell proliferation in a dose-dependent manner (**Figure 4A**). By contrast, MO-MDSC from TNF KO were not able to reduce CD4 T cell proliferation, while MDSC from tmTNF KI inhibited CD4 T cell proliferation similar to WT cells (**Figure 4A**). In addition, co-cultures of both WT and tmTNF KI MO-MDSC and splenocytes reduced IL-2 and IFN-γ production but not of TNF KO MO-MDSC (**Figures 4B,C**). Pleural PMN-MDSC ($Ly6G^+GR1^{high}$) from WT mice inhibited CD4 T cell proliferation in a dose-dependent manner (**Figure 4D**). PMN-MDSC from TNF-KO did not inhibit CD4 T cell proliferation, whereas tmTNF KI PMN-MDSC inhibited CD4 T cell proliferation (**Figure 4D**). However, PMN-MDSC co-cultures did not suppress IL-2 and IFN-γ responses as is the case for MO-MDSC, but surprisingly, TNF KO MDSC activated IFN-γ production with an 80-fold increase in a dose-dependent manner (**Figures 4E,F**). Together, these data show that tmTNF mediates the suppressive function of MDSC on CD4 T cells.

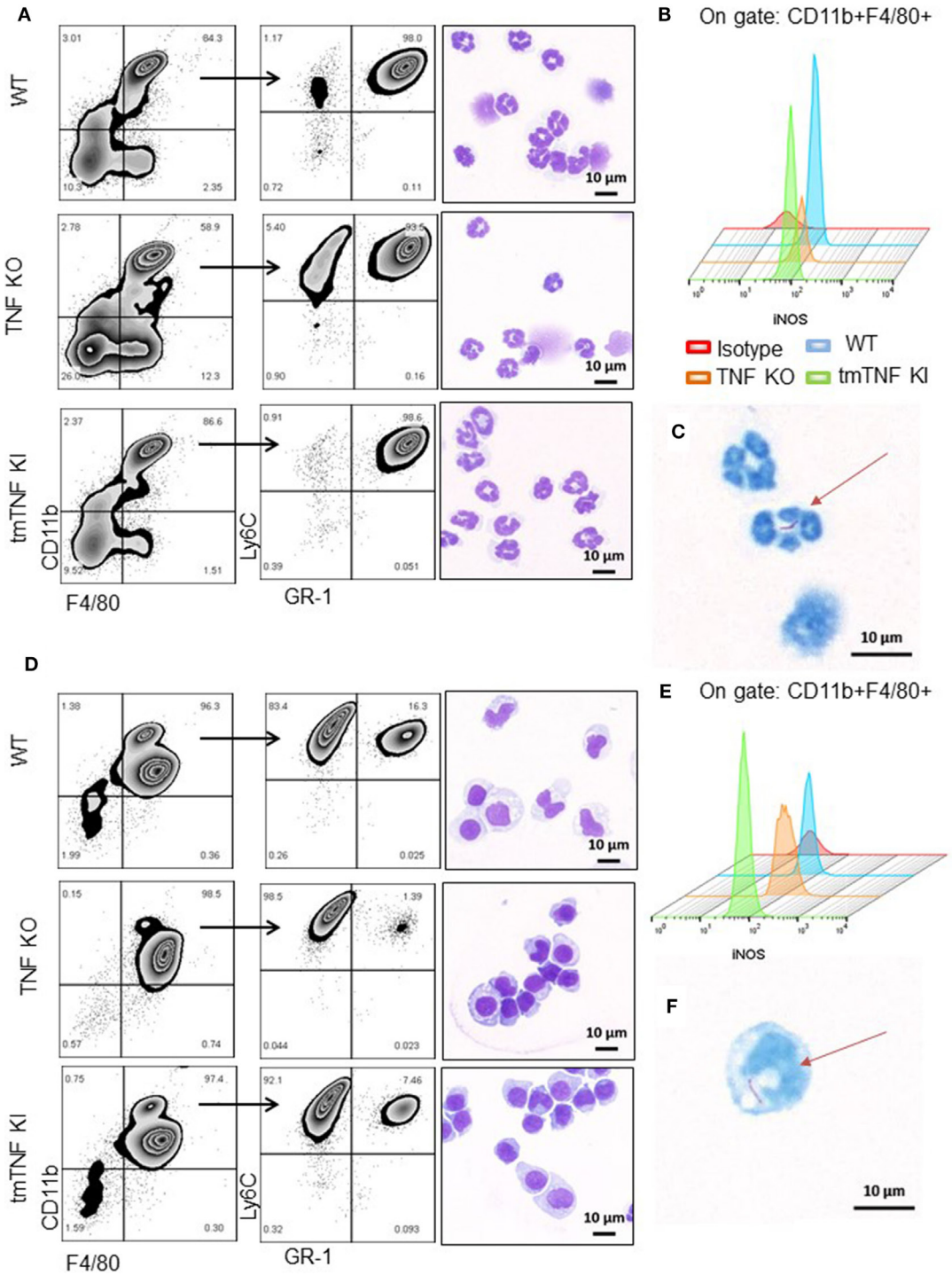

FIGURE 3 | Presence of tmTNF restores the frequency of myeloid-derived suppressor cells and iNOS expression. MDSC from a pool of pleural cells (n = 5–7 mice per group) were flow-sorted using a MDSC kit. **(A)** Representative zebra plot corresponding to the analysis used to evaluate the purity of sorted PMN-MDSC by flow cytometry and morphology after staining with May-Grunwald-Giemsa (MGG). **(B)** Representative Stagger Offset histogram depicting the frequency of iNOS$^+$ cells inside the gate of CD11b$^+$ F4/80$^+$ cells and comparison between wild-type (WT) (blue), tumor necrosis factor (TNF) KO (orange), and tmTNF KI (green) pleural MDSC. **(C)** Flow-sorted PMN-MDSC from TNF KO mice stained with Ziehl-Neelsen (ZN) and illustration of the presence of one BCG in the cell indicated by the arrow. **(D)** Representative zebra plot corresponding to the analysis used to evaluate the purity of MO-MDSC by flow cytometry and morphology after MGG stain. **(E)** Representative Stagger Offset histogram showing the frequency of iNOS$^+$ cells inside the gate of CD11b$^+$ F4/80$^+$ pleural MDSC and comparing WT (blue), TNF KO (orange) and tmTNF KI (green) cells. **(F)**. Flow-sorted MO-MDSC from TNF KO mice stained with ZN and illustration of the presence of one BCG in the cell indicated by the arrow. Data are representative of two experiments.

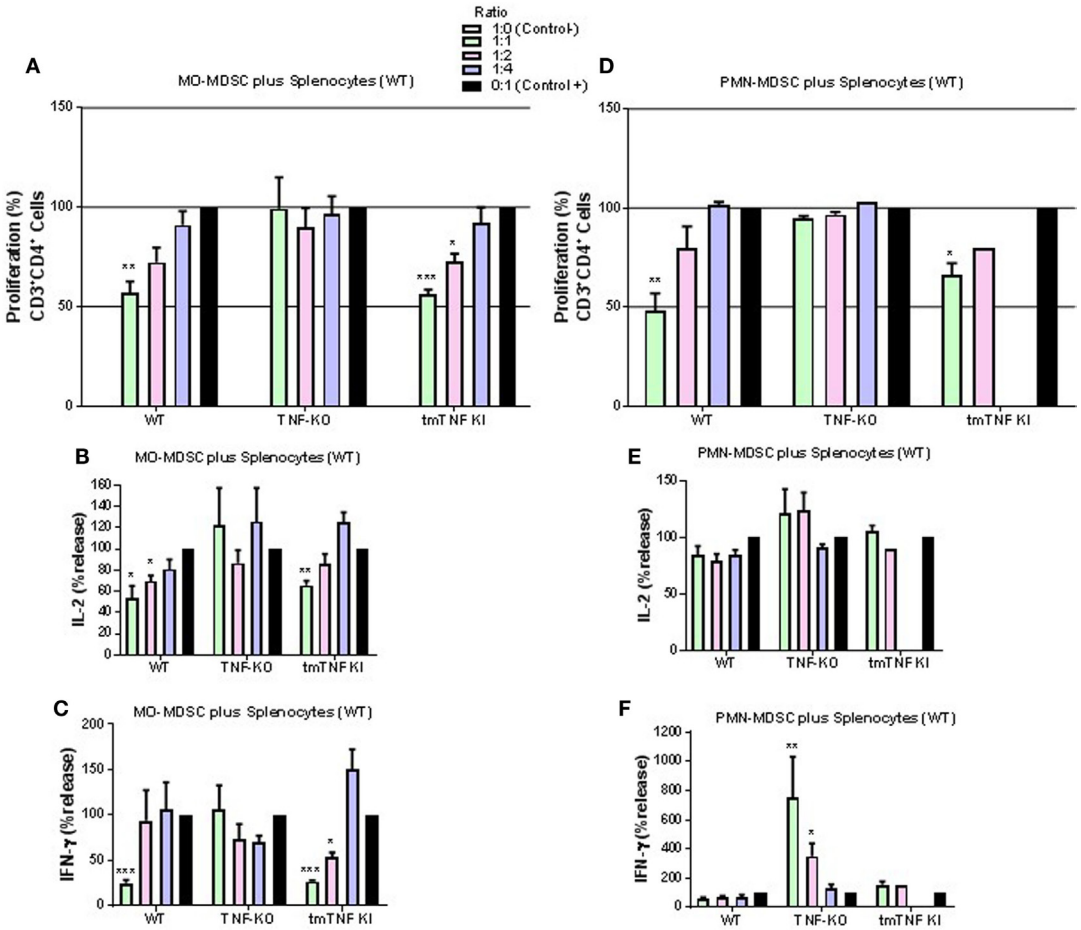

FIGURE 4 | Expression of tmTNF restores myeloid-derived suppressor cells (MDSC) suppressive function on CD4 T cells. **(A)**. Proliferation of CD3 CD4 T cells after polyclonal stimulation and in the presence or absence of flow-sorted pleural mononuclear MO-MDSC (ratio MDSC:Splenocytes, 1:1, 1:2, and 1:4) was measured by flow cytometry using KI-67 after 48 h of co-culture. Pools of pleural cells were from 5 to 7 mice per group. **(B)** IL-2 and **(C)** IFN-γ production from supernatants of splenocytes and MO-MDSC co-cultures at different ratio. **(D)** Proliferation of CD3 CD4 T cells after polyclonal stimulation and in the presence or absence of flow-sorted pleural polymorphonuclear PMN-MDSC co-cultured with splenocytes for 48 h. **(E)** IL-2 and **(F)** IFN-γ production from co-cultures of PMN-MDSC and splenocytes. MDSC alone were used as the negative control and activated splenocytes as positive controls (100%). Bar graphs show means ± SEM. Data are representative of three independent experiments (n = 3–6 per group, $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs positive control. ANOVA and Bonferroni *post hoc* test).

Interactions of MDSC Expressing tmTNF with TNFR2 on CD4 T Cells Is Required for MDSC Suppressive Function

We further asked if a specific TNFR is required for the CD4 T cell interaction with MDSC expressing tmTNF. Pleural MDSC cells from BCG-infected mice were co-cultured with activated splenocytes from mice whose CD4 T cells do not express TNFR2 (TNFR2-CD4 KO). We observed that MO-MDSC from either WT or tmTNF KI mice did not exhibit any suppressive activity on activated CD4 T cells and surprisingly, lymphocytes appeared to proliferate with increasing amounts of MO-MDSC (**Figure 5A**). The levels of IL-2 were not changed and the level of IFN-γ increased in a dose-dependent from MO-MDSC (**Figures 5B,C**). Similarly, PMN-MDSC from WT mice or tmTNF KI did not suppress CD4 T cell proliferation, but rather PMN-MDSC increased the frequency of CD4 T cell proliferation (**Figure 5D**). The levels of IL-2 were not affected and IFN-γ levels increased with increasing amounts of PMN-MDSC (**Figures 5E,F**).

We then tested whether absence of TNFR1 on CD4 T cells would affect responses to MDSC. MO-MDSC from WT and tmTNF KI mice induced suppression on T cell proliferation and on IL-2 effects but not on IFN-γ in the absence of TNFR1 on CD4 T cells (**Figures 6A–C**). PMN-MDSC also showed suppressive activity on TNFR1 KO T cell proliferation and on IL-2 but not on IFN-γ production (**Figures 6D–F**). These data suggest that TNFR1 expression on CD4 T cells is not essential for interaction between MDSC and CD4 T cells to exert suppressive function. Previous reports have shown that TNF signaling drives MDSC accumulation and favors tumor cell evasion. To examine if TNF signaling was also important for MDSC activity during acute BCG infection, we used sorted pleural MDSC from BCG-infected TNFR1/TNFR2 KO mice. These mice were shown to be highly sensitive to both

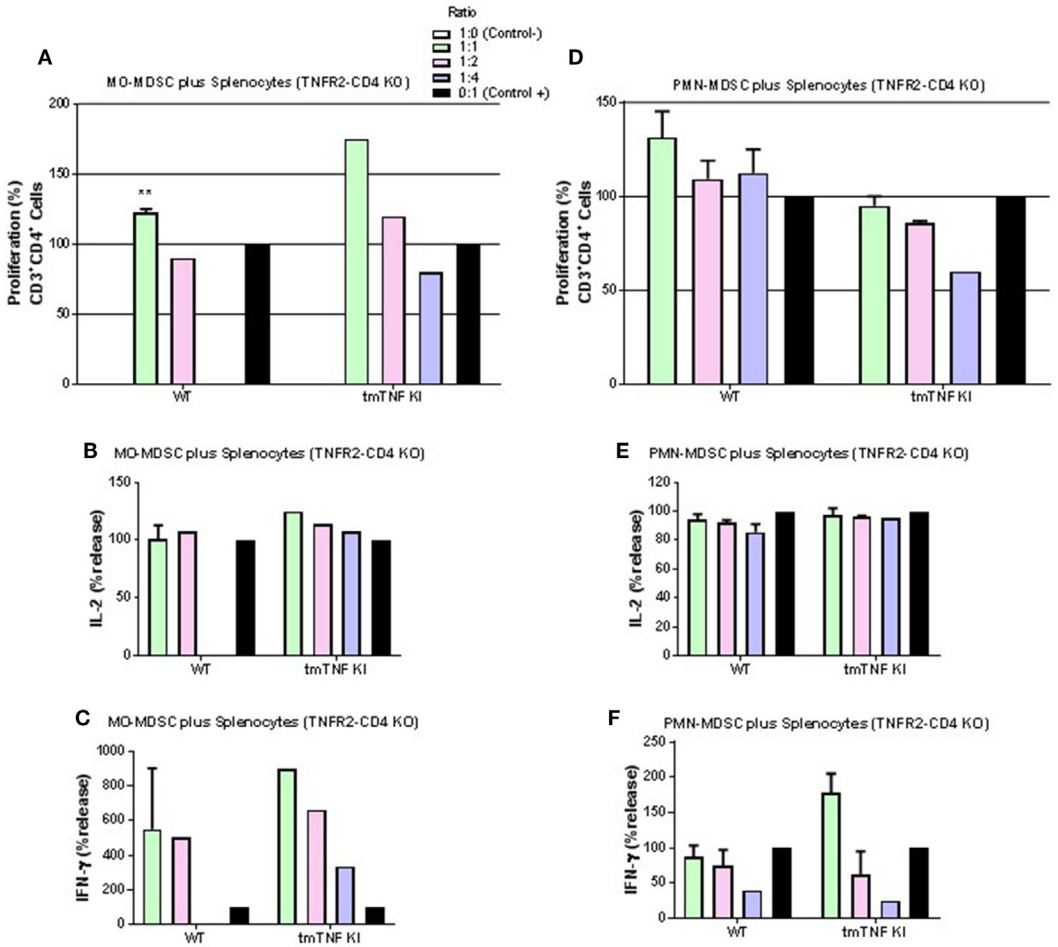

FIGURE 5 | Interaction of tmTNF and TNF receptor 2 (TNFR2) is required for suppressive function of myeloid-derived suppressor cells (MDSC) on CD4 T responses. **(A)** Proliferation of CD3 CD4 T cells without TNFR2 (TNFR2-CD4 KO) after polyclonal stimulation and in the presence or absence of flow-sorted pleural mononuclear MO-MDSC (ratio MDSC:Splenocytes, 1:1, 1:2 and 1:4) was measured by flow cytometry using KI-67 after 48 h co-culture. **(B)** IL-2 and **(C)** IFN-γ production from splenocyte and MO-MDSC co-cultures at different ratios. **(D)** Proliferation of CD3 CD4 T cells without TNFR2 (TNFR2-CD4 KO) after polyclonal stimulation and in the presence or absence of flow-sorted pleural polymorphonuclear PMN-MDSC co-cultured with splenocytes for during 48 h. **(E)** Il-2 and **(F)** IFN-γ production from co-cultures of PMN-MDSC and splenocytes. MDSC alone were used as negative control and activated splenocytes as positive controls. (Bar graphs show means ± SEM of $n = 4$–6 from two independent experiments, $^{**}P < 0.001$ vs positive control. ANOVA and Bonferroni *post hoc* test).

systemic and pleural BCG infection (31, 33). Both MO- and PMN-MDSC displayed complete absence of suppressive activity on CD4 T cells and even an enhancement of the proliferation of CD4 T cells co-cultured with MO-MDSC was observed (Figure S5 in Supplementary Material). Our results indicate that TNFR2 expression on lymphocytes is essential for the interaction with tmTNF to drive MDSC effector functions. In addition, absence of TNFRs on MDSC not only abolishes suppressive activity of MDSC but also activates proliferation and IFN-γ production of CD4 T cells.

Transmembrane TNF Down-Regulates Excessive Inflammation during Acute BCG-Induced Pleurisy

We have reported that BCG-induced pleurisy causes overt inflammation in the pleural cavity of TNF KO and TNFR1R2 KO, but not in WT mice (33). Indeed, at day 14 post-infection, the amounts of IFN-γ were 100-fold higher in TNF KO than in WT mice (33). We further explore whether tmTNF controls overt inflammatory environment within the pleural cavity. Following BCG-induced pleurisy, inflammation was confirmed in the pleural cavity of TNF KO mice and was controlled in tmTNF mice that had similar levels of pleural IFN-γ than WT mice (**Figure 7A**). In contrast to IFN-γ, IFN-α was reduced in TNF KO mice but the levels were similar in tmTNF KI and WT mice (**Figure 7B**). As the main producers of IFN-γ are CD4 T cells, we analyzed the frequency of CD4 T cells expressing IFN-γ and also IL-17 in the pleural cavity and in the spleen. We found similar results for WT and tmTNF KI cells, but TNF KO had an increased proportion of CD4 T cells expressing IFN-γ and lower frequency of cells producing IL-17 (**Figures 7C,D**). Splenic CD4 T cells showed only in TNF KO mice an increased

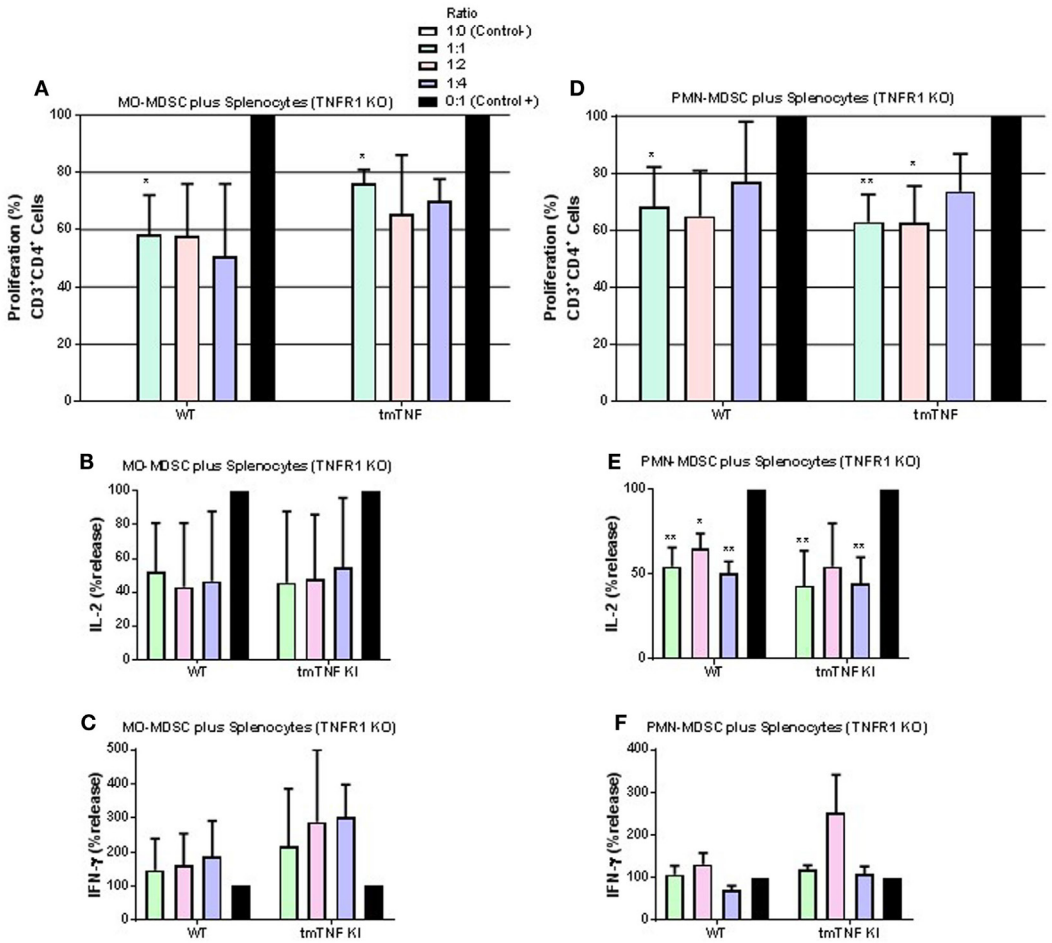

FIGURE 6 | TNFR1 is not necessary for suppressive function of myeloid-derived suppressor cells (MDSC) on CD4 T cell responses. **(A)** Proliferation of CD3 CD4 T cells from TNFR1 KO mice after polyclonal stimulation and in the presence or absence of flow-sorted pleural mononuclear MO-MDSC (ratio MDSC:Splenocytes, 1:1, 1:2, and 1:4) was measured by flow cytometry using KI-67 after 48 h of co-cultures. **(B)** IL-2 and **(C)** IFN-γ production from supernatants of splenocytes and MO-MDSC co-cultures at different ratio. **(D)** Proliferation of CD3 CD4 T cells from TNF KO mice after polyclonal stimulation and in the presence or absence of flow-sorted pleural polymorphonuclear PMN-MDSC co-cultured with splenocytes for 48 h. **(E)** IL-2 and **(F)** IFN-γ production from co-cultures of PMN-MDSC and splenocytes. MDSC alone were used as the negative control and activated splenocytes as positive controls (100%). (Bar graphs show means ± SEM of $n = 5–7$ mice per group from two independent experiments, $^*P < 0.05$ and $^{**}P < 0.001$ vs positive control. ANOVA and Bonferroni *post hoc* test).

frequency IFN-γ producing cells but no differences were observed for cells producing IL-17 (**Figures 7E,F**). Spleen CD4 T cells were then flow-sorted and activated with BCG (MOI 1) or anti-CD3/anti-CD28. Our results showed that BCG activation of CD4 T cells induced similar amounts of IFN-γ in WT and tmTNF KI cells, while TNF KO cells were over activated producing higher amounts of IFN-γ (**Figure 7G**). The second activation of CD4 T cells with anti-CD4/anti-CD28 antibodies also enhanced IFN-γ production by tmTNF KI cells compared to WT cells but TNF KO cells produced substantially higher amounts than tmTNF KI cells. This result suggests that TNF KO CD4 T cells are highly responsive to both antigen specific and polyclonal stimuli but tmTNF KI cells have attenuated responses as WT cells (**Figure 7H**). In conclusion, tmTNF is sufficient for downregulating hyperactivated TNF KO CD4 T cells, thus controlling cell-mediated inflammatory responses.

DISCUSSION

Tumor necrosis factor is a pleiotropic cytokine pivotal for the development of several human immunopathologies, and also involved in immunoregulatory functions and host defense mechanisms against many pathogens. TNF has been considered a major pro-inflammatory cytokine, however, from accumulating studies it appears that during mycobacterial infection TNF exerts both pro and anti-inflammatory activities that are necessary, first, for a rapid recruitment of cells to infected sites and second to attenuate this process in order to limit tissue injury. *In vitro* and *in vivo* studies suggested that TNF acts as a negative regulator of Th1 immune responses and that TNFR1 signaling is the receptor mediating anti-inflammatory activities during chronic mycobacterial infections (17, 35, 36). However, the underlying mechanisms involved in innate immunity against mycobacteria requiring

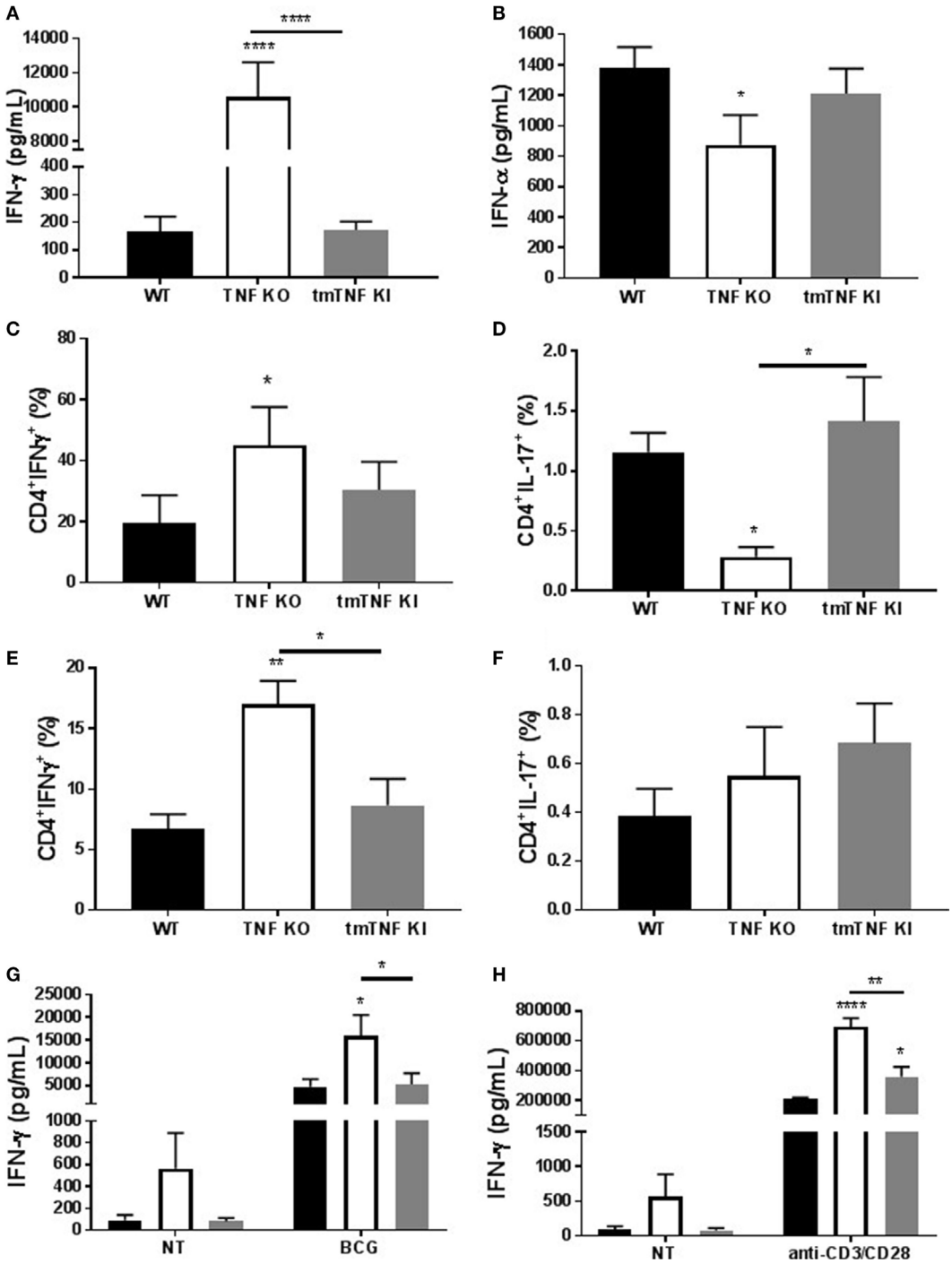

FIGURE 7 | Transmembrane tumor necrosis factor (TNF) controls the excessive inflammatory response mediated by CD4 T cells. **(A)**, IFN-γ and **(B)** IFN-α levels in the pleural fluid of mice infected with BCG for 14 days (n = 9–15 mice per group). **(C)** The frequency of CD4 T cells producing IFN-γ or **(D)** IL-17 was assessed in pleura cells by flow cytometry. **(E)** The frequency of CD4 T cells producing IFN-γ or **(F)** IL-17 was assessed in splenocytes from mice infected with BCG. **(G)** IFN-γ levels from flow-sorted CD4 T cells from BCG-infected mice and cultured for 24 h with or without BCG at MOI 1. **(H)** IFN-γ levels from flow-sorted CD4 T cells from BCG-infected mice and cultured for 36 h with or without anti-CD3/CD28 beads. Bar graphs show means ± SEM (n = 6–8/per group, $^{*}P < 0.05$, $^{**}P < 0.001$, $^{****}P < 0.00001$ vs wild-type (WT), ANOVA and Bonferroni *post hoc* test).

either tmTNF or solTNF are not elucidated. Our previous studies have shown that the heightened inflammatory reaction during acute *M. tuberculosis* infection caused by the absence of TNF was prevented by the tmTNF form binding to both TNFRs. On the contrary, during chronic infection tmTNF was not sufficient and mice died from overt inflammation and tissue necrosis in

spite of the low bacterial burden in infected organs at late stage of infection. This suggests a requirement of solTNF interacting with TNFR1 for disease resolution during chronic infection (18, 19). Our previous study revealed that TNFR1 expressed by myeloid cells, but not by T cells controlled chronic *M. tuberculosis* infection as the absence of TNFR1 on myeloid cells recapitulated the marked impairment in host protection and exacerbated pathology of mice without TNFR1 during *M. tuberculosis* infection (37). Studies assessing the role of TNFR2 during chronic tuberculosis infection have shown that TNFR2 can mediate deleterious effect by soluble TNFR2 shedding inhibiting TNF-associated activities on DC (38). These data suggest that tmTNF interacting with TNFR2 exerts differential activities during acute and chronic infections that depend on several cell types expressing different TNF receptors as well as of the time course of the infection. This study investigates how tmTNF controls the acute inflammatory process generated by BCG-induced pleurisy and reveals that MDSC accumulate in TNF KO mice, but these cells are not functional. However, MDSC expressing tmTNF recover MDSC suppressive function on CD4 T cells, attenuate inflammation limiting tissue injury and rescue tmTNF KI mice. Monocytic MDSC from WT and tmTNF KI mice inhibited CD4 T cells proliferation in association with inhibition of IL-2, IFN-γ and iNOS production. Granulocytic MDSC also inhibited CD4 T cells, however, the cytokine profile was not as clearly defined as for MO-MDSC, but iNOS was also expressed at much lower levels in TNF KO cells. We then examine the specific receptor sustaining MDSC function. We find that proliferation of activated CD4 T cells deprived of TNFR2 were not inhibited by MDSC, suggesting that the interaction of tmTNF expressed by MDSC and TNFR2 on CD4 T cells is critical for MDSC-mediated T cell suppression. It is important to note that absence of TNFR2 on T cells led to contrary effects as CD4 T cells display enhanced proliferation and enhanced IFN-γ production when co-cultured with MDSC. The proliferation capacity of TNFR2-deficient CD4 T cells was lower than that of WT cells as previously reported (39) which was shown to be normal in other report (40). Our data show that the proliferation CD4 T cells deficient in TNFR2 was not influenced by the presence of either WT or tmTNF KI MDSC. By contrast, the proliferation CD4 T cells deficient in TNFR1 was inhibited by both WT and tmTNF KI MDSC, suggesting the importance of TNFR2 on CD4 T cell suppressive activity. Thus, MDSC expressing tmTNF appears to control BCG-induced pleurisy *via* TNFR2 on CD4 T cells. Expression of tmTNF on MDSC has not been explored so far. To our knowledge, we describe here for the first time that tmTNF expressed by MDSC exerts suppressive activity on T cells expressing TNFR2 during acute BCG-induced pleurisy.

The role of TNF has been shown to be critical for the generation of MDSC during several pathologies, including cancer and chronic inflammation (24, 26, 41). Suppressive function of MDSC on T cells was shown to be dependent on the presence of TNFR2 on MDSC which could help tumor cells to evade the immune system (26). Ectopic expression of tmTNF on tumor cells promoted suppressive activity of MDSC expressing TNFR2 (41). In mouse models of carcinogenesis, neutralization of TNF by etanercept and infliximab resulted in reduced MDSC accumulation and delayed growth of transplanted tumors (27). Inhibition of solTNF by dominant-negative TNF biologics, blocking solTNF but not tmTNF, decreased MDSC frequency, reduced tumor growth, and prolonged survival of mice with chemically induced tumors, suggesting that solTNF was responsible for MDSC accumulation during carcinogenesis (42). TNF has been shown to act as a pro and anti-tumorigenic molecule depending on the different phases of carcinogenesis (43). Lymphotoxin-alpha (LT-α) also signaling through TNFR1 and TNFR2 can also contribute and impact on MDSC accumulation and expansion which indicates that TNF/LT-α pathways are major and complex targets in carcinogenesis.

Tumor necrosis factor signaling through TNFR2 has been shown to be required for MDSC accumulation and suppressive activities as also reported for T regulatory cells (25, 26, 44–46). Our data confirm that this important pathway tmTNF-TNFR2 preferentially leads to the activation of tolerogenic MDSC that are involved in anti-inflammation and infection resolution. TNFR2 expressed by T cells has been reported to act as a co-stimulatory molecule for antigen-driven T cell responses (39). TNF has been shown to activate suppression activity of regulatory T cells (Tregs) by inducing the expression of TNFR2 (47). BCG vaccination has been shown to activate Tregs mainly in the context of auto-immunity and diabetes (48). The exploration of Tregs functionality in the context of BCG pleural infection needs further investigation. Nevertheless, we found a very low proportion of Tregs in the pleural cavity of infected mice (0.3–0.4%) (data not shown) and very low levels of IL-10 (20–50 pg/mL), indicating a relative contribution of Tregs at this time point of the infection.

Several reports have explored the role of granulocytic and monocytic MDSC during chronic mycobacterial infections in the mouse model and in TB patients; however, results in terms of T cell responses are not totally clear probably due to the different model systems (11, 13, 14). During chronic murine TB, MDSC accumulation in the lung was increased in susceptible mice and associated with heightened lethality, but depletion of MDSC during infection ameliorated disease (15). In patients with TB, MDSC accumulation was identified in the blood and after successful treatment the frequency of MDSC was decreased as seen in healthy controls (9). Pulmonary accumulation of granulocytic MDSC expressing NO was also reported in TB patients (10). In general, studies on MDSC in TB showed that MDSC may contribute to the pathogenesis of TB, and in particular in susceptible hosts, MDSC were associated with disease aggravation (16, 49). However, studies on the role of MDSC during the initial phase of the infection are still missing. As in the case for TNF requirement that needs to be at the right time with sufficient levels to be efficient, MDSC can also exert protective activity during a specific time of the infection to prevent overt inflammation whereas they can be deleterious during chronicity. Contrary to previous results performed during chronic phase infection, our study on acute infection suggests that MDSC play a beneficial role by attenuating T-cell-mediated inflammatory responses.

Previous studies have shown that TNF acts as a negative regulator of Th1 immune responses as in the absence of TNF expansion of T cells and uncontrolled Th1 type immune responses

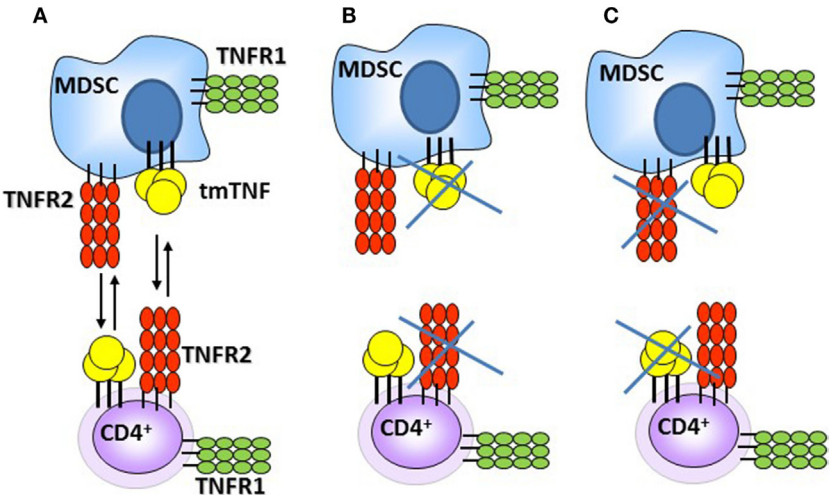

FIGURE 8 | Schematic representation of the interactions between myeloid-derived suppressor cells (MDSC) and CD4 T cells during acute BCG pleural infection. **(A)** Interactions triggering suppressive effects of MDSC on CD4 T cells *via* tmTNF and TNFR2. **(B,C)** Absence of tmTNF or TNFR2 abrogates MDSC suppressive activities [presented data and Ref. (25, 26, 41)].

caused tissue destruction (35). Our previous report also pointed out that the functional tmTNF$^{\Delta 1-9,K11E}$ controlled the exacerbated serum IFN-γ levels observed in TNF KO. By contrast, a second mouse strain (tmTNF$^{\Delta 1-12}$ KI mice) expressing a different mutant tmTNF$^{\Delta 1-12}$ were unable to control the BCG infection and exhibited high IFN-γ levels associated with aggravation of the disease and death as TNF KO mice (31). In this study, BCG-infected TNF KO mice exhibited excessive levels of IFN-γ, as previously observed, and impaired response in IFN-α in the pleural cavity. We show that expression of tmTNF$^{\Delta 1-9,K11E}$ regulated both IFN-γ and IFN-α with attenuation of the Th1 cell-mediated inflammatory responses in the pleural cavity of BCG-infected mice. This anti-inflammatory effect would result from the interaction of tmTNF on MDSC with TNFR2 on CD4 T cells. We also examined whether TNF signaling was needed for pleural MDSC suppressive activity and showed that TNF signaling on MDSC is important for CD4 T cell suppressive function during acute pleural BCG infection. TNFR1R2 KO MDSC trigger a contrary effect enhancing CD4 T cell proliferation and production of IFN-γ which recapitulates the effects observed with co-cultures of CD4 T cells deficient in TNFR2. These results suggest that MDSC requires the presence of tmTNF and also of TNFRs, most probably TNFR2 as previously reported (25, 26, 41). Based on our data and previous report, we propose that MDSC–CD4 T cell interactions can be mediated through tmTNF-TNFR2 and cells can express both tmTNF and TNFRs (**Figure 8**). Interaction of tmTNF with TNFR1 or TNFR2 can result in the transmission of different signals, including reverse signaling which remains to be investigated in MDSC – T cell interactions (50).

In conclusion, our study provides insights into the protective role of MDSC during acute mycobacterial infection that involves tmTNF signaling through TNFR2. Tm-TNFR2 interaction attenuates cell-mediated inflammatory responses associated with the infection and favors adaptive immunity and disease resolution.

ETHICS STATEMENT

This study was approved by Cantonal veterinary office from Geneva (authorization No. GE167/14).

AUTHOR CONTRIBUTIONS

Conception and drafting of the article: LC-G and IG. Performance and analysis of experiments: LC-G, IG, HU, DV, and GB. MB for discussions of the data and critical revision of the article: VQ and BR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00999/full#supplementary-material.

FIGURE S1 | Cytokine profile of pleural fluid WT and tmTNF KI mice. **(A)** IFN-γ, **(B)** IL-12p70, **(C)** IL-6, and **(D)** IL-10 cytokine levels and **(E)** the chemokine MCP-1 (CCL2) were evaluated in the pleural fluid of naive mice and after 2 and 14 weeks of BCG-induced pleurisy [bar graphs show means ± SEM, n = 6–14/per group, $^*P < 0.05$ vs WT, ANOVA and Bonferroni *post hoc* test from two experiments].

FIGURE S2 | Transmembrane TNF controls $CD11^+$ $GR1^+$ cell expansion in the pleural cavity of BCG-infected mice. **(A)** Representative zebra plot showing pleural cells expressing CD11b and GR1 from naïve mice and BCG-infected mice at day 14 post-infection. **(B)** Absolute number of pleural $CD11b^+$ $GR1^+$ cells obtained from total cell number recovered from pleural cavity per individual mouse [bar graphs are means ± SEM, $n = 3–5$ naïve condition and $n = 6–8$ for infected mice/per group from 2 experiments, $^*P < 0.05$ vs WT. ANOVA and Bonferroni *post hoc* test].

FIGURE S3 | Absence of TNF induces low frequency of MDSC with ability to produce iNOS and Arginase 1 in the pleural cavity of BCG-infected mice, even using CD11b and GR1 as main markers. MDSC were flow-sorted from total pleural cells using a MDSC kit. **(A)** Representative zebra plot with the analysis used to evaluate the purity of PMN-MDSC by flow cytometry, using CD11b and GR1 to identify MDSC cells. **(B)** Western blot of flow-sorted PMN-MDSC showing expression of iNOS and arginase-1 (Arg 1) in WT and tmTNF KI cells but less in TNF KO cells. **(C)** Representative Stagger Offset histogram showing the proportion of PMN-MDSC expressing iNOS inside the gate of $CD11b^+$ $GR1^+$ cells and comparison between WT (blue), TNF KO (orange) and tmTNF KI (green) mice. **(D)** Histogram representing western blot quantification compared to β-actin **(E)** Representative zebra plot with the analysis used to evaluate the purity of MO-MDSC by flow cytometry, using as main molecules CD11b and GR1 to identify MDSC. **(F)** Western blot of flow-sorted MO-MDSC showing expression of iNOS and Arg 1 in WT and tmTNF KI cells but not in TNF KO cells. Beta actin was used as control and TNF KO cells are over loaded. **(G)** Representative Stagger Offset histogram showing the proportion of MO-MDSC expressing iNOS inside the gate $CD11b^+$ GR1 (left) and comparison between WT (blue), TNF KO (orange), and tmTNF KI (green) mice. **(H)** Histogram representing western blot quantification compared to β-actin.

FIGURE S4 | Gating strategy for evaluation of CD4 T cell proliferation. Flow cytometry analysis to evaluate CD4 T cell proliferation following activation with anti CD3 1 μg/mL (Plate-immobilized) plus anti CD28 1 μg/mL and after 48 h of culture and using KI-67 proliferation marker.

FIGURE S5 | Expression of TNFRs on MDSC is required MDSC suppressive function on CD4 T cells. **(A)**. Proliferation of CD3 CD4 T cells after polyclonal stimulation and in the presence or absence of flow-sorted pleural mononuclear MO-MDSC (ratio MDSC:Splenocytes, 1:1, 1:2, and1:4) was measured by flow cytometry using KI-67 after 48 h of co-culture. Pools of pleural cells were from 5 to 7 mice per group. Sorted MDSC were from WT BCG-infected mice or from TNFR1TNFR2 KO mice. **(B)** IL-2 and **(C)** IFN-γ production from supernatants of splenocytes and MO-MDSC co-cultures at different ratio. **(D)** Proliferation of CD3 CD4 T cells after polyclonal stimulation and in the presence or absence of flow-sorted pleural polymorphonuclear PMN-MDSC co-cultured with splenocytes for 48 h. **(E)** IL-2 and **(F)** IFN-γ production from co-cultures of PMN-MDSC and splenocytes. MDSC alone were used as the negative control and activated splenocytes as positive controls (100%). Bar graphs show means ± SEM. Data are representative of two independent experiments ($^*P < 0.05$ vs positive control. ANOVA and Bonferroni post hoc test).

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Interleukin 6 Present in Inflammatory Ascites from Advanced Epithelial Ovarian Cancer Patients Promotes Tumor Necrosis Factor Receptor 2-Expressing Regulatory T Cells

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Background: Epithelial ovarian cancer (EOC) remains a highly lethal gynecological malignancy. Ascites, an accumulation of peritoneal fluid present in one-third of patients at presentation, is linked to poor prognosis. High levels of regulatory T cells (Tregs) in ascites are correlated with tumor progression and reduced survival. Malignant ascites harbors high levels of Tregs expressing the tumor necrosis factor receptor 2 (TNFR2), as well as pro-inflammatory factors such as interleukin 6 (IL-6) and tumor necrosis factor (TNF). IL-6 is also associated with poor prognosis. Herein, we study the effect of IL-6 and TNF present in ascites on the modulation of TNFR2 expression on T cells, and specifically Tregs.

Methods: Ascites and respective peripheral blood sera were collected from 18 patients with advanced EOC and soluble biomarkers, including IL-6, sTNFR2, IL-10, TGF-β, and TNF, were quantified using multiplexed bead-based immunoassay. Peripheral blood mononuclear cells (PBMC) from healthy donors were incubated with cell-free ascites for 48 h (or media as a negative control). In some experiments, IL-6 or TNF within the ascites were neutralized by using monoclonal antibodies. The phenotype of $TNFR2^+$ Tregs and $TNFR2^-$ Tregs were characterized post incubation in ascites. In some experiments, cell sorted Tregs were utilized instead of PBMC.

Results: High levels of immunosuppressive (sTNFR2, IL-10, and TGF-β) and pro-inflammatory cytokines (IL-6 and TNF) were present in malignant ascites. TNFR2 expression on all T cell subsets was higher in post culture in ascites and highest on $CD4^+CD25^{hi}FoxP3^+$ Tregs, resulting in an increased $TNFR2^+$ Treg/effector T cell ratio. Furthermore, $TNFR2^+$ Tregs conditioned in ascites expressed higher levels of the functional immunosuppressive molecules programmed cell death ligand-1, CTLA-4, and GARP. Functionally, $TNFR2^+$ Treg frequency was inversely correlated with interferon-gamma

(IFN-γ) production by effector T cells, and was uniquely able to suppress TNFR2+ T effectors. Blockade of IL-6, but not TNF, within ascites decreased TNFR2+ Treg frequency. Results indicating malignant ascites promotes TNFR2 expression, and increased suppressive Treg activity using PBMC were confirmed using purified Treg subsets.

Conclusion: IL-6 present in malignant ovarian cancer ascites promotes increased TNFR2 expression and frequency of highly suppressive Tregs.

Keywords: epithelial ovarian cancer, malignant ascites, interleukin 6, tumour necrosis factor 2, FoxP3, regulatory T cells, effector T cells, inflammation

INTRODUCTION

Ovarian cancer is one of the most lethal types of cancer in women globally (1, 2). This is because the majority of ovarian cancer patients are diagnosed in late stages, with up to one-third of patients presenting with a prominent peritoneal accumulation of fluid called "ascites." Ascites development is associated with chemo-resistance, disease recurrence (3, 4), and poorer survival in ovarian cancer patients (5–9). Ovarian cancer ascites further contains a complex reservoir of immune cells and cytokines, harboring immunosuppressive cells as well as inflammatory soluble factors (7, 10, 11). This unique milleau has been proposed to help tumor cells evade host immunosurveillance, so that tumor cells can continue growing without restriction (3, 7, 9, 10). In ovarian cancer, similar to other cancers, the immune system is hampered in controlling the tumor due to the presence of regulatory T cells (Tregs) that inhibit T effector (Teff) cell-mediated antitumor responses (9).

Tumor necrosis factor receptor type II (TNFR2) stimulates the activation and proliferation of Tregs from a resting to an activated state (12). Expression of TNFR2 on Tregs is reported to identify the maximally suppressive and functional Treg population in both mice and humans (13–15). Overabundance of TNFR2+ Tregs creates a potent immunosuppressive microenvironment associated with negative patient outcomes in diverse cancers, such as acute myeloid leukemia, lung cancer, ovarian cancer, and colorectal cancer (16–20). Decreasing TNFR2+ Treg levels using cyclophosphamide in mice (21) or panobinostat and azacitidine in humans (19) is associated with improved antitumor immune responses and prolonged survival. Lenalidomide has also been shown to both decrease TNFR2+ Treg levels and enhance Teff function in patients with acute myeloid leukemia. The high levels of TNFR2+ Tregs in ovarian cancer ascites can be driven by their preferential migration into the ascites, given their high levels of expression of the CCR4 chemokine receptor (18). It is also possible that cytokines present in ascites may promote TNFR2 expression on Tregs. Once TNFR2 is expressed on Tregs, tumor necrosis factor (TNF) in ascites can further stabilize FoxP3 expression, the hallmark transcription factor associated with Treg suppressive capabilities (22).

Tumor necrosis factor receptor 2 expression is elevated on peripheral blood mononuclear cells (PBMCs) of ovarian cancer patients, as well as on mononuclear cells present in ovarian cancer ascites (18). In a previous study looking at TNFR2+ Tregs from ovarian cancer ascites, Govindaraj and colleagues observed that TNFR2+ Tregs extracted from ascites express higher levels of immunosuppressive molecules CTLA-4 and GARP, and are functionally more suppressive when compared to peripheral blood TNFR2+ Tregs (18). Induction of CTLA-4 and GARP expression on human Tregs is dependent on the transcription factor FoxP3 (23–26). In the present study, we have assessed whether soluble components present in cell-free ascites can promote upregulation of these functional immunosuppressive molecules on Tregs, and their association with a TNFR2+ Treg phenotype.

Apart from these immunosuppressive check-point inhibitor receptors, it is also important to assess whether ascites may modulate the expression of other immunosuppressive molecules currently being explored for ovarian cancer immunotherapy. Programmed cell death ligand-1 (PD-L1), a member of B7 superfamily, is a negative immunoregulatory molecule that inhibits effector T cell activity and is highly expressed on cancer cells (27–31) and immune cells including Tregs (32, 33). PD-L1 expression on Tregs is associated with upregulated FoxP3 expression and promotes maximally suppressive Treg activity (34). PD-L1 inhibitors have demonstrated promising antitumor efficacy in several cancer types, including melanoma, non-small cell lung cancer, renal cell carcinoma, bladder carcinoma, and Hodgkin's lymphoma (35–37). Recent studies have shown that PD-L1 can be upregulated by pro-inflammatory cyokines such as TNF (38).

Elevated interleukin 6 (IL-6) in ascites and in the serum of patients with advanced ovarian cancer has been most strongly correlated with poor survival (39–41) as it has in multiple other cancers (42). IL-6 is a pleiotropic cytokine and an essential biomarker in the cytokine cascade that is involved in the initiation and regulation of inflammation (43, 44). It can be synthesized by dendritic cells, macrophages (45, 46), lymphocytes (47–50), somatic cells (e.g., fibroblasts, keratinocytes, endothelial cells) (51–53), and multiple cancer cell types including breast, lung, head and neck, colorectal, hepatobiliary, pancreatic, as well as ovarian cancer cells (54–62).

Accumulated Tregs in ascites and tissue have been found to be higher in patients with ovarian cancer and linked to advanced ovarian disease and poor prognosis (9). The production of TNF and IL-6 are concomitantly increased in these conditions (63); therefore, the potential effect of these pro-inflammatory cytokines on Tregs is of interest. The relationship between Tregs, IL-6, and TNF is likely to be complex. Experiments using murine cells with autoimmune disease reported that TNF promotes proliferation and maintains suppressive activity of Treg cells both *in vitro* and

in vivo (13, 64). In contrast, there are conflicting reports of the activity of TNF on human Tregs. Some studies suggest that TNF promotes a reduction in the expression of FoxP3 and inhibits the suppressive activity of human Tregs (65, 66). Conversely, a recent study showed that TNF, in the presence of IL-2, increases the expression of human Tregs (both CD25 and FoxP3), and their suppressive activity in a 3-day culture (67). TNFR2 is agreed to be the primary receptor for TNF on both murine and human Treg cells.

The effect of IL-6 on Tregs similarly has been a source of significant controversy. IL-6 has been reported to promote differentiation into T helper type 2 differentiation cells (68) and influence the balance between IL-17 producing cells (Th17) and Tregs (69). While IL-6 alone is unable to induce Th17 cells, culturing of IL-6 in combination with TGF-β (70–73) has been reported to promote murine and human naïve T cells to become Th17 and inhibit conversion into Tregs. In contrast, inducible Tregs activated in the presence of IL-2 and TGF-β did not differentiate into Th17 when cultured with IL-6 (74). In a murine study mimicking excessive IL-6 as seen in chronic inflammatory disorders and several cancers, T cells isolated from peripheral lymphoid organs in IL-6 transgenic mice not only had increased levels of Th17 but also Tregs which further were shown to have retained suppressive activity (75). This *in vivo* study, therefore, suggests that excessive IL-6 conditions do not negatively affect development and function of Tregs and may potentially promote them under specific conditions (75).

To explore the relationship between Tregs, TNF, and IL-6 in ovarian cancer ascites, we created an *in vitro* system to study the effect of IL-6 and TNF within cell-free ovarian cancer ascites on TNFR2$^+$ Treg and on TNFR2$^+$ Teff frequency and function. Our results suggest a critical role for IL-6, present in ovarian cancer ascites, in promoting highly functional TNFR2$^+$ Tregs, which are shown to be the only Treg subset capable of suppressing TNFR2$^+$ Teffs in ovarian cancer ascites cultures.

MATERIALS AND METHODS

Trial Design and Patient Details

This study was carried out in accordance with the recommendations of an Immunity and Ovarian Cancer trial (Project 13/32), HREC of Royal Women's Hospital with written informed consent from all patients. All patients gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the HREC of the Royal Women's Hospital, Melbourne. Ascites and peripheral blood serum samples were prospectively obtained from 18 patients with newly diagnosed advanced epithelial ovarian cancer (EOC) seen in the Oncology Unit, Royal Women's Hospital, Melbourne, Australia following informed consent. All relevant clinical information including demographic status, medical and drug history, clinical diagnosis, and disease extent and status were prospectively collected. Blood samples were obtained immediately prior to surgery and general anesthesia. Ascites samples were collected either during peritoneal tapping prior to chemotherapy or at the time of surgery. Histologic diagnosis of the study patients was confirmed independently by senior hospital pathologists, and all histologic data were prospectively collected and stored in a computerized hospital database. For healthy blood samples, 40 buffy coats were obtained from blood donated by healthy adult volunteers acquired at Australian Red Cross Blood Bank Service.

Isolation of Peripheral Blood Serum

Pre-operative venous blood was drawn from ovarian cancer patients into serum collecting (SST) vacutainer tubes (BD). Following collection, the tubes were left undisturbed at room temperature for 30 min to allow blood to clot. The clots were removed by centrifugation at 1,000 *g* for 10 min in a refrigerated centrifuge. All sera were stored at −80°C until use.

Isolation of Ascites Supernatant

Ascites samples from ovarian cancer patients were first filtered through a 100-μm cell strainer and centrifuged to remove the cellular component. The cell-free supernatant layer of the ascites was collected and stored at −80°C until use.

Isolation of PBMCs

Healthy donor (Australian Red Cross Blood Services) PBMCs were isolated by Ficoll (Amersham Pharmacia Biotech, Sweden) density gradient centrifugation. The isolated PBMCs were suspended in cryovials containing a freeze medium mixture of 10% DMSO (Sigma-Aldrich, USA) and 90% heat-inactivated fetal calf serum (GIBCO, Life Technologies, USA) and frozen at a speed of −1°C/min in a −80°C freezer then subsequently stored in liquid nitrogen. Prior to cell culture, each vial of frozen PBMCs was rapidly thawed in a 37°C water-bath and resuspended in complete AIM V media [AIM V (Life Technologies, USA) supplemented with 5% normal human serum (Sigma-Aldrich, USA)].

In Vitro Conditioning with Ascites

Peripheral blood mononuclear cells from healthy donors were cultured in a 96-well culture plate with 150 μl/well of either complete AIM V media alone or with 50% ascites supernatant (obtained from patient ascites *via* centrifugation) at a final concentration of 2×10^6 cells/ml. The cells were then incubated in a humidified incubator at 37°C with 5% CO_2. After 48 h, the cells were harvested, antibody labeled, and further analyzed by flow cytometry.

In Vitro Blockade of Cytokines within Ascites with Monoclonal Antibodies (mAbs)

Peripheral blood mononuclear cells from healthy donors were isolated by Ficoll density centrifugation and incubated *in vitro* in either complete AIM V media or cell-free ascites from an advanced EOC patient. IL-6 and TNF within the ascites was blocked with murine anti-human IL-6 monoclonal antibody (final concentration at 2.5 μg/ml, R&D, USA) and murine anti-human TNF monoclonal antibody at 500 ng/ml (R&D, USA). Mouse IgG1 immunoglobulins (isotype control) (R&D USA) were used as a negative control. Isotype control and mAbs were added into media and ascites, respectively, in a 10-ml tube, gently suspended and incubated at room temperature for 10 min. PBMCs

from healthy donors were then added into a 96-well culture plate with 150 µl/well of either complete AIM V media alone or with 50% ascites supernatant (blocked IL-6 or TNF or both) at a final concentration of 2×10^6 cells/ml. Following 48 h of incubation in a humidified incubator at 37°C with 5% CO_2, cells were washed, stained, and analyzed with flow cytometry.

Flow Cytometric Analysis

To determine the frequency and phenotype of T cell populations in PBMCs, multicolor flow cytometry was performed using the following surface antibodies: anti-CD3 PerCP (BD Pharmingen, USA) and anti-CD8 FITC (Biolegend, USA); anti-CD4 APC-Cy7 (BD Pharmingen, USA), and anti-CD25 PECF584 (BD Pharmingen, USA), anti-CD127 BV650 (Biolegend, USA), anti-TNFR2 biotinylated followed by conjugation with Streptavidin PECy7 (BD Pharmingen, USA), anti-PD-L1 PE (Biolegend, USA), anti-CTLA-4 BV605 (Biolegend, USA), and anti-GARP BV711 (BD Pharmingen, USA). Following primary staining, a fixable dead cell marker (Life Technologies, USA) was also used to distinguish between dead and live cells. Intracellular levels of FoxP3 and IFN-γ were determined following fixation and permeabilization of cells using a fixation/permeabilization buffer kit (eBioscience, USA) then staining the cells with anti-FoxP3 APC (eBioscience, USA) and anti- IFN-γ v450 antibody (eBioscience, USA). Flow cytometry data were acquired on a Becton Dickinson LSR II flow cytometer using FACS Diva software, acquiring a minimum of 100,000 events per sample. Fluorescence minus one (FMO) controls and isotype-matched immunoglobulins were used to enable accurate gating. Data were analyzed using Flow jo software (TreeStar, USA).

Intracellular Cytokine Analysis

Peripheral blood mononuclear cells from healthy donors cultured in a 48-well plate at final concentration of 2×10^6 cells/ml per well in either AIM V media alone or with 50% ascites supernatant for 48 h. Cells were washed and then stimulated for 6 h with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml of ionomycin (Sigma-Aldrich, USA) at 37°C in a 5% CO_2, humidified incubator. Brefeldin A (eBioscience, USA) was added for the last 5 h of incubation at a concentration of 1 µg/ml. After stimulation, the cells were washed and labeled for surface markers as above, followed by intracellular staining for FoxP3 and IFN-γ and then prepared for flow cytometric analysis. Unstimulated cells, FMO, and isotype-matched immunoglobulins were also used as controls. Flow cytometry data were acquired on a Becton Dickinson LSR II flow cytometer, and data were analyzed using Flowjo software (TreeStar, USA).

Cell Sorting and Culture

Flow cytometric cell sorting was performed using BD Influx to isolate Teff and Tregs cells as well as Tregs TNFR2$^+$ and Tregs TNFR2$^-$ subsets. PBMCs from healthy donors were stained with the following surface antibodies: anti-CD3 PerCP (BD Pharmingen, USA), anti-CD4 APC-Cy7 (BD Pharmingen, USA), anti-CD127 BV650 (Biolegend, USA), and anti-CD25 PECF584 (BD Pharmingen, USA). For isolation of Tregs TNFR2$^+$ and Tregs TNFR2$^-$ subsets in some experiments, pre-sort PBMCS were additionally stained for biotinylated followed by conjugation with Streptavidin PECy7 (BD Pharmingen, USA). A fixable dead cell marker (Life Technologies, USA) was used to distinguish between dead and live cells. Following exclusion for doublet and dead cells, cells for sorting were initially gated on the CD3$^+$CD4$^+$ population. Within CD4$^+$ gates, cells were then gated on CD127 and CD25 to identify and isolate CD25^{hi}CD127^{lo} (Tregs) and CD25$^-$CD127$^+$ (Teff) population, respectively. Additionally, further gating on TNFR2 was performed to isolate Treg TNFR2$^+$ and Tregs TNFR2$^-$ subsets. Prior to culture, purity was assessed on post-sort Tregs and were confirmed to be 95 ± 3% FoxP3$^+$ by flow cytometry. Samples containing the sorted populations were then suspended at a ratio of 10^5 cells per 50 µl of complete AIM V media and were cultured in either complete AIM V media alone or in cell-free ascites for 48 h. The cells were then incubated in a humidified incubator at 37°C with 5% CO_2. After 48 h, the cells were harvested, antibody labeled to assess phenotype, and then analyzed by flow cytometry.

In Vitro Suppression Assay

To determine the difference in ratio at which Tregs incubated in ascites are suppressive compared Tregs incubated in media, add-back suppression assays were performed. Following *in vitro* conditioning of PBMCS in either complete AIM V media or ascites for 48 h, the cells were washed, harvested, and labeled with anti-CD3 PerCP (BD Pharmingen, USA), anti-CD4 APC-Cy7 (BD Pharmingen, USA), anti-CD127 BV650 (Biolegend, USA), and anti-CD25 PECF584 (BD Pharmingen, USA). Following primary staining, a fixable dead cell marker (Life Technologies, USA) was used to distinguish between dead and live cells. The cultured cells underwent cell sorting as described in Section "Cell Sorting and Culture." Three populations consisting of Teff incubated in media, Tregs incubated in media, and Tregs incubated in ascites were isolated. Teff were further labeled with Carboxyfluorescein Diacetate Succinimidyl Ester (CSFE) (Molecular Probes, US) at 0.5 µM to monitor cell proliferation. The labeled effector cells were cultured alone, or at a 1:2, 1:4, or 1:8 and 1:16 ratio with autologous Tregs already conditioned in either complete AIM V media or ascites. The cells were cultured (usually in triplicates) in a 96-well plate pre-coated with anti-CD3 (1.0 µg/ml clone OKT3, Biolegend). This was followed by the addition of soluble anti-CD28 (1.0 µg/ml clone CD28.2, BD Pharmingen) for 3 days at 37°C with 5% CO_2. Cells were harvested, stained, and further analyzed by flow cytometry.

Multiplexed Bead-Based Immunoassay

Cell-free ascites were collected from 18 patients with advanced EOC and soluble biomarkers were quantified using BD™ Cytokine cytometric bead arrays (flex sets for IL-10, IFN-γ, IL-2, IL-6, TNF, TGF-β, and sTNFR2, BD USA), following manufacturer's protocol. Ascites supernatants were used at 1 in 4 and 1 in 8 dilution. Ascites samples were analyzed in duplicates and results were calculated as the average of two values. Samples were then acquired by flow cytometry on a Becton Dickinson LSR II flow cytometer collecting 300 events per analyte. Flow cytometry

standard data files were exported and was analyzed by FCAP Array software version 3.0.

For simultaneous measurement of multiple cytokines in serum, multiplex magnetic bead immunoassay kits were used as per manufacturer's protocol (Invitrogen). Human cytokine 25-Plex panel was used to determine quantitative measurement for IL-10, IFN-γ, IL-2, IL-6, and TNF in study serum. sTNFRII and TGF-β were analyzed separately using singleplex bead kits. The serum samples were randomly assigned to the plates to avoid assay bias and to determine inter-assay differences. Five ascites samples which were quantified using cytometric bead array were also analyzed to determine limits of agreement. All samples were analyzed in duplicate and results were calculated as the average of two values. The samples were analyzed using a Luminex® 200™ analyser (Luminex Corp.) as per standard protocol. Data were analyzed using a five-parametric-curve fitting within the manufacturer's software.

Assessment of ascites and serum samples were on different platform as the samples were from part of a larger trial samples, where the respective platforms were used for cytokine assessment. The platforms were chosen based on the cytokines being analyzed. We formally compared the same samples run across both platforms to provide cross validation of the two using Bland–Altman method comparison study observed mean differences of estimated bias 2.04 ± 9.05 between two tests, and the 95% limits of agreement were between −15.7 and 19.8. Therefore, results from both the platforms were confirmed to be within good limits of agreement.

Statistical Analysis

Comparison of two groups of data were analyzed by Wilcoxon matched-paired *t*-test, while three-group data were analyzed with one-way ANOVA with Dunn's multiple comparison test (*post hoc*). Pearsons' correlations were employed for correlation analyses. $p < 0.05$ was significant. Data were always shown as mean ± SEM. Statistical analyses were performed using Graphpad prism 7.0.

RESULTS

Culturing PBMCs in Cell-Free Ovarian Cancer Malignant Ascites Increases TNFR2$^+$ Expression on T Cell Subsets

To quantify potential general changes in cell population frequencies in response to ovarian cancer ascites, we isolated peripheral mononuclear cells (PBMCs) from healthy donors ($n = 30$) and incubated them *in vitro* in AIM V media or cell-free ascites from advanced EOC patients, followed by cell staining and analysis by flow cytometry. T-lymphocytes were identified using an anti-CD3 antibody, a pan T-cell marker. The percentages of CD4$^+$ and CD8$^+$ T cells were unchanged following incubation of PBMCs in ascites (**Figures 1A–B**). Within the CD4$^+$ gate, Tregs were further identified as CD25^{hi}FoxP3$^+$, while effector T cells (Teff) were identified as CD25$^-$FoxP3$^-$ (**Figure 1C**). CD25^{hi}Foxp3$^+$ cells gated using this strategy were further confirmed to be CD127^{lo} (Figure S1 in Supplementary Material). Malignant ascites compared to media

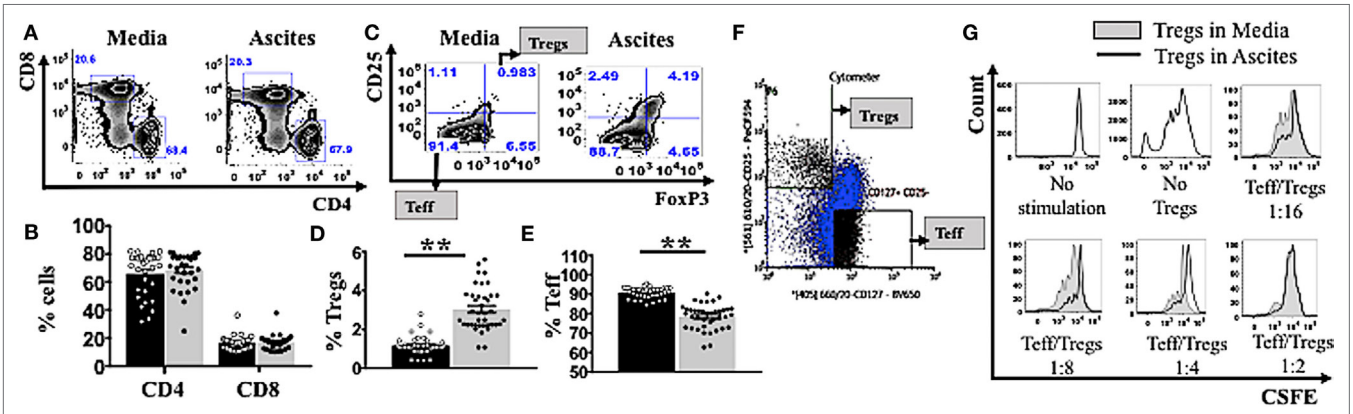

FIGURE 1 | Ascites increases frequency of functional regulatory T cells (Tregs). **(A–E)** Peripheral blood mononuclear cells (PBMCs) from healthy donors ($n = 30$) were isolated by Ficoll density centrifugation and incubated *in vitro* in either AIM V media or cell-free ascites from advanced epithelial ovarian cancer patients for 48 h. Cells were washed, stained with anti-CD3, CD4, CD8, CD25, tumor necrosis factor receptor 2 (TNFR2), and FoxP3, and analyzed with flow cytometry. **(F,G)** PBMCs from two healthy donors were isolated by Ficoll density centrifugation. Cells were labelled anti-CD3, anti-CD4, anti-CD25, and anti-CD127, and flow cytometric cell sorting was performed. **(A)** Flow cytometry plots of CD4$^+$ and CD8$^+$ T cells within CD3$^+$ T cells incubated in media and ascites. **(B)** The frequency (%) of CD4$^+$ and CD8$^+$ T cells within media (black bar) and ascites (gray bar) ($n = 30$). **(C)** Tregs were identified as CD25^{hi}FoxP3$^+$ and effector T cells (Teff) were identified as CD25$^+$FoxP3$^+$ within CD4$^+$ T cells. Flow cytometry plots of Tregs and Teff cells within CD4$^+$ T cells incubated in media and ascites. **(D,E)** The frequency (%) of Tregs **(D)** and Teff **(E)** within media (black bar) and ascites (gray bar) ($n = 30$). **(F)** Tregs and Teff were identified by gating on CD3$^+$CD4$^+$ population, followed by CD127 and CD25 to identify CD25^{hi}CDl27^{lo} and CD25$^-$CD127$^+$ T cells respectively ($n = 2$). **(G)** Prior to suppression assay, Teff were labelled with carboxyfluorescein diacetate succinimidyl ester (CSFE) and incubated in media, while Tregs were cultured in either complete AIM V media or in cell-free ascites for 48 h ($n = 2$). Cells were then harvested and washed. The labelled effector cells were cultured either alone or at a 1:2, 1:4, 1:8, and 1:16 ratio with autologous pre-conditioned Tregs in a 96-well plate with anti-CD3/CD28 for 3 days. Cells were washed, stained, and analyzed by flow cytometry. Proliferation of Teff by Tregs incubated in ascites (black line) compared to Tregs incubated in media (gray line and shaded), cultured alone (no Tregs) or in co-culture with several ratios of Teff to Tregs. *$p < 0.05$ and **$p < 0.0001$, Wilcoxon matched-pair *t*-test. Data from PBMCs were pooled from three independent experiments (error bars-SEM), while data of sorted cells are replicates of two donors.

induced higher frequencies of Tregs (**Figure 1D**) and conversely decreased frequencies of Teff (**Figure 1E**). As Treg-related cell markers can be expressed on non-Treg cells upon cell stimulation including transient expression of FoxP3 (76), we used purified Tregs to confirm that the phenotype of $CD25^{hi}FoxP3^{+}$ observed in ascites cultures identifies functional suppressors. We purified Tregs from PBMCs of two healthy donors (already conditioned in media or ascites for 48 h) by using flow cytometry cell sorting. Following gating on $CD3^{+}CD4^{+}$ lymphocyte population, Tregs were then sorted as $CD25^{hi}CD127^{lo}$ and Teff as $CD25^{-}CD127^{+}$ T cells (**Figure 1F**). The purity of post-sort Tregs were confirmed to be 95 ± 3% $FoxP3^{+}$ by flow cytometry. We performed a suppression assay using these purified Tregs conditioned in ascites, to formally determine their ability to suppress autologous effector T cells compared to media. Purified $CD4^{+}CD25^{-}$ effector T cells were labeled with CSFE to monitor cell proliferation and were cultured alone (without Tregs) or at a 1:2, 1:4, or 1:8 and 1:16 ratio with autologous Tregs (pre-conditioned in either AIM V media or ascites for 48 h) in a 96-well plate with mAb stimulants anti-CD3 and soluble anti-CD28 for 3 days. As shown in **Figure 1G**, both media and ascites culture derived Tregs suppressed proliferation of Teffs. Moreover, on a cell-for-cell basis Tregs cultured in ascites showed higher suppressive capacity on autologous Teffs compared to Tregs conditioned in media (**Figure 1G**). The above result confirmed that malignant ascites caused increases in functional Tregs.

We next investigated whether culturing in ascites also influenced changes in the phenotypes of Tregs and Teffs. We incubated PBMCs from healthy donors ($n = 30$) in AIM V media or cell-free malignant ascites, followed by cell staining and analysis by flow cytometry. We found that culture with ascites strongly upregulated TNFR2 expression on both $CD4^{+}$ and $CD8^{+}$ T cell subsets (**Figure 2A**), resulting in a higher proportion (%) of $TNFR2^{+}$ CD4 and CD8 T cells (**Figure 2A**). Additionally, stimulation with malignant ascites significantly upregulated the median fluorescence intensity of TNFR2 on Tregs as well as the frequency of $TNFR2^{+}$ Tregs (**Figure 2F**) ($p < 0.05$). This effect was also found consistently in 18 separate EOC patient derived ascites affecting PBMCs from a single healthy volunteer (**Figure 2F**) or a single cell-free ascites affecting PBMCs from 30 healthy volunteers (data not shown). We also used purified Tregs and Teff from healthy donors ($n = 2$) and cultured them in a similar *in vitro* system. We observed similar increased in TNFR2 expression on these purified T cell subsets, particularly Tregs (**Figures 2B,C**). Although ascites decreased the frequency of Teff (**Figure 1E**), ascites significantly increased the frequency of $TNFR2^{+}$ Teffs, as well as the level of expression of TNFR2 on the effectors (**Figures 2D–F**). However, the fold change in TNFR2 expression in ascites compared to media was significantly lower for Teffs compared to Tregs (1.97 ± 0.37 vs 3.37 ± 0.48, $p < 0.001$) resulting in a significantly increased ratio of $TNFR2^{+}$ Treg/Teff (1.14 ± 0.21 vs 1.98 ± 0.32, $p < 0.0001$) (**Figure 2F**). Together the

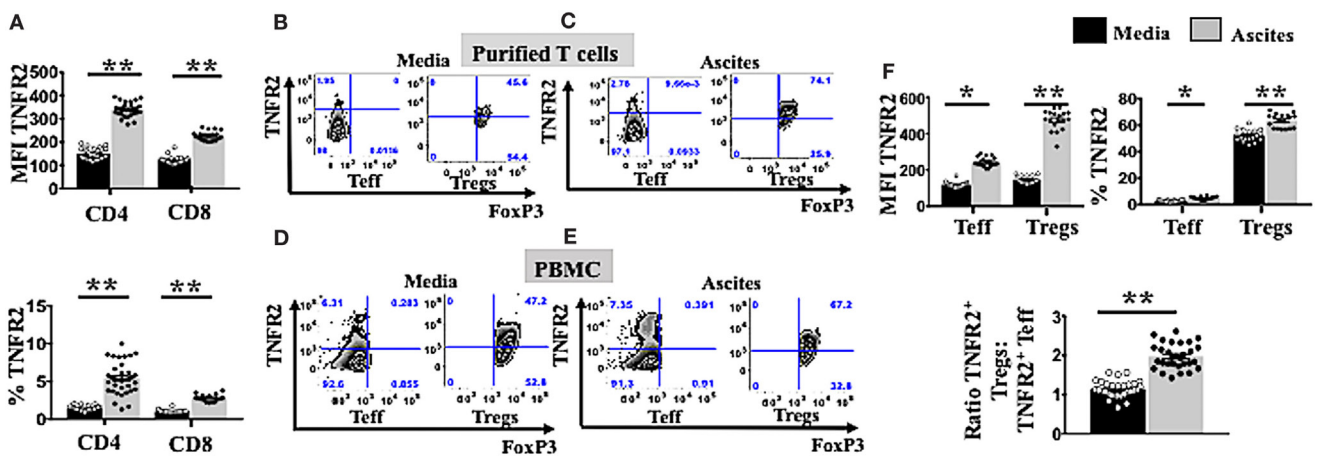

FIGURE 2 | Ascites upregulates tumor necrosis factor receptor 2 (TNFR2) expression on both T effector (Teff) and regulatory T cells (Tregs). **(A)** Peripheral blood mononuclear cells (PBMCs) from healthy donors ($n = 30$) were isolated by Ficoll density centrifugation and incubated *in vitro* in either AIM V media or cell-free ascites from advanced epithelial ovarian cancer (EOC) patients for 48 h. Cells were washed, stained with anti-CD3, CD4, and CD8 and TNFR2 and analyzed with flow cytomcery **(A)**. The level of TNFR2 expression (median fluorescence intensity, MFI) and frequency (%) of TNFR2 within $CD4^{+}$ and $CD8^{+}$ T cells in media (black bar) and ascites (gray bar) ($n = 30$) **(B,C)**. PBMCs from two healthy donors were isolated by Ficoll density centrifugation. Cells were labelled anti-CD3, anti-CD4, anti-CD25, and anti-CD127 and flow cytometric cell sorting for Tregs and Teff was performed. Isolated Tregs and Teff were cultured in either complete AIM V media or in cell-free ascites for 48 h. Cells were then washed and labeled for surface markers with CD3, CD4, CD25, and TNFR2 followed by intracellular staining for FoxP3 and prepared for flow cytometric analysis **(B,C)**. Representative flow cytometry expression of TNFR2 and FoxP3 on sorted Tregs and Teff cells as well incubated in media **(B)** and ascites **(C)**. **(D–F)** PBMCs from a single healthy donor were incubated *in vitro* for 48 h in either AIM V media or cell-free ascites from 18 patients with advanced EOC ($n = 18$). Cells were washed, stained with CD3, CD4, CD25, TNFR2, and FoxP3, and analyzed with flow cytometry. **(D,E)** Expression of TNFR2 and FoxP3 was analyzed with flow cytometry by gating on Tregs and Teff cells derived from PBMCs incubated in media and ascites. **(F)** The level of TNFR2 expression (MFI) within Teff and Tregs and percentages (%) of $TNFR2^{+}$ Teff and Tregs and the ratio of $TNFR2^{+}$ Tregs to $TNFR2^{+}$ Teff in media and ascites ($n = 18$). $^{*}p < 0.05$ and $^{**}p < 0.0001$, Wilcoxon matched-pair *t*-test. Data from PBMCs were pooled from three independent experiments (error bars-SEM), while data of sorted cells are replicates of two donors.

above data demonstrate conditioning in ascites promotes higher expression of TNFR2 on Tregs and Teffs.

TNFR2$^+$ Tregs Conditioned in Malignant Ascites Express Higher Levels of Immunosuppressive Molecules PD-L1, CTLA-4, and GARP and Are Negatively Correlated with Total and TNFR2$^+$ Teff Activity

TNFR2$^+$ Tregs have been demonstrated to be more suppressive compared to TNFR2$^-$ Tregs in various diseases including chronic inflammatory conditions (16–19). Highly suppressive Tregs usually express elevated levels of functional immunosuppressive molecules CTLA-4, GARP (13, 18, 19), and PD-L1 (34). We explored whether ascites could further induce other immunosuppressive molecules on TNFR2$^+$ and TNFR2$^-$ Tregs. We used purified Tregs TNFR2$^+$ and Tregs TNFR2$^-$ subsets isolated from PBMC of healthy donors ($n = 2$) by flow cytometric cell sorting. These purified Treg subsets were initially identified by gating on CD3$^+$CD4$^+$ for lymphocytes population and followed by CD25^{hi}CD127^{lo} (**Figure 3A**). Two subsets of Tregs were then derived by sorting on TNFR2$^+$ and TNFR2$^-$ population. Following incubation in either AIM V media or cell-free ascites of an advanced EOC patient for 48 h, purified Tregs were stained for surface markers with CD3, CD4, CD25, PD-L1, CTLA-4, GARP followed by intracellular staining for FoxP3 and prepared for flow cytometric analysis. This exploratory data using purified Treg subsets suggested that TNFR2$^+$ Tregs conditioned in ascites had increased expression of immunosuppressive molecules, including PD-L1, CTLA-4, and GARP when compared to TNFR2$^+$ Tregs in media as well as when compared to TNFR2$^-$ Tregs (**Figure 3B**).

We, therefore, performed an experiment to determine if this upregulation pattern would also be reproducibly found when using PBMC. We incubated PBMCs from healthy donors ($n = 30$) in either AIM V media or ascites supernatant of an advanced EOC patient for 48 h followed by cell labeling with anti-CD3, CD4, CD25, TNFR2, PD-L1, CTLA-4, GARP, and FoxP3 and subsequent flow cytometric analysis. TNFR2$^+$ Tregs conditioned in malignant ascites showed significantly higher levels of expression of PD-L1 (1,550 ± 66.0 vs 1,103 ± 34. 2, $p < 0.0001$), CTLA-4 (1,116 ± 36.6 vs 795 ± 39.5, $p < 0.0001$), and GARP (612.5 ± 34.0 vs 315.7 ± 20.9, $p < 0.0001$) compared to media (**Figure 3C**). Moreover, within PBMCs conditioned in ascites, TNFR2$^+$ Tregs expressed higher levels of immunosuppressive molecules compared to TNFR2$^-$ Tregs, including PD-L1 (1,898 ± 59.9 vs 689 ± 29.5, $p < 0.0001$), CTLA-4 (1,082 ± 32.6 vs 465.5 ± 37.8, $p < 0.0001$), and GARP (544.3 ± 35.3 vs 308.8 ± 10.2, $p < 0.0001$) (**Figures 3C–F**). These differences were likely to be driven by significantly higher expression of FoxP3 on

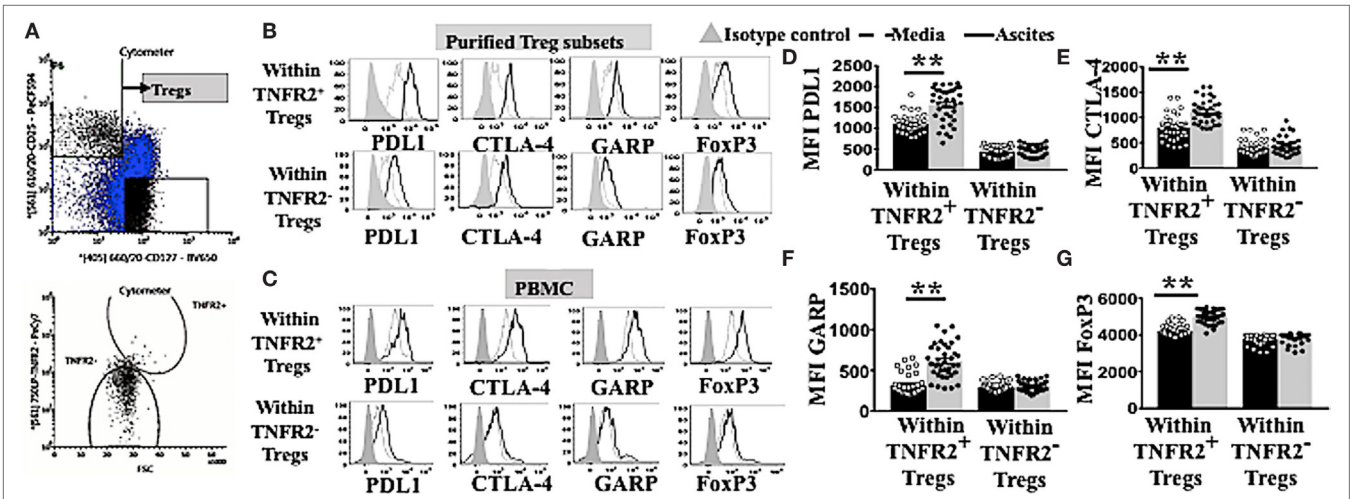

FIGURE 3 | TNFR2$^+$ regulatory T cells (Tregs) conditioned in ascites expressed higher levels of functional immunosuppressive molecules, such as programmed cell death ligand-1 (PD-L1), CTLA-4, and GARP. **(A)** Peripheral blood mononuclear cells (PBMCs) from two healthy donors were isolated by Ficoll density centrifugation and labelled with anti-CD3, CD4, CD25. CD127 and tumor necrosis factor receptor 2 (TNFR2) and flow cytometric cell sorting was performed. **(A)** Tregs were identified by gating on CD3* CD4* population, followed by CD127 and CD25 to identity CD25^{hi} CD127^{lo}. Two populations of Tregs were sorted by gating on TNFR2$^+$ and TNFR2$^-$. Isolated Tregs subsets were cultured in either complete AIM V media or in cell-free ascites for 48 h. Cells were washed and labeled with anti-CD3, CD4. CD25, PD-L1, CTLA-4. GARP and FoxP3 and prepared for flow cytometric analysis. **(C–G)** PBMCs from healthy donors ($n = 30$) were incubated in either AIM V media or ascites supernatant from an advanced epithelial ovarian cancer patient for 48 h. Cells were washed, stained for anti-CD3, CD4, CD25, TNFR2. PD-L1, CTLA-4, GARP, and FoxP3, and prepared for flow cytometric analysis. **(C,B)** Expression of PD-LI, CTLA-4, GARP, and FoxP3 expression were analyzed in sorted TNFR2$^+$ Tregs and TNFR2$^-$ Tregs, **(B)** and in Tregs from PBMCs by gating on Tregs TNFR2$^+$ and Tregs TNFR2$^-$ cells incubated in media (dashed line) and ascites (solid line). The shaded histogram represents staining with an isotype control. **(D–G)** The level of expression (in MFI) PD-L1, CTLA-4, GARP, and FoxP3 within Tregs TNFR2$^+$ and Tregs TNFR2$^-$ cells incubated in media (black bar) and ascites (gray bar) ($n = 30$). *$p < 0.05$ and **$p < 0.0001$, Wilcoxon matched-pair t-test. Data using PBMCs were pooled from three independent experiments (error bars-SEM), while data of sorted cells are replicates of two donors.

TNFR2$^+$ Tregs conditioned in ascites than media (5,048 ± 48.4 vs 4,238 ± 56.5, $p < 0.0001$), and in TNFR2$^+$ compared to TNFR2$^-$ Tregs within ascites conditioned cells (**Figures 3C,G**) (5,048 ± 48.4 vs 3,842 ± 32.0, $p < 0.0001$). The above data support a more potent immunosuppressive regulatory phenotype for TNFR2$^+$ Tregs conditioned in ascites as compared to media, as well as for TNFR2$^+$ compared to TNFR2$^-$ Treg induced by culturing PBMCs in ascites.

The latter hypothesis was specifically tested by determining the capacity of the ascites induced TNFR2$^+$ Tregs to be associated with decreased CD4 effector T cell function. PBMCs from healthy donors ($n = 30$) were incubated in either AIM V media or cell-free malignant ascites in two plates in parallel. One plate remained unstimulated to assess the proportion of T cell subsets, while another plate underwent PMA and ionomycin stimulation to detect intracellular cytokine production. As expected, Tregs were induced at a higher frequency by ascites compared to media in unstimulated PBMCs cultures with ascites, and this increase in Tregs (fold change) (media or ascites cultured) was inversely correlated with the ability of Teff to produce IFN-γ (**Figure 4A**). Moreover, only increases TNFR2$^+$ Tregs (**Figure 4B**), but not TNFR2$^-$ Tregs (**Figure 4C**) were inversely correlated with effector CD4 T cell function. Given we found ascites also increased TNFR2$^+$ Teffs, and these cells are reported as hyperactive cytokine producers (77), we tested whether conventional Tregs would be able to suppress their cytokine production. **Figure 4D** shows that there was no inverse relationship between conventional Tregs increases and TNFR2$^+$ Teff IFN-γ production, showing they could not suppress this effector T cell subset. Previous studies by Chen and colleagues have suggested only TNFR2$^+$ Tregs can suppress TNFR2$^+$ T cell effectors (78). Consistent with these studies the fold change of TNFR2$^+$ Tregs (**Figure 4E**), but not TNFR2$^-$ Tregs (**Figure 4F**) was strongly inversely correlated with the ability of TNFR2$^+$ Teffs to produce IFN-γ. We next determined whether the proportion of Tregs, TNFR2$^+$ Tregs, and TNFR2$^-$ Tregs correlated with the total production of IFN-γ within CD4$^+$ T cells to ensure that the inverse correlation seen is not influenced by reciprocal gating for Teff and Tregs subpopulations following incubation in ascites. There was inverse correlation between total IFN-γ within CD4$^+$ T cells to Tregs and TNFR2$^+$ Tregs, but not TNFR2$^-$ Tregs. There was also increase in the fold change of IFN-γ producing CD4$^+$TNFR2$^+$ T cells which inversely correlated to Tregs as well as TNFR2$^+$ Tregs but not TNFR2$^-$ Tregs (Figure S2 in Supplementary Material). Similar correlation patterns were also observed when the level of IFN-γ expression measured by mean fluorescence intensity (MFI) was used instead of proportion of IFN-γ$^+$ T cells (data not shown). We also looked at whether CD8 T cell function was affected by the induced TNFR2$^+$ Tregs.

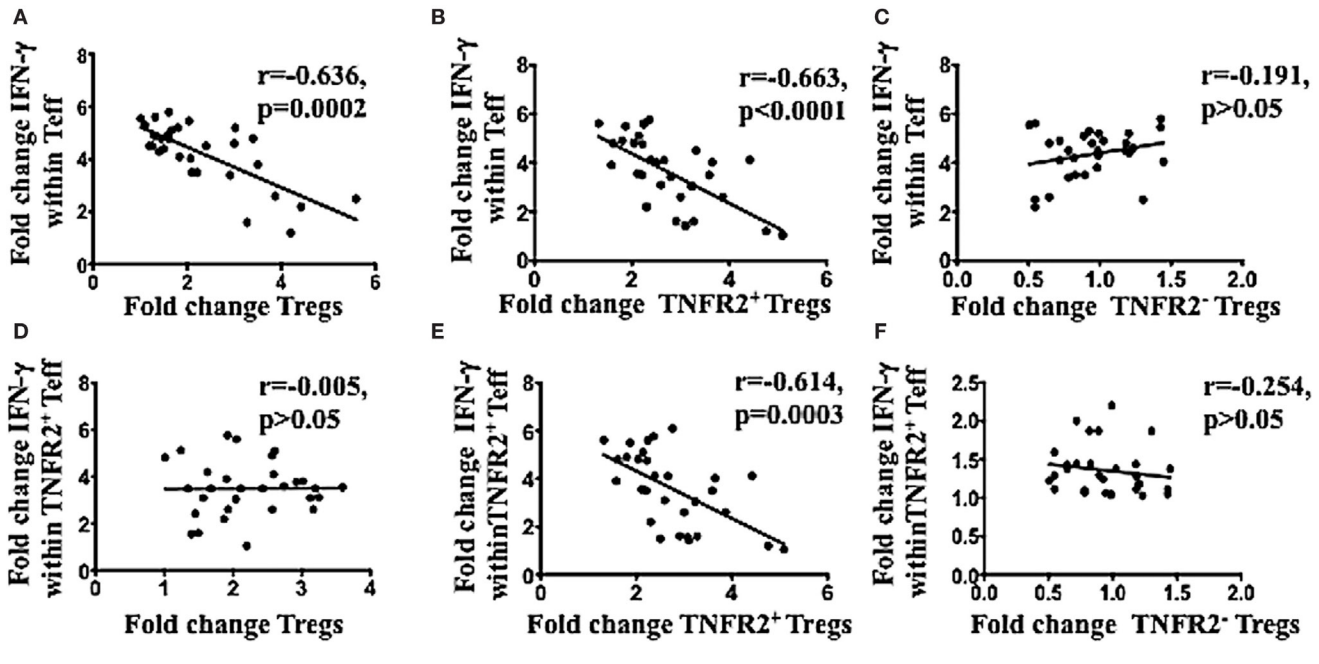

FIGURE 4 | TNFR2$^+$ regulatory T cells (Tregs) conditioned in ascites, compared to media had a higher frequency and were inversely correlated with IFN-γ production by effector T cells in the same donor. Peripheral blood mononuclear cells from healthy donors from three independent experiments ($n = 30$) were incubated in either AIM V media or ascites supernatant of an advanced epithelial ovarian cancer patient for 48 h in two plates simultaneously. One of plate was left unstimulated for quantification of T cell subsets, while another plate was incubated with PMA and ionomycin for the last 6 h, and Brefeldin A was added at 1 μg/ml for the last 5 h of incubation, to detect intracellular cytokine production. Cells were washed, labeled for surface markers CD3, CD4, CD25. Tumor necrosis factor receptor 2 (TNFR2) followed by intracellular staining for FoxP3 and IFN-gamma (IFN-γ) and then prepared for flow cytometric analysis. **(A–C)** Correlation of the fold change in Tregs **(A)**. TNFR2$^+$ Tregs **(B)**, and TNFR2$^-$ Tregs **(C)**, following conditioning in ascites with the fold change in IFN-γ production by T effector (Teff) respectively. **(D–F)** Correlation of the fold change in Tregs **(D)**, TNFR2$^+$ Tregs **(E)**, and TNFR2$^-$ Tregs **(F)** conditioned in ascites with the fold change in IFN-γ production by TNFR2$^+$ Teff respectively. $p > 0.05$ is not significant.

There was no inverse correlation between conventional Tregs or TNFR2$^+$ Tregs with the ability of CD8 to produce IFN-γ (data not shown).

Higher Levels of Immunosuppressive (sTNFR2, IL-10, and TGF-β) and Pro-inflammatory Cytokines (IL-6 and TNF) Are Present in Malignant Ascites Compared to Serum of Advanced Ovarian Cancer Patients

We next determined the pro-inflammatory soluble factors present in malignant ascites that may promote TNFR2$^+$ Tregs. Ascites and respective peripheral blood serum were collected from 18 patients with ovarian cancer. The mean age at the time of cytoreductive surgery was 60.2 ± 7.82 years (range 49–78 years). All patients had advanced disease including 16 patients with Stage III (88.8%) and 2 patients (11.2%) with Stage IV disease. Specimens were collected prior to surgery and before chemotherapy. Final pathology was consistent with high-grade (poorly differentiated) serous type ovarian cancer. We performed multiplexed bead-based immunoassay on cell-free pre-treatment ascites and on respective sera of all 18 patients. Ascites specimen demonstrated a significant elevated level of immunosuppressive (sTNFR2, IL-10 and TGF-β) and pro-inflammatory cytokines (IL-6 and TNF) compared to respective serum (**Figure 5**). TNFR2 expression has been shown to be induced by either TNF (15, 79) or TNF in combination with IL-6 in cancer cell lines (20). We next explored whether blockade of either of these soluble factors would influence overall Treg induction by malignant ascites, as well as TNFR2 expression.

TNFR2$^+$ Tregs Are Decreased in Frequency and in their Suppressive Phenotype following Blockade of IL-6 in Ascites

Following conditioning of PBMCs from healthy donors in malignant cell-free ascites for 48 h, soluble factors IL-6 and TNF within the ascites were blocked with neutralizing mAb against IL-6 or TNF. Mouse IgG1 immunoglobulins (isotype control) (R&D USA) were used as a negative control. Cells were washed, stained, and analyzed using flow cytometry. The overall fraction of CD4$^+$ and CD8$^+$ T cells following conditioning in ascites and blockade with either IL-6 or TNF mAbs showed no significant differences compared to isotype control (**Figures 6A,B**). By contrast, within CD4 T cells, there was a clear increase in Teff and decrease in Tregs following blockade of IL-6 within ascites compared to isotype control (**Figures 6C,D**). The ratio of Teff to Tregs was, therefore, greatly increased following blockade of IL-6 within ascites (**Figure 6E**). These effects were not observed following blockade of TNF within ascites. Moreover, blockade of bioactive IL-6 in ascites, but not TNF, also decreased the level of TNFR2 expression in both CD4$^+$ and CD8$^+$ T cells (**Figures 6F,G**) as well as Teff and Tregs (**Figures 6H,I**), with the most prominent effect found on Tregs, overall increasing the ratio of TNFR2$^+$ Teffs to TNFR2$^+$ Tregs (**Figure 6J**). Following the blockade of bioactive IL-6 within ascites with monoclonal antibody, the level of expression of PD-L1 (1,550 ± 66.0 vs 803 ± 104.8, $p < 0.0001$), CTLA-4 (1,116 ± 36.6 vs 203.2 ± 42.0, $p < 0.0001$), and GARP (612.5 ± 34.0 vs 58.0 ± 32.8, $p < 0.0001$) decreased within TNFR2$^+$ Tregs conditioned in ascites compared to media (**Figure 6K**). The expression of FoxP3 on TNFR2$^+$ Tregs following blockade of IL-6 was decreased compared to TNFR2$^+$ Tregs within ascites conditioned cells (**Figure 6K**). The above

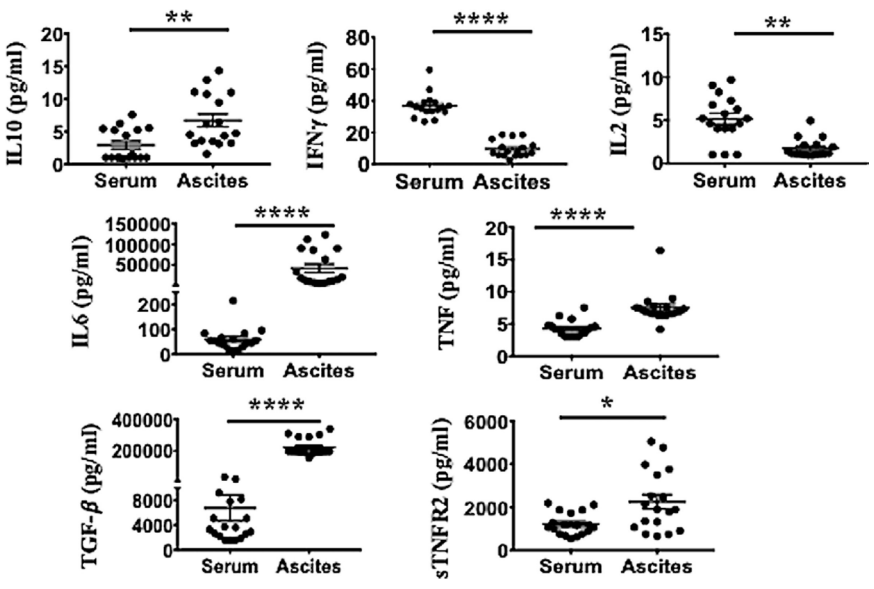

FIGURE 5 | Ascites of epithelial ovarian cancer (EOC) patients contain higher levels of immunosuppressive (sTNFR2, IL-10 and TGF-β) and pro-inflammatory cytokines [interleukin 6 (IL-6) and TNF], but lower levels of IFN-γ compared to their respective serum. Cell-free ascites and corresponding peripheral blood serum were collected from 18 patients with advanced EOC and soluble biomarkers including IL-10, IFN-γ, IL-2, IL-6, TNF, TGF-β, and sTNFR2 quantified using multiplexed bead-based immunoassay. *$p < 0.05$, **$p = 0.001$–0.01, ***$p = 0.0001$–0.001, ***$p < 0.0001$, Wilcoxon matched-paired *t*-test (error bars-SEM).

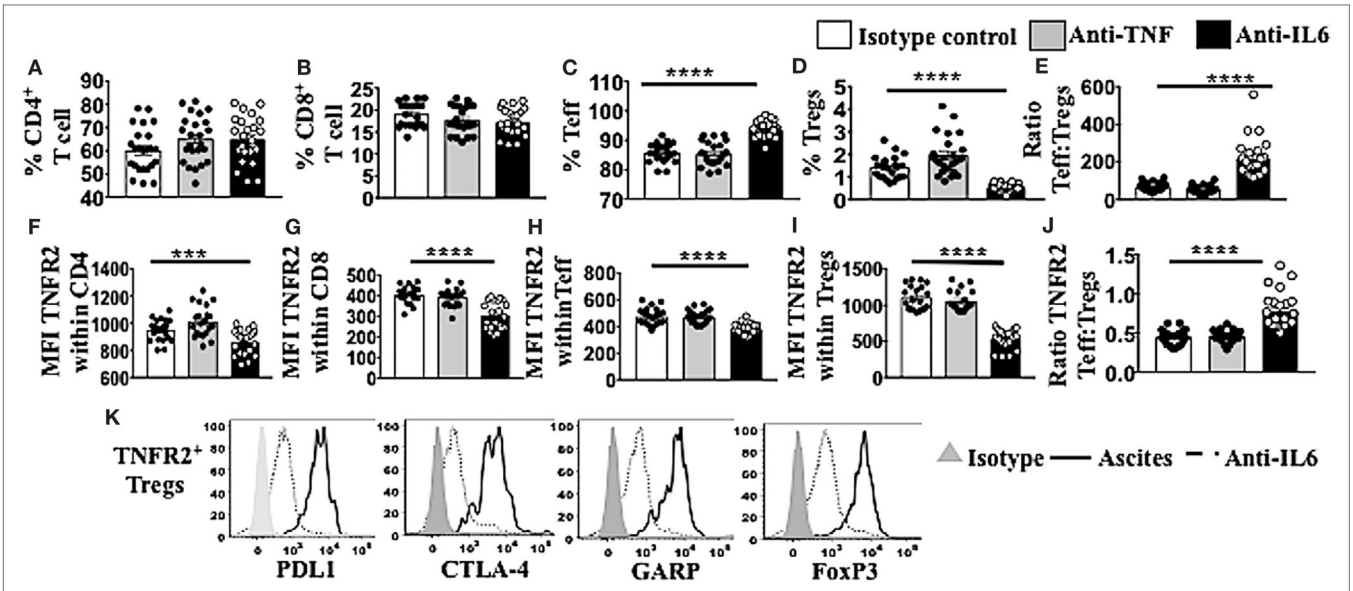

FIGURE 6 | Blockade of bioctive interleukin 6 (IL-6), but not TNF, within ascites of epithelial ovarian cancer (EOC) patients decreases percentage of regulatory T cells (Tregs) as well as TNFR2$^+$ Tregs, as well as tumor necrosis factor receptor 2 (TNFR2) expression. Peripheral blood mononuclear cells (PBMCs) from healthy donors (n = 30) were isolated by Ficoll density centrifugation and incubated *in vitro* in either AIM V media or cell-free ascites from advanced EOC patients for 48 h. IL-6 and tumor necrosis factor (TNF) within the ascites were blocked with mouse anti-human IL-6 monoclonal antibody (mAb) at 2.5 μg/ml and mouse anti-human TNF at 500 ng/ml. Mouse IgG1 immunoglobulins (isotype control) were used as a negative control. Cells were washed, labeled for surface and intracellular markers CD3, CD4, CD8, CD25, TNFR2, programmed cell death ligand-1 (PD-L1), CTLA-4, GARP, and FoxP3, and analyzed with flow cytometry. **(A–E)** The frequency (%) of CD4, CD8, T effector (Teff), and Treg **(A–D)** and the ratio of Teff to Tregs **(E)** within ascites following blockade with IL-6 (black bar) or TNF (gray bar). **(F–J)** The level of TNFR2 expression (median fluorescence intensity, MFI) within CD4, CD8, Teff, and Tregs **(F–I)** and ratio of TNFR2 expressing Teff to Tregs **(J)** in ascites following IL-6 or TNF blockade. **(K)** PD-L1, CTLA-4, GARP, and FoxP3 expression on TNFR2$^+$ Tregs cells incubated in ascites (solid line) and following blockade with IL-6 mAb (dashed line). The shaded (gray) histogram represents staining with an isotype control. $^*p < 0.05$, $^{**}p = 0.001$–0.01, $^{***}p = 0.0001$–0.001, $^{****}p < 0.0001$, One-way ANOVA and Dunn's multiple comparison test (post hoc). Data are pooled from three independent experiments (error bars-SEM).

data support an active role of IL-6 within ascites on promoting TNFR2 expression on Tregs.

DISCUSSION

The present study shows for the first time that culture with ovarian cancer-associated malignant ascites promotes increased frequencies of T cells expressing high levels of TNFR2. Moreover, it shows a critical role for IL-6 in promoting TNFR2 expression on T cells, and particularly on Tregs. These findings could provide a mechanistic link between IL-6 and Tregs, where both have been independently associated with disease progression, for example in breast, lung, renal, and colorectal cancer (80–83). These results also suggest that IL-6 blocking therapy may have additional beneficial therapeutic effects (62). Specifically, and similarly to our *in vitro* studies, it may preferentially decrease TNFR2$^+$ expressing Tregs, leading to an increase in IFN-γ production by Teffs. This is particularly important in the case of TNFR2$^+$ Teffs, since our studies show that, similarly to what has been demonstrated in autoimmunity (84), that these cells may only be suppressed by TNFR2$^+$ Tregs within ovarian cancer ascites.

The high levels of TNFR2$^+$ Tregs within patient ascites have been suggested to be the consequence of preferential migration of TNFR2$^+$CCR4$^+$ Tregs into ascites (9, 18). Herein, we show for the first time that a higher fraction of TNFR2$^+$ expressing T cell subsets, particularly on CD4$^+$CD25^{hi}FoxP3$^+$ (Tregs) can also be induced *de novo* from healthy donor PBMCs conditioned with cell-free ovarian cancer ascites. In addition, the increase in TNFR2 expression also demonstrated on purified Treg subsets cultured in ascites, further suggests the direct induction of TNFR2 by soluble factors in the ascites in the absence of other immune cells. The ovarian cancer ascites used in our study from patients in advanced disease stages showed a mixture and levels of soluble factors similar to previous studies (11, 18, 85, 86), with a predominance toward Th2 vs Th1 type cytokines, and high levels of IL-6 and TNF compared to serum. We have consistently observed similar effects across 18 different ovarian cancer ascites and on cells derived from different human volunteers, confirming the robustness of this effect.

In addition to being induced at high frequencies by ovarian cancer ascites, TNFR2$^+$ Tregs were also potent suppressors when compared to the TNFR2$^-$ T cell fraction, which was in agreement with previous studies (13, 18). The higher frequency of TNFR2$^+$ Tregs induced from malignant ascites was inversely correlated with IFN-γ production by effector T cells in our study. TNFR2$^+$ Tregs conditioned in ovarian cancer ascites, compared to media, also expressed higher levels of functional immunosuppressive molecules such as PD-L1, CTLA-4, and GARP. These differences were likely to be driven by the significantly higher expression

of FoxP3 on TNFR2$^+$ Tregs compared to TNFR2$^-$ Tregs within ascites conditioned cells. Our observation that only TNFR2$^+$ Tregs suppress TNFR2$^+$ Teff in these cultures is highly likely to be mediated by the higher levels of these immunosuppressive molecules. Future studies may be able to distinguish the relative contribution of each one of these molecules on their ability to suppress TNFR2$^+$ effectors. The present study shows that the high levels of CTLA-4 and GARP, previously shown to be present on Tregs derived from human cancer ascites (18), may be directly induced by soluble factors within this ascites, and specifically IL-6. Moreover, PD-L1 expression is also upregulated by ovarian cancer ascites. Recent human trials have demonstrated promising antitumor efficacy for immunosuppressive checkpoint inhibitors in several cancer types (35–37); however, their role as ovarian cancer therapeutics is still not well-established. As blockade of bioactive IL-6 within ascites decreased TNFR2 expression on Tregs, IL-6 may potentially play a combined role with other pro-inflammatory cytokines such as TNF, as seen in past studies (38), to help decrease immunosuppressive molecules such as CTLA-4, GARP, and PD-L1.

Tumor necrosis factor receptor 2 expression can also be induced on Teff by soluble factors within the tumor microenvironment or by TCR stimulation according to Chen (78), and this was also observed in our study. The expression of TNFR2 on Teffs rendered these cells resistant to suppression by Tregs, whereas TNFR2-expressing Teff were highly proliferative, secreted high levels of effector cytokines (IFN-γ), and were more resistant to Treg-mediated inhibition (78). This was also demonstrated in our study where TNFR2$^+$ effector T cells were resistant to suppression by TNFR2$^-$ Tregs, but susceptible to inhibition by TNFR2$^+$ Tregs.

Tumor necrosis factor and IL-6 are pleiotropic pro-inflammatory cytokines that modulate growth, differentiation, and proliferation of various types of cells. Both these cytokines, secreted by cancer cells as well as immune cells, are also involved in the inflammation process and play a functional role in malignancy promotion and progression in various cancers including ovarian cancer (61, 63, 87–89). The present study shows a new critical role for IL-6 present in ascites in promoting TNFR2 expression on T cells, particularly Tregs. In contrast to previous studies, TNF did not appear to be similarly critical. The role of TNF and IL-6, independently or in combination, on TNFR2 expression on diverse cell types is controversial (20, 64, 79, 90). Addition of exogenous TNF, but not IL-6 or IL-1β, have been found to upregulate TNFR2 on murine Treg cells *in vitro* (64). TNF alone has no effect on TNFR2 expression on human colon cancer cell lines *in vitro*; however, the combination of IL-6 and TNF can upregulate TNFR2 on such cells (90). In the same study, the regulation of TNFR2 by IL-6 was confirmed using an *in vivo* murine model. The lack of IL-6 in TCR/IL-6 KO double mutant (IL-6 DKO) mice resulted in markedly reduced TNFR2 expression in colonic epithelial cells compared with TCR KO mice, even in the presence of mild colitis in both groups (90). It was, therefore, possible that, even if we could not detect direct effects for TNF, TNF would still potentiate TNFR2 induction by IL-6. However, we did not observe an enhancement of the decrease in TNFR2 expression by blockade of both IL-6 and TNF compared to IL-6 blockade alone (data not shown).

Tumor necrosis factor receptor 2 upregulation is associated with increased suppressive activity of Tregs. Chen and colleagues used purified T cell subsets from lymph nodes of C57/BL6 mice and performed a suppression assay by coculturing Tregs and Teff with exogenous TNF (10 ng/ml) and IL-2 (10 ng/ml) for up to 72 h. The authors found that prolonged exposure to TNF increased Treg suppressive activity, but shorter duration did not demonstrate this effect (64). We did not find a prominent role for TNF in our study, most likely given we used different methods (ascites vs pure cytokines), species (murine vs human), measurements (TNFR2 expression vs suppression add-back assays), and timepoints (48 vs 72 h). Ascites may contain other soluble factors such as TGF-β that may synergize with IL-6 to promote TNFR2 expression. TGF-β has been suggested to play a role in the generation and expansion and stability of Tregs (91). The exploration of the influence of these cytokines in promoting TNFR2 expression is worthwhile, but beyond the scope of this paper. Preliminary results suggest TGF-β may not play a substantial role in the upregulation of TNFR2 on Tregs (manuscript in preparation). In the present paper, we found that blockade of the elevated IL-6 level within ascites, but not TNF, decreased TNFR2 expression, particularly on Tregs.

Therefore, the findings from this study suggest IL-6 may play an active role in promoting early TNFR2 upregulation, which may in turn enable these Tregs to respond to TNF. The nature of our short term cultures (we do not add IL-2 or other factors which may extend cell survival) does not allow us to test this hypothesis directly. However, taking together with the findings from a previous study (64) and the present data, we propose IL-6 may promote early activation of TNFR2 expression on Tregs, while TNF may be responsible for long term maintenance of TNFR2 expression and Tregs suppressive activity. Consistent with this hypothesis, Mizoguchi and colleagues have observed in their *in vitro* system using colonic epithelial cells from murine colitis model that while TNFR2 upregulation is seen on day 8, upregulation of IL-6/STAT3 is observed earlier by day 4, indicating that the IL-6/STAT3 signaling cascade is activated before upregulation of TNFR2 expression (90). Another recent study looking at human colon cancer cell lines (20), confirmed the critical role for the IL-6/STAT3 pathway in enabling TNFR2 upregulation (20, 90).

Future studies could also address the influence of IL-6 on different subsets of TNFR2 expressing effector T cells. Previous studies have shown that TNFR2$^+$ effectors were capable upon anti-CD3 and anti-CD28 stimulation of producing a range of cytokines including IFN-y, IL-2 and IL-10 (77, 78). It would be particularly interesting to study the effect of IL-6 on cytokine producing Th17 cells given literature suggesting that Th17 plays an important role in the pathogenesis of diverse group of autoimmune diseases as well inflammatory diseases and cancers, including ovarian cancer (92, 93).

The immune system is capable of effective antitumor responses against many cancers including ovarian cancer. As ovarian cancer has been identified as an immunogenic tumor, immunotherapy should be optimized to be included as part of ovarian cancer therapeutics. Our study demonstrates that in

ovarian cancer, TNFR2 expression can be selectively decreased on all T cells, and predominantly on Tregs, by blockade of bioactive IL-6 within ascites. Our findings support future research into two potential interactive immunotherapeutic targets, TNFR2$^+$ Tregs and IL-6, to help enhance effective antitumor responses in patients with ovarian cancer.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Immunity and Ovarian Cancer trial (Project 13/32), HREC of Royal Women's Hospital with written informed consent from all patients. All patients gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by HREC of the Royal Women's Hospital, Melbourne.

AUTHOR CONTRIBUTIONS

Concept and design: NK, MM, OM, MQ, and MP. Development of methodology: NK, MM, MQ, and MP. Acquisition of data (acquired and managed patients, provided facilities, etc.) and writing, review, and/or revision of the manuscript: NK, MM, OM, AS, MQ, and MP. Analysis and interpretation of data (e.g., statistical analysis, computational analysis): NK, MM, and MP. Administrative, technical, or material support (i.e., organizing data, constructing databases): NK, OM, AS, MQ, and MP. Study supervision: OM, MQ, and MP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01482/full#supplementary-material.

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Chronic Inflammation Increases the Sensitivity of Mouse Treg for TNFR2 Costimulation

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TNF receptor type 2 (TNFR2) has gained attention as a costimulatory receptor for T cells and as critical factor for the development of regulatory T cells (Treg) and myeloid suppressor cells. Using the TNFR2-specific agonist TNCscTNF80, direct effects of TNFR2 activation on myeloid cells and T cells were investigated in mice. *In vitro*, TNCscTNF80 induced T cell proliferation in a costimulatory fashion, and also supported *in vitro* expansion of Treg cells. In addition, activation of TNFR2 retarded differentiation of bone marrow-derived immature myeloid cells in culture and reduced their suppressor function. *In vivo* application of TNCscTNF80-induced mild myelopoiesis in naïve mice without affecting the immune cell composition. Already a single application expanded Treg cells and improved suppression of CD4 T cells in mice with chronic inflammation. By contrast, multiple applications of the TNFR2 agonist were required to expand Treg cells in naïve mice. Improved suppression of T cell proliferation depended on expression of TNFR2 by T cells in mice repeatedly treated with TNCscTNF80, without a major contribution of TNFR2 on myeloid cells. Thus, TNFR2 activation on T cells in naïve mice can lead to immune suppression *in vivo*. These findings support the important role of TNFR2 for Treg cells in immune regulation.

Keywords: inflammation, immune regulation, costimulation, MDSC, TNFR2, regulatory T cell

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INTRODUCTION

TNF is a key inflammatory cytokine regulating the immune system. It induces inflammation and tissue injury via the activation of TNF receptor type 1 (TNFR1). Currently, TNF blockade is used as anti-inflammatory intervention in patients with chronic inflammatory diseases such as rheumatoid arthritis or inflammatory bowel diseases (1–3). However, there is also evidence for adverse side effects from experimental and clinical studies (4–7). The interaction of TNF with its two functionally different receptors TNFR1 and TNF receptor type 2 (TNFR2) partly explains the complexity of TNF effects. Selective inhibition of soluble TNF or of TNFR1 has been suggested to avoid detrimental TNFR1 activation but to preserve the interaction of endogenous membrane TNF with TNFR2 (8). Activation of TNFR2 has gained attention, in particular, in conferring immune suppression (9) and, recently, by inducing regulatory T (Treg) cells (10–13). The T cell costimulatory effect of several TNF family members, including TNFR2, seems to be important for the promotion of the

development of Treg cells (14). Also, expansion of suppressive Treg cells *in vitro* was improved by activation of TNFR2 (15, 16). Thus, TNFR2 proved to be critically involved in generation and function of regulatory T (Treg) cells, offering the opportunity for a more specific immune regulatory treatment of autoimmune diseases (13, 17, 18).

The role of TNFR2 in immune suppression conferred by myeloid-derived suppressor cells (MDSC), a not so well characterized immature subpopulation of myeloid cells, is less clear. Generation of functional MDSC seems to depend on TNFR2 signaling by arresting their differentiation to mature macrophages (19, 20). In addition, activation of TNFR2 is also required for the optimal suppressive function of MDSC (21, 22).

We and others have previously shown that TNFR2 signaling impacts both on T cell and myeloid cell populations. So far, however, no specific activation of the TNFR2 was applied, but indirect models of TNFR2-deficiency were used. Here, we present a study of effects induced by a TNFR2-specific agonist on the cellular level. The contribution of TNFR2 activation on T cells, Treg cells, and MDSC was analyzed *in vitro* as well as *in vivo* in naïve mice and in mice with chronic inflammation. This comparative study of healthy and diseased animals with focus on multiple immune cell populations aims at a better assessment of the TNFR2 agonist as a possible therapeutic agent. While TNFR2 signaling is crucial for induction of suppressive Treg cells (10–13), we show here that, by contrast, activation of TNFR2 on myeloid cells interfered with the maturation of MDSC and reduced their suppressive capacity. However, expression of TNFR2 on T cells was critical for the dominating immune suppressive effect of TNFR2 agonist in chronically inflamed mice. Thus, the level of inflammation and therefore the targeted pathology seem to be critical parameters for the therapeutic use of the TNFR2 agonist.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Janvier (LeGenest, France). TNFR2-deficient mice (C57BL/6-Tnfrsf1btm1Mwm) (23) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6N Ly5.1 (CD45.1) (24) mice were kindly provided by Petra Hoffmann, University of Regensburg. Mice carrying the conditional $TNFR2^{flox/flox}$ allele ($TNFR2^{fl/fl}$) were generated by breeding Tnfrsf1b/tm1a(EUCOMM)Wtsi mice to FLPe delete mice (25). Location and orientation of both loxP sites and deletion of the beta-galactosidase reporter gene and the neomycin resistance cassette were verified by cloning of the corresponding PCR products and subsequent sequence analysis. For genotyping the following primers were used: 5′ TGTGAGTGCAAGGACACACGGTGC 3′ and 5′ GGCCAGGAAGTGGGTTACTTTAGGGC 3′. Cell-specific ablation of TNFR2 on T cells (CD4cre/$TNFR2^{fl/fl}$) was achieved by breeding $TNFR2^{fl/fl}$ mice to CD4-Cre mice (26). CD4cre/$TNFR2^{fl/fl}$ lack the expression of TNFR2 on T cells while the expression on myeloid cells is not changed. To generate macrophage- and neutrophil-specific TNFR2-deficient mice (LysMcre/$TNFR2^{fl/fl}$), $TNFR2^{fl/fl}$ mice were crossed with LysM-Cre mice (27). Fewer myeloid cells express TNFR2 in these mice and the expression is mainly seen on immature myeloid cells of the MO-MDSC subtype. Mice were bred and housed in an animal facility with barrier conditions at the University of Regensburg. This study was carried out in accordance with institutional guidelines. The protocol was approved by the district government of Lower Franconia, Würzburg (Az: 54-2532.1-27/10, AZ: 54-2532.1-37/13).

TNFR2 Agonist

Generation of tenascin-trimerized single-chain mouse TNF receptor p80 (TNFR2)-specific TNF (TNCscTNF80) as a TNFR2-specific agonist has been described recently as STAR2 (13). The TNCscTNF80 expression cassette was subcloned into pT2/SV-Neo and transfected into HEK293 cells together with the Sleeping Beauty Transposon plasmid pCMV(CAT)T7-SB100 [Addgene, Cambridge, MA, USA (28)] to produce TNCscTNF80 from HEK293 transfectants. TNCscTNF80 contains a Flag epitope and was purified from cell supernatants by affinity chromatography on anti-FlagM2 Agarose and eluted with Flag-peptide (Sigma, Deisenhofen, Germany). After dialysis (Spectra/Por, Serva, Heidelberg, Germany), the protein concentration was determined by scanning (Typhoon 9200, GE Health Care, Solingen, Germany) a SyproRed (Invitrogen, Carlsbad, CA, USA)-stained polyacrylamide gel (10% SDS-PAGE) and comparing the intensity of the TNCscTNF80 band with that of a BSA protein standard (Invitrogen, Life Technologies, Darmstadt, Germany) using the Image Quant TL 7.0 Analysis software (GE Health Care). Biological activity and specificity was routinely tested in a T cell proliferation costimulator test: carboxyfluorescein succinimidyl ester (CFSE, eBioscience, Frankfurt, Germany)-labeled spleen cells (2×10^6/ml) were cultured with anti-CD3 (0.1 µg/ml) with or without TNCscTNF80 (50 and 5 ng/ml) for 72 h. Proliferation of CD4 and CD8 T cells was quantified by FACS analysis. Lipopolysaccharide contamination was excluded in control experiments with heat-inactivated TNCscTNF80. TNCscTNF80 exclusively and specifically binds to and activates TNFR2 but not TNFR1 (13).

Cells

Cell separation out of cell suspensions was performed with magnetic beads following the instructions of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach). Bone marrow-derived myeloid cells were generated from bone marrow as described (29). For evaluation of NO production capacity, these cells were stimulated with LPS (*E. coli* O127:B8, 0.1 µg/ml, Sigma) and IFNγ (120–240 IU/ml, PeproTech GmbH, Hamburg) for 48 h.

Flow Cytometry

Single cell suspensions were prepared from spleens, and pooled lymph nodes and red blood cells were lysed, or cells were harvested from cell culture. Unspecific antibody binding was blocked by anti-FcRII/III-antibody (BD Biosciences, Heidelberg, Germany) and cells stained with fluorochrome-labeled antibodies. Antibodies for flow cytometric analyses were purchased from either eBiosciences (Frankfurt, Germany) or BD Bioscience. Fluorescence was measured on a BD LSR-II

cytometer and analyzed using FACSDiva software (BD Biosciences). Living single cells were gated based on forward/sideward scatter properties.

T Cell Proliferation

Single cell suspensions from spleens were prepared, and red blood cells lysed. For proliferation assays, splenocytes were labeled with 1 μM CFSE (Invitrogen). CFSE-labeled splenocytes (2×10^5) or purified T cells (5×10^4) were activated with 0.1 μg/ml anti-CD3ε antibody (clone 145.2C11, purified from hybridoma supernatant) with or without additional stimulation as indicated for 72 h. In an experiment to test the requirements for CD28, T cells were purified from splenocytes before CFSE-labeling. CFSE-labeled T cells were then stimulated with 0.5 μg/ml anti-CD3ε antibody, with or without 2.5 μg/ml anti-CD28 (clone 37.51), with or without blocking anti-CD80 (clone 16-10A1, 10 μg/ml) + anti-CD86 (clone GL-1, 10 μg/ml) antibodies. Cell proliferation was analyzed after 72 h by assaying CFSE dilution by flow cytometry.

Treg Cell Expansion

Treg cells with a purity of more than 98% $CD4^+CD25^{high}CD62L^+$ from wild-type as well as $TNFR2^{-/-}$ mice were cultured in the presence of anti-CD3ε and anti-CD28 antibodies (MACSiBead particles, Miltenyi Biotec) and recombinant human IL-2 (Proleukin S, Novartis Pharma, Basel, Switzerland) with or without TNCscTNF80 according to the instructions of the manufacturer of the Treg cell expansion kit (mouse, Miltenyi Biotec) for 7 days.

T Cell Suppression

Carboxyfluorescein succinimidyl ester-labeled effector spleen cells ($0.5–1.5 \times 10^5$) were cultured with or without Treg cells for 72 h as described (10, 30). Labeled effector cells from TNFR2-deficient mice were used to avoid interferences by activation of the TNFR2 on effector cells in the cultures. Duplicate or triplicate cultures were stimulated with soluble anti-CD3ε antibodies (0.5 μg/ml, BD Bioscience) for 72 h. Interleukin 10 (IL-10) was quantified using the Duo Set ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. To determine the suppressive activity of myeloid suppressor cells, CFSE-labeled spleen cells (2×10^5) were stimulated with anti-CD3ε (0.25 μg/ml, BD Bioscience) and anti-CD28 (0.125 μg/ml, BD Bioscience) and cultured with or without different numbers of bone marrow-derived myeloid cells. Proliferation of CD4 or CD8 T cells was determined by flow cytometry. Nitrite concentrations in the supernatants were determined using Griess reagent measuring the optical density at 540 nm.

In Vivo Analysis of T Cell Activation

Naïve $TNFR2^{fl/fl}$, $CD4cre/TNFR2^{fl/fl}$, $LysMcre/TNFR2^{fl/fl}$, and $TNFR2^{-/-}$ mice were injected six times (ip) with either TNCscTNF80 (75 μg/mouse) or PBS every other day. Two days after treatment cessation, between 1.5×10^6 and 1×10^7 CFSE-labeled T cells from untreated wild-type mice were adoptively transferred and *in vivo* activated with anti-CD3ε antibody (10 μg/mouse, purified from hybridoma supernatant of clone 145.2C11, iv) the next day. Three days later, splenocytes and the axial, brachial, and inguinal lymph node cells were analyzed by flow cytometry for T cell proliferation.

Model of Chronic Inflammation

To induce chronic inflammation in mice, the model described by Sade-Feldman was used (20). In brief, mice were immunized three times with *Mycobacterium tuberculosis*-BCG (231141, 50 μg, Difco Laboratories, Detroit) in incomplete Freund's adjuvant (Sigma) every 7 days with the last injection without Freund's adjuvant. Two days later, mice were analyzed.

Statistical Analyses

GraphPad Prism 5 was used as statistical software for the data analyses. For comparing two groups, Student's *t*-test was used. For comparing multiple groups, one-way ANOVA with Tukey *post hoc* test, or two-ANOVA with Bonferroni *post hoc* test were used. For analysis of experiments with several dilutions of anti-CD3-antibody, the area-under-the-curve was calculated and used for the statistical analysis. Statistical significant results ($p < 0.05$) are indicated with asterisks (*) in the figures.

RESULTS

Effect of TNFR2 Activation on T Cells

Treatment with a recombinant agonistic fusion protein (TNCscTNF80) with selective activity for mouse TNFR2 induced expansion of Treg cells in mice as reported recently (13). To analyze the effect in more detail on a cellular and molecular level, we tested TNCscTNF80 for costimulation of T cell activation. The costimulatory effect of TNF can be measured as facilitated induction of T cell proliferation and has been described to be TNFR2-specific (31, 32). Mouse spleen cells were cultured with limiting concentrations of anti-CD3ε agonistic antibody (to activate the TCR) in the presence or absence of recombinant human TNF, mouse TNF, or TNCscTNF80, and the proliferation of CD4 and CD8 T cells was analyzed. **Figure 1** shows data of CD4 T cells; CD8 T cells responded in a similar way (data not shown). Consistent with the concept of TNFR2 as a costimulatory receptor, TNCscTNF80 induced proliferation of T cells (**Figure 1A**) only in combination with parallel activation of the TCR. Such requirement of TCR activation for the effect of TNCscTNF80 on T cells has already been described recently (13). Costimulation by TNCscTNF80 was superior over mouse TNF at low protein concentrations. Human TNF, known not to activate the mouse TNFR2 (31, 33), did not affect T cell proliferation. TNCscTNF80 improved the proliferation of CD4 as well as CD8 T cells from wild-type mice in a dose-dependent fashion (**Figure 1B**). CD8 T cells reacted at about four times lower anti-CD3 concentrations than CD4 T cells to the TNFR2 costimulation. TNCscTNF80 had no costimulatory effect on T cells from TNFR2-deficient mice confirming the TNFR2-specificity of the agonistic agent (**Figure 1C**). TNCscTNF80 in the presence of 0.5 μg/ml anti-CD3ε also strongly enhanced the IFNγ release in spleen cell cultures from 28.8 pg/ml in controls without TNCscTNF80 to 1,165.2 pg/ml in cultures with TNCscTNF80 (10 ng/ml).

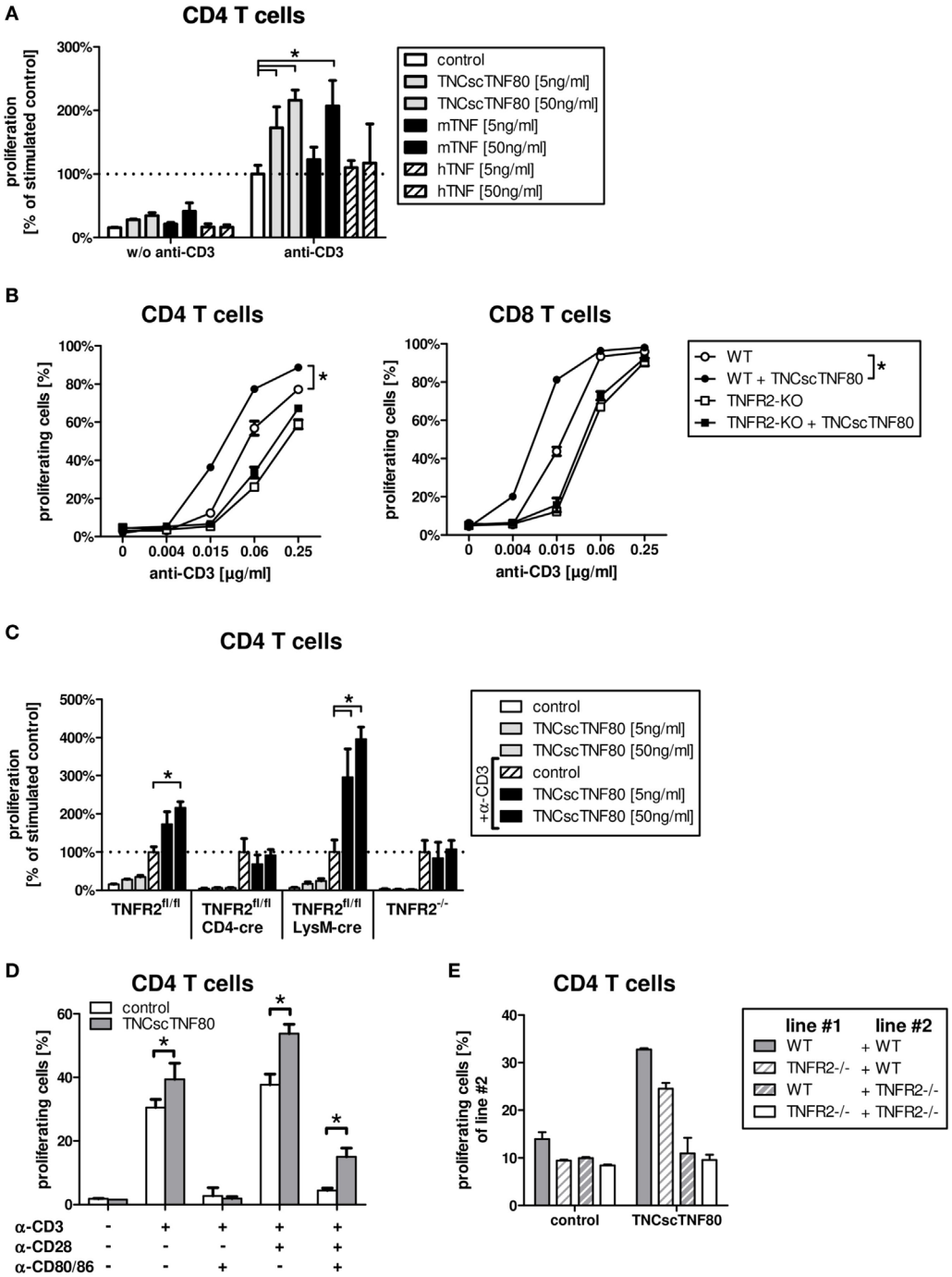

FIGURE 1 | TNCscTNF80-increased proliferation *in vitro*. The relative proliferation of stimulated CD4 T cells from WT mice without or with 5 or 50 ng/ml of either TNCscTNF80, mouse TNF, or human TNF is shown. Data are relative to anti-CD3ε-activated T cells without any TNF variant. **(A)** The proliferation of CD4 (left) or CD8 (right) T cells from either WT (round symbols) or TNFR2-deficient (square symbols) mice is shown in the presence (filled symbols) or absence (empty symbols) of TNCscTNF80 (10 ng/ml) and increasing concentrations of stimulating anti-CD3ε antibodies. Data shown are mean + SD of culture replicates from one representative experiment of five. For statistical analysis, the area-under-the-curve was calculated for all five experiments, and the combined data were analyzed. **(B)** The relative proliferation of stimulated CD4 T cells from different mouse lines without or with 5 or 50 ng/ml of TNCscTNF80 is shown. For each mouse line, proliferation was calculated relative to the respective control of anti-CD3ε-activated T cells without TNCscTNF80 **(C)**. Purified CD4 T cells from WT mice were stimulated with various combinations of anti-CD3ε (0.5 µg/ml), anti-CD28 (2.5 µg/ml) antibody, and blocking anti-CD80/CD86 antibodies (10 µg/ml). The proliferation of CD4 T cells cultured with or without TNCscTNF80 (10 ng/ml) is shown **(D)**. Splenocytes from WT and TNFR2-deficient mice were split in two parts, one labeled with carboxyfluorescein succinimidyl ester (CFSE) and one unlabeled. Unlabeled splenocytes (line #1) were combined with CFSE-labeled splenocytes (line #2) at a ration of 1:1 and stimulated with anti-CD3ε antibody. The proliferation of CFSE-labeled CD4 T cells (line#2) in the presence or absence of TNCscTNF80 (10 ng/ml) is shown **(E)**. For panels **(A,C–E)**, mean + SD of culture replicates from one out of two independent experiments is given.

Similar to T cells from TNFR2-deficient mice, T cells from CD4cre/TNFR2$^{fl/fl}$ mice, lacking TNFR2 on all T cells (**Figure 2A**), did not react to TNCscTNF80 costimulation (**Figure 1C**). By contrast, T cells from LysMcre/TNFR2$^{fl/fl}$ mice—with reduced TNFR2 expression on myeloid cells and in particular on immature myeloid cells of the MO-MDSC subtype (**Figures 2B,C**)—showed a proliferative pattern similar to wild-type T cells upon TNCscTNF80 application.

The costimulatory effect of TNCscTNF80 for T cell proliferation was prevented by blocking the activation of CD28 by using anti-CD80 antibodies, demonstrating that TNCscTNF80 was not able to compensate for lack of CD28 activation (**Figure 1D**).

The requirement for direct activation of TNFR2 on T cells for costimulation by TNCscTNF80 was validated further using mixed cultures of splenocytes from wild-type and TNFR2-deficient mice. The proliferation of TNFR2-deficient T cells was not enhanced by TNCscTNF80 even in cultures also containing T cells from wild-type mice (**Figure 1E**).

Effect of TNFR2 Activation on Treg Cell Function

To test the influence of the TNFR2 agonist on the suppressive function of Treg cells, TNCscTNF80 was added to Treg cell-containing cultures of proliferating T cells. Cells from TNFR2-deficient mice were used as CFSE-labeled effector T cells to avoid any costimulatory effect by the activation of the TNFR2 on the effector T cells. Added to such suppression cultures, TNCscTNF80 diminished the Treg-induced suppression of CD4 T cells (**Figure 3A**). In this experimental setup, CD8 T cells were not suppressed by Treg cells. Hence, a possible reduction of CD8 T cell suppression was not assessed.

To further study the impact of TNCscTNF80 on Treg cells, total splenocytes were cultured in the presence of agonistic anti-CD3ε antibody. Interestingly, phenotypic analysis demonstrated that TNCscTNF80 increased the expression levels of CD25 and Foxp3 of Treg cells after 72 h in these cultures (**Figure 3B**). However, TNCscTNF80 downregulated the percentages of Treg cells positive for CD39, GITR, or CD25 within 48 h, while CD73, OX40L, and CTLA-4 were not affected. No effects were observed at 3 h of culture (**Figure 3C**). Furthermore, we did not observe an impact on TNFR2 levels of Treg in such cultures (data not shown). The TNFR2 agonist had marginal effects on the IL-2-induced activation of STAT5 and did not affect pZAP70 in Treg cells during T cell activation (data not shown). This is in full agreement with results of Kim et al. suggesting discrete effects of TNFR2 on the signaling pathways of T cells (2, 34, 35).

Since the presence of TNCscTNF80 seemed to improve viability and expansion of Treg cells, highly purified Treg cells were stimulated and cultured in the presence of IL-2 with or without TNFR2 agonist. As CD8 T cells would overgrow costimulated T cell cultures, single contaminating CD8 T cells were avoided by very careful sorting of purified Treg cells. Treg cells with a purity of more than 98% of CD4$^+$CD25^{high}CD62L$^+$ cells from wild-type as well as TNFR2$^{-/-}$ mice were cultured in the presence of anti-CD3ε and anti-CD28 antibodies and recombinant

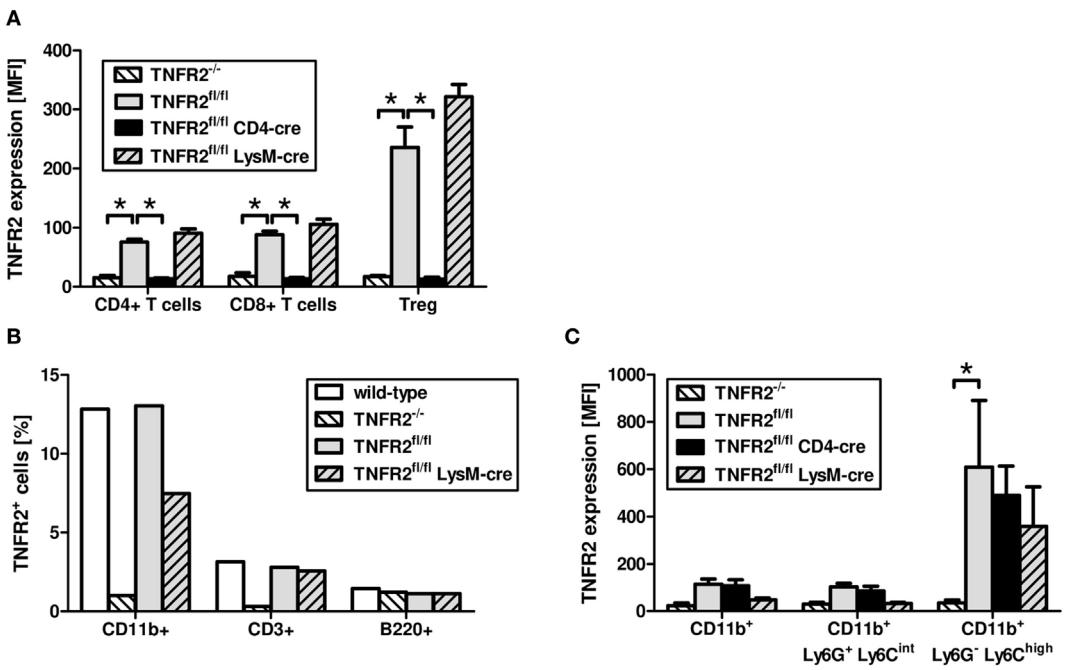

FIGURE 2 | Expression of TNFR2 on cells from genetically modified mouse lines. Splenocytes of naïve male mice of each mouse genotype were analyzed by flow cytometry for the expression levels [mean fluorescent intensity (MFI)] of TNFR2 on CD4$^+$, CD8$^+$, and Treg (CD4$^+$Foxp3$^+$) cells **(A)**. The frequency of cells expressing TNFR2 on CD11b$^+$, CD3$^+$, and B220$^+$ cells was analyzed by flow cytometry **(B)**. Bone marrow cells of naïve male mice of each mouse genotype were analyzed by flow cytometry for the MFI of TNFR2 expression on two subtypes of CD11b$^+$ myeloid cells, PMN-MDSC (Ly6G$^+$Ly6C^{int}), and MO-MDSC (Ly6G$^-$Ly6C^{high}) **(C)**. Results derived from three individual mice per group are expressed as mean values + SD. The data are representative of one out of three experiments.

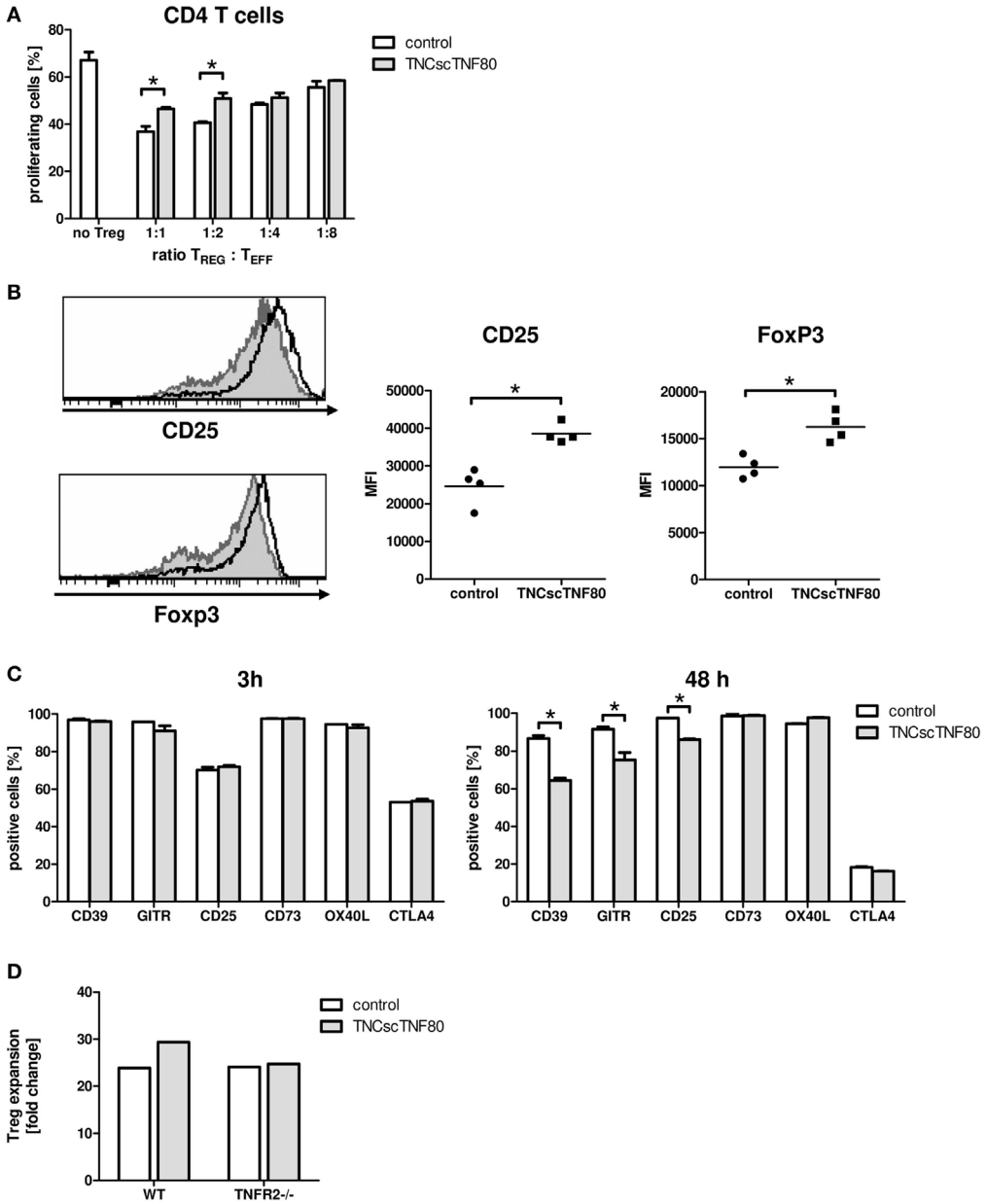

FIGURE 3 | Effects of TNCscTNF80 on Treg cells. Treg cells from wild-type (CD45.1) mice were cultured with anti-CD3 activated TNFR2-deficient splenocytes (CD45.2) with or without TNCscTNF80 (10 ng/ml) at the indicated ratios. The percentage of proliferating CD4 T effector cells (CD45.2) was determined by flow cytometry after 72 h. Data are given as mean of culture replicates + SD from one out of two independent experiments **(A)**. Treg cells from four CD45.2 mice were separately cultured with activated CD45.1 splenocytes with or without TNCscTNF80 (10 ng/ml). After 72 h, the expression profile (left) and the mean of the fluorescent intensity (MFI, right) for CD25 and Foxp3 of Treg cells (CD45.2) from these cultures were analyzed. The left panels show the marker expression profile of CD45.2 Treg cells in one representative culture without (gray histograms) and one culture with (black line histograms) TNCscTNF80. In the right panels, each symbol represents one mouse. Data from one out of four independent experiments are shown **(B)**. Activated total splenocytes were cultured for 3 or 48 h with (gray bars) or without (white bars) TNCscTNF80 (10 ng/ml), and the expression of the surface markers CD39, GITR, CD25, CD73, OX40L, and CTLA-4 were determined on Treg cells ($CD4^+Foxp3^+$) by flow cytometry. Data from one experiment are given as mean of culture replicates + SD **(C)**. Purified $CD4^+CD25^{high}CD62L^+$ Treg cells from wild-type as well as TNFR2-deficient ($TNFR2^{-/-}$) mice were cultured in the presence of antibodies to anti-CD3ε, anti-CD28, and recombinant human IL-2 with or without TNCscTNF80. After 7 days, the cell yield of Treg ($CD4^+Foxp3^+$) cells from wild-type and TNFR2-deficient mice was determined, and the expansion calculated. Representative data of one experiment out of two with similar results are shown **(D)**.

human IL-2 with or without TNCscTNF80 for 7 days. By the end of the expansion period, Treg cells had expanded 23.9-fold without TNCscTNF80. This expansion was increased to 29.4-fold by the presence of the TNFR2 agonist (**Figure 3D**). As expected, TNFR2-deficient Treg cells did not profit from the enhancing effect of TNCscTNF80 in the same experiment (yield

24.1 and 24.7-fold, respectively; similar effects were observed in a second experiment). The expanded cells of both groups consisted of at least 97% Treg cells and expressed similar levels of various Treg signature markers, e.g., CD25, FoxP3, and GITR (data not shown).

Effect of TNFR2 Activation on Myeloid Cells

TNFR2 also plays a role during the differentiation of myeloid precursor cells from the bone marrow to mature myeloid cells as has been shown previously (21). Lack of TNFR2 retarded differentiation of bone marrow cells from TNFR2-deficient mice and led to reduced suppressor activity of TNFR2-deficient immature myeloid cells for T cells. To test the influence of direct TNFR2 activation during myeloid cell differentiation, bone marrow cell cultures from wild-type mice containing GM-CSF with or without TNCscTNF80 were analyzed. Intriguingly, activation of TNFR2 by the TNFR2 agonist reduced the cellular yield and retarded the maturation of cells from such cultures in a similar way as seen with cells of TNFR2-deficient mice (**Figures 4A,B**). TNCscTNF80 also reduced their T cell suppressive activity (**Figure 4C**) and their capacity to produce nitrite (**Figure 4D**).

Furthermore, reduced nitrite production capacity following activation by LPS and IFNγ was found for bone marrow-derived myeloid cells from LysMcre/TNFR2$^{fl/fl}$ compared with wild-type mice (**Figure 4E**). Thus, suppressive activity of

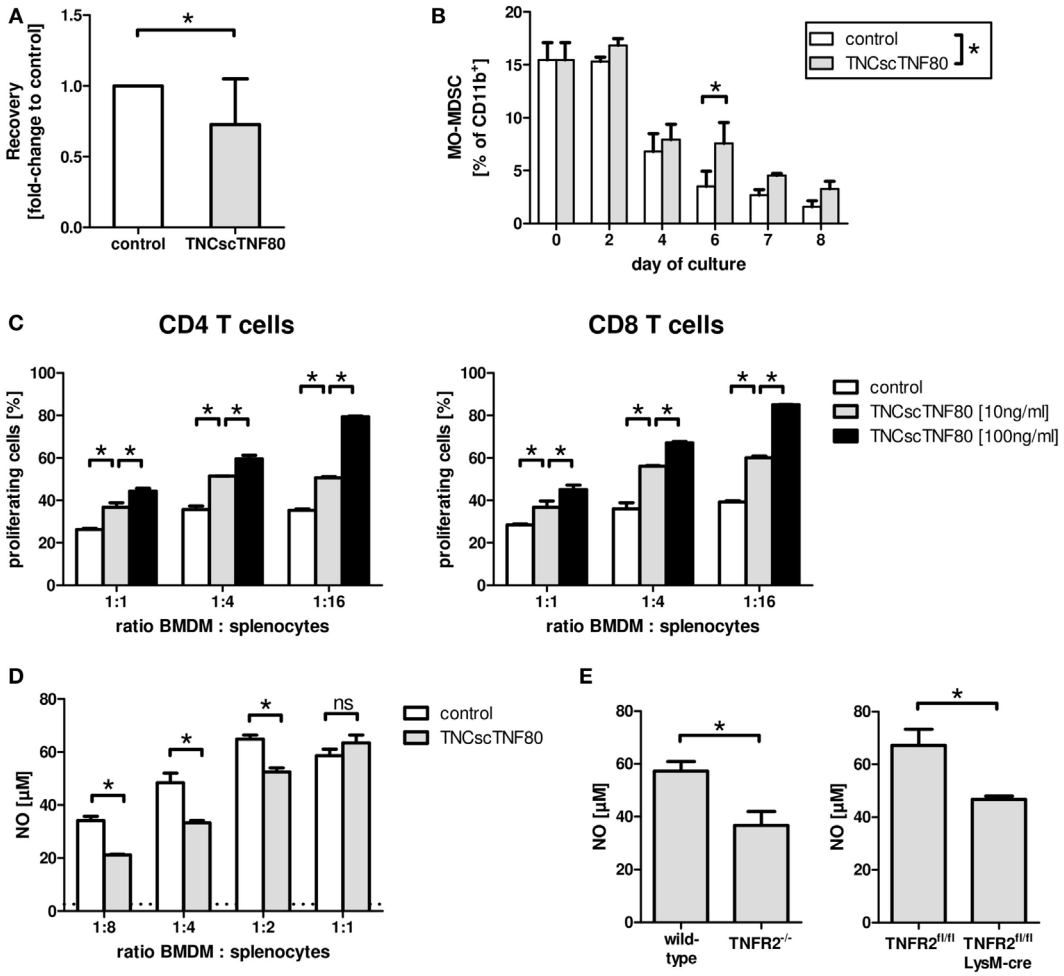

FIGURE 4 | Effects of TNCscTNF80 on myeloid cells. The cell yield of bone marrow-derived myeloid cells was determined after 7 days of culture in the presence of GM-CSF with or without TNCscTNF80 (10 ng/ml). Data of 10 experiments are shown as mean + SD of the normalized values. **(A)** The percentage of immature MO-MDSC (Ly6G$^-$Ly6C^{high}) in cultures of bone marrow-derived myeloid cells with or without TNCscTNF80 (10 ng/ml) at the indicated time points is shown. Data shown as mean + SD of four mice from one experiment **(B)**. Suppressive activity of graded numbers of bone marrow-derived myeloid cells (BMDM) generated over 6 days in the presence or absence of TNCscTNF80 (10 and 100 ng/ml) was measured. Proliferation of CD4 (left) and CD8 T (right) cells was determined by cytometry **(C)**. The concentration of generated nitrite in supernatants of these cultures (containing 100 ng/ml of TNCscTNF80) was determined. The horizontal dotted line indicates the background NO levels of splenocytes cultured without additional BMDM. Data from one experiment are shown **(D)**. Bone marrow-derived myeloid cells from the indicated mouse lines were generated over 8 days, and the capacity to produce nitrite following stimulation with LPS and IFNγ was determined. Data shown as mean + SD of four mice from one experiment **(E)**.

myeloid suppressor cells seems to depend on TNFR2 expression on myeloid cells since bone marrow-derived suppressor cells from TNFR2-deficient mice have also been described to be less suppressive (21).

Effect of TNFR2 Activation *In Vivo*

In the *in vitro* experiments described earlier, we have observed that TNFR2 activation has different effects on the functions of Treg cells and myeloid cells. To evaluate the *in vivo* impact of the TNFR2 on these cell populations on Treg cell function, Treg cells were isolated from wild-type ($TNFR2^{fl/fl}$) and TNFR2-deficient mice as well as from CD4cre/$TNFR2^{fl/fl}$ and LysMcre/$TNFR2^{fl/fl}$ mice, and their suppressive activity was compared in a T cell suppression assay. Treg cells from TNFR2-deficient mice clearly suppressed the T cell proliferation to a lower degree compared with Treg cells derived from wild-type mice as previously shown (21). Surprisingly, TNFR2-deficient Treg cells from CD4cre/$TNFR2^{fl/fl}$ mice suppressed T cell proliferation as good as wild-type Treg cells. By contrast, Treg cells from the LysMcre/$TNFR2^{fl/fl}$ mice, with TNFR2-deficient myeloid cells, seemed to be less suppressive than wild-type Treg cells (data not shown). These experiments were not repeated since the *in vitro* suppression test might not reflect the *in vivo* situation.

To further analyze the impact of TNFR2 on leukocyte function, naïve mice were treated with TNCscTNF80. A single injection had no measurable effect on the spleen; two injections of TNFR2 agonist given within 24 h induced mild splenomegaly (**Figure 5A**). After two or three injections of TNCscTNF80, no change in the composition of splenocytes was observed while changes were observed in the bone marrow: mice developed first signs of increased numbers of myeloid cells after two injections, thus, indicating only a very mild peripheral inflammatory reaction to specific TNFR2 activation. A mild transient myelopoiesis was observed in the bone marrow, with an increase of $CD11b^+$ myeloid cells from 42.4% in untreated mice to 62.0% in TNCscTNF80-treated mice, of $CD11b^+Ly6G^+$ immature myeloid cells from 26.2 to 43.2%, and of $CD11b^+Ly6C^{high}$ from 4.1 to 7.1%, respectively, after three injections of TNFR2 agonist.

When TNCscTNF80 was injected six times every other day into naïve mice, significantly enhanced spleen weight and increased myelopoiesis in the bone marrow were observed. In addition, repeated TNFR2 activation expanded Treg cells in the spleen and lymph nodes in a TNFR2-dependent manner (**Figure 5B** and data not shown). To test whether such mice after repeated TNCscTNF80 treatment are immune suppressed, wild-type T cells were adoptively transferred into different mouse lines that do not express TNFR2 on specific cell types. Proliferation of T cells was only reduced in wild-type ($TNFR2^{fl/fl}$) and in LysMcre/$TNFR2^{fl/fl}$ recipient mice. TNCscTNF80 treatment did not lead

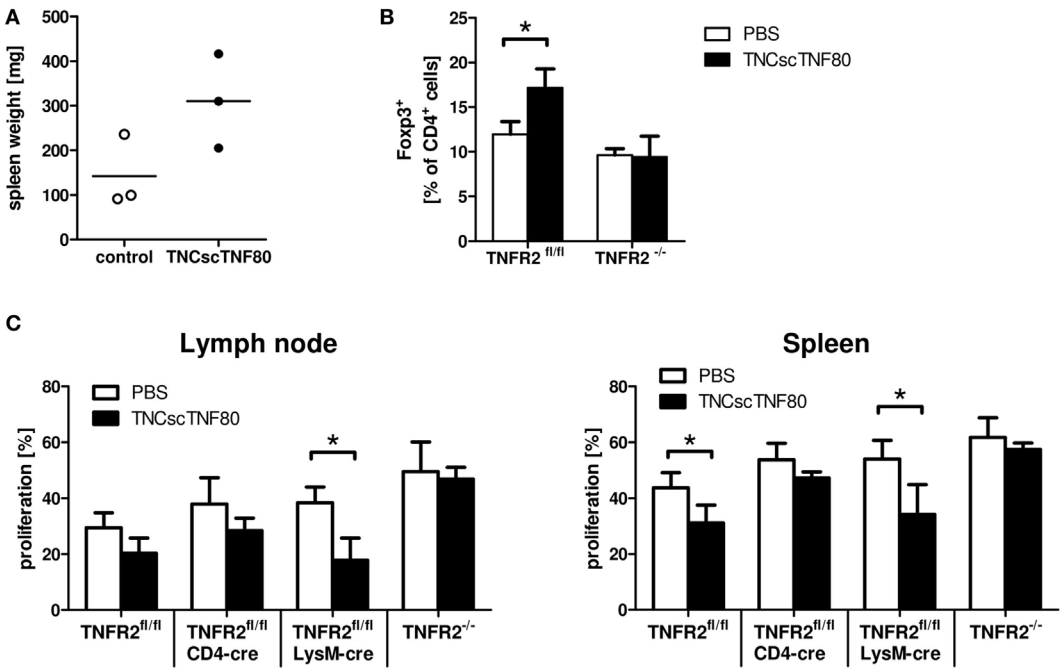

FIGURE 5 | Effects of TNCscTNF80 *in vivo*. Naive mice received TNCscTNF80 (75 μg/mouse) two times (day 5 and 3). Spleen weight was determined on day 0. Data shown are from one experiment; each symbol represents a mouse **(A)**. Naïve $TNFR2^{fl/fl}$ and $TNFR2^{-/-}$ mice were treated six times with either TNCscTNF80 (75 μg/mouse) or PBS. Three days after treatment, the percentages of splenic Treg cells ($Foxp3^+$) were analyzed by flow cytometry. Results are shown as mean + SD of three individual mice per group. Data shown are from one experiment **(B)**. Naïve $TNFR2^{fl/fl}$, CD4cre/$TNFR2^{fl/fl}$, LysMcre/$TNFR2^{fl/fl}$, and $TNFR2^{-/-}$ mice were treated six times with either TNCscTNF80 (75 μg/mouse) or PBS. Two days after treatment cessation, carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells were adoptively transferred and activated. Three days later, pooled cells from axial, brachial, and inguinal lymph nodes (left) and spleen cells (right) were analyzed by flow cytometry. Percentages of proliferating cells of CFSE-positive T cells in the indicated mouse lines were determined **(C)**. Data are derived from one experiment and are shown as mean + SD of three to four individual mice per group.

to a reduction of T cell proliferation in case of ablated systemic TNFR2 expression in TNFR2$^{-/-}$ mice or T cell-specific TNFR2 deficiency in CD4cre/TNFR2$^{fl/fl}$ mice (**Figure 5C**). These findings indicate that TNFR2 on host T cells but not host myeloid cells is required for immune suppression.

To test the *in vivo* effects of TNFR2 stimulation in mice with ongoing inflammation, the model of chronic inflammation by Sade-Feldman was used (20). In this model, a challenge with BCG induces a strong splenomegaly in BCG-pretreated mice. The expansion of the myeloid cell fraction in bone marrow (data not shown) and spleen (**Figure 6A**) was paralleled by a compression of the lymphocyte compartment (**Figure 6B**) documenting ongoing myelopoiesis. BCG pretreatment of mice did not change the frequency of Treg cells in the CD4 T cell population compared with untreated mice (**Figure 6C**). Similar effects of BCG immunization were found in TNFR2-deficient mice and in mice from the CD4cre/TNFR2$^{fl/fl}$ and LysMcre/TNFR2$^{fl/fl}$ mouse lines indicating a TNFR2-independent inflammatory reaction upon BCG immunization (data not shown).

In contrast to injections of the TNFR2 agonist into naïve mice, already a single or two injections of TNCscTNF80 given before the last BCG challenge significantly enhanced the frequency of Treg cells in the splenic CD4 T cell population of BCG-immunized mice (**Figure 6C**). In addition, the activation

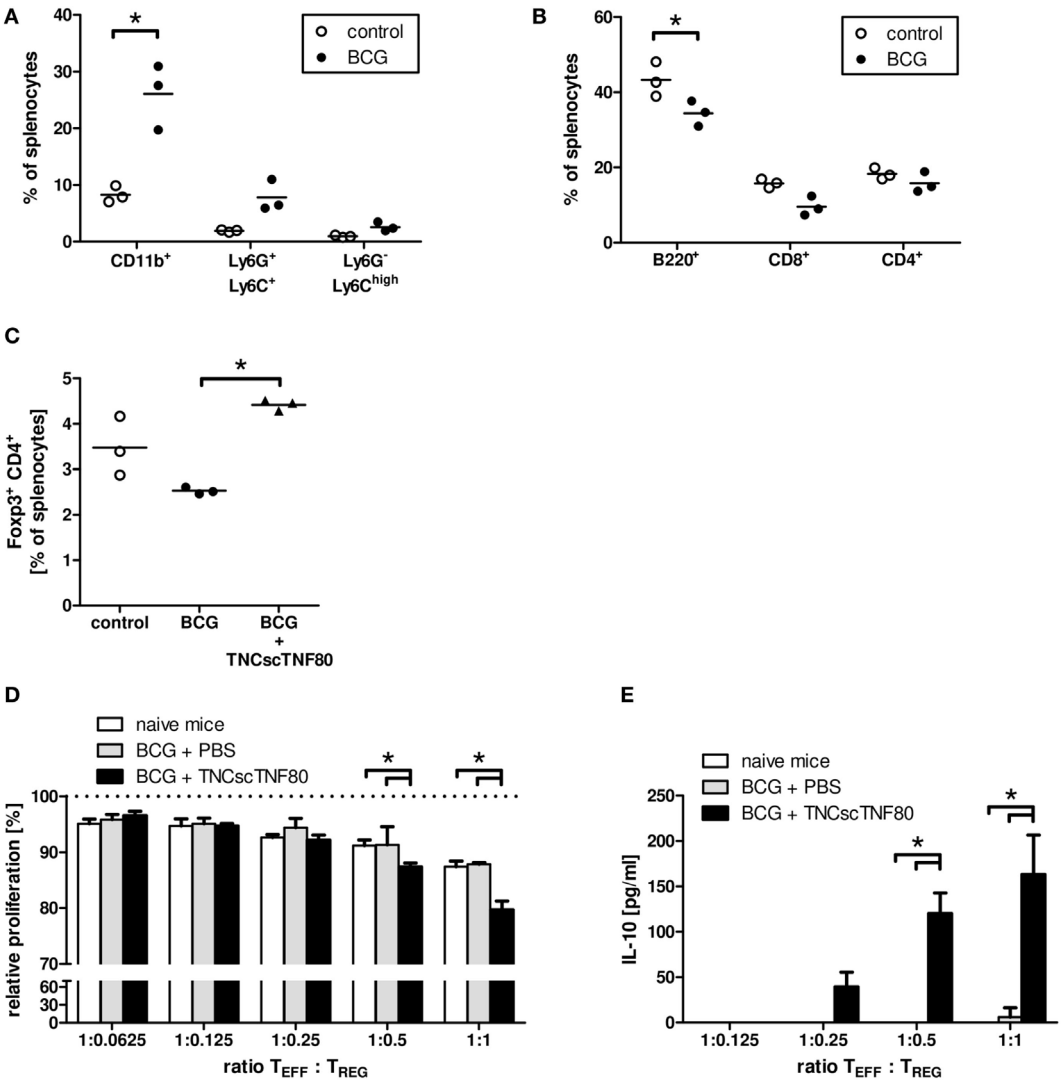

FIGURE 6 | Effects of TNCscTNF80 in the BCG *in vivo* model of chronic inflammation. The composition of spleen cells from mice immunized with BCG was analyzed. The fractions of myeloid (CD11b$^+$) cells and the immature myeloid cell populations PMN-MDSC (Ly6G$^+$Ly6C$^+$) and MO-MDSC (Ly6G$^-$Ly6C^{high}) **(A)**, as well as of B (B220$^+$) and T (CD8$^+$, CD4$^+$) **(B)** were determined by flow cytometry. The fraction of Treg (CD4$^+$Foxp3$^+$) cells in control mice and in BCG-immunized mice treated or not with TNFscTNF80 was determined by flow cytometry **(C)**. Each symbol represents one mouse. Data are shown from one experiment out of two **(A–C)**. The relative proliferation of activated T cells in the presence of graded numbers of Treg cells from either naïve mice (open bars), BCG-treated mice (gray bars), or BCG and TNCscTNF80-treated mice (black bars) was determined. Data are shown as mean + SD of three mice from one experiment. Proliferation data are normalized to proliferation in cultures without additional Treg cells **(D)**, and concentrations of IL-10 were determined in the supernatants **(E)**.

of TNFR2 enhanced the suppressive activity of Treg cells for CD4 T cell proliferation (**Figure 6D**). The immune regulatory cytokine IL-10 was also markedly increased in the suppression cultures containing Treg cells from TNCscTNF80 pretreated animals (**Figure 6E**). However, the frequency of Treg cells in BCG-immunized mice was not altered by two injections of the agonist when given 1 or 3 days after the BCG challenge (data not shown).

DISCUSSION

Intrigued by recent findings of TNFR2-mediated Treg cell expansion *in vivo* (13, 36), we analyzed the consequences of TNFR2 activation on the cellular level. We focused on three major cell populations: effector T cells, regulatory T cells and myeloid-derived suppressor cells (MDSC).

As we have shown previously, cultures containing GM-CSF and bone marrow cells from TNFR2-deficient mice showed retarded differentiation and a lower yield of mature myeloid cells and reduced nitrite production and suppressive activity of MDSC (21). Unexpectedly, in this study, addition of TNFR2 agonist to cultures of wild-type bone marrow precursor cells had similar effects as TNFR2-deficiency on MDSC. Since endogenous TNF as well as soluble TNFR2, an inhibitor of TNF, are produced in such cultures, the addition of TNCscTNF80 could act as a sink for the soluble TNFR2 thereby enhancing the effect of the endogenous TNF on TNFR1. Alternatively, a possibly bell-shaped response curve of the costimulatory effect could explain these seemingly contradictory results. TNFR2 signaling, however, is unquestionably modulating generation and suppressive functions of MDSC as well as of Treg cells.

The TNFR2-specific agonist TNCscTNF80 improved activation of CD4 and CD8 T cells confirming earlier findings (31, 32). CD4 T cells and, even more sensitive, CD8 T cells showed stronger proliferation upon agonistic TNFR2 activation. TNFR2 expression was required for the TNFR2 agonistic signaling and, as expected, human TNF was not able to induce this costimulatory effect (31). TNCscTNF80, therefore, is a veritable TNFR2-specific agonist providing a costimulatory signal to the T cell receptor. In our hands, this TNFR2-specific costimulation was not able to compensate for the lack of CD28 activation by CD80/CD86. By contrast, Kim and Teh (32) previously suggested that TNFR2 might provide costimulation for CD28-independent T cell activation. However, that study employed a markedly different methodology, such as no specific blockade of CD28 or CD80/CD86, much higher concentrations of plate-bound anti-CD3 for T cell stimulation and the use of TNFR2-deficient cells instead of TNFR2 activation. Therefore, a direct comparison to our approach is difficult.

In contrast to its costimulatory activity, the TNFR2 agonist TNCscTNF80 reduced suppressive activity of Treg cells *in vitro* while, interestingly, at the same time increased the expression of their CD25 and Foxp3. However, prolonged exposure of Treg to the TNFR2 agonist did not change the expression of CD73, OX40L, and CTLA-4 while downregulating markers known to be involved in Treg functions such as CD25, CD39, and GITR. This might explain the reduced suppressive activity of Treg cells after prolonged exposure to TNCscTNF80. Highly purified Treg cells expanded stronger *in vitro* upon addition of TNFR2 agonist, supporting recent data demonstrating the improved expansion of mouse and human Treg cells *in vitro* by additional activation of TNFR2 (15, 16). The expanded Treg cell population was homogenous and did not change the expression levels of CD25, Foxp3, GITR, and notably also not of TNFR2. However, these results obtained under cell culture conditions might not be predictive for the impact of TNCscTNF80 on the Treg cell population *in vivo*.

The analysis of systemic TNFR2-deficient mice had previously demonstrated that TNFR2 is critical for frequency and function of Treg cells [own data and Ref. (12)]. In this context, TNFR2-bearing myeloid cells might interact with newly generated natural Treg cells in the thymus to influence the generation and level of suppressive activity as suggested previously (14). To find out whether TNFR2-bearing myeloid cells cooperate with TNFR2-bearing T cells for generation of Treg cells with maximal suppressive activity, mice with cell type-specific expression of TNFR2 were used in this study. Such a cooperation has been suggested by the data recently shown by Nguyen and Ehrenstein (35). In this study, the suppressive activity of Treg cells from mice with TNFR2-deficient T cells or with myeloid cells expressing low levels of TNFR2 could not be determined unambiguously in suppression tests *in vitro*. However, TNFR2-bearing T cells were crucial for the induction of T cell suppression *in vivo* while a reduction of the presence of TNFR2 on the surface of myeloid cells had no measurable influence. Thus, TNFR2-activity on myeloid cells does not seem to be necessary for the induction of natural Treg cells.

TNFR2-specific activation by the agonist had distinct effects in naive mice and mice undergoing chronic inflammation (BCG model). First signs of increased myelopoiesis in naïve mice were only observed after repeated treatment with TNCscTNF80 indicating a very mild peripheral inflammatory reaction to specific TNFR2 activation in naïve mice. The agonist showed stronger effects in mice with chronic inflammation, induced by BCG immunization, where already a few applications led to increased Treg cell numbers and function. The characteristically strong increase in myeloid cells in the BCG model, as in other models of chronic inflammation, might mask the effects of TNCscTNF80 on myelopoiesis that was seen in mice in steady state. Thus, chronic inflammation seems to increase the sensitivity of Treg cells for TNFR2 costimulation. Possibly, endogenous TNF produced in inflammation sets the stage for effective Treg cell induction by TNFR2 activation. Several studies point to the impact of inflammation and TNF on the Treg population. Inflammation-induced Treg increase has also been shown in a TNF-induced model of rheumatic arthritis (37). Furthermore, activated effector $CD4^+$ T cells can boost Treg cell expansion and suppressive function through TNF as shown by Baeyens et al. (38), and in models of autoimmune diabetes (39) and experimental graft-versus-host disease (40). For human Treg cells, Zaragoza et al. (41) recently suggested that TNF does not impair their function *in vitro*. This finding was challenged by Nie et al. (42), who reinforced the earlier

concept that TNF reduces human Treg function. The authors discussed that the findings by Zaragoza et al. might be caused by different methodology, such as Treg purification using a positive selection method that might have led to activation of the Treg. This discussion suggests that the impact of TNF on Treg cell function depends on the current activation state of the Treg cells.

Taken together, our data support the use of a TNFR2-specific agonist to utilize TNFR2-specific functions, such as the previous finding of TNFR2-dependent induction of Treg cell expansion *in vivo* (13). However, the joined analysis of effector T cells, Treg cells, and MDSC in this study highlights the pluripotent function of TNFR2 in costimulation and regulation of multiple immune cell populations. Some effects are seemingly contradictory, such as increasing expansion of Treg cells, but inhibiting their suppressive function *in vitro* at the same time. Hence, the anti-inflammatory *in vivo* effects of a TNFR2 agonist depend on the specific model studied, on the level of inflammation and, therefore, the targeted pathology. For therapeutic TNFR2-agonistic treatment, the balance of costimulating effector T cells and expanding Treg cells requires careful *in vivo* investigation as well as analysis of the disease-specific inflammatory state of the immune system.

ETHICS STATEMENT

Mice were bred and housed in an animal facility with barrier conditions at the University of Regensburg. This study was carried out in accordance with institutional guidelines. The protocol was approved by the district government of Lower Franconia, Würzburg (Az: 54-2532.1-27/10, AZ: 54-2532.1-37/13).

AUTHOR CONTRIBUTIONS

Conception and drafting of the article: DM and SM. Performance and analysis of experiments: TS, LF, SW, NM, KM, DZ, DW-S, TH, and HW. Discussions of the data and critical revision of the article: HW, SM, and DM.

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REVIEW
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Forward and Reverse Signaling Mediated by Transmembrane Tumor Necrosis Factor-Alpha and TNF Receptor 2: Potential Roles in an Immunosuppressive Tumor Microenvironment

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Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine produced mainly by activated macrophages, lymphocytes and other cell types. Two distinct forms of TNF-α have been identified: soluble TNF-α (sTNF-α) and transmembrane TNF-α (mTNF-α). mTNF-α, which is the precursor of sTNF-α, can be cleaved by the TNF-α converting enzyme (TACE) and is released as sTNF-α. sTNF-α binds primarily to TNF receptor 1 (TNFR1) and plays an important role in the inflammatory immune response, whereas mTNF-α interacts primarily with TNF receptor 2 (TNFR2) and mediates the promotion of cellular proliferation and survival and other biological effects. It has been reported that the interaction between mTNF-α and TNFR2 induces bi-directional (forward and reverse) signaling in both mTNF-α- and TNFR2-expressing cells. Increasing evidence shows that the forward and reverse signaling mediated by mTNF-α and TNFR2 might play a significant role in the tumor microenvironment. In this review, the role of the crosstalk between mTNF-α and TNFR2 in the tumor microenvironment will be discussed.

Keywords: transmembrane tumor necrosis factor-alpha, TNF receptor 2, tumor microenvironment, forward signaling, reverse signaling

Tumor necrosis factor-alpha is also known as cachexin or cachectin and is a potent inflammatory cytokine produced mainly by activated macrophages, lymphocytes, and other cell types (1, 2). It was first demonstrated that serum from bacillus Calmette–Guérin (BCG)-infected mice treated with lipopolysaccharide (LPS) could cause hemorrhagic necrosis in tumors in animals; this effect was mediated by a "tumor-necrotizing factor" (3). In the following years, more details about the signaling pathways triggered by TNF-α and the functions of TNF-α were revealed.

Although TNF-α was first described as a soluble molecule that induces hemorrhagic necrosis in tumor tissues in experimental animals, the following studies reported that TNF-α exerts anti-tumor effects and pro-tumor effects in different circumstances. It has been demonstrated that there are two different forms of this type of cytokine, soluble TNF-α (sTNF-α) and transmembrane TNF-α (mTNF-α) (4, 5). mTNF-α, the precursor of soluble TNF-α, can be cleaved by TACE and

released as sTNF-α. Both forms of TNF-α can bind to TNFR1 or TNFR2 and exert pleiotropic effects on various cell types (6). TNFR1 is expressed on most cell surfaces and mediates cytotoxic effects and pro-inflammatory and pro-apoptotic effects, whereas TNFR2 is expressed primarily on lymphocytes and is involved in the activation and proliferation of lymphocytes (7–9). It has been reported that sTNF-α binds primarily to TNFR1 and plays an important role in inflammatory immune responses (10). mTNF-α, however, interacts primarily with TNFR2 (both soluble and transmembrane forms) and mediates effects that are overlapping and contrary to those of sTNF-α (7, 11). Notably, the crosstalk between mTNF-α and TNFR2 triggers bi-directional signaling in target cells and mTNF-α-expressing cells (12–14). Increasing evidence has shown that in the tumor microenvironment, the interaction between mTNF-α and TNFR2 plays a significant role in tumor progression (15). The expression of mTNF-α, its signaling pathway and the biological effects triggered by the interaction between mTNF-α and TNFR2 will be discussed in this review.

mTNF-α EXPRESSED ON DISTINCT CELL TYPES EXERTS DIFFERENT EFFECTS

In the 1980s, the gene coding for TNF-α was cloned and expressed by different teams who used different methods; their results marked profound progress in TNF-α research (16–19). In 1988, Kriegler et al. (20) announced that they had identified and characterized a novel, rapidly inducible cell surface cytotoxic integral transmembrane form of TNF-α that could explain the complex physiology of the molecule. mTNF-α is a stable homotrimer and is the precursor form of sTNF-α. mTNF-α can be cleaved by TNF-α converting enzyme (TACE) and then released as sTNF-α into the circulation to exert its biological function *via* type 1 and 2 TNF-α receptors (6). The two forms of TNF-α functions in two fundamentally different ways. mTNF-α is mainly expressed on monocytes/macrophages, lymphocytes, and some other cell types. In addition, mTNF-α acts as a bipolar molecule that transmits signals both as a ligand and as a receptor in a cell-to-cell contact-mediated manner, which means that mTNF-α not only mediates the forward signals to the target cells through cell-to-cell contact but also transmits the reverse (outside-to-inside) signals back into the mTNF-α-bearing cells (5, 21).

As a ligand, mTNF-α expressed on monocytes/macrophages and lymphocytes exhibits stronger cytotoxic activity than sTNF-α, because it is cytotoxic not only to sTNF-α-sensitive target cells but also to sTNF-α-resistant target cells (22, 23). mTNF-α expressed on T cells mediates the host defense against intracellular pathogens and the activation of endothelial cells and B cells and contributes to monocyte cytokine production (5). mTNF-α on dendritic cells can enhance the proliferation and cytotoxic activity of NK cells (24, 25). Activated $CD8^+$ T cells express mTNF-α and expand $V\beta5^+$ regulatory T cell populations through the transduction of signaling *via* TNFR2 on the Tregs (26). Moreover, some mTNF-α-bearing tumor cells can recruit immunosuppressive cells to the tumor microenvironment *via* the interaction between mTNF-α and TNFR2 to facilitate the evasion of tumor cells (27). On the other hand, as a receptor, mTNF-α mediates reverse signals back into the mTNF-α-bearing cells, such as T cells, monocytes/macrophages, and NK cells, to regulate the immune responses of these different cell types (5). mTNF-α-bearing tumor cells are stimulated by TNFR2 to induce reverse signaling to activate the NF-κB pathway, which can promote tumor cell survival and apoptosis resistance (21, 28). In summary, the current studies indicate that mTNF-α is expressed on monocytes/macrophages, lymphocytes, and even tumor cells and exerts different effects through its interaction with TNFR2.

THE CROSSTALK BETWEEN mTNF-α-BEARING CELLS AND TNFR2-EXPRESSING CELLS EXERTS DISTINCT EFFECTS ON THE TUMOR MICROENVIRONMENT

Increasing evidence shows that in the tumor microenvironment, the interaction between mTNF-α and TNFR2 plays a significant role in tumor progression. However, the effects and mechanisms of mTNF-α/TNFR2 interaction in the tumor microenvironment are not identical. On the one hand, it has been reported that the mTNF-α/TNFR2 interaction could promote the progression of cancer by recruiting immunosuppressive cells to the tumor microenvironment or by enhancing survival, metastasis, and apoptosis resistance of tumor cells (15, 21, 27, 28). On the other hand, as mentioned above, the interaction between mTNF-α and TNFR2 causes cytotoxicity toward not only sTNF-α-sensitive target cells but also sTNF-resistant tumor cells (28).

mTNF-α/TNFR2 Interaction Promotes Immunosuppressive Cell Accumulation in Tumor Microenvironments

Myeloid-derived suppressor cells (MDSCs) are important immunoregulatory cells in the cancer microenvironment. MDSCs are a heterogeneous group of immune cells from the myeloid lineage; they include immature precursors of macrophages, granulocytes, and dendritic cells. MDSCs are characterized by Gr1 and CD11b expression on the cell surface in mice, while in humans, they are identified as HLA^-DR^- $CD11b^+CD33^+$ cells (27, 29). MDSCs possess strong immune suppressive activity, which defines their functions in modulating the immune response and immune tolerance. The expansion and suppressive functions of MDSCs are relevant to chronic inflammatory conditions, especially in neoplastic disorders. The spectrum of action of MDSC activity encompasses that of T cells, NK cells, dendritic cells, and macrophages, which explains the ability of MDSCs to facilitate tumor evasion (8).

Tumor necrosis factor-alpha/TNF receptor 2 signaling is involved in the regulation of recruitment, differentiation, and suppressive activities of this cell population (29). Previous studies have shown that in tumor-bearing mice, multiple inflammatory mediators, including interleukin-1β (IL-1β), IL-6, and prostaglandin E2 (PEG2), produced by tumor cells induce the accumulation of MDSCs in the tumor microenvironment

of bone marrow (9, 30, 31). It has been identified that TNFR2$^+$ MDSCs are recruited to tumor sites, and in addition to inflammatory factors, mTNF-α expressed by tumor cells can also promote MDSC accumulation *via* TNFR2 expressed by MDSCs (15, 27, 32). Further, in a mouse model implanted with breast cancer 4T1 cells expressing an uncleavable mTNF-α mutant, greater accumulation of regulatory T cells was found in the tumor site (15). TNFR deficiency in MDSCs results in a decrease in CXCR4 expression and the impaired recruitment of MDSCs to tumor tissue (27). mTNF-α/TNFR2 signaling also promotes MDSC survival *via* the upregulation of FLICE-inhibitory protein (c-FLIP) leading to the inhibition of caspase-8 activity (32). It has been identified that mTNF-α, rather than sTNF-α, can also enhance the immunosuppressive activity of MDSCs *via* TNFR2 (15). This action is related to the upregulation of arginase-1 and inducible NO synthase transcription, the promotion of NO, reactive oxygen species, IL-10, and TGF-β secretion, and the enhanced inhibition of lymphocyte proliferation. Upregulated expression of mTNF-α in 4T1 cells promotes tumor progression and angiogenesis in animal models and results in greater MDSC accumulation, increased NO, IL-10, and TGF-β levels, and poor lymphocyte infiltration. It has been demonstrated that p38 phosphorylation and NF-κB activation triggered by mTNF/TNFR2 are the most important mechanisms through which mTNF-α regulates MDSCs (15).

Although many studies have reported that mTNF/TNFR2 can enhance tumor progression by recruiting and activating MDSCs, Ardestani et al. (33) reported that mTNF-α-expressing tumor cells induced tumor-associated myeloid cell death. In a mouse model implanted with Lewis lung cancer cells or B16F10 melanoma cells expressing mTNF-α, tumor growth was decreased and related to significantly reduced tumor-associated myeloid cell infiltration. mTNF-α triggers ROS production in myeloid cells and induces necrotic cell death, but the mechanism by which mTNF-α induces ROS production needs to be further studied (34, 35). In another study, the mTNF-α-producing transformed tumor cell line HeLa was used as a "vaccine" to induce tumor rejection by stimulating macrophages to exert an anti-tumor effect; this strategy is believed to be a promising and safe cytokine gene therapy (36).

mTNF-α/TNFR2 Regulates Survival, Apoptosis, and Metastasis of Tumor Cells through Forward and Reverse Signaling

In addition to regulating the accumulation and activation of immune cells in the tumor microenvironment, mTNF-α/TNFR2 also affects the survival, apoptosis, and metastasis of tumor cells directly. It has been reported that Raji cells, a human Burkitt lymphoma cell line, express both mTNF-α and TNFR2, which could mediate forward signaling or reverse signaling to induce cell death or survival *via* the NF-κB pathway (28). On the one hand, when mTNF-α acts as a ligand binding to TNFR2 on tumor cells, NF-κB activity is downregulated, which is followed by the subsequent inhibition of anti-apoptotic gene transcription, such as cIAP-1 and Fas-associated death domain-like IL-1β-converting enzyme-like inhibitory protein. On the other hand, when mTNF-α on tumor cells acts as a receptor to trigger reverse signaling, the activation of NF-κB is induced, and the production of anti-apoptotic proteins is further enhanced. Constitutive NF-κB activation causes Raji cells to be resistant to TNF-α-mediated cytotoxicity and sustains tumor cell survival (28, 37). These data indicate that there is a balance between forward and reverse signaling, but that reverse signaling is always dominant; consequently, this balance maintains constitutive NF-κB activation to sustain tumor cell viability (28). However, it was reported that the transfection of mTNF-α into the murine hepatic carcinoma cell line H22 upregulated Fas expression and induced tumor cell apoptosis *via* the Fas/FasL pathway. Moreover, mTNF-α inhibits CD44v3 expression to suppress tumor metastasis (38). We predict that in different types of tumor environment, the signaling pathways mediated by the mTNF-α/TNFR2 interaction are different, which may facilitate tumor survival or induce tumor cell apoptosis.

Since mTNF-α signaling influences survival and apoptosis of tumor cells directly, the expression of mTNF-α and its relationship with clinical characteristics was analyzed in cancer patients. It has been reported that mTNF-α expression is much higher in breast cancer compared with atypical dysplasia and hyperplasia, but mTNF-α is absent in normal breast tissue (37). In addition, the expression levels of mTNF-α are increased in acute leukemia (AL) and leukemia stem cells (LSCs). The high levels of mTNF-α expression in AL and LSCs correlate with poor risk stratification, extramedullary infiltration, and adverse clinical parameters (39). Targeting mTNF-α using a mAb inhibits leukemia cell growth and prevents the recurrence of leukemia in secondary serial transplantation into NOD-SCID mice (39). The *in vivo* and *in vitro* mAb experiments suggest that mTNF-α is a promising candidate for treating mTNF-α-positive tumors, especially in patients who are not sensitive to TNF antagonists (37, 39).

From the above findings, it can be concluded that different cell types are involved in the interaction between mTNF-α and TNFR2 in the tumor microenvironment and can influence tumor progression. On the one hand, the interaction facilitates tumor growth and progress. Tumor cells expressing mTNF-α recruit immune suppressive cells to the tumor microenvironment *via* TNFR2, which can suppress the anti-tumor immune response (15). Moreover, constitutive NF-κB activation triggered by reverse signaling protects Raji cells from sTNF-α-mediated cytotoxicity and sustains tumor cell survival (28). On the other hand, it has been reported that the mTNF-α expressed on Lewis lung cancer cells or B16F10 melanoma cells is related to reduced tumor-associated myeloid cell infiltration and decreased tumor growth (33). In addition, Raji cells can induce forward signaling in neighboring tumor cells through the interaction between mTNF-α and TNFR2, which leads to inhibition of NF-κB activation and mTNF-α-induced cell death. However, forward signaling is not dominant in Raji cells (28).

FORWARD SIGNALING OF mTNF-α/TNFR2

Unlike the pathways triggered by mTNF-α/TNFR1, the downstream signaling pathways triggered by TNFR2 have not been

clearly clarified. TNFR2 has no enzymatic activity by itself, thus any signal transduction needs the recruitment of adaptor proteins (40, 41). Current studies suggest that the mTNF-α/TNFR2 interaction mediates most of the biological behaviors by recruiting TNFR2-associated factor (TRAF2) to bind to the cytoplasmic domain of TNFR2, which induces the activation of NF-κB, c-Jun N-terminal kinase (JNK), or AP-1 pathways (38, 42–44). TRAF proteins have seven members and they act as adaptor proteins between TNFR2 and the kinases involved in the activation of JNK and NF-κB (45, 46). Among the seven members, TRAF2 is the key mediator in the signaling pathways of TNFR2 (42). The intracellular region of TNFR2 contains several highly conserved sequences, including TRAF2-binding sites (comprising 402-SKEE-405 and amino acids 425–439) and module III (amino acids 338–379), which contains no TRAF2 binding sites but a region (amino acids 343–379) related to TRAF2 degradation (40, 43). Upon TNFR2 activation, TRAF2 translocates to a Triton X-100 insoluble compartment where TRAF2 is K48-linked ubiquitinated and finally degraded by the proteasome (42).

NF-κB is a transcriptional factor composed of homodimeric and heterodimeric complexes of related proteins from the Rel superfamily. The inhibitory subunit IκB-α stabilizes NF-κB (28, 47). IκB kinase (IKK) is activated upon the interaction between TNFR2 and TRAF2 (45, 46). Once phosphorylated by IKK, IκB-α is recognized for ubiquitination and is degraded by the proteosome, and the IκB-α/NF-κB complex can release NF-κB for translocation into the nucleus. In the nucleus, NF-κB binds to target gene promoters and induces the expression of these genes (47, 48). The activation of NF-κB can promote tumor cell survival and apoptosis resistance and MDSC activation (21, 27, 28). When TNFR1 and TNFR2 are co-expressed, TRAF2 degradation results in an enhanced TNFR1 cytotoxicity that is associated with the inhibition of NF-κB (43).

Jun N-terminal kinase is an important kinase that initiates a signaling pathway. JNK belongs to the mitogen-activated protein kinase family (49). In the context of TRAF2 interaction with TNFR2, JNK can be activated transiently by TRAF2 and prolonged activation can occur in a TRAF2-independent fashion. Module III (amino acids 338–379), which is a region on TNFR2 that contains no TRAF2 binding sites, is able to activate JNK in a TRAF2-independent manner (40, 43). Deletion of TRAF2-binding sites can eliminate TRAF2-induced NF-κB but not JNK activation. In the process of JNK activation, ASK1 interacting protein 1 (AIP1), which is an adaptor molecule, interacts with the amino acids 338–355 within module III to regulate the JNK pathway (50). The interaction between TRAF2 and TNFR2 induces both NF-κB and JNK activation to transmit proliferation signals. Then, TRAF2 degradation induced by TNFR2 contributes to inhibition of NF-κB and TRAF2-dependent JNK signaling, but TNFR2 is still able to activate a TRAF2-independent JNK pathway, which is responsible for TNFR2-dependent cell death in some cell types (51, 52). A recent study indicated that a new adaptor molecule known as aminopeptidase P3 (APP3, also known as XPNPEP3) was identified in the TNFR2 signaling complex. One of its two isoforms, mitochondrial APP3 (APP3m) is recruited to TNFR2 and induces TNF-TNFR2-dependent phosphorylation of JNK1 and JNK2, which exerts an anti-apoptotic function (53).

During the recruitment of MDSCs to tumor sites, mTNF-α/TNFR2 can activate both the NF-κB and p38 MAPK pathways (27). Research has demonstrated that both NF-κB and p38 MAPK mediate mTNF-induced MDSC activation. In this process, the level of p38 phosphorylation is significantly increased. Upon preincubation of MDSCs with a p38 MAPK inhibitor or NF-κB inhibitor, the immune suppressive function of MDSCs is abrogated. P38 MAPK activation is TRAF2-dependent as well. The interaction between mTNF-α and TNFR2 induces NF-κB and p38 MAPK activation and then CXCR4 expression increases, which contributes to the recruitment and activation of MDSCs (15, 27). Moreover, the p38 MAPK pathway regulates NF-κB transactivation *via* direct acetylation of p65 and is necessary for TNF-mediated NF-kB activation (54).

In addition to the signaling pathways mentioned above, the interaction between TRAF2 and TNFR2 induces the activation of the transcription factors AP-1 through MAPK3s (40). Moreover, tumor expressing mTNF-α can stimulate the Fas expression that mediates tumor cell apoptosis *via* the Fas/FasL pathway. However, the involved pathways need to be explored in greater detail (37).

REVERSE SIGNALING OF mTNF-α/TNFR2

Increasing evidence suggests that mTNF-α can act as a receptor when interacting with sTNFR2, anti-TNF antibody, or TNFR2-expressing cells, thus activating intracellular signaling pathways. The outside-to-inside signaling mediated by mTNF-α is called reverse signaling. Reverse signaling is proven to be profoundly important in the activation of immune cells and apoptosis of macrophages (55, 56).

Take the interaction between monocytes and T cells in rheumatoid arthritis (RA) for example: TNFR2 on T cells behaves as a ligand and binds to mTNF-α on monocytes to trigger reverse signaling back into the monocytes, which contributes to the activation of monocytes. The reverse signaling mediated by mTNF-α/TNFR2 induces the activation of ERK1/2, which results in the dephosphorylation of the small cytoplasmic domain of mTNF-α and increases calcium concentrations. The reverse signaling transduced from mTNF-α to the nucleus activates monocytes to increase the production of TNF-α (57). It was reported that activated T cells in the synovial membrane of RA patients exhibiting a pathological phenotype that strongly induced the production of pro-inflammatory cytokines by monocytes through the interaction between mTNF-α and TNFR2 (57–59).

In addition, mTNF-α can mediate negative regulatory signaling that induces monocytes/macrophages to become resistant to inflammatory responses triggered by LPS (60). This negative regulatory response is mediated by the MAPK/ERK pathway (61). Pallai et al. (62) demonstrated that exposure of macrophages to LPS can induce the reverse signaling pathway *via* mTNF-α expressed on macrophages, after which, the reverse signaling activates the MAPK kinase (MKK) 4 pathway to induce TGF-β production. TGF-β then activates the ERK kinase pathway and mediates resistance to LPS-induced inflammation by inhibition of pro-inflammatory cytokines. In addition, the AKT pathways are also activated and are likely to act as a negative regulator of TGF-β production. However, the production of TGF-β mediated

by mTNF-α reverse signaling is not a universal response. For example, when TNFR2-expressing T cells interact with mTNF-α-expressing monocytes/macrophages, the monocytes/macrophages are induced to produce TNF-α rather than TGF-β,as described above (57, 62). Because of the important role of TNF-α, anti-TNF agents have already been used in clinical treatment. TGF-β, which is induced by reverse signaling, plays an essential role in determining the therapeutic efficacy of TNF-α antagonists (63–69).

CONCLUSION

Increasing evidence indicates that sTNF-α and mTNF-α play an essential role in the regulation of immune responses and tumor progression. In the tumor microenvironment, mTNF-α-expressing tumor cells contribute to the accumulation and activation of immunosuppressive cells to suppress the anti-tumor immune responses mediated by immune cells, which facilitates the growth and evasion of tumor cells. In addition, reverse signaling triggered by the interaction between mTNF-α and TNFR2 also plays a significant role in maintaining tumor cell survival and contributing to the metastasis of tumor cells. Targeting mTNF-α *via* mAbs is a promising strategy for treating mTNF-α-positive tumors. Notwithstanding its pro-tumor effect, the interaction between mTNF-α and TNFR2 can cause anti-tumor effects in indirect and direct ways. The mTNF-α/TNFR2 interaction suppresses tumor cells through the induction of tumor-associated myeloid cell death or the direct activation of the Fas/FasL pathway in tumor cells. The effects and mechanisms of mTNF-α/TNFR2 interaction in the tumor microenvironment, which include either regulating immunosuppressive cells or directly acting upon tumor cells, need to be further explored.

ETHICS STATEMENT

This study was approved by Ethic committee of Tianjin Medical University.

AUTHOR CONTRIBUTIONS

YQ was responsible for the data collection and the draft of the manuscript. GZ gave assistance to finish the manuscript. HL came up to the idea of the research, conceived the manuscript, and modified it.

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Synthetic Nanoparticles That Promote Tumor Necrosis Factor Receptor 2 Expressing Regulatory T Cells in the Lung and Resistance to Allergic Airways Inflammation

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Synthetic glycine coated 50 nm polystyrene nanoparticles (NP) (PS50G), unlike ambient NP, do not promote pulmonary inflammation, but instead, render lungs resistant to the development of allergic airway inflammation. In this study, we show that PS50G modulate the frequency and phenotype of regulatory T cells (Treg) in the lung, specifically increasing the proportion of tumor necrosis factor 2 (TNFR2) expressing Treg. Mice pre-exposed to PS50G, which were sensitized and then challenged with an allergen a month later, preferentially expanded TNFR2$^+$Foxp3$^+$ Treg, which further expressed enhanced levels of latency associated peptide and cytotoxic T-lymphocyte associated molecule-4. Moreover, PS50G-induced CD103$^+$ dendritic cell activation in the lung was associated with the proliferative expansion of TNFR2$^+$Foxp3$^+$ Treg. These findings provide the first evidence that engineered NP can promote the selective expansion of maximally suppressing TNFR2$^+$Foxp3$^+$ Treg and further suggest a novel mechanism by which NP may promote healthy lung homeostasis.

Keywords: nanoparticles, tumor necrosis factor 2, asthma, PS50G, lung, lymph nodes, animal model

INTRODUCTION

Nanoparticles (NP), defined as particles with a diameter less than 100 nm, comprise the dominant type of particles in ambient airborne particulate matter (1). NP can be divided into three categories: naturally occurring, anthropogenic, and engineered nanoparticles (ENP) that are manufactured for industrial or consumer applications (2). The increasing use of NP for pulmonary drug delivery (3, 4) continues to drive the debate on their potential to negatively or positively modulate lung immune homeostasis (5).

The lung is confronted by a diverse range of natural and man-made NP, on a daily basis, and must maintain a state of immune ignorance or "tolerance" to retain pulmonary homeostasis and prevent undesirable immunopathology. As nanotechnology develops, it is clear that it will also be important to understand the impact of ENP on the lung. ENP by themselves have the capability to induce beneficial or detrimental effects on lung immune homeostasis, depending on their characteristics

(2). For example, nickel NP (6) and titanium dioxide NP (7) can exacerbate existing allergic airway inflammation (AAI). However, inert glycine-coated polystyrene 50-nm NP (PS50G) behave differently from most of the ubiquitous particles in the environment, *in vivo* and *in vitro* (8–12). Of note, PS50G also induce the secretion of chemokines involved in recruitment and/or maturation of monocytes and dendritic cells (DCs), and pre-exposure to PS50G prevents the subsequent elicitation of AAI (8). Furthermore, immune imprinting by PS50G in the lung leads to subsequently modified pulmonary immune responses to allergens (9).

Immune imprinting or "innate training" is a phenomenon wherefore non antigenic stimuli (e.g., toll-like receptor ligands or NP) alter the capacity of the immune system to react to subsequent unrelated stimuli (13, 14). Some innate training mechanisms include impairment of pulmonary antigen-presenting cell (APC) function (9, 15), altered antigen delivery (16), and induction of regulatory myeloid-derived suppressor cells (12). Previously, we demonstrated that PS50G not only negatively imprint inflammatory CD11b^{hi} dendritic cell (DC) but also increase the frequency of CD103$^+$ DC in the lung (9), a population that contributes to airway homeostasis by inducing Foxp3$^+$ regulatory T cells (Treg) (17), through a Treg-independent production of IL-10 (18) or IL-12 (19). By using AAI murine models, Treg were demonstrated to play a major role in controlling lung homeostasis and its responsiveness to environmental allergens (20, 21). Therefore, we hypothesized that PS50G innate training would also substantially change the homeostasis of Treg in the lung, particularly inflammation related Treg expressing tumor necrosis factor (TNF) receptor 2 (TNFR2$^+$Foxp3$^+$ Treg), reported as maximally suppressive in other disease settings (22–24).

MATERIALS AND METHODS

Mice

Female BALB/c mice aged 6–8 weeks were obtained from the Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia and housed in the Alfred Medical Research and Education Precinct (AMREP) animal house. All studies with mice were approved by the AMREP Animal Ethics Committee.

Particle Preparation, Instillation, and Immunization

Polybead carboxylate microspheres (unlabeled, nominally 0.05 mm; no. 15913; Polysciences, Warrington, PA, USA) were glycine coated, as described (25) and referred to as PS50G. To investigate the long-term effects of PS50G on the innate immune response, mice received saline or PS50G (200 μg/50 μl) intratracheally on day 0 and lymph nodes (LN) and lungs were collected on days 1, 3, 7, and 30 post instillation. In some experiments, 10 μg lipopolysaccharide derived from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) were used as a positive "inflammatory" control. The effects of PS50G on acute allergic asthma were investigated by intratracheally instilled PS50G (200 μg/50 μl) into mice on days 0 and 2 prior to intraperitoneal sensitization with ovalbumin (OVA) (50 μg; Sigma-Aldrich, St. Louis, MO, USA) in aluminum hydroxide (General Chemical, Parsippany, NJ, USA) on days 12 and 22 and intranasal OVA challenge (25 μg) on days 32, 34, 37, and 39. Tissue sampling was performed 24 h after the final lung allergen challenge (day 40) as described (8, 9).

Antibodies, Surface, and Intracellular Staining

Cells (1×10^6) were stained on ice for 20 min with combinations of the following antibodies: CD3 (APC-Cy7 and Qdot 605) (Life technologies, Grand Island, NY, USA); CD4 (V450 and V500) (BD Biosciences, San Jose, CA, USA); CD25 (PE-Cy7 and APC-Cy7), CD120b/tumor necrosis factor 2 (TNFR2) (PE), latency associated peptide (LAP) (Per-CP), cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) biotin or their respective immunoglobulin isotypes (all eBioscience, San Diego, CA, USA). For intracellular staining of Foxp3 (APC) and Ki67 (FITC) (eBioscience, San Diego, CA, USA), cells were first permeabilized according to the manufacturer's instructions. The following antibodies were used to identify CD103$^+$ DC: CD103 (PE) (BD Biosciences), CD11c (APC) and MHCII (APC-eFluor 780) (eBioscience), CD11b (AF700) and CD86 (Brilliant Violet Blue) (BioLegend), and Live/Dead cell stain kit-Aqua (Invitrogen). Acquisition was on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analysis was performed using FlowJo (Tree Star, Ashland, OR, USA).

Statistical Analysis

Data were analyzed for normality and log-transformed as necessary prior to analysis by Student's *t*-test or ANOVA with Bonferroni posttests, depending on the number of experimental groups. Spearman's correlations were used for the comparison of continuous variables. The Spearman's *r* value for the correlation between the two variables was stated in each result. Statistical analysis was performed using Graph Pad Prism v5.02 software. Group sizes are indicated in the figure legends. Data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

PS50G Instillation Increased TNFR2$^+$Foxp3$^+$ Treg in the Lung

Intratracheal instillation of PS50G into the lungs of mice increased frequencies of Treg, peaking at day 7, which decreased but remained significantly higher than the saline control group by day 30 (**Figures 1A,B**). TNFR2$^+$ Treg are maximally suppressive (26), and TNFR2$^+$ T effector cells (Teff) are maximal cytokine producers (27). PS50G instillation significantly increased the proportion of TNFR2$^+$Foxp3$^+$ Treg within the total Treg population (CD3$^+$CD4$^+$CD25$^+$) from ~9% at day 1 to ~20% at day 3, peaking at day 7 (>30%), and this increase remained significantly elevated above the saline control group even up to day 30 (**Figure 1C**). Conversely, TNFR2$^-$Foxp3$^+$ Treg decreased from day 3 to 7, remaining low to day 30 (**Figure 1D**). By contrast, PS50G did not change the proportion of TNFR2$^+$Foxp3$^-$ cells within Teff (CD3$^+$CD4$^+$CD25$^-$ cells) (**Figure 1E**). The total numbers of TNFR2$^+$Foxp3$^+$ Treg in the lung also increased following

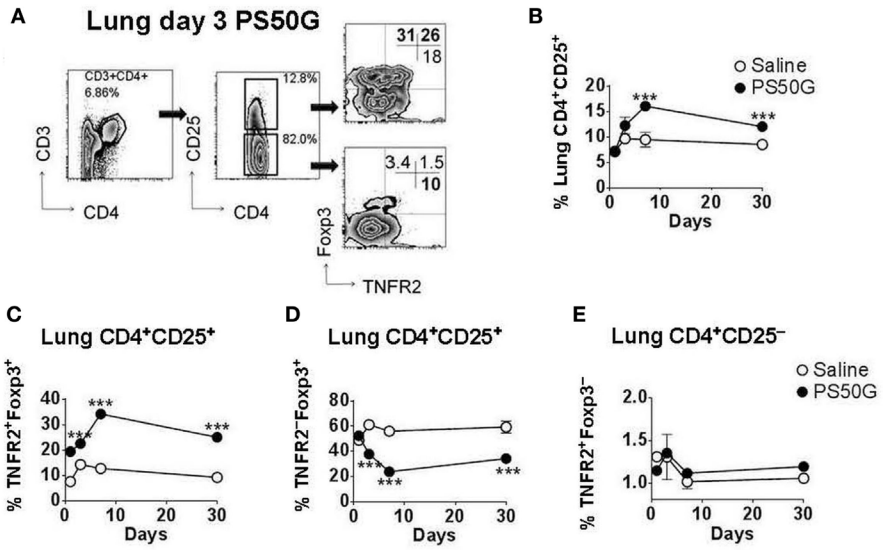

FIGURE 1 | PS50G instillation selectively increases lung CD3+CD4+CD25+ cells that are TNFR2+Foxp3+. Naïve mice (n = 5–7 per group per time point) received PS50G intratracheally on day 0 or saline as control. Samples were collected on days 1, 3, 7, and 30. **(A)** Stained lung cells were gated on viable CD3+CD4+CD25+ and CD3+CD4+CD25− cells, followed by gating on TNFR2 co-expressed with Foxp3. Representative FACS contour plots showing TNFR2+ cells in the lung on day 3 from PS50G treated mice. Percentages of **(B)** CD3+CD4+CD25+ cells; **(C)** TNFR2+Foxp3+ regulatory T cells (Treg); **(D)** TNFR2−Foxp3+ Treg; and **(E)** TNFR2+Foxp3− Teff. Data represent the mean ± SEM of at least three experiments. ***$p < 0.001$.

PS50G instillation (**Table 1**), while the absolute numbers of the other subsets remained unaltered. Overall, PS50G was shown to preferentially promote the induction of TNFR2+Foxp3+ Treg in the lung.

PS50G Instillation Increased the Percentages of TNFR2+Foxp3+ Treg in the Lung-Draining LN

While Treg in the lung play a substantial role in controlling lung inflammation, the priming, activation, and expansion of T cells associated with airway inflammation also involves the LN that drain the lungs. To investigate the effects of PS50G on Treg in the lung-draining LN, we applied a similar gating strategy to that used for lung Treg (**Figure 2A**). Instillation of PS50G did not significantly affect the percentages of CD3+CD4+CD25+ Treg at day 1 and 3, but increased the frequency from ~9 to ~15% at day 7, returning to saline control levels at day 30 (**Figure 2B**). A similar pattern was observed in the percentages of TNFR2+Foxp3+ Treg (increasing from ~ 20 to ~ 30% at day 7) (**Figure 2C**). In contrast, the percentages of TNFR2−Foxp3+ Treg were significantly increased as early as day 1 and returned to saline control levels from day 3 to day 30 (**Figure 2D**). PS50G instillation did not affect the frequency of TNFR2+Foxp3− Teff, being <2% for all time points in both saline and PS50G groups (**Figure 2E**). Absolute numbers of CD3+CD4+ T cells, CD4+CD25+ Treg, TNFR2+Foxp3+ Treg, TNFR2−Foxp3+ Treg, and TNFR2+Foxp3− Teff increased on day 3 (**Table 1**), as reflected by approximately fivefold increase in total lung-draining LN numbers (data not shown). Overall, we saw an increase in the percentages of CD3+CD4+CD25+ Treg, and TNFR2+Foxp3+ Treg, but not TNFR2−Foxp3+Treg on day 7 in the lung-draining LN after instillation of PS50G.

TABLE 1 | PS50G alter the numbers of CD3+CD4+ T cells, total regulatory T cells (Treg), and tumor necrosis factor 2 cells in the lung and lung-draining lymph nodes (LN).

Cells	Groups	Cell numbers (10^4) lung	Cell numbers (10^4) LN
Total cell numbers	Saline	1,054 ± 252	593 ± 207
	PS50G	2,030 ± 162	2,085 ± 109***
CD3+CD4+ cells	Saline	72.1 ± 5.0	476 ± 29.8
	PS50G	70.2 ± 8.2	867 ± 73.2***
CD3+CD4+CD25+ Treg	Saline	8.84 ± 0.36	59.6 ± 6.1
	PS50G	8.32 ± 2.1	105.8 ± 14.3***
TNFR2+Foxp3+ within CD3+CD4+CD25+ Treg	Saline	0.87 ± 0.23	9.2 ± 2.3
	PS50G	2.43 ± 0.91***	16.2 ± 2.9***
TNFR2− Foxp3+ within CD3+CD4+CD25+ Treg	Saline	5.1 ± 0.9	28.0 ± 6.4
	PS50G	5.01 ± 1.09	54.4 ± 7.21***
CD3+CD4+CD25− Teff	Saline	60.7 ± 2.87	501.4 ± 20.5
	PS50G	62.3 ± 11.2	902 ± 13.6***
TNFR2+Foxp3− within CD3+CD4+CD25− Teff	Saline	0.89 ± 0.32	11.02 ± 3.3
	PS50G	1.12 ± 0.82	20.09 ± 3.6***

Naïve mice (n = 5–7 per group per time point) received PS50G intratracheally on day 0 or saline as control. Samples were collected on day 3. Lung and lung-draining LN were analyzed for cell numbers and percentages. Data represent the mean ± SEM of at least three experiments.
$^{***}p < 0.001$.

PSG50-Induced Inhibition of AAI Is Associated with Increased Local Efficiency in the Induction of TNFR2+Foxp3+ Treg Upon Allergen Challenge

We previously showed that PS50G instillation inhibits the elicitation of subsequent AAI in atopic mice (8, 9). Herein, we

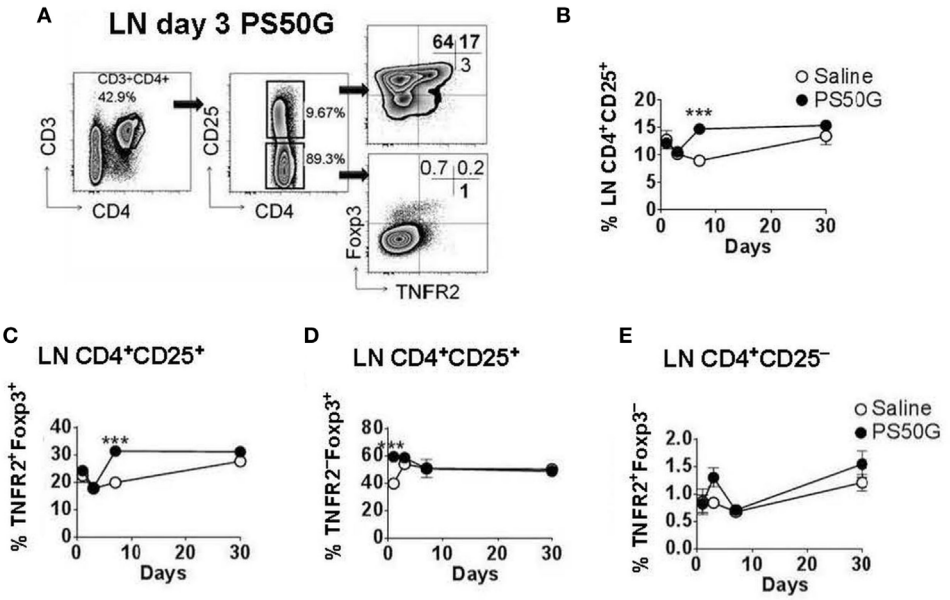

FIGURE 2 | PS50G instillation selectively increases lung-draining lymph nodes (LN) $CD3^+CD4^+CD25^+$ cells that are $TNFR2^+Foxp3^+$ on day 7. Naïve mice (n = 5–7 per group per time point) received PS50G intratracheally on day 0 or saline as control. Samples were collected on days 1, 3, 7, and 30. **(A)** Stained lung-draining LN cells were gated on viable $CD3^+CD4^+CD25^+$ and $CD3^+CD4^+CD25^-$ cells, followed by gating on TNFR2 co-expressed with Foxp3. Representative FACS contour plots showing $TNFR2^+$ cells in the lung-draining LN at day 3 from PS50G-treated mice. Percentages of **(B)** $CD3^+CD4^+CD25^+$ cells; **(C)** $TNFR2^+Foxp3^+$ regulatory T cells (Treg); **(D)** $TNFR2^-Foxp3^+$ Treg; and **(E)** $TNFR2^+Foxp3^-$ Teff. Data represent the mean ± SEM of at least three experiments. ***$p < 0.001$.

hypothesized that the re-elicitation of Treg, and particularly $TNFR2^+$ Treg, by a subsequent allergen challenge, would differ between PS50G vs saline pretreated animals. We also wanted to address whether the proportion of $TNFR2^+$ Teff elicited by the allergen would be impacted by prior PS50G exposure. The results showed that PS50G pre-instillation resulted in an increased ability of the lungs to respond by $TNFR2^+Foxp3^+$ Treg upregulation to a subsequent allergen challenge (**Figure 3**). Specifically, the proportion of $TNFR2^+Foxp3^+$ Treg within total T cells and within Treg was significantly increased in the PS50G/OVA/OVA group compared to the control groups (Sal/Sal/Sal and Sal/OVA/OVA) (**Figure 3B**). The percentages of lung $TNFR2^-Foxp3^+$ Treg in PS50G/OVA/OVA and Sal/OVA/OVA groups decreased markedly from ~60 to ~30%, an approximately twofold decrease as compared to the saline negative control group (Sal/Sal/Sal) (**Figure 3C**). On the other hand, the percentages of $TNFR2^+Foxp3^-$ Teff significantly increased after allergen challenge (approximately fourfold), regardless of whether the animals had been pretreated with PS50G or saline (**Figure 3D**). Thus, PS50G selectively increased $TNFR2^+Foxp3^+$ Treg proportions, without affecting $TNFR2^-Foxp3^+$ Treg or $TNFR2^+Foxp3^-$ Teff, resulting in an increased ratio of $TNFR2^+Foxp3^+$ Treg to $Foxp3^-$ Teff compared to the Sal/OVA/OVA group (**Figures 3E,G**). Furthermore, the ratio of $TNFR2^-Foxp3^+$ Treg to $Foxp3^-$ Teff was unchanged compared to the Sal/OVA/OVA group (**Figures 3F,H**). Given that a portion of the $Foxp3^-$ effector cells is $CD25^+$, we also analyzed this subset to assure that the ratio of $TNFR2^+Foxp3^+$ Treg to $Foxp3^-$ Teff would not be affected. Interestingly, similar patterns were observed for Treg/Teff ratio (**Figures 3E–H**) indicating that in the lung the total Treg/Teff ratio remains consistent regardless of CD25 expression.

PSG50-Induced Inhibition of Elicitation of AAI Is Associated with Increased $TNFR2^+Foxp3^+$ Treg in Lung-Draining LN

In the lung draining LN, allergen challenge (Sal/OVA/OVA) was followed by a decrease in frequency of $TNFR2^+Foxp3^+$ Treg relative to the saline control group (Sal/Sal/Sal) (**Figure 4B**). PS50G pretreatment (PS50G/OVA/OVA) prevented this decrease. Although the observed differences were small, the levels of $TNFR2^+Foxp3^+$ Treg were significantly higher in the PS50G/OVA/OVA group (**Figure 4B**) and showed an increased ratio of $CD25^+TNFR2^+Foxp3^+$ Treg to $CD25^+Foxp3^-$ Teff (**Figure 4G**), even though the ratio of $CD25^+TNFR2^+Foxp3^+$ Treg to $CD25^-TNFR2^+Foxp3^-$ Teff did not significantly change in any of the groups (**Figure 4E**). By contrast, no significant differences were observed in the frequencies and ratios of $TNFR2^-Foxp3^+$ Treg to $Foxp3^-$ Teff (**Figures 4C,F,H**). No differences were observed in $TNFR2^+Foxp3^-$ Teff (**Figure 4D**).

Mechanisms Underlying the PS50G Induced Increase in $TNFR2^+Foxp3^+$ Treg in the Lung during AAI

To gain insight into whether the increase in $TNFR2^+Foxp3^+$ Treg proportions and numbers in the lung and lung-draining LN after allergen challenge was driven by increased proliferation, we analyzed expression of the proliferative marker Ki67. PS50G

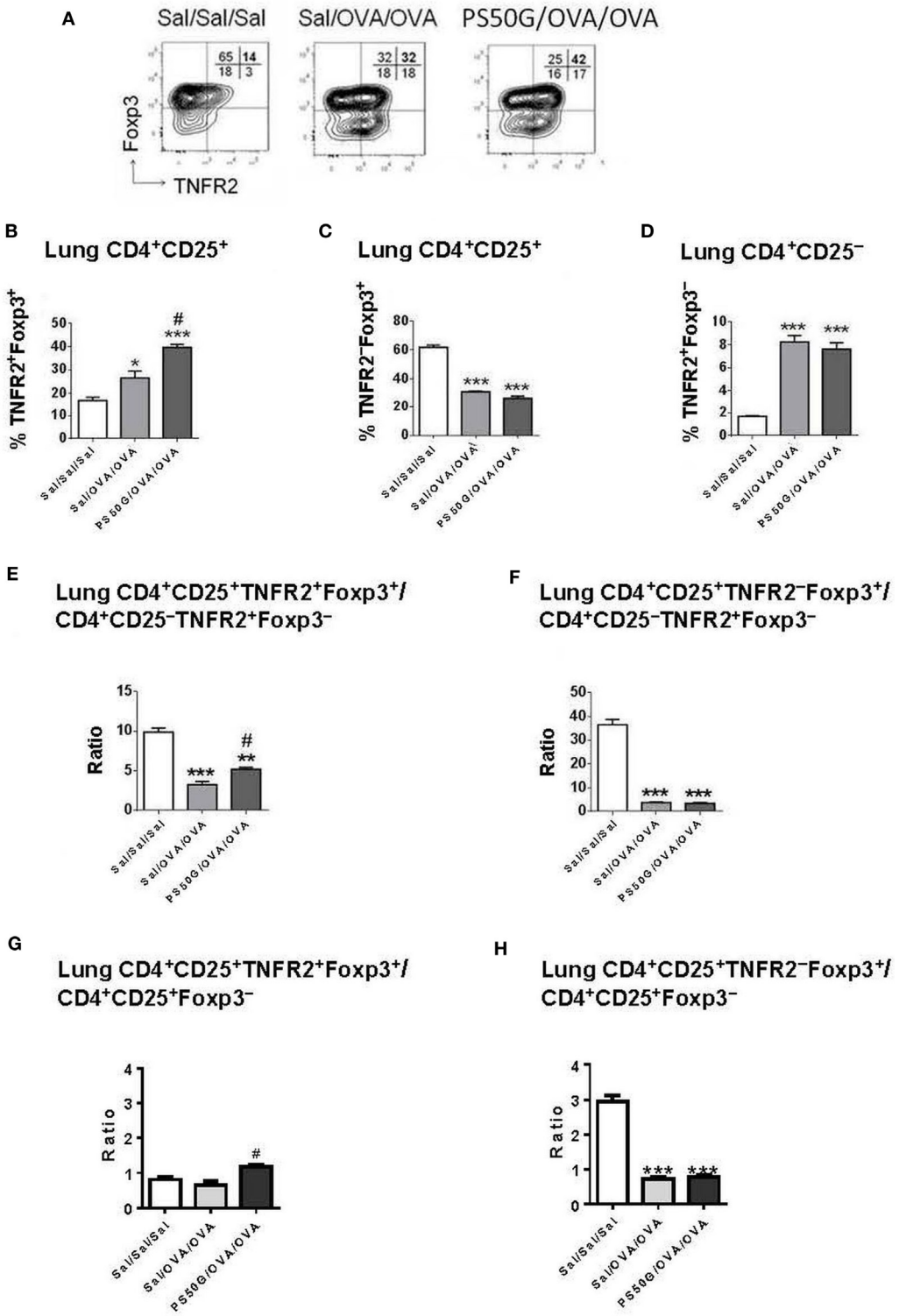

FIGURE 3 | PS50G instillation selectively increases lung CD3+CD4+CD25+ T cells that are TNFR2+Foxp3+ in allergic airway inflammation mouse model. **(A)** Stained lung cells were gated on viable CD3+CD4+CD25+ and CD3+CD4+CD25− cells, followed by gating on tumor necrosis factor 2 co-expressed with Foxp3. Percentages of **(B)** TNFR2+Foxp3+ regulatory T cells (Treg); **(C)** TNFR2−Foxp3+ Treg and **(D)** TNFR2+Foxp3− Teff. Ratios of **(E,G)** TNFR2+Foxp3+ Treg and **(F,H)** TNFR2−Foxp3+ Treg to Foxp3− Teff. Data represent the mean ± SEM of at least three experiments with four to six mice per group. *$p < 0.05$ and ***$p < 0.001$ compared with saline negative control group (Sal/Sal/Sal); #$p < 0.01$, compared with OVA positive control group (Sal/OVA/OVA).

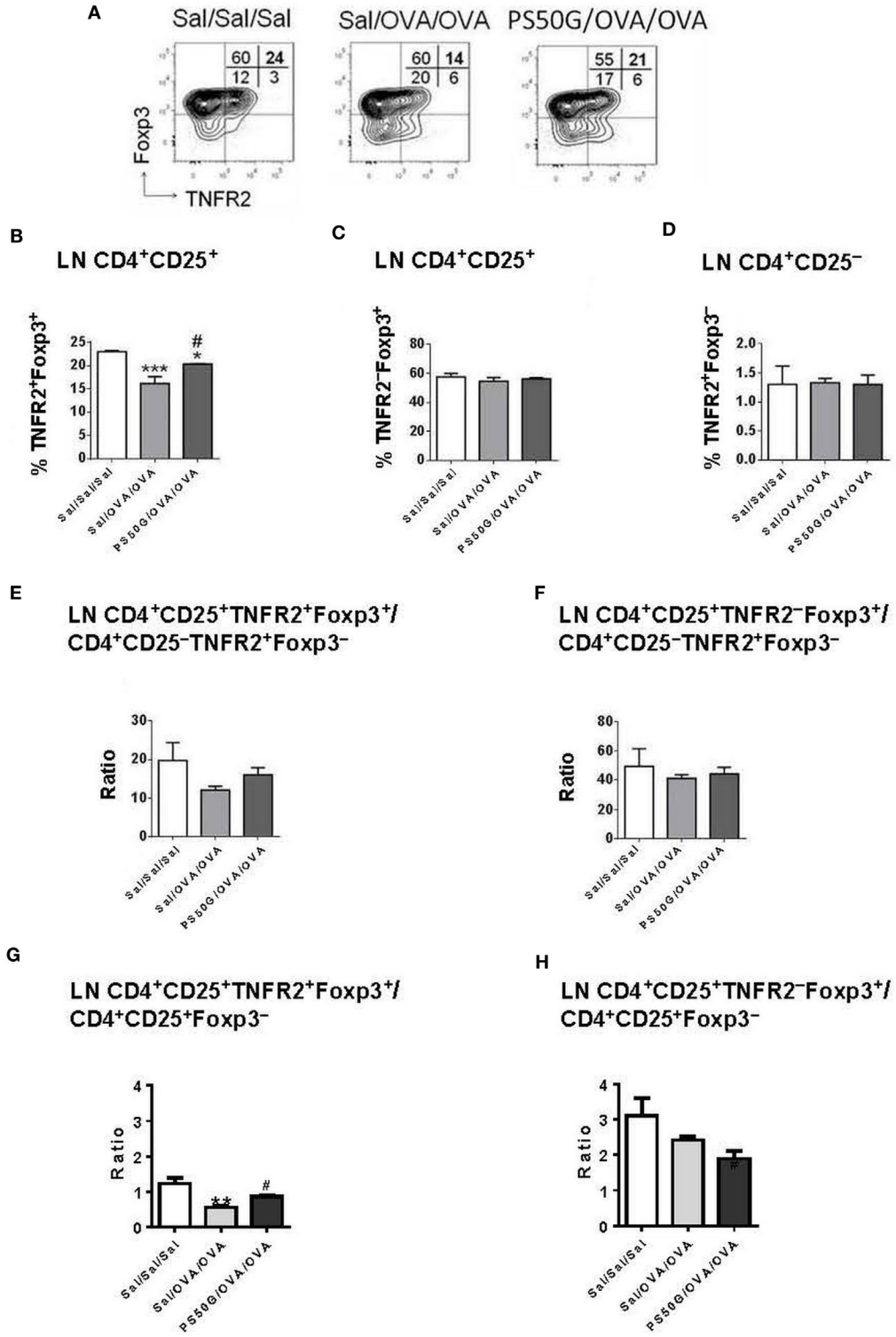

FIGURE 4 | PS50G instillation selectively increases lung-draining lymph nodes $CD3^+CD4^+CD25^+$ that are $TNFR2^+Foxp3^+$ in allergic airway inflammation mouse model. **(A)** Stained lung cells were gated on viable $CD3^+CD4^+CD25^+$ and $CD3^+CD4^+CD25^-$ cells, followed by gating on tumor necrosis factor 2 co-expressed with Foxp3. Percentages of **(B)** $TNFR2^+Foxp3^+$ regulatory T cells (Treg); **(C)** $TNFR2^-Foxp3^+$ Treg; and **(D)** $TNFR2^+Foxp3^-$ Teff. Ratios of **(E,G)** $TNFR2^+Foxp3^+$ Treg and **(F,H)** $TNFR2^-Foxp3^+$ Treg to $Foxp3^-$ Teff. Data represent the mean ± SEM of at least three experiments with four to six mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with saline negative control group (Sal/Sal/Sal); $^\#p < 0.05$, compared with OVA positive control group (Sal/OVA/OVA).

pretreatment (PS50G/OVA/OVA) significantly increased the percentages of Ki67$^+$ cells preferentially within TNFR2$^+$Foxp3$^+$ Treg in the lung post allergen challenge in the AAI model, when compared to Sal/OVA/OVA group (**Figure 5A**). By contrast, the frequency of proliferated cells within TNFR2$^+$Foxp3$^-$ Teff in the lung after allergen challenge in mice with a PS50G pretreatment (PS50G/OVA/OVA) were similar to that of the saline negative control group indicated by the dashed line (Sal/Sal/Sal) (**Figure 5A**). No significant differences in the proportion of Ki67$^+$ cells within TNFR2$^-$Foxp3$^+$ Treg or TNFR2$^+$Foxp3$^-$ Teff were found between PS50G/OVA/OVA and Sal/OVA/OVA groups, showing that PS50G preferentially induced proliferative expansion of TNFR2$^+$Foxp3$^+$ Treg. PS50G did not affect the frequency of Ki67$^+$ cells for any of the populations examined in the lung-draining LN. However, we observed that the frequency of Ki67$^+$ cells within the TNFR2$^-$Foxp3$^+$ Treg was threefold to fourfold lower than within the TNFR2$^+$Foxp3$^+$ Treg and TNFR2$^+$Foxp3$^-$ Teff subsets (**Figure 5B**). These results suggest that changes in Treg subset frequencies upon PS50G instillation and subsequent allergen challenge increase the TNFR2$^+$Foxp3$^+$ Treg proliferative state in the lung.

PS50G Pretreatment Increased LAP$^+$ and CTLA-4$^+$ Cells within TNFR2$^+$Foxp3$^+$ Treg in Lung and Lung-Draining LN of AAI Mouse Model

Previous studies in other disease models have shown that Treg have maximal suppressive capacity, which is associated with higher expression of immunosuppressive molecules such as CTLA-4 (28, 29). To confirm that the TNFR2$^+$Foxp3$^+$ Treg elicited during AAI could also exhibit a suppressive potential, we analyzed their expression of the TGF-β binding molecule LAP and of CTLA-4. Moreover, we tested whether PS50G pre-exposure could further alter the expression of these functional molecules on the TNFR2$^+$Foxp3$^+$ Treg. We found that PS50G treatment significantly increased the proportion of LAP$^+$ cells within the TNFR2$^+$Foxp3$^+$ Treg subset, but not within the TNFR2$^-$Foxp3$^+$ Treg and TNFR2$^+$Foxp3$^-$ Teff subsets in the lung (**Figure 6A**). PG50G did not alter the proportion of LAP$^+$ cells in any cell population in the lung-draining LN (**Figure 6B**). Consistent with the finding that LAP expression is not associated with Teff, we identified the lowest frequency of LAP$^+$ cells within TNFR2$^+$Foxp3$^-$ Teff both in the lung and in the lung-draining LN (<0.3%) (**Figures 6A,B**). Although PS50G did not alter the proportion of CTLA-4 positive Treg (TNFR2$^+$Foxp3$^+$ Treg and TNFR2$^-$Foxp3$^+$ Treg) in the lung (**Figure 6C**), they induced a twofold increase in the frequency of CTLA-4$^+$ cells exclusively within TNFR2$^+$Foxp3$^+$ Treg in the lung-draining LN (**Figure 6D**). Therefore, PS50G increased expression of molecules associated with the suppressive function of TNFR2$^+$Foxp3$^+$ Treg in the lung and lung-draining LN.

Activation of CD103$^+$ DC Positively Correlates with TNFR2$^+$Foxp3$^+$ Treg Expansion

Our previous data showed that the expression of CD86 on CD103$^+$ DC is positively correlated with PS50G uptake (9). To investigate the possible relationship between PS50G uptake by tolerogenic CD103$^+$ DC and Treg in the lung, we analyzed the correlation between activated CD103$^+$ DC (based on CD86 expression) with TNFR2$^+$Foxp3$^+$ Treg. As predicted, PS50G increased the frequency of activated CD103$^+$ DC (**Figure 7A**), which positively correlated with overall increases of TNFR2$^+$ Treg (**Figures 7B,C**, left panel) and the proportion of TNFR2$^+$Foxp3$^+$ Treg that proliferated after PS50G administration (based on Ki67 expression) (**Figure 7B**, right panel). By contrast, activated

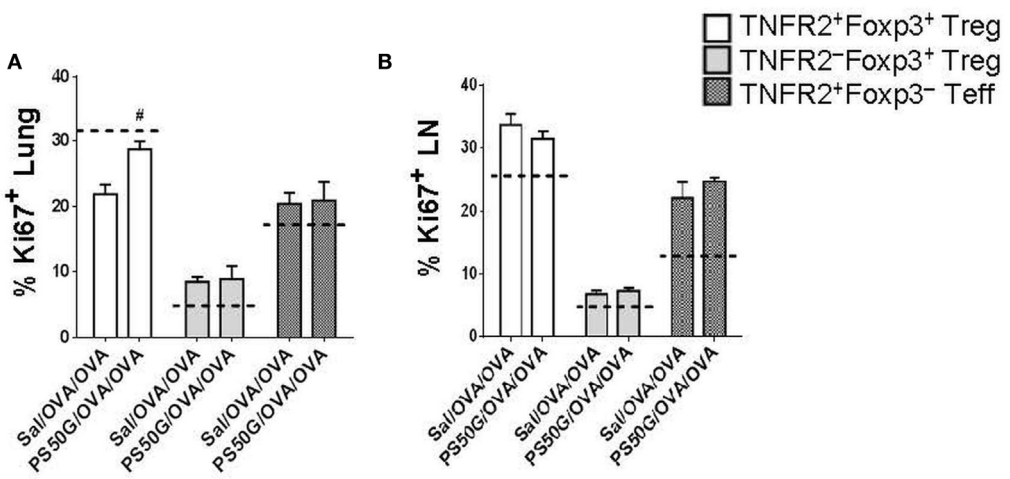

FIGURE 5 | PS50G instillation increases the percentages of Ki67$^+$ cells within lung TNFR2$^+$Foxp3$^+$ regulatory T cells (Treg) during allergic airway inflammation. Stained lung and lung-draining lymph nodes (LN) cells were gated as in **Figures 3A** and **4A**, respectively followed by gating on Ki67. Percentages of **(A)** lung Ki67$^+$ and **(B)** lung-draining LN Ki67$^+$ within TNFR2$^+$Foxp3$^+$ Treg; TNFR2$^-$Foxp3$^+$ Treg and TNFR2$^+$Foxp3$^-$ Teff. Data represent the mean ± SEM of at least three experiments with four to six mice per group. $^{\#}p < 0.05$, compared with OVA positive control group (Sal/OVA/OVA). The dashed lines denote the percentages of cells expressing the respective markers derived from the saline negative control group (Sal/Sal/Sal).

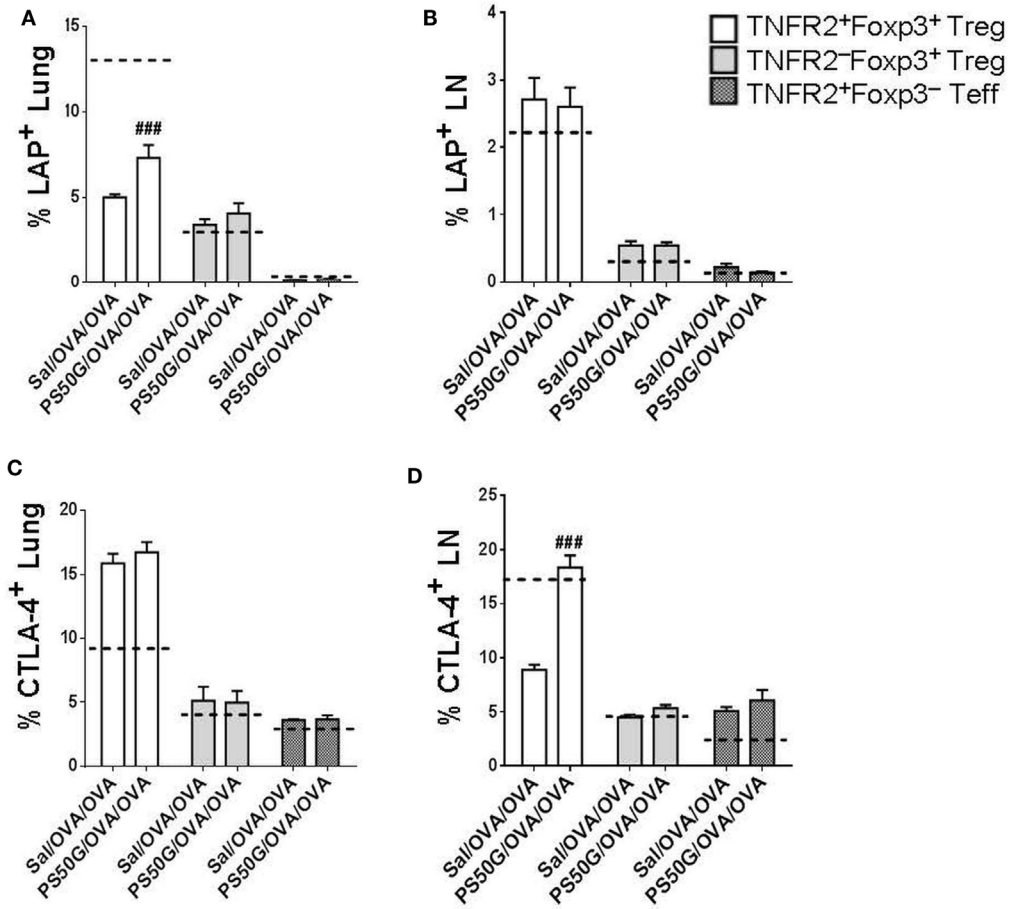

FIGURE 6 | PS50G instillation increases the percentages of lung LAP+ and lung-draining lymph nodes (LN) CTLA-4+ cells within TNFR2+Foxp3+ regulatory T cells (Treg) during allergic airway inflammation. Stained lung cells and lung-draining LN cells were gated as in **Figures 3A** and **4A**, respectively, followed by gating on latency associated peptide and CTLA-4. Percentages of **(A)** lung LAP+; **(B)** lung-draining LN LAP+; **(C)** lung CTLA-4+; and **(D)** lung-draining LN CTLA-4+ within TNFR2+Foxp3+ Treg; TNFR2−Foxp3+ Treg; and TNFR2+Foxp3− Teff. Data represent the mean ± SEM of at least three experiments with four to six mice per group. ###$p < 0.001$, compared with OVA positive control group (Sal/OVA/OVA). The dashed lines denote the percentages of cells expressing the respective markers derived from the saline negative control group (Sal/Sal/Sal).

CD103+ DC negatively correlated with TNFR2−Foxp3+ Treg (**Figure 7C**, right panel). Together this data suggest that CD103+ DC activation promotes the expansion of highly proliferative TNFR2 expressing Treg.

DISCUSSION

A number of ambient, anthropogenic, and ENP have been described that exert detrimental effects on lung immune homeostasis and exacerbate the symptoms of asthma and lung inflammation upon allergen challenge in susceptible individuals (30, 31). However, our studies suggest a radically different role for non-toxic particles such as PS50G: promoting homeostasis and preventing the elicitation of inflammation upon allergen challenge in atopic individuals (8, 9). Our initial studies suggested that PS50G can modulate pulmonary DC function (8, 9). Here, we reveal a novel role for PS50G, leading to augmented elicitation of lung TNFR2+Foxp3+ Treg upon allergen challenge in sensitized animals, associated with increased control of allergic lung airway inflammation. Furthermore, for the first time, we show that NP can be engineered to induce the upregulation of TNFR2+Foxp3+ Treg in the lung.

Increased frequencies of Treg in the periphery or lymphoid organs indicate that Treg have either proliferated (32, 33) or migrated into the tissue (34, 35). Previously, it was shown that the size of the Treg pool is critical for maintaining immunological balance, and even relatively minor modulation of Treg numbers alters immunity, with preferential effects on T helper 2 (Th2) immunity (36). Our present data are in agreement with Tian et al., showing efficient prevention of Th2 cell elicitation by allergens in the lung after allergen challenge. Chen et al. have demonstrated that, in peripheral lymphoid organs, Treg and Teff expressing TNFR2 exhibit greater proliferative capacity than the non-TNFR2 expressing subsets (27). Here, we show for the first time that, in the lung, even in a largely non-inflammatory environment (during homeostasis),

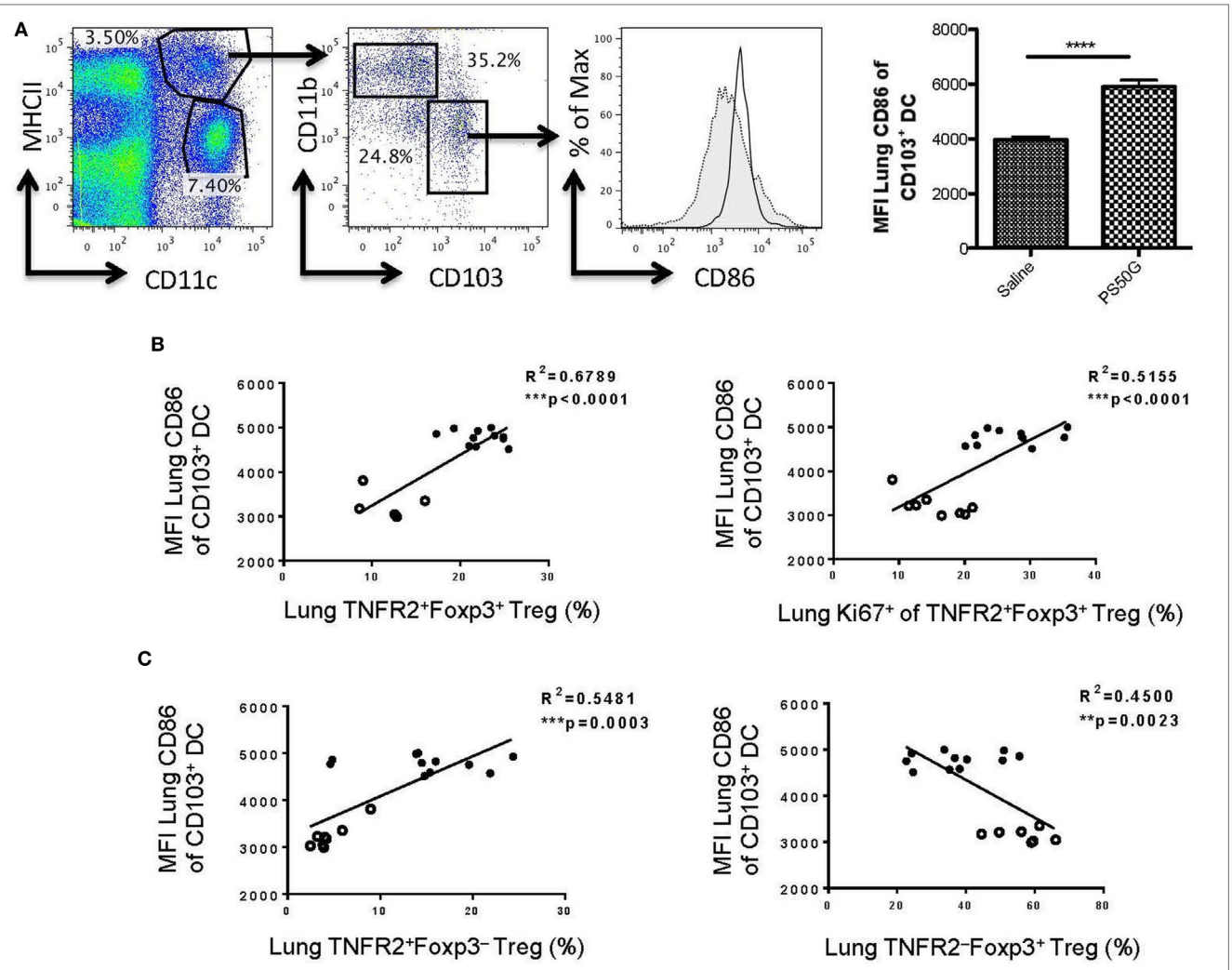

FIGURE 7 | CD86 expression on lung CD103+ dendritic cell (DC) positively correlates with the proportions of TNFR2+ regulatory T cells (Treg) and TNFR2+Foxp3+ that are expanded (Ki67+). Naïve mice (n = 5–7 per group per time point) received PS50G i.t on day 0 or saline as control. Samples were collected on days 1, 3, 7, and 30. **(A)** Stained lung cells were gated on viable MHCII^{high}CD11c+, followed by gating on CD11b−CD103+ cells on day 3 post instillations. Expression of CD86 population in saline (gray line, filled histogram) and PS50G (black line, open histogram). MFI of MHCII^{high}CD11c+CD11b−CD103+ DC on day 3 post instillations. Data represent the mean ± SEM. ***$p < 0.001$ of at least three experiments. The percentages of **(B)** TNFR2+Foxp3+ Treg (left panel), TNFR2+Foxp3+Ki67+ Treg (right panel), and **(C)** TNFR2+FoxP3− Treg (left panel) positively correlated with MFI of CD86 on CD103+ DC (MHCII+CD11c+CD11b−). The percentages of **(C)** TNFR2−FoxP3+ Treg (right panel) negatively correlated with CD103+ DC. Open circles correspond to saline group (n = 5–8) and closed circles correspond to PS50G (n = 11) data partially from days 1, 3, 7, and 30.

TNFR2+Foxp3+ Treg and TNFR2+Foxp3− Teff both exhibit a greater proliferative capacity. Therefore, TNFR2+Foxp3+ Treg with strong proliferative capacity in the lung may be critical to respond rapidly to inflammatory stimuli in this environment, in addition to effectively controlling the activation of Th2 effectors. Previous studies have shown that only TNFR2+Foxp3+ Treg are able to suppress highly bioactive TNFR2+Foxp3− Teff (26), which we observed are also highly elicited upon allergen challenge. Therefore, TNFR2+Foxp3+ Treg are a pivotal determinant of the level and type of immunity elicited in response to inflammatory environmental stimuli in the lung. Overall, our findings suggest that TNFR2+Foxp3+ Treg proliferate in the lung to maintain homeostasis and limit inflammatory responses, while maintaining "appropriate" responses to harmless airborne stimuli.

After allergen challenge in atopic animals, the proliferation of TNFR2+Foxp3+ Treg in the lung was increased in animals that had been previously pretreated with PS50G. CTLA-4 expression in LN, but not the periphery, is critical to prevent elicitation of adaptive immunity (37). In turn, LAP expression is associated with potent peripherally activated Treg immunosuppressive phenotypes (38). Pretreatment with PS50G resulted in higher levels of expression of LAP (in the lung) and CTLA-4 (in the lung-draining LN) specifically on TNFR2+Foxp3+ Treg, suggesting they

can promote increases in the relevant, organ-specific, maximally suppressive phenotypes. Thus, the increases in TNFR2$^+$Foxp3$^+$ Treg during AAI, promoted by PS50G pretreatment, were associated with their increased proliferative expansion capacity. Together, these results show how PS50G can increase the long-term capacity of the lungs to maintain a normal response following an allergen challenge. In addition, the response occurred without allergic Th2 driven exacerbations even in atopic animals, by preferentially expanding TNFR2$^+$Foxp3$^+$ Treg. A recent study further demonstrated the critical nature of TNFR2 driven regulation in the lung, by showing that aberrant TNFR2 signaling exacerbates airway inflammation in an AAI mouse model, specifically by promoting Th2 and Th17 cell polarization while inhibiting Th1 and CD4$^+$CD25$^+$ T cells differentiation (39). In this context, PS50G may improve the capacity of the lungs to control inflammation through TNFR2 signaling. In addition, as shown by our results, PS50G can also increase the TNFR2$^+$Foxp3$^+$ Treg pool and its proliferative potential. The latter may support the maintenance of an effective immunoregulatory pool size for homeostatic control in inflammatory environments.

Previously, we demonstrated that PS50G are preferentially taken up by DC in the lung and may affect their long-term function. The present study extends these findings by showing that PS50G increase the frequency and enhance the suppressor phenotype of TNFR2$^+$Foxp3$^+$ Treg. Such a broad immunological imprint has important consequences on adaptive immune responses in the lung, especially in controlling allergen-induced Th2 cells in AAI. How different subsets of effector T cells, relevant to diverse lung diseases, ranging from Th2 cells in inflammatory allergic diseases, to Th1 and Th17 cells in cancers, are affected by TNFR2$^+$Foxp3$^+$ Treg expansion in the lung induced by particles such as PS50G or other stimuli, will be a useful question to address in future studies in diverse lung disease models. From a practical point of view, such properties need to be understood if NP is to be rationally deployed as carriers to deliver drugs and vaccines into the lungs (2). Indeed, like PS50G particles, gold NP have more recently been observed to promote homeostatic imprints in the lung (40) and silver NP for the overall homeostasis of the intestinal tract (41). Conversely, toxic and pro-inflammatory NP such as those derived from diesel exhaust fumes promote increased susceptibility to allergic airways inflammation (42). Although the immunological basis of such imprints was not explored in many of these studies, we speculate that DC functional impairment and altered TNFR2$^+$Foxp3$^+$ Treg function are likely to be critically involved.

Tolerogenic and migratory CD103$^+$ DC travel toward lung-draining LN to prime the differentiation of naïve CD4$^+$ T cells into Treg (17), whereas lung macrophages are involved in maintaining Foxp3 expression by Treg, once these cells populate the lung tissue (21). Our data support previous findings on CD103$^+$ DC in establishing airway tolerance (17–19) and further suggest that CD103$^+$ DC might promote TNFR2$^+$Foxp3$^+$ Treg expansion. As this is the first study investigating the effects of non-toxic ENP on TNFR2$^+$Foxp3$^+$ Treg in the lung, further studies should follow to evaluate the role of CD103$^+$ DC in priming and/or inducing TNFR2$^+$Foxp3$^+$ Treg both in the lung and lymphoid organs.

While PS50G were used as a new model to show that TNFR2$^+$Foxp3$^+$ Treg can be preferentially expanded in the lung, these NP are not biodegradable, which may complicate their direct clinical translatability. Nevertheless, our previous studies have shown that PS50G are biocompatible, non-toxic and non-inflammatory even at high doses in the lung. While conventional materials such as nickel or titanium oxide cannot be used at high doses in the lung given toxicity concerns (43, 44), the development of new types of biodegradable NP may open to door to new classes of immunoregulators to help control inflammatory lung disease. In this context, some polymers such as poly(lactide-*co*-glycolide) (3) and fullerenes (45) hold significant promise and biodegradable NP of a larger (46) or smaller size (47, 48) have already been shown to suppress inflammation and to induce Treg. Furthermore, distinct nanoparticle sizes may induce different anti-inflammatory responses according to the type of disease. For example, Miller and colleagues showed that intravenous infusion of poly(lactide-*co*-glycolide) microparticles (500 nm) promote anti-inflammatory effects in the periphery in mice with relapsing experimental autoimmune encephalomyelitis (46). However, our previous studies in the lung showed that PS50G (50 nm) particles are capable of providing a broader anti-inflammatory imprint than PS500G (500 nm) particles, potentially associated by their preferential uptake by DC (rather than macrophages), including CD103$^+$ DC. In future studies, it will be important to map the extent to which particle size preferences will influence anti-inflammatory activity in diverse peripheral inflammation models.

Collectively, the present study proposes a novel mechanism by which NP such as PS50G, modulate the adaptive immune response by altering TNFR2$^+$Foxp3$^+$ Treg homeostasis, thereby decreasing susceptibility to allergic disease. In summary, together with our previous findings, these results implicate an important role of non-toxic ENP in establishing and preserving Treg numbers and functions through mechanisms such as (a) increasing the proliferative rate of Treg, thus altering the ratio between Treg and Teff, (b) maintenance of a self amplification loop by TNF/TNFR2 interaction, and (c) indirectly targeting CD103$^+$ DC to modify Treg.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Victorian Prevention of Cruelty to Animals Act 1986 and its incorporated Australian code of practice for the care and use of animals for scientific purposes 2013. The protocol was approved by the Alfred Medical Research and Education Precinct (AMREP) animal ethics committee.

AUTHOR CONTRIBUTIONS

RM, JL, JLS and CH performed experiments and analyzed data; MP, CH, RO, and JR supervised RM and JL. MP, CH designed experiments and analyzed data; MP, CH, RO, and JR designed the overall project; RM, MP, CH, and JB interpreted data and wrote

the manuscript; MP, CH, JB, JR, and RO edited the manuscript. MP, CH, JR, and RO provided funding for the project. CH and MP contributed equally.

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MINI REVIEW
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The Effect of TNF-α on Regulatory T Cell Function in Graft-versus-Host Disease

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FoxP3$^+$ regulatory T cells (Tregs) are a subset of CD4$^+$ T cells that can suppress proliferation and effector functions of T cells, B cells, NK cells, and antigen-presenting cells. Treg deficiency causes dramatic immunologic disease in both animal models and humans. As they are capable to suppress the function and the proliferation of conventional CD4$^+$ and CD8$^+$ T cells, Treg-based cell therapies are under evaluation for the treatment of various autoimmune diseases and are currently employed to prevent graft-versus-host disease (GvHD) in clinical trials of hematopoietic stem cell transplantation. Even though tumor necrosis factor-α (TNF-α) is well known for its pro-inflammatory role, recent studies show that it promotes Treg activation and suppressive function. In the present review, we discuss the role of TNF-α in Treg function and the possible implications on the actual treatments for immune-mediated diseases, with a particular attention to GvHD.

Keywords: TNF-α, regulatory T cells, TNFR2, immune regulation, tolerance, hematopoietic stem cell transplantation, graft-versus-host disease

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INTRODUCTION

The recent discoveries of immune suppressive cells such as natural FoxP3$^+$ regulatory T cells (Tregs) (1–3), invariant natural killer T cells (4), myeloid derived suppressor cells (5), and others prove the complexity of the mechanisms that underlie the immune response. These findings have prompted studies of the role of immune suppressive cells in different physiologic and pathologic conditions. Tregs are a subset of CD4$^+$ T cells that express the alpha chain of the IL-2 receptor (CD25) and a nuclear transcription factor termed forkhead box P3 (FoxP3) (1–3). They can suppress proliferation and function of many other immune cells such as CD4$^+$ FoxP3$^-$ T cells, CD8$^+$ T cells, B cells, NK cells, and antigen-presenting cells. Studies on mouse models and on patients affected by immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, a genetic disease with Treg deficiency due to mutations in *FOXP3* gene, demonstrated that Tregs are required for immune homeostasis and for survival (1–3). These discoveries provided key insights on the cellular mechanisms of immune regulation. Tregs are critical for maintenance of tolerance toward the self in secondary lymphoid organs and peripheral tissues and play an important role in the control of the inflammatory response (1–3, 6). Recently, we and others demonstrated that Tregs can build an immunological niche in the bone marrow for hematopoietic stem cells and B cell precursors allowing for their maintenance and differentiation (7, 8).

As Tregs suppress the function of conventional T cells (Tcons) and other immune cells, Treg-based cell therapies are under evaluation for the treatment of immune-mediated diseases. Recent studies showed that adoptive transfer of Tregs prevents graft-versus-host disease (GvHD), a life-threatening

immune complication of allogeneic hematopoietic stem cell transplantation (HSCT). In this setting, donor Tcons mediate alloreactions that eradicate tumor cells [graft-versus-tumor (GvT)], but that are also directed against normal tissues (mainly skin, gut, and liver), causing GvHD (9, 10). Studies in preclinical models and the results of clinical trials prove that infusion of Tcons under the control of Tregs prevents GvHD, while preserving GvT effects (11–14). As Tregs constitute only 1–5% of total peripheral blood $CD4^+$ T cells, their paucity and the complexity of their isolation limit further clinical applications. Thus, different strategies have been tested to expand Treg number and/or enhance Treg function *in vitro* and *in vivo* (15).

Tumor necrosis factor-α (TNF-α) is widely known for its pro-inflammatory activity (16–20). In the clinic it is used to enhance immune responses against tumors (21, 22) and several drugs have been developed to limit its function for treating autoimmune diseases (23–28). Its role in the pathogenesis of GvHD has been extensively described: TNF-α is released in patients after conditioning regimens with chemotherapy and/or radiotherapy and during the active phase of acute GvHD, and it is believed to enhance $CD8^+$ T cell mediated alloreactivity exacerbating immune destruction of GvHD target tissues (10, 29). Following these studies TNF-α-inhibitory drugs such as the monoclonal antibodies infliximab and adalimumab and the competitive soluble TNF-α receptor etanercept are now in use in the clinic for the treatment of steroid-refractory GvHD (30).

TNF-α is synthetized as a trimeric type II transmembrane protein, which can be cleaved to give rise to soluble extracellular TNF-α. Both membrane and soluble TNF-α are biologically active (16–20). TNF-α can bind two receptors, TNF receptor 1 (TNFR1) and 2 (TNFR2). Membrane TNF-α acts preferentially through TNFR2. TNFR1 is widely expressed on a variety of cells and its engagement triggers pro-inflammatory responses. TNFR2 expression is almost exclusively restricted to immune cells and its binding promotes cell survival and proliferation (17–20). TNFR1 contains a cytoplasmic "death domain," which recruits the adaptor molecule TNFR1-associated death domain protein (TRADD). TNFR1 interacts with different signaling complexes through TRADD, leading to either cell survival or cell death, depending on cellular context and signaling regulation. TNFR2, that lacks the cytoplasmic death domain sequence, binds directly TNFR-associated factor 2 and activates the nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (19, 20). TNFR1-deficient mice display defects in immunity to infection and in inflammatory response. In contrast, TNFR2-deficient mice show signs of exacerbated inflammation (31). In line with these data, TNFR1-deficient mice are resistant to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, that is a model of multiple sclerosis, while TNFR2-deficient mice exhibit more severe disease (32–34). In the same model, TNF-α-deficient mice also show extensive inflammation, demyelination, and high mortality (35). As Tregs preferentially express TNFR2, recent studies explored TNF-α impact on Treg function (36–38). Many of them highlight, not without controversies, the possibility that TNF-α could enhance Treg suppressive activity, suggesting a new regulatory function for TNF-α.

In this review, we will describe how TNF-α impacts on Treg phenotype and function and how Treg immune responses can be modified by TNF-α exposure over time. We will report controversial studies on human Tregs where TNF-α role is not fully elucidated yet. We will also discuss the possible implications of these studies on the actual treatments for immune-mediated diseases mainly focusing on GvHD and we will propose future clinical directions.

IMMUNE-REGULATORY ROLE OF TNF-α IN Treg FUNCTION

Role of TNF-α in Mouse Treg Function

The first clear indication of a role of the TNF-α/TNFR2 pathway in Treg function derived from studies in mice (36). *In vitro*, TNF-α in the presence of IL-2 increases the expression of CD25 and FoxP3, enhances the proliferation of Tregs and the suppression they exert on effector T cell proliferation. Mouse Tregs express higher levels of TNFR2 than $CD4^+$ $CD25^-$ T cells, while both subsets barely express TNFR1 (36). $CD4^+$ $CD25^+$ $TNFR2^+$ Tregs display an activated phenotype ($CD45RB^{low}$, $CD62^{low}$, $CD44^{high}$, high levels of CD69, CD103, GITR, and CTLA-4) and are more suppressive *in vitro* than $CD4^+$ $CD25^+$ $TNFR2^-$ cells (39). *In vitro*, TNF-α in combination with IL-2 selectively upregulates the expression of TNFR2 and other members of the TNF-α receptor superfamily, including OX40, 4-1BB, and FAS on Tregs (40). Studies in TNFR2-deficient mice showed that TNFR2 is required for natural Treg optimal function *in vivo*. In fact, wild-type Tregs controlled colitis induced by the transfer of naïve $CD4^+$ T cells into $Rag1^{-/-}$ mice, while TNFR2-deficient Tregs did not (41, 42). Similarly, neutralization of TNF-α exacerbated skin inflammation and was associated with a reduction of Tregs in the draining lymph nodes in a murine model of psoriasis-like disease (43).

Several reports show that Treg activation through the TNF-α/TNFR2 pathway can be exploited to enhance protection from GvHD in mouse models of allogeneic HSCT. In a mouse model of HSCT serum of mice during acute GvHD contained high levels of TNF-α that induced Treg proliferation and suppressive function. Donor TNF-α-primed Tregs prevented GvHD and prolonged mouse survival at an unfavorable Treg:Tcon ratio compared with unprimed Tregs. Importantly, the donor T cell mediated GvT effect against a leukemia cell line was unaffected (44). In another study, donor Treg-mediated protection from GvHD was abrogated by using a TNFR2 blocking mAb, or when either TNFR2-deficient Tregs or TNF-α-deficient T cells were infused (45). Finally, Chopra et al. showed that treatment of irradiated recipient mice with a TNFR2 specific agonist protein successfully expanded radiation-resistant host Tregs *in vivo*, resulting in prolonged survival and reduced GvHD severity after transplantation. The GvT effect and the function of donor T cells against pathogens (e.g., cytomegalovirus) were preserved even after host–Treg expansion induced by the TNFR2 agonist. The beneficial effects of the TNFR2 agonist were abrogated in TNFR2-deficient mice (46).

Role of TNF-α in Human Treg Function

Like mouse Tregs, human Tregs preferentially express TNFR2 (37, 38). The majority of human $CD4^+$ $CD25^+$ $TNFR2^+$ cells express FOXP3 and high levels of CD45RO, a marker of activated effector and memory T cells. *In vitro*, they suppress Tcon proliferation and function more efficiently than $CD4^+$ $CD25^+$ $TNFR2^-$ cells (38). Okubo et al. showed that TNF-α or a TNFR2 agonist antibody promote *in vitro* expansion of $TNFR2^+$ Tregs when added to standard expansion protocols (culturing medium with anti-CD3/CD28 stimulus and IL-2, in the presence or not of rapamycin) (47). TNFR2 stimulated and expanded Tregs had a striking homogeneous phenotype ($CD4^+$ $CD25^{high}$ $FOXP3^+$ $CTLA4^+$ $CD127^-$ $CD62L^+$ Fas^+ $HLA\text{-}DR^+$ $CD45RO^+$ $CCR5^-$ $CCR6^-$ $CCR7^-$ $CXCR3^-$ $ICOS^-$), they were endowed with a greater suppressive function, and produced lower levels of IFN-γ and IL-10. In fact, such highly suppressive Tregs co-expressing TNFR2 could ameliorate the onset of autoimmune diseases. For example, in type 1 diabetic patients the same authors observed an increase in resting $CD45RA^+$ Tregs and a decrease in activated $CD45RO^+$ Tregs. *In vitro* treatment with TNF-α or TNFR2 agonist antibody corrected the activation defect of Tregs in these patients (48). Thus, TNFR2 activation could trigger survival and proliferation of human Tregs through the NF-κB and MAPK pathways.

Despite the similarities between mouse and human Tregs expressing TNFR2, there are conflicting data on the effects of TNF-α on human Tregs. Some studies suggest that Treg function is impaired in rheumatoid arthritis (RA) patients and treatment with anti-TNF-α antibodies restores it (49–52). Tregs from patients with active RA or with active Systemic Lupus Erythematosus have been showed to express reduced levels of FOXP3 but increased levels of TNFR2 and to have defective function *in vitro* (50, 53). The mechanisms underlying Treg defective function are not well understood and TNFR2 expression levels could not be involved in the pathogenesis of these autoimmune disorders. Moreover, function of Tregs from patients with various autoimmune diseases could have been affected by many factors, including disease status and previous treatments. Furthermore, anti-TNF-α therapy has been shown to be associated with the induction of a population of $CD62L^-$-induced Tregs rather than a recovery in natural Treg function (54). In another study, the anti-TNF-α antibody adalimumab was shown to bind to membrane TNF-α expressed by monocytes and to promote Treg expansion by paradoxically enhancing TNFR2-mediated signaling in RA patients (55).

Conflicting data also arose from *in vitro* analyses of TNF-α effects on Tregs from healthy donors. Some studies showed that suppression of Tcon proliferation exerted by Tregs was impaired in the presence of TNF-α (50–52, 56, 57). On the other hand, other authors reported that TNF-α in combination with IL-2 increased CD25 and FOXP3 expression and induced Treg proliferation and function (47, 48, 58). Different experimental conditions could account for these inconsistencies, such as Treg selection methods and purity, length of Treg exposure to TNF-α and its concentration, and TNF-α effects on effector T cells in coculture experiments. Our personal observations support the notion that TNF-α upregulates the expression of Treg specific markers and it does not impair Treg function *in vitro*. However, these contradictory results highlight the need for an extensive investigation of the role of TNF-α in Treg function *in vivo*, in humanized preclinical models.

ANTI-TNF-α THERAPIES AND TNFR2 PATHWAY BLOCKADE

The intrinsic pro-inflammatory role of TNF-α and its ability to induce production of other inflammatory cytokines (e.g., IL-1, IL-6, GM-CSF, IFN-γ) made TNF-α an ideal therapeutic target for conditions where a reduction of inflammatory response was needed (23–28, 59, 60). Thus, drugs that block or reduce TNF-α activity have been developed to treat autoimmune diseases, such as RA, inflammatory bowel diseases, psoriasis, ankylosing spondylitis, and others. The recombinant anti-TNF-α antibody infliximab, which blocks both soluble and membrane TNF-α, demonstrated clear clinical efficacy in the treatment of Crohn's disease and RA. Following this initial success several other anti-TNF-α drugs were tested in the clinic and anti-TNF-α treatment is now a fundamental step in the treatment of autoimmunity (25, 61).

Studies on GvHD after HSCT showed that TNF-α levels are increased in patients with acute GvHD and tend to correlate with disease onset and progression (10, 29, 30, 62). TNF-α is rapidly released by tissue macrophages after the conditioning regimen and it induces donor T cell activation and further proliferation possibly triggering GvHD. Thus, anti-TNF-α therapy was rapidly considered in this condition: infliximab and etanercept (a human recombinant TNF-α receptor that competes for and inactivates soluble and membrane TNF-α) have been used to treat steroid-refractory GvHD (30, 63, 64). After initial studies that were suggesting clinical efficacy, the lack of response in a big portion of patients, the high-risk of life-threatening infections that may follow the treatment, and the possibility of GvHD exacerbations or rapid progression after treatment, are limiting their clinical use and leave doubts on their application in this setting (63).

The clinical effects of anti-TNF-α therapy should be reconsidered by virtue of the new insights on TNF-α/TNFR2 pathway in Treg function. As TNF-α inhibition can reduce Treg *in vivo* suppressive function, the potential benefit of the treatment in inflammatory conditions could be limited. Anti-TNF-α treatments are not effective in some of the autoimmune diseases in which TNF-α is involved. Moreover, patients treated with anti-TNF-α drugs can develop other immune-mediated complications (65, 66). In fact, in multiple sclerosis, whose pathogenesis appears to be sustained by TNF-α (67), TNF-α blockade resulted in unexpected disease progression and onset of new lesions with demyelination (68).

As Tregs are critical for GvHD protection and control over time, limiting Treg function could be a potential pitfall of TNF-α blocking therapy in the HSCT setting. TNF-α that is produced after conditioning regimens with radiotherapy and/or chemotherapy can bind TNFR2 and at the same time activate Tregs and alloreactive T cells (44, 69). The higher TNFR2 expression in Tregs in comparison to the other T cell subsets makes them avid of the cytokine and could favor their activation (**Figure 1**). Furthermore, Tregs prevent GvHD mainly during the early

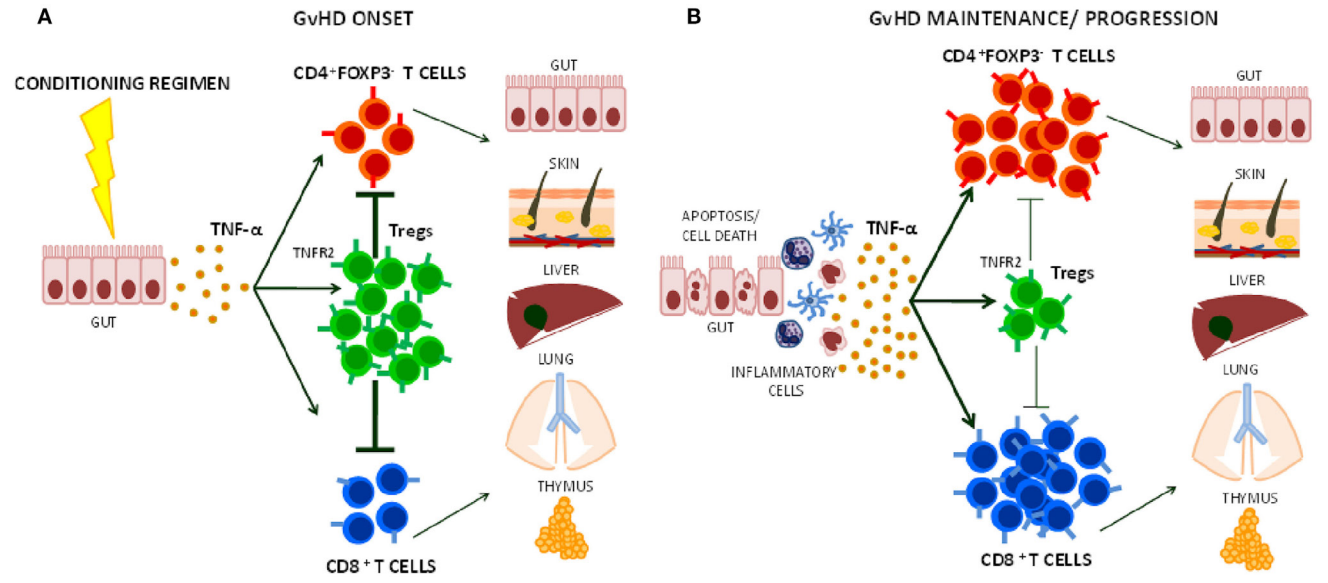

FIGURE 1 | Effects of TNF-α on Treg function during graft-versus-host disease (GvHD) onset, maintenance, and progression. **(A)** The release of TNF-α that follows tissue damage (e.g., gut) due to conditioning regimens with chemotherapy and/or radiotherapy in HSCT induces T cell activation. Its preferential action on Tregs through TNFR2 helps limiting $CD4^+$ and $CD8^+$ effector T cell function during the early phases of GvHD. **(B)** At later stage, TNF-α may further activate alloreactive T cells contributing to GvHD maintenance and/or progression.

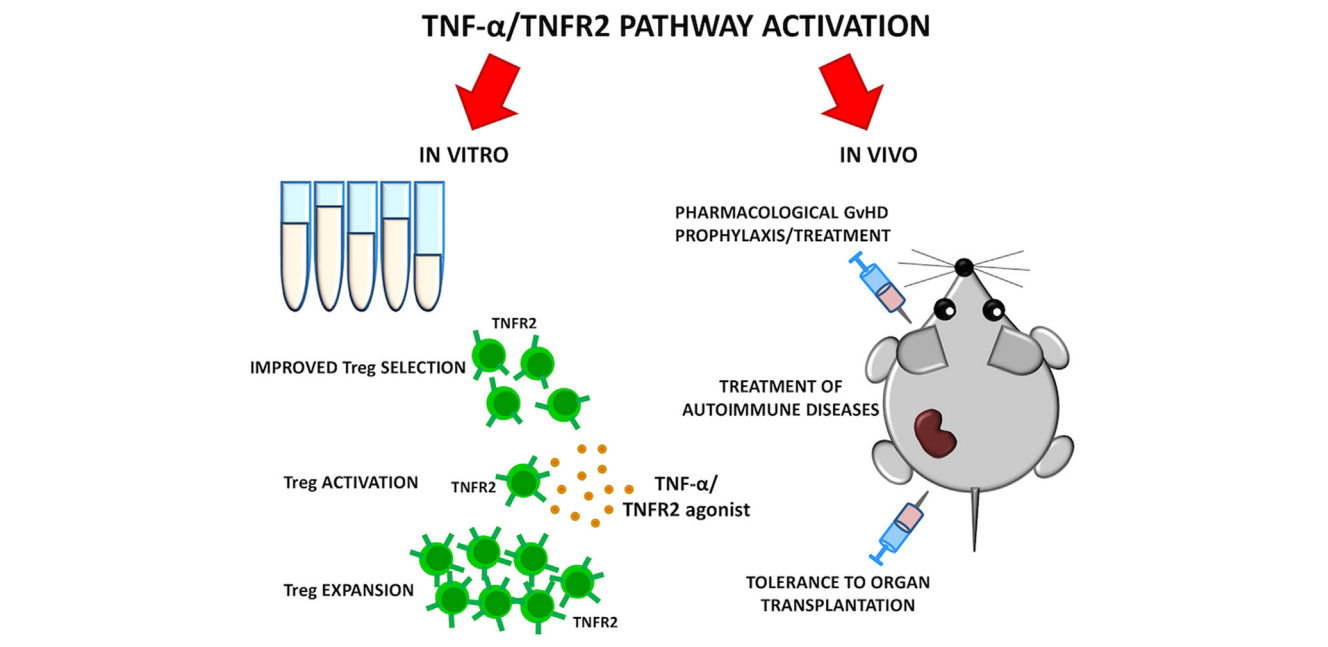

FIGURE 2 | Goals of selective TNFR2 activation on Tregs. TNF-α/TNFR2 pathway could be activated *in vitro* to ameliorate quality of Treg cellular products. Selective TNFR2 agonists may result in preferential Treg activation and expansion *in vivo*. Such strategies could be explored for graft-versus-host disease (GvHD) prevention, treatment of autoimmune diseases, and tolerance induction to organ transplantation.

phase after transplant (15, 70). Thus, the use of anti-TNF-α drugs as GvHD prophylaxis may be particularly counteractive as it could block Treg-mediated suppression of donor alloreactive T cell proliferation in secondary lymphoid tissues (30, 71, 72). Anti-TNF-α drugs are usually used in steroid-refractory GvHD (63). At this stage, TNF-α may have a limited role in sustaining the function of cytotoxic donor alloreactive T cells, which have been already activated and expanded. Moreover, it could be

possible that TNF-α blockade limits Treg residual function, thus contributing to disease progression or loss of clinical response in some patients. An optimal window for the use of anti-TNF-α therapy could be the very onset of GvHD when TNF-α recruits and activates donor cytotoxic T cells.

CLINICAL PERSPECTIVES

Based on the data discussed above, stimulation of the TNF-α/TNFR2 pathway is expected to activate and expand Tregs (36, 38, 39, 44, 47). $CD4^+$ $FoxP3^+$ Tregs, thanks to their high expression of TNFR2, are preferentially activated when the whole $CD4^+$ T cell pool is exposed to TNF-α. In these conditions, they acquire a proliferative and functional advantage in comparison to the effector $CD4^+$ $FoxP3^-$ T cells suggesting selectivity of the TNF-α/TNFR2 pathway in the $CD4^+$ T cell subset (44). Vaccination with Bacillus Calmette Guérin, a strong inducer of TNF-α secretion, promoted in vivo specific expansion of $CD4^+$ $CD25^{high}$ $FOXP3^+$ Tregs in one subject (47). On the other hand, $CD4^+$ $CD25^-$ effector T cells upregulate TNFR2 expression after TCR stimulation and become more resistant to Treg-mediated suppression (69). In addition, TNFR2 expression by effector $CD4^+$ T cells was required to induce full-fledged experimental colitis in one study (73). Thus, the effects of the activation of the TNF-α/TNFR2 pathway should be carefully evaluated *in vivo*.

Compared with the available anti-TNF-α drugs, blocking antibodies that selectively inhibit TNFR1 or TNFR2 could be used for different clinical purposes (61). Anti-TNFR1 antagonists could be more effective for the treatment of autoimmune diseases, as they do not interfere with Treg function. As Tregs co-expressing TNFR2 are abnormally abundant in human and murine tumors and can support their growth (39, 74), blocking TNF-α/TNFR2 pathway could be a therapeutic option in cancer (75). Indeed, a TNFR2 antagonist antibody has been shown to concomitantly suppress Treg function and promote effector T cell proliferation *in vitro* (76).

As TNFR2 is highly expressed by a Treg subset with maximal suppressive function, it could be used as a marker for Treg selection for adoptive therapy purposes. At the same time, treatments that specifically stimulate TNFR2 could selectively boost Treg function. TNFR2 agonists can activate and expand Tregs *ex vivo* and possibly *in vivo* (47). The use of Treg-based cellular therapies is limited by the paucity of Tregs in the periphery and the complexity of *in vitro* manipulation required for their expansion while preserving function and purity. TNFR2 agonists may represent an alternative strategy to expand *in vitro* a Treg population endowed with higher purity and enhanced activity, thus improving results of current Treg-based clinical trials for GvHD prevention in HSCT. Moreover, the ability of TNFR2 agonists to expand highly suppressive Tregs *in vivo* should be carefully evaluated in preclinical models. Such studies could open the possibility of Treg-based immunotherapies for autoimmune diseases where regulation of T cell response is impaired or for tolerance induction to organ-transplantation (**Figure 2**).

CONCLUDING REMARKS

TNF-α has been widely known for its pro-inflammatory activity, but the effects that follow the stimulation of its two main receptors should be carefully taken in consideration when evaluating the pathogenesis and the treatment of immune-mediated diseases. In this context, new discoveries on the role of TNF-α/TNFR2 pathway may provide relevant tools for a correct use in the clinic of anti-TNF-α treatments and for improving Treg-based therapies. While the blockade of this pathway is under investigation for cancer treatment, TNFR2 stimulation could be used to induce and expand Tregs thus controlling detrimental immune responses. Further studies are needed to evaluate whether Treg activation *via* TNFR2 could enhance yield, purity, and efficacy of Treg-based cell therapies. Such approach could have a potential for quick clinical translation in HSCT trials where Tregs are in use to prevent GvHD and boost immune reconstitution. The rising growth of studies on mouse and human Treg function strongly support a new role for TNF-α and TNFR2 as key players in the complex interplay between immune cells during immune regulation and tolerance.

AUTHOR CONTRIBUTIONS

AM, SP, and AP wrote the manuscript. AV provided overall guidance and reviewed the manuscript.

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PERSPECTIVE
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Association of *TNFRSF1B* Promoter Polymorphisms with Human Disease: Further Studies Examining T-Regulatory Cells Are Required

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The TNFR2 receptor is expressed by highly active regulatory T cells, and thus constitutes an important therapeutic target for the treatment of autoimmune disease and cancer. Disease susceptibility as well as the potential response to therapies directed at TNFR2 could be significantly impacted by genetic variation in the promoter of the *TNFRSF1B* gene that codes for the TNFR2 protein. To date, only a few studies have examined the association of *TNFRSF1B* promoter variation with disease, and the potential impact on T-regulatory cell (Treg) number and function has not been examined. We propose that copy number variation of a key transcription factor binding site has a significant effect on *TNFRSF1B* promoter activity, and should be considered in studies of disease susceptibility and especially with regard to variation in the level of TNFR2 expression on Tregs.

Keywords: tumor necrosis factor, TNFR2, T-regulatory cells, promoter, variable number tandem repeat, transcription

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INTRODUCTION

Tumor necrosis factor (TNF) is a multifunctional cytokine that can affect multiple cellular responses, such as inflammation, tumorigenesis, viral replication, septic shock, and autoimmunity (1). These functions are mediated through binding of TNF to either the TNFR1 (*TNFRSF1A* gene) or TNFR2 (*TNFRSF1B* gene) receptors. TNFR1 is expressed by most cells, and likely accounts for the pleiotropic effects of TNF administration, including the severe side effects associated with systemic administration (2). TNFR2 expression is more restricted, with significant levels expressed by a subset of highly suppressive T-regulatory cells (Tregs) in both mouse and human (3, 4). TNFR2 has also been detected in the central nervous system (5), on cardiac myocytes (6), and on thymocytes (7). TNF signaling through TNFR1 has been associated with apoptosis, while TNFR2 receptor stimulation generally leads to a proliferative response (8). Accordingly, TNFR1 and TNFR2 have differences in their signaling pathways, although there is some overlap (9). TNF binding to TNFR1 triggers apoptosis through the activation of the TNFR1-associated death domain and Fas-associated death domain adaptor proteins. By contrast, TNFR2 signaling uses TRAF2, leading to NF-κB activation, resulting in enhanced growth and survival (10). However, IL-2 stimulation of T cells induces both TNFR2 and RIP expression that results in apoptotic cell death in response to TNFR2 signaling (11). The more restricted expression of TNFR2 makes it a more attractive molecular target for drug development than TNFR1. TNFR2-expressing Tregs are abundant in human and murine tumors (12), and TNFR2 is also expressed by multiple tumors and promotes their growth (13). It therefore appears that TNFR2 can play an important role in tumor development by suppressing the immune response in addition to promoting tumor cell growth. A recent study has demonstrated that an

antagonistic anti-TNFR2 antibody was capable of inhibiting Treg proliferation and directly killed the OVCAR3 ovarian cancer cell line that has high levels of TNFR2 expression, suggesting that targeting TNFR2 may be an effective anti-tumor therapy (14).

ASSOCIATION OF TNFR2 ALTERATIONS WITH DISEASE

The level of TNFR2 signaling would be expected to have a significant effect on the proliferation of T cells. A recent study of TNFR2-deficient effector T cells (Teffs) demonstrated the importance of this receptor for the proliferative expansion of Teffs (15). Whole exome sequencing of patients with cutaneous T cell lymphomas (Mycosis fungoides and Sézary syndrome) revealed that 38% had alterations that would positively effect TNFR2 signaling (16). 14% of patients demonstrated a copy number gain of the *TNFRSF1B* gene that was correlated with increased *TNFRSF1B* mRNA levels and increased TNFR2 protein in a cell line with increased gene copy number. 4% of patients had a mutation at codon 377 of TNFR2 that enhanced NF-κB signaling.

Genome-wide association studies have implicated the *TNFRSF1B* gene in two human diseases, systemic lupus erythematosus [SLE (17)] and anti-neutrophil cytoplasmic antibody (ANCA) in inflammatory bowel disease (18). In SLE, a M196R variant was associated with the disease: however, the functional relevance of this change was not determined. In ANCA, a SNP in the first intron was associated with decreased TNFR2 levels in carriers of the SNP associated with susceptibility, but the mechanism behind the decrease in TNFR2 was not investigated.

Although changes in TNFR2 levels were associated with disease susceptibility, none of the studies cited above looked directly at variation within the *TNFRSF1B* promoter region. A 15 bp insertion/deletion has been identified within the core promoter region that affects the copy number of a repeated 15 bp sequence (19), and it is referred to as a variable number tandem repeat (VNTR). The repeated sequence contains a predicted SP1-binding site, and therefore the number of repeats might have an effect on promoter activity. Several studies have examined the effect of variation in the promoter VNTR on disease (20–22). A study of patients with hematologic malignancies treated with prolonged chemotherapy (20) showed that the susceptibility to develop invasive pulmonary aspergillosis (IPA) was decreased in individuals that were homozygous for *TNFRSF1B* alleles containing two copies of the repeat (2/2 genotype). 43% of the patients that developed IPA had the 2/2 genotype, whereas 69% of patients without IPA were 2/2 ($p = 0.03$). In a study of bone mineral density, individuals that were homozygous for a single copy of the repeat (1/1 genotype) had a lower rate of lumbar spine bone loss than subjects that had at least one allele with two copies of the element (21). Individuals possessing either the 2/2 or 1/2 genotype had bone loss of 0.84%, whereas individuals with the 1/1 genotype lost 0.39% ($p = 0.017$). In lupus (SLE) patients, patients with the 2/2 genotype had reduced disease activity, as measured by reduced renal involvement and higher C3 levels (22). 71% of patients with the 1/1 genotype were renal disorder positive, as compared to 29% for the 1/2 or 27% for the 2/2 genotypes ($p = 0.05$). The increased copy number of an element with a putative SP1-binding site could result in a higher level of *TNFRSF1B* transcription, however, none of these studies examined the level of TNFR2 expression.

FUNCTIONAL EFFECT OF COPY NUMBER VARIATION OF AN SP1-BINDING ELEMENT IN *TNFRSF1B*

In order to further investigate a possible functional effect of the copy number variation on promoter activity, we performed

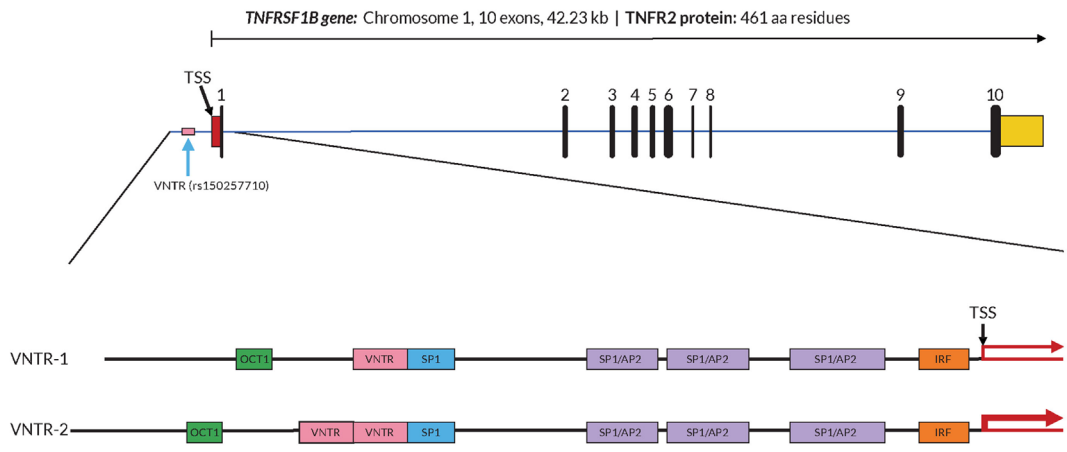

FIGURE 1 | Structure of the *TNFRSF1B* gene and core promoter region. A schematic representation of the exon–intron organization of the human *TNFRSF1B* gene. The exons are indicated by the numbered black rectangles. The 5′-untranslated and 3′-untranslated regions are indicated by red boxes and yellow boxes, respectively. The position of the variable number tandem repeat (VNTR) element is shown, and the dbSNP reference number is indicated in parentheses. The 419 bp 5′-flanking region of the human proximal *TNFRSF1B* promoter analyzed is shown in an expanded view, with the positions of putative transcription factor binding sites shown as colored boxes. Promoter variants with either 1 (VNTR-1) or 2 (VNTR-2) copies of the VNTR are shown. The transcription start site (TSS) is marked with a black arrow, and the relative levels of transcription are indicated by red arrows of differing thickness.

an *in vitro* analysis of the core *TNFRSF1B* promoter region. **Figure 1** shows a schematic of the *TNFRSF1B* gene and the identification of potential transcription factor binding sites. The two copies of the perfect 15 base pair (bp) repeat that constitutes the VNTR are shown, followed by a partial repeat containing the first 10 bp of the repeat, but including a predicted SP1-binding site that is also present in the VNTR. The 419 bp region starting 386 bp upstream of the transcription start site (TSS) to 33 bp downstream of the TSS was cloned into the pGL3 luciferase reporter vector, and the relative luciferase activity of promoter fragments containing either one or two copies of the element was determined. As shown in **Figure 2A**, two copies of the repeat had a significantly higher promoter activity than 1 copy in HeLa cells and the Jurkat T cell line. The 293 cell line showed a small effect, but this was not statistically significant. This suggests that individuals bearing two copies of the 15 bp repeat should

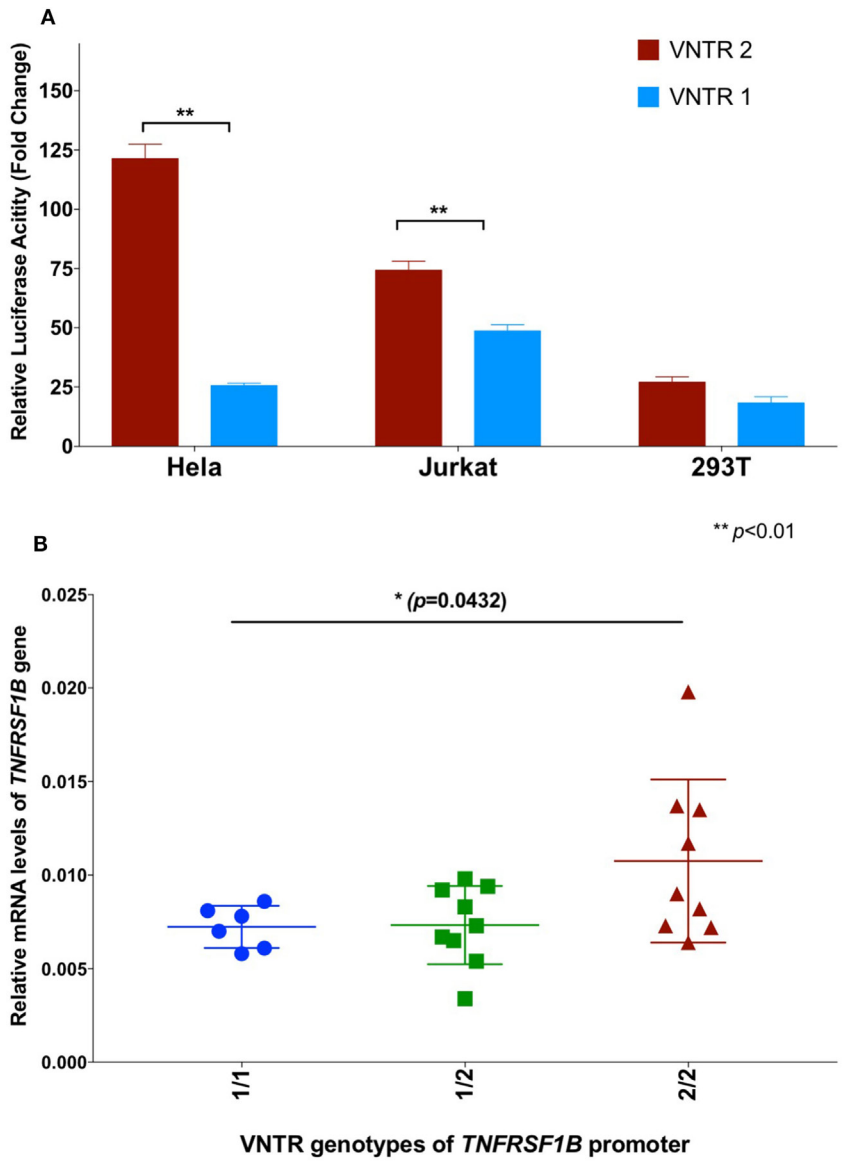

FIGURE 2 | **(A)** Effect of variable number tandem repeat (VNTR) number on *TNFRSF1B* promoter activity. Activity of pGL3-luciferase reporter constructs containing the genomic sequence of the *TNFRSF1B* gene from −475 to −57 relative to the start codon, were transfected into Hela, Jurkat and 293T cells. Promoter fragments with either two copies of the VNTR (VNTR-2) or one copy (VNTR-1) were compared. The average fold activity of constructs relative to empty pGL3 vector from at least three independent experiments is shown. Error bars represent ±1 SEM. An unpaired *t*-test with Welch's correction was used to calculate statistical significance. **(B)** QPCR of *TNFRSF1B* transcripts. Total cellular RNA was purified from peripheral blood mononuclear cells isolated from healthy donors (NCI-Frederick Research Donor Program; http://ncifrederick.cancer.gov/programs/science/rdp/default.aspx) and cDNA synthesis was carried out using random hexamer primer. Real time RT-PCR primers were: *TNFRSF1B* exon1-For 5′-CTGGGCTGCGGCGCACGCCTTG-3′; TNFRSF1B exon2-Rev 5′-GCAGCACATCTGAGCTGTCTGG-3′. HPRT1-For 5′-TGAGGATTTGGAAAGGGTGT-3′; HPRT1-Rev 5′- GAGCACACAGAGGGCTACAA-3′. Relative mRNA levels of *TNFRSF1B* were normalized to HPRT1 by the delta CT method. ANOVA was used to calculate statistical significance.

have a higher rate of transcription of the *TNFRSF1B* gene, and increased expression of TNFR2. The predicted effect of VNTR copy number on transcription was tested by comparing *TNFRSF1B* transcript levels in peripheral blood lymphocytes from donors with either the 1/1, 1/2, or 2/2 VNTR genotypes (**Figure 2B**). Donors with the 2/2 genotype had a significantly higher level of *TNFRSF1B* transcript than donors with the 1/1 genotype.

CONCLUSION AND PERSPECTIVE

Although genetic variation in the TNFR2 receptor has been associated with several human diseases, we believe that additional studies examining variation in the copy number of the VNTR bearing a predicted SP1-binding site in the *TNFRSF1B* promoter region are warranted. We have shown that there is a direct functional effect on promoter activity and transcript levels, which would be predicted to affect receptor levels. Several studies have associated changes in TNFR2 levels with susceptibility to disease, so one would expect the VNTR to also show association in these diseases. Previous studies that have used exon sequencing would of course have missed the effect of this genetic variation. Given that the frequency of *TNFRSF1B* alleles lacking one of the repeats is in the range 20–30% depending on the population studied, there is likely substantial variation in TNFR2 levels due to the VNTR that may be associated with susceptibility to multiple diseases. The majority of previous work on the genetic association of *TNFRSF1B* variation with disease was performed before the importance of this receptor in Treg function was appreciated (3, 4). The effect of *TNFRSF1B* gene variation on the number and function of Tregs represents an avenue of research that could shed considerable light on the mechanisms behind the disease associations observed. If the *TNFRSF1B* gene variant containing two copies of the VNTR leads to increased expression of TNFR2 on Treg cells, this could result in increased Treg cell numbers and activity. Higher Treg levels could potentially explain the observation that lupus patients with two copies of the VNTR had reduced disease activity (22).

In addition to the predicted role of variation in TNFR2 expression on Treg function and the control of autoimmunity, there may also be significant effects on the response of T effector cells in cancer patients treated with checkpoint inhibitors. If TNFR2-directed reagents are eventually introduced into the clinic, it may be informative to look for associations between the response to such agents, and the VNTR status of the individual.

AUTHOR CONTRIBUTIONS

SA performed data analysis and wrote the manuscript. HL performed experiments, analyzed data, and wrote the manuscript.

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Tumor Necrosis Factor α and Regulatory T Cells in Oncoimmunology

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Tumor necrosis factor α (TNF) is a potent pro-inflammatory cytokine that has deleterious effect in some autoimmune diseases, which led to the use of anti-TNF drugs in some of these diseases. However, some rare patients treated with these drugs paradoxically develop an aggravation of their disease or new onset autoimmunity, revealing an immunosuppressive facet of TNF. A possible mechanism of this observation is the direct and positive effect of TNF on regulatory T cells (Tregs) through its binding to the TNF receptor type 2 (TNFR2). Indeed, TNF is able to increase expansion, stability, and possibly function of Tregs *via* TNFR2. In this review, we discuss the role of TNF in graft-versus-host disease as an example of the ambivalence of this cytokine in the pathophysiology of an immunopathology, highlighting the therapeutic potential of triggering TNFR2 to boost Treg expansion. We also describe new targets in immunotherapy of cancer, emphasizing on the putative suppressive effect of TNF in antitumor immunity and of the interest of blocking TNFR2 to regulate the Treg compartment.

Keywords: tumor necrosis factor α, TNFR2, regulatory T cells, cancer, graft-versus-host disease, immunotherapy

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TNFR2 ON REGULATORY T CELL (Treg): STATE OF THE ART

Immunosuppressive Feature of Tumor Necrosis Factor α (TNF)

Tumor necrosis factor α is a pleiotropic cytokine produced by various cell types and involved in a wide range of pathological processes [for review, see Ref (1, 2)]. It has been initially considered as a pro-inflammatory molecule. However, preclinical and clinical data have shown that it also mediates a paradoxical anti-inflammatory and immunomodulatory effect. Indeed, in murine models of type 1 diabetes or lupus nephritis, TNF may have a protective effect (3–7). Moreover, new onset or exacerbation of chronic inflammatory and autoimmune diseases has been observed in patients treated with anti-TNF therapies (8–14). We will describe below in detail the case of graft-versus-host disease (GVHD) as an example of the ambivalent action of TNF in an immunopathology.

Different Possible Mechanisms for the Suppressive Action of TNF

Tumor necrosis factor α binds to two receptors, namely, TNF receptor type 1 (TNFR1) and TNFR2 (**Figure 1**). Unlike TNFR1 that has a ubiquitous expression, TNFR2 is expressed by some immune cells, preferentially by a fraction of Tregs, some endothelial cells, and cells of the nervous tissue (2, 15). Several mechanisms have been proposed to explain the suppressive action of TNF. It was shown that chronic stimulation with TNF may inactivate TCR signaling (16) or induce T cell

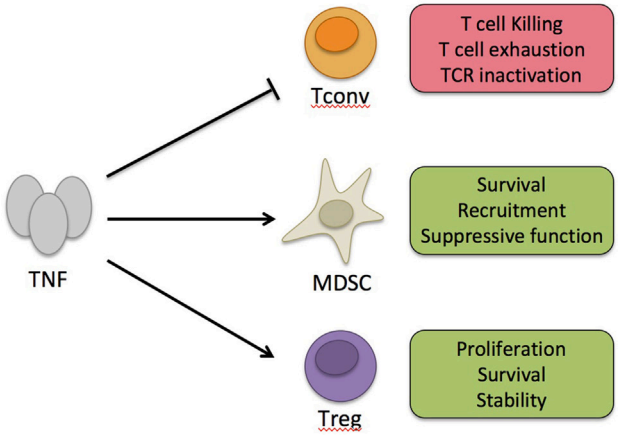

FIGURE 1 | Immunosuppressive action of tumor necrosis factor α (TNF). TNF can exert its immunosuppressive activity by intrinsic negative effect on conventional T cells (Tconvs) activation or by boosting suppressive cells, such as myeloid-derived suppressor cells (MDSC) or regulatory T cells (Tregs). On Tconvs, long-term effect of TNF may promote killing, exhaustion, or TCR inactivation. On MDSC, TNF may boost their activity by promoting their survival, local recruitment, or suppressive function. On Tregs, TNF may promote their proliferation, survival, and stability.

exhaustion (17). Alternatively, the cytokine may kill CD8+ T cells, a phenomenon emphasized for autoreactive cells (18). Besides these cell-intrinsic mechanisms, TNF may exert its suppressive activity by stimulating cells that have immunosuppressive action, such as myeloid-derived suppressor cells (MDSCs) (19, 20). Finally, the pioneer works of Chen and Oppenheim suggested that this immunosuppressive effect of TNF could be related to a direct activation of Tregs (15, 21). This latter mechanism, which is the most studied one and supported by data obtained by different groups, is detailed below. Generally, the suppressive action of TNF is considered to be mediated by its interaction with TNFR2 since TNFR2 signaling appears to be protective in various immunopathologies and several of the mechanisms described earlier are TNFR2 dependent (22). However, whereas TNF/TNFR1 interaction has been mostly described to be pro-inflammatory, TNFR1 triggering may also inhibit IL-12/IL-23 p40 expression by macrophages (23). This mechanism may explain the paradoxical expansion of Th1/Th17 cells following anti-TNF treatment in patients with autoimmune diseases who do not respond to this therapy (24, 25).

TNFR2 Expression by Tregs

TNFR2 expression is upregulated in activated Tregs and can be detected in activated conventional T cells (Tconvs), although at lower levels than in activated Tregs. Some other members of the TNFR family, such as GITR, OX40, or 4-1BB, are also preferentially expressed by Tregs, and their expression is also upregulated upon activation (26). Remarkably, in transcriptomic analyses, comparing Tregs and Tconvs of lymphoid tissues, TNFR2, OX40, and GITR belong to the Treg signature and their expression correlates with low DNA methylation in Tregs suggesting that their transcription is at least partly regulated at the epigenetic level (27, 28). These three molecules are expressed early in the Treg lineage, since the thymic Treg progenitor stage, and their expression is essential for Treg development (29). In mice lymphoid tissues or in human blood, TNFR2 is expressed by the fraction of activated Tregs expressing high levels of other activation markers such as CTLA-4 (30). TNFR2 expression remarkably identifies a subset of Tregs with the highest suppressive capacity (21, 30, 31).

Stimulating Effect of TNF on Tregs *via* TNFR2

The direct effect of TNF on TNFR2-expressing Tregs has been studied by Chen and Oppenheim *in vitro* and has been reviewed elsewhere (32). Briefly, TNF increases proliferation, survival, stability, expression of CD25, Foxp3, and activation markers, as well as suppressive function of mouse Tregs (15, 26, 30, 31). Many of these effects of TNF, notably on proliferation, could be reproduced with human Tregs (32–35). However, some studies claim that TNF inhibits the suppressive activity of human Tregs (36–39). The interpretation of some of these studies was complicated by the fact that TNF can render Tconvs more refractory to the Treg-mediated suppression. After extensive and careful exploration of this question, we could conclude that TNF does not inhibit the suppressive activity of human Tregs (35).

Role of TNFR2 on Treg Biology *In Vivo*

The *in vivo* role of TNFR2 on Treg biology has been more difficult to evaluate because of the absence of a conditional knockout of TNFR2 in Tregs. However, there is strong evidence that TNF can boost Treg expansion in different inflammatory contexts (40). We showed that TNF, probably produced by Tconvs, stimulated Treg proliferation during type 1 diabetes (41). Others observed a similar phenomenon during septic shock, infectious disease, or immune response (15, 42, 43). Also, TNFR2-deficient Tregs lost their capacity to control colitis, which was associated with reduced survival and stability compared with wild-type control Tregs (31, 44). The critical role of TNFR2 expressed by Tregs has been also studied in the context of GVHD and cancer and will be specifically discussed below. Overall, among all the effects of TNF on Treg biology, its capacity to increase proliferation is the most convincing since it has been reported in many *in vitro* and *in vivo* studies performed by different groups using mouse and human Tregs. The evidence that this cytokine also increases Treg survival and stability is quite convincing and its effect on Treg function requires further investigation.

HOPE AND DISAPPOINTMENT IN TARGETING TNF IN GVHD

TNF and TNFR1 As Predictive Biomarkers in GVHD

Tumor necrosis factor α plays a key role in acute GVHD (aGVHD), a systemic and highly inflammatory complication that occurs after allogeneic hematopoietic stem cell transplantation (allo-SCT) (45). TNF indeed plays a major role at different steps of this pathological process in which donor T cells recognize as foreign host healthy tissues and eventually cause

their destruction (**Figure 2**). In this line, clinical studies have clearly demonstrated a positive correlation between soluble TNFR1 levels measured 7 days after transplant and the time to onset and severity of aGVHD (46, 47). The increase in TNFR1 levels between baseline and day 7 was not only an independent predictor of aGVHD but also of transplant-related mortality and overall survival. Also, a rise in TNF, as measured by protein levels in peripheral blood, RNA transcription levels, or flow cytometry, precedes the onset of aGVHD, before peaking at the time of its development (48–50). Overall, the results of these clinical studies have led to the integration of TNFR1 as part of a biomarker panel that can discriminate patients with and without aGVHD, and predict survival (51).

Anti-TNF Clinical Trials in GVHD

The key role of TNF in aGVHD pathophysiology logically led researchers and physicians to try to block this cytokine to decrease inflammation and consequently to prevent or treat aGVHD. Along this line, most of the clinical trials focused on two molecules: infliximab—a monoclonal antibody (mAb) that binds TNF—and etanercept—a soluble TNFR that competes with cellular receptors for TNF binding. The great hope risen by TNF targeting in aGVHD during the first decade of this century has unfortunately faded rapidly due to somewhat disappointing results of clinical studies. Indeed, clinical trials failed to prove any benefit in adding infliximab to standard treatment, both for aGVHD prophylaxis and treatment (52, 53). Only small retrospective studies have shown promising response rates for the treatment of steroid-refractory aGVHD, mostly in case of intestinal tract involvement (54–57). However, the benefit of infliximab in steroid-refractory aGVHD does not seem to be superior to the one observed with other drugs available (58), even though prospective randomized trials are lacking. Moreover, other studies have shown that responses after infliximab therapy are poorly sustained and have raised concern over a heightened risk of severe infections (59, 60).

Regarding etanercept, a single center prospective study showed a promising response rate when combining etanercept with standard high-dose corticosteroids for first-line treatment of aGVHD compared with a cohort of contemporaneous case-matched patients treated with high-dose corticosteroids alone (61). However, the higher response rate observed with etanercept did not translate into a significantly superior survival at 6 months from aGVHD onset. Moreover, a multicenter prospective randomized "pick the winner" study comparing four promising molecules in combination with corticosteroids for first-line aGVHD treatment identified mycophenolate mofetil, and not etanercept, as the most promising agent (62). However, mycophenolate mofetil failed to prove any benefit in the subsequent multicenter, randomized, double-blinded, and placebo-controlled phase 3 trial evaluating its addition to standard corticosteroids (63). In the setting of steroid-refractory aGVHD, two small single center studies have shown only a modest effect of etanercept with few complete responses (64, 65). As for infliximab, efficacy seemed to be higher in case of gut involvement. Finally, a phase 2 study involving 100 patients also evaluated etanercept as part of aGVHD prophylaxis, in combination with tacrolimus and low-dose methotrexate (66). Once again, the benefit of etanercept was not obvious, as its addition to standard prophylaxis did not affect the overall risk of grade 2–4 aGVHD, as compared with a control

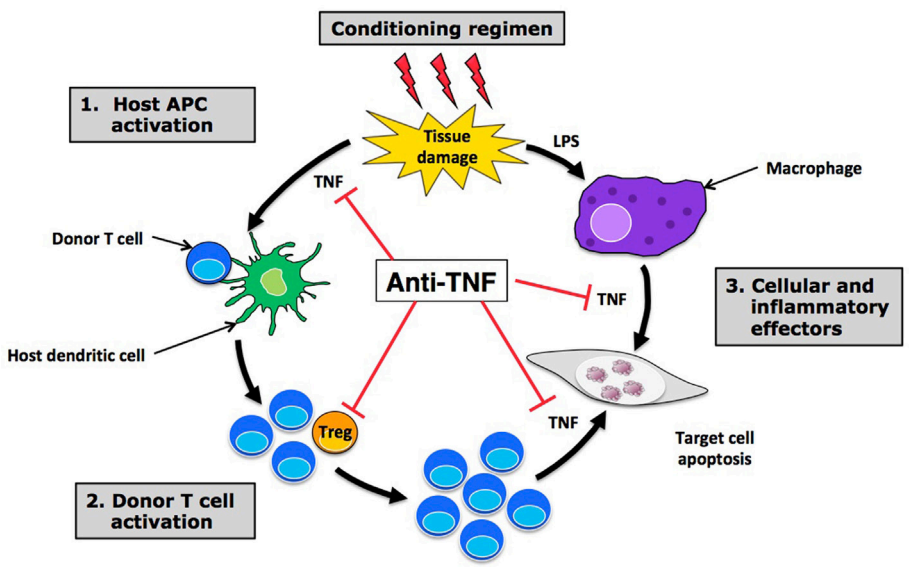

FIGURE 2 | Hope and disappointment in targeting tumor necrosis factor α (TNF) in graft-versus-host disease (GVHD). Anti-TNF treatments are able to block the effect of TNF at different steps of acute GVHD pathophysiology, including initial host APC activation (1), effector T cell recruitment and activation in target tissues (2), and direct cell necrosis (3). By inhibiting TNF ligation to TNFR2 expressed by regulatory T cells (Tregs), anti-TNF treatments could also have a deleterious effect on these suppressive cells, leading to an increased expansion and activation of alloreactive donor T cells that may be responsible for the disappointing results observed with anti-TNF treatments in this setting. Abbreviations: APC, antigen-presenting cell; LPS, lipopolysaccharide.

cohort of 161 previously reported patients. Only a potential benefit among non-total-body-irradiated patients was suggested in this study. TNFR1 plasma level monitoring can also be used to evaluate and/or predict response to treatment with etanercept, as a significant reduction in these levels has been observed in responding patients (61, 66). To summarize, the current place of anti-TNF treatments in the arsenal of aGVHD is only limited to a therapeutic option for steroid-refractory disease, mostly in case of intestinal tract involvement. A possible explanation of this failure is that blocking TNF would also impact on the TNFR2-dependent Treg boost that is protective in GVHD as suggested by experimental data discussed below.

HOPE IN TARGETING TNFR2 (AND Tregs) IN GVHD

Regulatory T cells modulate alloreactivity during allo-SCT. Cell therapy using Tregs efficiently control GVHD (67, 68) whereas Treg deletion can be used to boost the graft-versus-leukemia (GVL) effect (69). Thus, some research teams envisioned TNFR2 as a potential target to act directly on Tregs in this setting and modulate alloreactivity with either TNFR2 agonists or antagonists. In this regard, three important studies were published almost simultaneously in 2016 (70–72).

In a murine model of aGVHD prevention relying on Treg infusion, we have clearly shown using three different experimental approaches that the protective effect mediated by therapeutic Tregs was dependent on TNF produced by pathogenic Tconv and TNFR2 expressed by Tregs (71). Indeed, when blocking the TNF/TNFR2 interaction with an anti-TNFR2 mAb, or when using either TNFR2-deficient therapeutic Tregs or TNF-deficient Tconvs, aGVHD prevention was abolished in all cases, highlighting a boost of alloreactivity after TNF/TNFR2 blockade. Moreover, Treg and Tconv phenotypes were also modified, with the former displaying decreased expression of activation and suppression markers while the latter showed increased production of pro-inflammatory cytokines.

The second study was published by Chopra and colleagues, who developed a TNFR2 agonist called STAR2 (70). *In vitro*, STAR2 was able to stimulate expansion and activation of Tregs, an effect not observed with Tconvs. This selective Treg expansion and activation was also triggered *in vivo*, when mice were treated with STAR2 intraperitoneal injections. Most of all, in a murine model of aGVHD, pretransplant administration of STAR2 to recipient mice protected from aGVHD and significantly increased survival. The protective effect of STAR2 was associated with a preserved GVL effect and had no deleterious effect on posttransplant anti-cytomegalovirus immune reconstitution.

Finally, in the study of Pierini and colleagues, therapeutic Tregs were preincubated *in vitro* with TNF (+IL-2) for a short period (72). This TNF priming resulted in a higher expression of Foxp3 and activation/suppression markers by Tregs and a higher proliferation rate. Most interestingly, when such "TNF-primed Tregs" were infused to recipient mice in an aGVHD murine model, this resulted in prolonged survival, increased weight gain, and improved GVHD clinical score, even at the very low 1:10 Treg:Tconv ratio. In this study also, the beneficial effect of TNF priming did not come with a detrimental loss of the GVL effect.

Altogether, the results of these three studies pave the way for TNFR2 targeting to modulate alloreactivity after allo-SCT (**Figure 3A**). Additional preclinical data are needed, especially regarding the effect of TNFR2 agonists and antagonists on various human cell types (Tconvs, Tregs, and cancer cells) *in vitro*, before the start-up of clinical trials evaluating their efficacy and safety for prevention and/or treatment of aGVHD and posttransplant relapse of hematologic disease, respectively. Notably, in the setting of aGVHD prevention, single center clinical trials have shown the high potential of Treg cell therapy (73, 74). However, adoptive transfer of such cells is limited by the small proportion of Tregs among peripheral blood mononuclear cells (PBMCs) that necessitates an *ex vivo* culture for expansion before infusion to the patient. In this regard, the direct administration of a TNFR2 agonist to the patient to selectively activate and expand *in vivo* Tregs with the highest suppressive capacity holds the promise of a more simple, costless, less time consuming, and possibly more efficient method.

NEW CHECKPOINT INHIBITORS IN IMMUNOTHERAPY OF CANCERS AND ROLE OF Tregs

New Targets in Immunotherapy of Cancers

Several clinical trials have clearly demonstrated that modulation of the immune response can improve the overall survival of advanced stage cancer patients (**Figure 4**) (75, 76). Indeed, since the approval of a-CTLA-4 antibody treatment for metastatic melanoma in 2011 (77–79), the field has witnessed the advent of numerous therapeutic approaches modulating the immune response (80, 81). Blockade of programmed death 1 and its major ligand PD-L1 has given impressive and durable clinical results (82–84) and fueled clinical evaluation (85) of (i) new inhibitory checkpoint targets, such as LAG-3 (86), TIM-3 (87), VISTA (88), and TIGIT (89), (ii) agonistic antibodies targeted to co-stimulatory receptors, such as 4-1BB (90), GITR (91), CD40 (92), and OX40 (93), (iii) cell-based therapies using dendritic cells, tumor-infiltrating lymphocytes (TILs), and genetically engineered T cells (CAR-T cells) (94), (iv) immune modulators such as innate ligands (95), and (v) vaccines, notably directed to neo-epitopes (94, 96). Along these lines, dozens of antitumor immunotherapeutic approaches have been already approved by regulatory agencies and thousands of such clinical trials are currently ongoing. Nevertheless, only 20–40% of patients benefit from these therapies, and some cases of resistance have been described (97–99).

Most of the abovementioned treatments are thought to work mainly by (re)-activating the cytotoxic arm of the immune response (100–102), namely, CD8+ T cells and NK cells; and by rescuing them from exhaustion (103, 104). Nevertheless, as the antitumoral immune response is also highly curtailed by Tregs, overcoming Treg-mediated immunosuppression in the tumor microenvironment (105, 106) represents a sound

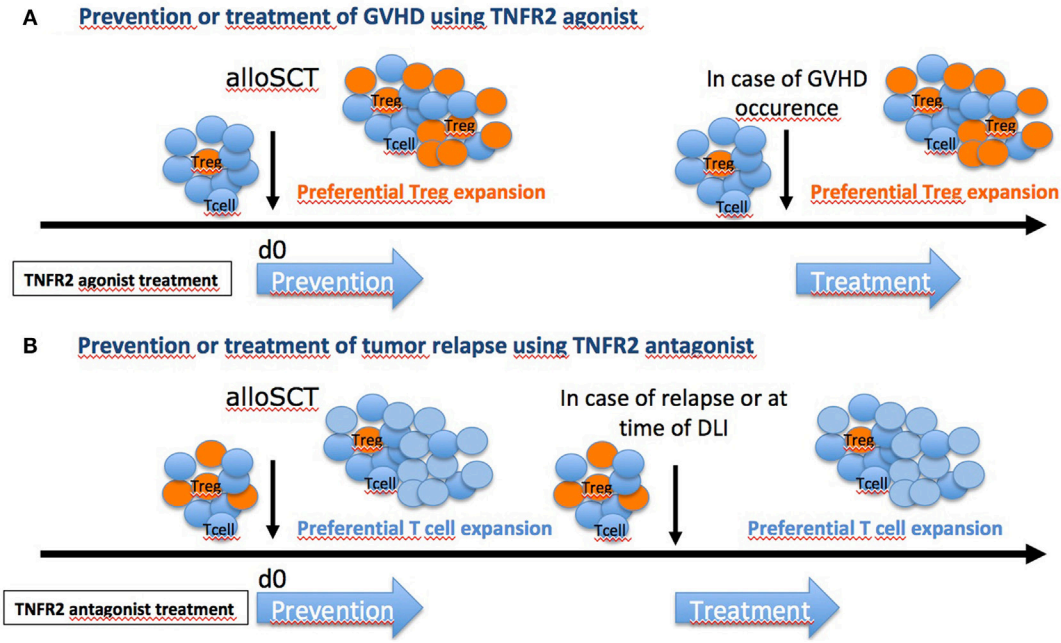

FIGURE 3 | Hope in targeting TNFR2 [and regulatory T cells (Tregs)] in graft-versus-host disease (GVHD). Depending on the clinical situation and the risk for the patient to develop or not GVHD, different therapeutic strategies could be envisaged. **(A)** For patients with elevated risk of GVHD (unrelated donor or one or several HLA mismatch), TNFR2 agonist could be administered to recipients before allo-SCT, as shown previously (73), or at time of grafting to boost Tregs. Patients could also be treated at time of GVHD occurrence. **(B)** For patients with elevated risk of relapse (aggressive leukemia, geno-identical allo-SCT), anti-TNFR2 could be administered to recipients at time of grafting to inhibit Tregs. In case of tumor relapse, patients could also be treated at time of donor lymphocyte infusion (DLI) to block Treg effect.

alternative for successful cancer immunotherapy. Of note, this can be obtained either by depleting Tregs or by inhibiting their function *in vivo* (107).

Can We Treat Cancer by Depleting Tregs?

The first proof of the beneficial effect of Treg depletion on the antitumoral response was brought forward by Onizuka et al. (108). They showed that administration of an anti-CD25 antibody (mAb; PC61) had prophylactic, although not therapeutic efficacy, probably due to the concurrent elimination of CD25-expressing activated effector lymphocytes. More recently, the group of Quezada (109) has shown that anti-CD25 antibody-mediated Treg depletion can be ineffective due to the high expression of the inhibitory Fc receptor FcgRIIb by cells present in the tumor microenvironment. Consequently, anti-CD25 antibodies designed to avoid FcgRIIb binding induced massive Treg depletion in the tumor and led to impressive tumor regression. Also, specific depletion of Tregs in transgenic DEREG mice (110), which express a diphtheria toxin receptor under the control of the *Foxp3* regulatory sequences, resulted in a partial regression of established melanoma that correlated with CD8+ T cell accumulation in the tumor. Furthermore, mouse studies point out that anti-CTLA-4 antibodies mainly act by eliminating or inhibiting the tumor-associated Tregs (which highly express this molecule) rather than by reinvigorating exhausted T cells (111, 112). Indeed, controversial results have been observed with Daclizumab (an anti-CD25 antibody) and with a fusion protein between the IL-2 and the diphtheria toxin (Ontak) (113–118). Thus, direct proofs of the beneficial effect of Treg depletion in human are still missing for solid cancers, and there are to date no clinical tools that specifically target this population. This point is more advanced in the field of onco-hematology. With the intent of preventing or treating post allo-SCT relapse of hematologic disease, GVL effect can be activated by donor lymphocyte infusion (DLI). In this setting, the harmful effect of Tregs after DLI was suggested by a study in which the authors quantified Tregs in DLI products and demonstrated that patients with a durable complete remission of their malignancy after DLI had received a lower number of Tregs (119). This observation led to the idea of depleting Tregs to improve responses to DLIs, an approach that was successfully tested in a clinical trial in which a magnetic depletion of CD25+ cells was performed on donor PBMCs before their infusion to recipients that were considered "alloreactivity resistant" (69).

Can We Treat Cancer by Modulating Treg Differentiation and Expansion?

Besides Treg depletion, tumor-associated Tregs can be therapeutically targeted by the modulation of the tumor microenvironment. Indeed, cancer cells produce metabolites, cytokines, and growth factors that can (i) promote Treg accumulation and expansion, (ii) enhance Treg function, and even (iii) induce

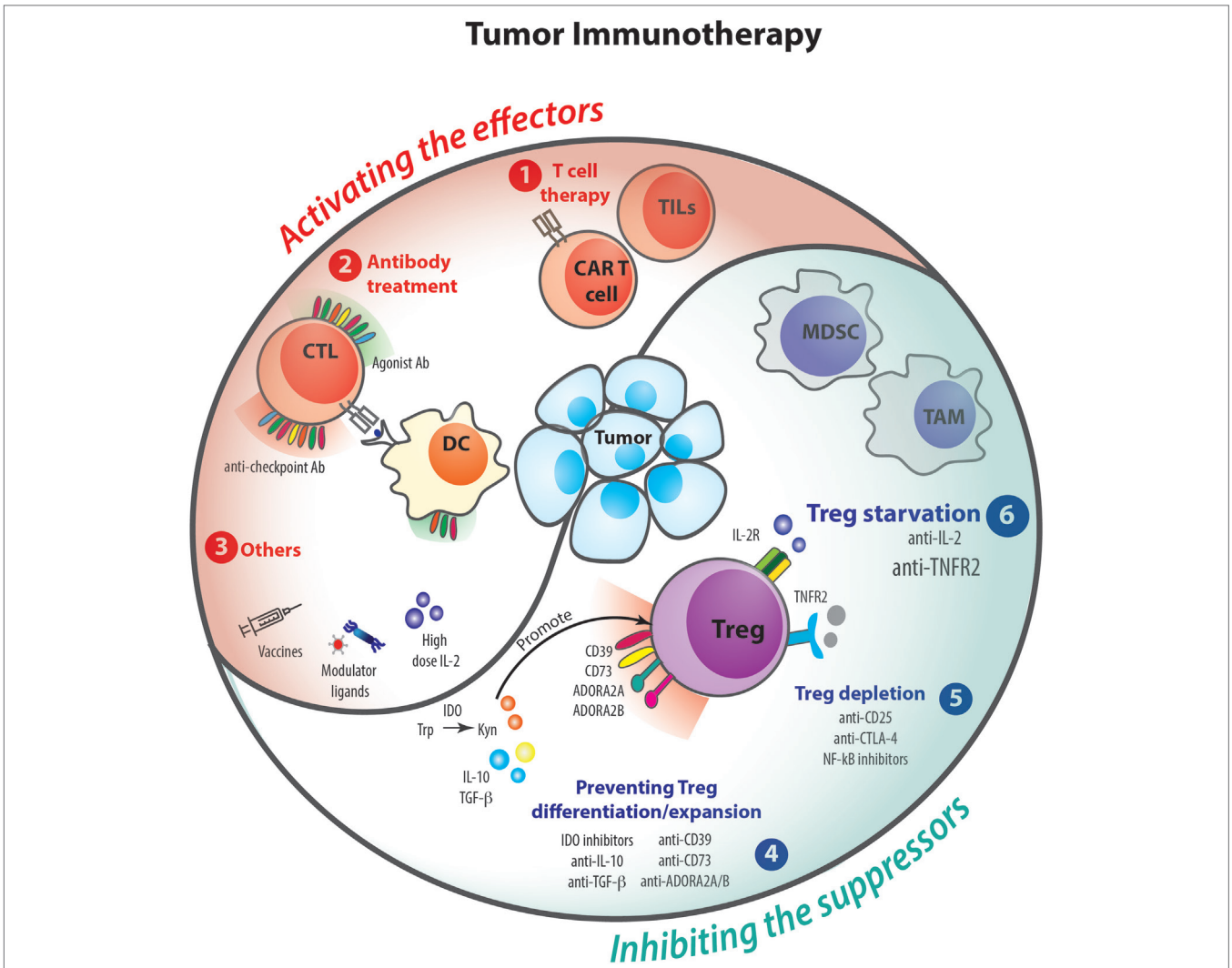

FIGURE 4 | Tumor immunotherapies and regulatory T cells (Tregs). Over the past decades, several clinical trials and animal models have demonstrated that therapies acting on the immune response can help to fight against cancer. To control tumoral process, immunotherapies can either activate the effector arm of the immune response (1–3) or inhibit the suppressor mechanisms (4–6). The following therapies that potentiate T cell responses have been proposed: tumor-infiltrating lymphocytes (TILs) and CAR T cell thrapies (1); agonist and anti-checkpoint antibody treatments (2); other therapies such as vaccines, modulator ligands, and high doses of IL-2 (3). On the other hand, cancers promote suppressor mechanisms involving Tregs or myeloid-derived suppressor cell (MDSC), which are able to inhibit effector cells. Some treatments are being tested to modulate Treg suppression by preventing their differentiation/expansion (4), Treg depletion (5), or starvation (6).

Treg conversion from conventional CD4+ T cells (96). Among them, adenosine—generated upon catabolism of ATP by the ectoenzymes CD39 and CD73—and kynurenines—generated upon catabolism of tryptophan by the indoleamine 2,3-dioxygenase (IDO) enzyme—favor the accumulation, conversion, and expansion of Tregs and suppression of effector T cells (120). Accordingly, IDO inhibitors and either antagonists of A2A/A2B adenosine receptor or anti-CD39 and anti-CD73 antibodies significantly decrease the rate of Treg peripheral conversion and impair tumor growth (108, 121–124). Furthermore, therapeutic agents targeting these molecules in combination with immune checkpoint inhibitors show additive or synergistic effects in experimental tumor models, and their combination is currently under clinical investigation (96, 125). In addition, therapies aiming at inhibition of CD4+ T cell differentiation into Tregs have been tested. Among them, the effects of neutralizing antibodies or pharmacologic inhibitors of IL-10 and TGF-β have been evaluated in preclinical and clinical settings (126–128). These studies have demonstrated both pro- and antitumoral effects, probably due to their complex involvement in immune and non-immune processes. Moreover, there are not consistent data on the effect of these therapies on Tregs. Overall, manipulation of Treg induction and function through inhibition of metabolic and biochemical pathways active in the tumor microenvironment represent an alternative immunotherapeutic approach. Nevertheless, the significant side effects associated with the involvement of these

pathways in different physiological processes must be taken into consideration.

Can We Treat Cancer by IL-2 Deprivation to Target Tregs?

On top of the abovementioned strategies designed to disarm Tregs for therapeutic aims, "cytokine starvation or cytokine deviation" represents an alternative promising approach. Namely, deprivation of Tregs from IL-2 and TNF—two key cytokines essential for their biology—should lead to Treg dysfunction or death. Clinical manipulation of IL-2 levels remains complex as IL-2 can act both as an immune stimulating or suppressive cytokine, depending on the dose. On one hand, low-doses of IL-2 favor Treg survival and suppressive function and lead to a better control of autoimmune and inflammatory diseases (129–131). On the other hand, high-dose IL-2 administration boosts effector immunity and, consequently, enhances antiviral or antitumoral responses (132, 133). Noteworthy, in the cancer setting, low efficacy of high-dose IL-2 administration (134) can be explained in part by the unwanted effect of IL-2 on Tregs, which constitutively express the high affinity IL-2 receptor [composed by three subunits: IL-2-Rα (CD25), IL-2Rβ and IL-2Rγ] (135). For efficient antitumoral effect, there is a need to activate CD8+ T and NK cells, which also respond to IL-2 through the intermediate affinity IL-2 receptor, composed of IL-2Rβ and IL-2Rγ (136, 137). Interestingly, to prevent the IL-2 critical signal on Tregs, IL-2/anti-IL-2 antibody complexes, formed by an anti-IL-2 antibody acting as a CD25 mimotope hampering IL-2 fixation to CD25, were used to redirect IL-2 action to CD8+ T and NK cells (138). Of note, mutant IL-2 proteins have been designed to bear reduced binding affinity to CD25 and preserved affinity for IL-2Rβ, endowing them with preferential action on NK and CD8+ T cells. As for IL-2, depriving Tregs from TNF may also impair their function and improve antitumoral responses as detailed below. Thus, starvation of cytokine, such as IL-2, may emerge as a new firearm among the arsenal of immunotherapeutic strategies, which either alone or in combination, enrich the picture of immune checkpoint inhibitors available to fight cancers.

CAN WE TREAT CANCER BY TNF DEPRIVATION TO TARGET Tregs?

TNF Is Pro-Tumoral

As suggested by its name, TNF was described initially as a killer of cancer cells. We now know that this cytokine plays a complex role in cancer and tumor immunity because of its pleiotropic effect and the fact that it has two receptors. Actually, TNF is even considered mostly as a pro-tumor cytokine. Numerous mouse studies have shown that anti-TNF drugs reduced tumor growth in different types of cancers. This deleterious effect of TNF was further supported in TNF knockout mice that display reduced tumor growth (139–146). The individual role of TNFR1 and TNFR2 was assessed in knockout mice in some of these studies.

TNF/TNFR1 Interaction Promotes Carcinogenesis and Pro-Tumoral Inflammation

The pro-tumoral effect of TNF has been explained by different mechanisms (**Figure 5**). TNF may directly promote cell transformation by activating oncogenes and inducing DNA damage (147). It may stimulate cell proliferation favoring cell transformation and neovascularization that is critical in cancer development (144, 146, 148). TNF may also promote growth of tumors that benefit from inflammatory cytokines and chemokines by recruiting neutrophils and macrophages (139, 141, 145, 149). Also, TNF may favor tumor invasiveness and metastasis by stimulating matrix metalloproteinase production

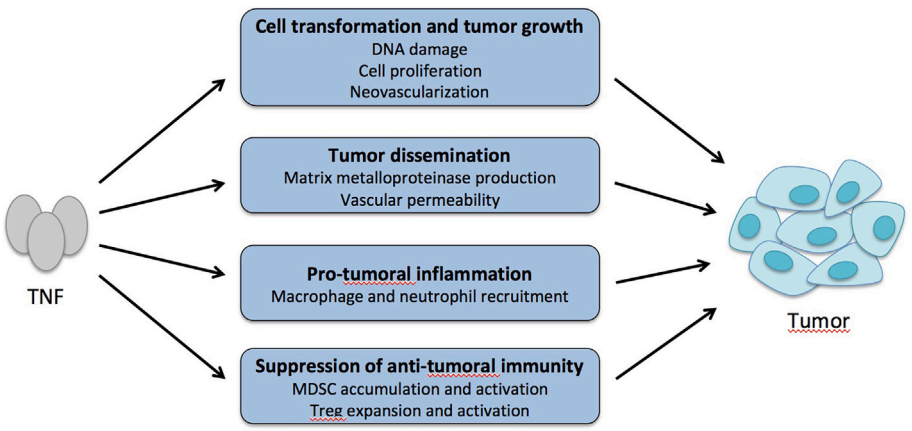

FIGURE 5 | Tumor necrosis factor α (TNF) is a pro-tumoral cytokine. TNF may promote cell transformation and tumor growth by increasing DNA damage and mutations, abnormal cell proliferation, and neovascularization. TNF may also favor tumor cell dissemination by increasing matrix metalloproteinase production and vascular permeability and leakiness. By recruiting macrophages and neutrophils in the tumor environment that release inflammatory cytokines and chemokines, TNF may promote growth of tumors that respond to these inflammatory factors. Finally, by boosting the activity of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) in the tumor environment, TNF may indirectly suppress antitumor immunity.

and vascular permeability (150). When analyzed, the role of TNFR1 rather than TNFR2 was involved in these different mechanisms.

TNF/TNFR2 Interaction Promotes Immunosuppression by Boosting MDSCs and Tregs

In mouse models of cancers, reduced tumor growth in mice treated by anti-TNF drugs or in TNFR2 knockout mice was associated with decreased numbers of MDSCs suggesting that TNF increases survival, recruitment, or function of MDSCs that suppress antitumor immunity (**Figure 5**) (20, 140, 143, 151). In a mouse model of melanoma, TNF injection favored tumor metastasis by acting on TNFR2-expressing hematopoietic cells, which was associated with an increase of Tregs (142). A similar mechanism was observed in models of colorectal cancer and hepatocarcinoma, since the tumor-dependent Treg expansion was abolished with an anti-TNFR2 mAb. Also, pretreatment of Tregs with TNF increased their capacity to suppress antitumor immunity after adoptive transfer (152).

In the setting of hematologic tumor relapse after allo-SCT, a similar approach using an anti-TNFR2 blocking mAb or TNFR2 antagonist could be considered to inactivate the deleterious effect of Tregs (**Figure 3B**). These molecules may be administered directly to the recipient to prevent or treat hematologic relapse, with or without a combined DLI, or even be used to preincubate donor PBMCs before infusion, to inactivate Tregs contained in the product.

What about the Role of TNF in Cancer Patients?

All the above studies were performed in mice. What do we know about the role of TNF in cancer in patients? It is well described that some cancers, such as colorectal cancer and hepatocarcinoma, benefit from chronic inflammation. Importantly, recent meta-analyses of patients receiving anti-TNF treatment because of their autoimmune diseases did not show an increased risk of cancer development (153, 154). Also, because of the beneficial effect of anti-TNF administration in preclinical mouse models, some patients with advanced cancers received TNF blockers. In this phase II trial, infliximab and etanercept were well tolerated (155, 156). The possible effects of these treatments have been studied *in vitro* or *in vivo* after xeno-transplantation in immunodeficient mice. Results indicated that blocking TNF may reduce tumor growth, which is associated with reduced tumor dissemination, angiogenesis, and infiltration with myeloid cells (157–159). Finally, TNF may suppress antitumor immunity by boosting Tregs *via* TNFR2 since high amounts of TNFR2+ Tregs were associated with more severe lung and ovarian cancer (160, 161).

It has to be emphasized that these studies that provide possible mechanisms to explain the supratumoral effect of TNF were only based on correlations or *in vitro* observations. None of them has provided definitive *in vivo* proofs because of the pleiotropic effect of TNF. This would have required, for instance, conditional deletion of TNFR in a cell subset. However, based on what is known on the effect of TNF on Tregs and of Tregs on antitumoral immunity (see above), the possibility that TNF inhibits antitumor immunity by boosting Tregs is a very attractive hypothesis that may play a major role in some cancer types.

CONCLUSIVE REMARKS

Immunotherapy of cancers is a promising land but unfortunately only a minority of patients responds to these treatments. Among multiple targets that are being tested, TNFR2 is an attractive one. Indeed, TNF blockade may have different impacts by limiting cell transformation, neovascularization, or pro-tumoral inflammation and may boost antitumor immunity by acting on MDSC or Tregs. Recent works suggest that targeting TNFR2-expressing Tregs would be a safe and efficient way to stimulate antitumor immunity. Future experiments and clinical trials are required to validate this new therapy.

AUTHOR CONTRIBUTIONS

BS, EP, and JC organized the plan and structure of the manuscript, and all the authors contributed to the redaction.

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An Autocrine TNFα–Tumor Necrosis Factor Receptor 2 Loop Promotes Epigenetic Effects Inducing Human Treg Stability *In Vitro*

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A crucial issue for Treg-based immunotherapy is to maintain a *bona fide* Treg phenotype as well as suppressive function during and after *ex vivo* expansion. Several strategies have been applied to harness Treg lineage stability. For instance, CD28 superagonist stimulation *in vitro*, in the absence of CD3 ligation, is more efficient in promoting Treg proliferation, and prevention of pro-inflammatory cytokine expression, such as IL-17, as compared to CD3/CD28-stimulated Treg. Addition of the mTOR inhibitor rapamycin to Treg cultures enhances FOXP3 expression and Treg stability, but does impair proliferative capacity. A tumor necrosis factor receptor 2 (TNFR2) agonist antibody was recently shown to favor homogenous expansion of Treg *in vitro*. Combined stimulation with rapamycin and TNFR2 agonist antibody enhanced hypo-methylation of the *FOXP3* gene, and thus promoting Treg stability. To further explore the underlying mechanisms of rapamycin and TNFR2 agonist-mediated Treg stability, we here stimulated FACS-sorted human Treg with a CD28 superagonist, in the presence of rapamycin and a TNFR2 agonist. Phenotypic analysis of expanded Treg revealed an autocrine loop of TNFα–TNFR2 underlying the maintenance of Treg stability *in vitro*. Addition of rapamycin to CD28 superagonist-stimulated Treg led to a high expression of TNFR2, the main TNFR expressed on Treg, and additional stimulation with a TNFR2 agonist enhanced the production of soluble as well as membrane-bound TNFα. Moreover, our data showed that the expression of histone methyltransferase EZH2, a crucial epigenetic modulator for potent Treg suppressor function, was enhanced upon stimulation with CD28 superagonist. Interestingly, rapamycin seemed to downregulate CD28 superagonist-induced EZH2 expression, which could be rescued by the additional addition of TNFR2 agonist antibody. This process appeared TNFα-dependent manner, since depletion of TNFα using Etanercept inhibited EZH2 expression. To summarize, we propose that an autocrine TNFα–TNFR2 loop plays an important role in endorsing Treg stability.

Keywords: regulatory T cells, tumor necrosis factor receptor 2, TNFα, rapamycin, tumor necrosis factor receptor 2 agonist antibody, Treg stability

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INTRODUCTION

$CD4^+FOXP3^+$ regulatory T cells (Treg) inhibit autoreactive effector T cells (Teff) and are important for immune homeostasis. The absence of Treg leads to lethal autoimmune disease in mice and humans, thereby highlighting their critical role in preventing autoimmunity (1). Notwithstanding the first successes of translation of Treg-based cell therapy into the clinic, a critical concern in

utilizing Treg is their stability. Treg lineage stability is defined by a stable expression of the transcription factor FOXP3, a highly demethylated Treg-specific demethylation region (TSDR), potent suppressive capacity and lack of pro-inflammatory cytokine production (2, 3). For the efficacy of Treg-based immunotherapy, the development of optimal *ex vivo* expansion protocols that yield high numbers of stable Treg is a prerequisite. Standard expansion protocols using anti-CD3/anti-CD28 mAb-coated microbeads plus exogenous rhIL-2 not only lead to high cell yields (4, 5) but also reveal Treg plasticity, whereby Treg loose FOXP3 and start producing IL-17A and IFNγ (6–8). Stimulating Treg with an anti-CD28 superagonist antibody (CD28-SA) results in efficient Treg expansion and reduced pro-inflammatory cytokine production *in vitro* (9). Since Treg are less susceptible to rapamycin-mediated inhibition of cell proliferation as compared to non-Treg cells, this mTOR inhibitor is often added to Treg expansion cultures to increase the purity of the final cell product (10–16). However, rapamycin does limit Treg growth both *in vitro* and *in vivo* (17, 18). It is of interest to note that the combined addition of a tumor necrosis factor receptor 2 (TNFR2) agonistic monoclonal antibody and rapamycin not only rescues rapamycin-mediated inhibition of Treg proliferation but also leads to a highly homogenous Treg phenotype as well as a stable suppressive function upon expansion (19, 20).

TNFα is initially expressed on cell surface as a membrane-bound TNFα (mTNFα), which can be cleaved by a metalloprotease TNF-alpha converting enzyme (TACE) to generate soluble TNFα (sTNFα) (21). Both sTNFα and mTNFα bind to TNFR2, but only mTNFα is capable to fully activate TNFR2 downstream signal events including NFkB pathway, which is involved in cytokine storm, cell survival and proliferation (22, 23). TNFR2 is constitutively expressed on both murine and human Treg, and TNFR2$^+$ Treg are the most suppressive Treg subpopulation (24–27). The TNFα–TNFR2 interaction is required for Treg mediated suppression in a mouse model of autoimmune-mediated colitis (28, 29). Several studies demonstrated that sTNFα preserved or even increased FOXP3 expression, as well as Treg suppressive capacity in both mice and humans (19, 25, 30, 31). But anti-TNF therapy of patients with active rheumatoid arthritis restored FOXP3 expression as well as suppressive function (32). Notably, the high serum levels of TNFα were associated with increased peripheral Treg numbers in patients with colorectal cancer and hepatocellular carcinoma, where blockade of TNFα/TNFR2 signals inhibited Treg cell expansion and benefited cancer therapy (33), thereby indicating that TNFα is capable of mediating Treg expansion.

Treg lineage stability is ultimately maintained by sustained expression of FOXP3 and Treg-specific epigenetic modification patterns (34). In response to inflammatory cues, FOXP3 recruits the histone methyltransferase EZH2 at the FOXP3-bound loci and selectively deposits the transcriptional suppression mark trimethylation of histone H3 at lysine 27 (H3K27me3) (35). In mice, it was shown that EZH2 expression was induced in a CD28-dependent manner and the mutant mice bearing Treg-specifically depletion of EZH2 developed fetal multi-organ autoimmunity with excessive T cell activation (36). Of note, EZH2-deficient FOXP3$^+$ murine T cells secreted pro-inflammatory cytokines (37). It is not yet clear whether human Treg show similar EZH2 expression metrics. Microarray analysis of human naïve T cells revealed that *EZH2* gene was the most highly induced CD28-dependent chromatin modifier (36).

Having previously established that a CD28 superagonist mAb (CD28-SA) acts as a very effective stimulus to support efficient Treg expansion (9), and that the combined use of rapamycin and TNFR2 agonist enhanced the demethylation of TSDR, thus harnessing Treg stability (20), we further explored Treg *ex vivo* stimulation and maintenance of stability by combining CD28 superagonist mAb, rapamycin and TNFR2 agonist mAb. We found that the harnessing effect of rapamycin and TNFR2 agonist on Treg stability was achieved through an autocrine loop of TNFα *via* TNFR2, whereby rapamycin enhanced TNFR2 expression and TNFR2 agonist increased the production of TNFα. Moreover, our data demonstrated that, similar to murine Treg, the histone methyltransferase EZH2 was induced in human Treg upon CD28 superagonist stimulation. Intriguingly, the combined addition of rapamycin and TNFR2 agonist maintained EZH2 expression in a TNFα-dependent manner.

MATERIAL AND METHODS

Isolation of Human Treg

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) of buffy coats that were purchased from Sanquin blood bank (Region South-East, Netherlands). All donors gave written informed consent for the use of these buffy coats for scientific research purposes, and according to Dutch law. CD4$^+$ T cells were enriched using the RosetteSep™ human CD4$^+$ T cell enrichment cocktail and processed according to manufacturer's recommendations (StemCell Technologies, Vancouver, BC, Canada). This typically resulted in a >95% purified CD4$^+$ T cell population in the absence of CD8$^+$ cells. To obtain high purity Treg, subsequent FACS sorting of CD4$^+$CD25^{high} Treg was performed using a BD FACSAria cell sorter (BD Biosciences, Erembodegem, Belgium) after labeling CD4$^+$ cells with CD25/Pe-Cy7 (M-A251; BD Biosciences).

Treg Cell Culture

FACS-sorted CD4$^+$CD25^{high} Treg were cultured for 7 days with IL-2 (200 U/mL) containing medium alone as non-stimulated control, or together with different combinations of CD28 superagonist (CD28-SA, 1 μg/mL, Clone ANC28.1/5D10, Cat# 177-820, preservative free; Ancell, Bayport, MN, USA), rapamycin (Rap, 1 μM, Sigma-Aldrich, St. Louis, MO, USA), and TNFR2 agonist mAb (2.5 μg/mL, Clone MR2-1, Hycult, Netherlands). Exogenous recombinant human (rh) TNFα (50 ng/mL, R&D, Minneapolis, MN, USA) was used to replace TNFR2 agonist where indicated. Etanercept (10 μg/mL, ETN-Enbrel®, Pfizer) was added to cell culture for the depletion of TNFα. Cells were harvested at day 7 of culture for phenotypic analysis, and culture supernatants were collected and stored for the subsequent cytokine analysis.

Flow Cytometry and Antibodies

Cells were phenotypically analyzed using a multicolor flow cytometer Navios (Beckman Coulter, Mijdrecht, Netherlands). The following conjugated mAb were used: CD25/Pe-Cy7 (M-A251), HLA-DR/FITC (L243) (both from BD Bioscience); TIGIT/PE (MBSA43, eBioscience, Vienna, Austria), CD3/ECD (UCHT1), CD4/PE-Cy5.5 (1388.2), CD8/APC-AF700 (B9.11) (all from Beckman Coulter), TNFR2/APC (#22235; R&D), and Fixable Viability Dye eFluor780 (eBioscience). To detect the expression of mTNFα, cells were first stained with biotin-labelled Infliximab followed with APC-conjugated streptavidin (eBioscience). For intracellular staining, EZH2/PE (11/EZH2, BD Bioscience), FOXP3/eFluor 450 (PCH101), and Helios/AlexFluor 647 (22F6) (both from eBioscience) were used after fix-perm-treatment of cells, according to the manufacturer's instructions. Isotype matched control antibodies were used to define marker settings. Data were analyzed using the software Kaluza (Beckman Coulter).

Cytokine Detection Assay

IL-17A, IFNγ, and TNFα were determined in the culture supernatants using Luminex cytokine assays (Invitrogen), according to the manufacturer's instructions. The lower levels of detectable cytokines were IL-17A (2 pg/mL), IFNγ (2.3 pg/mL), and TNFα (2.3 pg/mL).

Coculture Suppression Assays

FACS-sorted Treg cells were cultured under the stimulation conditions described above. Thereafter, cultured Treg were collected at day 7 of culture, washed, and added at different ratios to CFSE-labeled $CD4^+CD25^-$ responder T cells (Tresp). Coculture mixture was stimulated with anti-CD3/anti-CD28 mAb-coated microbeads at a bead-to-cell ratio of 1:5 for 3 days before analyzing the dilution of CFSE using flow cytometry.

Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted by using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany) followed by cDNA synthesis using the SuperScript III First-Strand Synthesis System and Oligo(dT)20 primers (Thermo Fisher Scientific, Waltham, MA, USA). Taqman gene expression assays were purchased from Thermo Fisher Scientific (see Table S1 in Supplementary Material). RT-qPCR cycle values (CT) obtained for specific mRNA expression in each sample were normalized to the CT values of the housekeeping gene HPRT1 (endogenous control). The relative mRNA expression of gene interested was calculated using $2^{-\Delta CT}$ formula.

Statistics

Statistical analysis was performed using the GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences were calculated using the Wilcoxon matched pairs signed rank test, or the non-parametric Friedman test or Kruskal–Wallis test plus Dunn's *post hoc* test for multiple comparisons, where applicable. Differences were considered statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

RESULTS

Rapamycin Increases the Expression of TNFR2 on CD28 Superagonist-Stimulated Treg

Tumor necrosis factor receptor 2 is known to be crucial for phenotypic and functional stability of Treg, especially in an inflammatory environment (29). We thus started off by examining the expression level of TNFR2 on human FACS-sorted $CD4^+CD25^{high}$ Treg stimulated with a CD28 superagonist mAb (CD28-SA) in the presence or absence of Rap and/or TNFR2 agonist. Treg cultured in IL-2 containing medium alone were used as non-stimulated control, and the expression of TNFR2 was determined by flow cytometry at day 7 of culture. In the absence of CD28-SA stimulation, TNFR2 agonist itself neither revealed a potential cytotoxic effect on cultured Treg nor the regulation of TNFR2 expression as compared to Treg cultured under the medium control condition (Figure S1A–B in Supplementary Material). Stimulation of Treg using CD28-SA significantly enhanced the expression of TNFR2 (92.5 ± 2.9 vs. $70.5 \pm 3.1\%$ for medium control, $p < 0.05$), while the addition of Rap to CD28-SA stimulated Treg resulted in the highest expression level of TNFR2, both in frequency ($95.5 \pm 1.7\%$; $p < 0.01$) and in median fluorescence intensity [median fluorescent intensity (MFI), 18.9 ± 3.9 vs. 2.1 ± 0.2 for medium control; $p < 0.001$] (**Figure 1A**). The addition of TNFR2 agonist to CD28-SA stimulated Treg hardly affected TNFR2 expression as compared to CD28-SA (MFI, 6.10 ± 1.2 vs. 7.93 ± 1.1, $p > 0.05$). Surprisingly, Treg cultured with the triple combination of CD28-SA + Rap + TNFR2 agonist expressed a similar level of TNFR2 ($77.9 \pm 6.3\%$) as that observed for control Treg ($70.5 \pm 3.1\%$, $p > 0.05$) (**Figure 1A**). The potential cytotoxic effect of TNFR2 agonist on cultured Treg is unlikely as we observed similar cell viability under all conditions tested (Figure S2 in Supplementary Material). Interference of the TNFR2 agonist with the subsequent detection of TNFR2 in this case was also not likely, as we selectively chose an APC-conjugated anti-TNFR2 mAb (Clone #22235) derived from a different clone than the TNFR2 agonist (Clone MR2-1). FITC-conjugated TNFR2 mAb derived from the same clone MR2-1 as TNFR2 agonist used in Treg culture failed to detect any expression of TNFR2, whereas APC-conjugated TNFR2 did (Figure S3 in Supplementary Material). Instead, we propose that binding of TNFR2 agonist might have caused the internalization of the TNFR2–ligand complex (38), leading to lower levels of detection. The rapamycin enhanced TNFR2 expression was also reflected at mRNA level since the highest *TNFRSF1B* (TNFR2) mRNA was observed under the condition of CD28-SA + Rap (**Figure 1B**). Taken together, the data suggest that Rap increases TNFR2 expression on Treg following cell stimulation.

The Addition of Rap and TNFR2 Agonist to CD28 Superagonist-Stimulated Treg Initiates an Autocrine TNFα–TNFR2 Loop

Loss of Treg stability implies that Treg acquire the capacity to produce effector cytokines upon stimulation. We therefore measured the amount of IL-17A, IFNγ, and TNFα in the culture

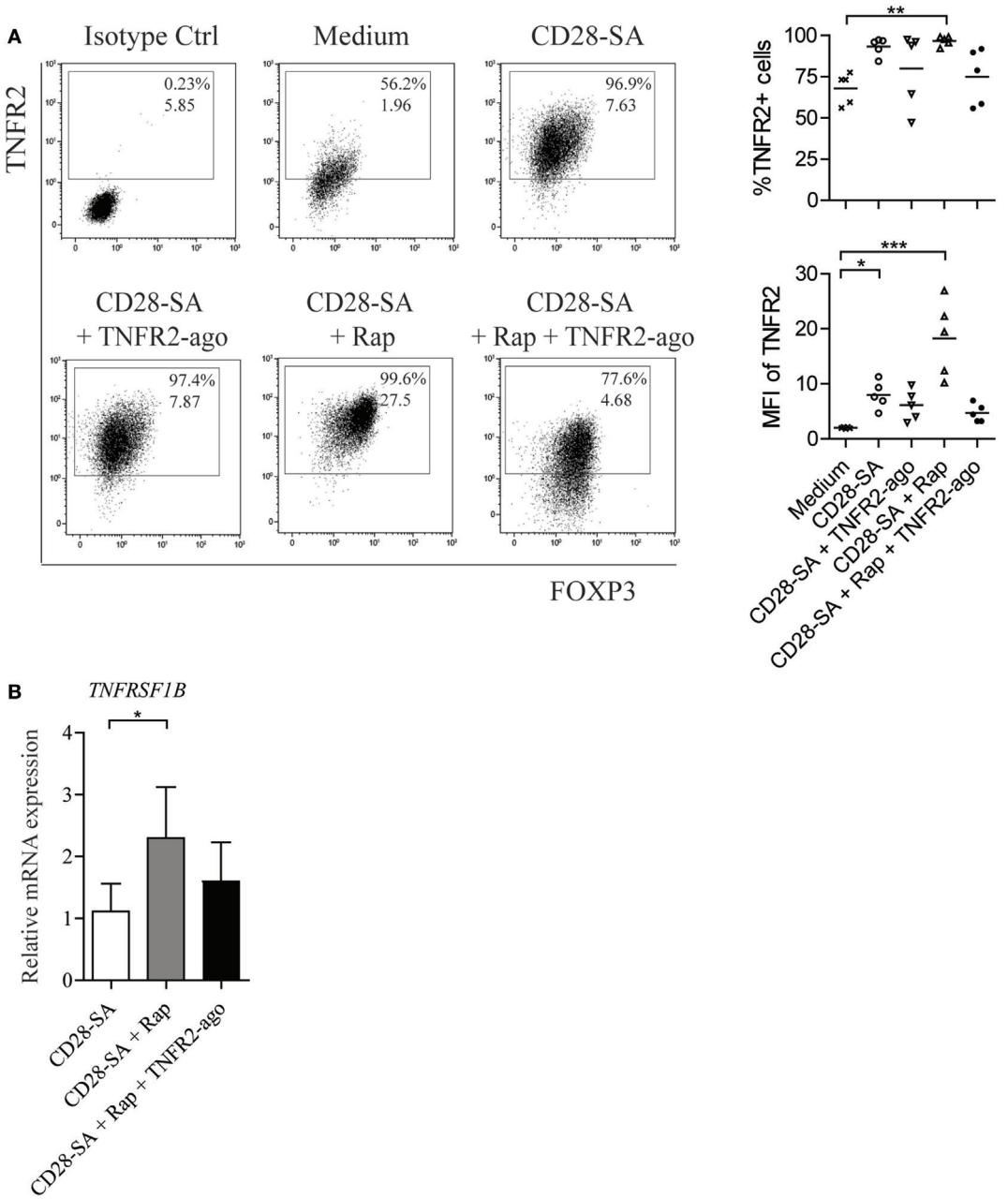

FIGURE 1 | Rapamycin increases TNFR2 expression on human Treg following the stimulation with CD28 superagonist. Flow cytometry of TNFR2 expression on human Treg that were expanded for 7 days under the indicated conditions (legends): non-stimulated medium control (Medium), stimulation with CD28 superagonist mAb (CD28-SA) with and without rapamycin in the absence or presence of TNFR2 agonist mAb (CD28-SA, CD28-SA + TNFR2-ago, CD28-SA + Rap, CD28-SA + Rap + TNFR2-ago). **(A)** Dot plots show TNFR2 vs. FOXP3 expression of one representative donor. Cumulative data showing the percentage as well as the median fluorescent intensity (MFI) of TNFR2 expression on Treg cultured under the conditions as indicated on the *X*-axis. $N = 5$. Lines show the mean values. **(B)** Relative mRNA expression of *TNFRSF1B* in Treg stimulated with the conditions as described on the *X*-axis. $N = 4$. All data are shown as mean ± SEM. Friedman with Dunn's *post hoc* test was used for statistical analysis. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$).

supernatants of Treg that were stimulated under distinct conditions. Neither Treg cultured in medium control condition nor that cultured in the presence of TNFR2 agonist produced any cytokines (Figure S1C in Supplementary Material), whereas upon CD28-SA stimulation, Treg started to produce low, but detectable amounts of IL-17A, IFNγ, and TNFα. The addition of Rap to the culture prevented CD28-SA stimulated Treg to produce IL-17A (0.1 ± 0.1 vs. 16.2 pg/mL ± 6.7, $p < 0.05$), as well as TNFα (9.7 ± 3.9 vs. 28.6 pg/mL ± 9.1, $p < 0.01$), but it marginally affected IFNγ production (**Figure 2A**). The addition of TNFR2 agonist to the culture minimally regulated CD28-SA induced IL-17A and IFNγ production, whereas it increased the amount of TNFα

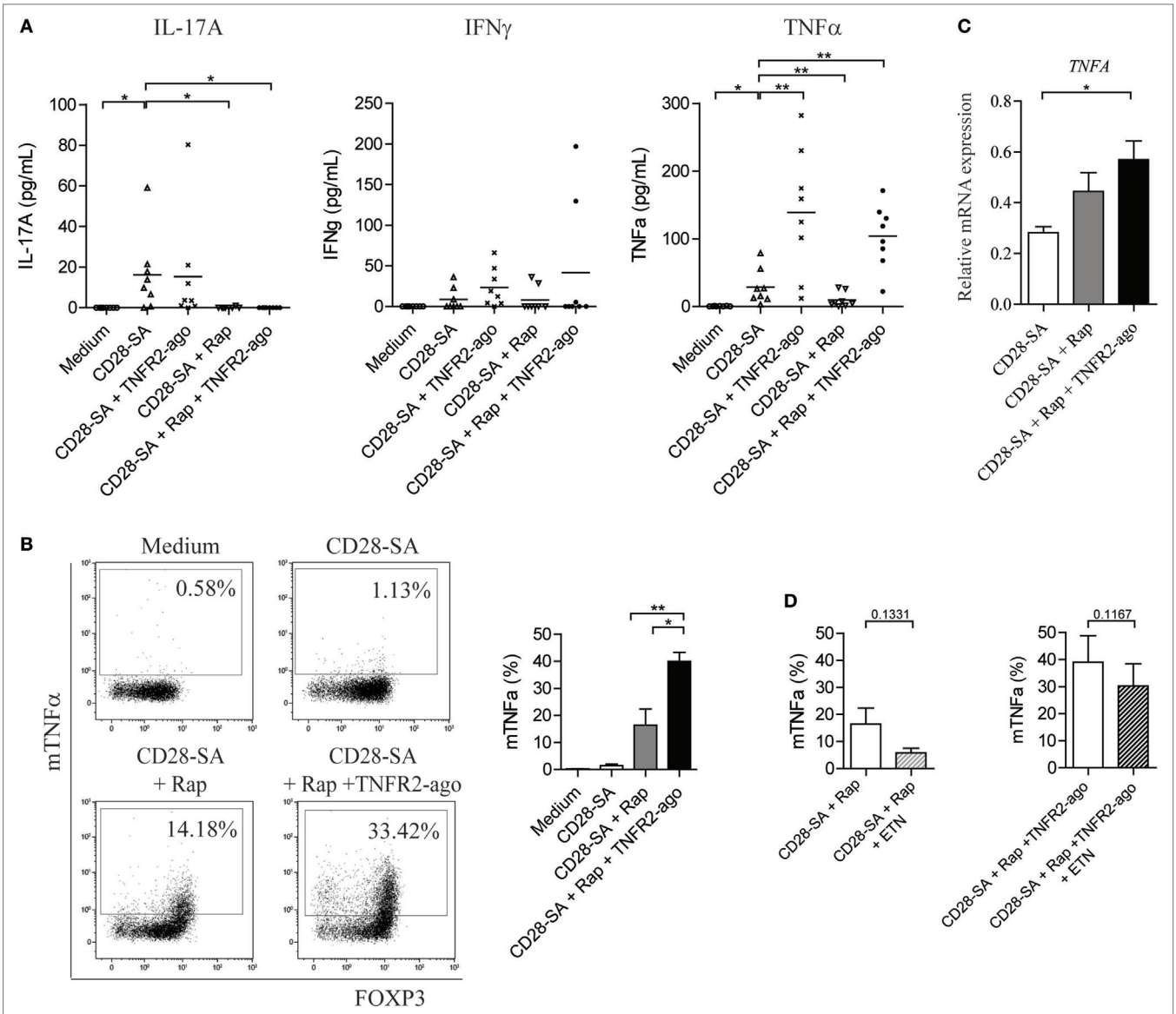

FIGURE 2 | Addition of rapamycin and TNFR2 agonist to CD28-superagonist stimulated Treg initiates an autocrine TNFα–TNFR2 loop. FACS-sorted human Treg were stimulated for 7 days as indicated on the *X*-axis; the culture supernatants were collected at day 7 and the presence of cytokines were determined using Luminex. **(A)** Cumulative data showing the amount of IL-17A, IFNγ, and TNFα produced by Treg cultured as described on the *X*-axis. *N* = 8. **(B)** Flow cytometry analysis of membrane-bound TNFα (mTNFα) as well as FOXP3 expression at day 7 of culture. Dot plots showing one representative donor. Graph shows the cumulative data. *N* = 4. Percentage of mTNFα-positive cells is indicated in the dot plots. **(C)** Relative mRNA expression of *TNFA* in Treg stimulated with the conditions as described on the *X*-axis. *N* = 4. **(D)** FACS-sorted human Treg were stimulated with CD28-SA + Rap or CD28-SA + Rap + TNFR2 agonist in the presence or absence of the TNFα-blocking agent Etanercept (ETN). Cumulative data showing the percentage of mTNFα$^+$ cells at day 7 of culture. *N* = 3. All data are shown as mean ± SEM. Friedman with Dunn's *post hoc* test were used for statistical analysis. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

(139.0 ± 32.85 vs. 28.62 pg/mL ± 9.1, $p < 0.05$). Notably, adding TNFR2 agonist to Rap treated CD28-SA stimulated Treg resulted in a similar high amount of TNFα (103.7 pg/mL ± 16.4) as that of Treg stimulated with CD28-SA + TNFR2 agonist (**Figure 2A**).

Soluble TNFα is derived from its precursor mTNFα, whereby mTNFα is cleaved by the TNFα-converting enzyme TACE to release its extracellular C-terminal portion (21). To test whether the enhanced soluble TNFα production was due to the increased conversion from its precursor, we analyzed the expression of mTNFα on Treg cultured under different combinations of CD28-SA, Rap, and TNFR2 agonist. At day 7 of culture, few mTNFα$^+$ cells were detected on CD28-SA stimulated Treg (1.5 ± 0.5%), whereas addition of Rap enhanced mTNFα expression (16.4 ± 5.9%). However, triple stimulation with CD28-SA + Rap + TNFR2 agonist further promoted the frequency of mTNFα$^+$ cells (40.0 ± 3.3%, $p < 0.05$, **Figure 2B**). Similarly, the highest expression of *TNFRA* (TNFα) mRNA was observed under triple stimulation with CD28-SA + Rap + TNFR2 agonist (**Figure 2C**).

This data indicates that the combined addition of Rap and TNFR2 agonist to CD28-SA stimulated Treg did increase their capacity to produce more TNFα. So, Rap treatment increased the expression of TNFR2 on CD28-SA stimulated Treg cells, while the additional treatment with a TNFR2 agonist significantly enhanced TNFα production. This might well result in an autocrine loop of TNFα *via* TNFR2, thus leading to stabilization of the Treg phenotype.

To find further support for this autocrine TNFα–TNFR2 loop, we depleted TNFα by using Etanercept. As shown in **Figure 2D**, regardless of the stimulation condition used, extra addition of Etanercept resulted in decreased mTNFα expression, albeit not statistically significant. Taken together, the data show that there is a positive feedback loop in the regulation of TNFα cytokine production upon TNFα–TNFR2 interaction.

The TNFα–TNFR2 Interaction Is Required for a Homogenous Treg Phenotype

Potent Treg function is associated with high expression of specific cell markers, including Treg lineage transcription factor FOXP3, Helios, and the co-inhibitory receptor TIGIT (39–41). We thus performed phenotypic analysis of Treg that were cultured for 7 days under distinct stimulatory conditions. TNFR2 agonist itself hardly influenced the Treg phenotype (Figure S1D in Supplementary Material). When cells were stimulated with CD28-SA, the addition of Rap preserved or even slightly increased the expression of CD25 as well as FOXP3, Helios, and TIGIT (**Figure 3A**). Intriguingly, the addition of TNFR2 agonist to CD28-SA stimulated Treg clearly enhanced the expression of HLA-DR (81.5 ± 5.9 vs. 44.8 ± 3.9%, $p < 0.01$) while it hardly regulated other markers tested (**Figure 3A**). The combined addition of Rap and TNFR2 agonist to CD28-SA stimulated Treg significantly enhanced the frequency of the HLA-DR, TIGIT and Helios positive fractions (**Figure 3A**, $p < 0.01$), and preserved the high expression of FOXP3. Of note, when exogenous soluble rhTNFα was used instead of the TNFR2 agonist, we observed a similar expression of CD25, FOXP3, TIGIT, and Helios, but not of HLA-DR, which was only enhanced by the presence of the TNFR2 agonist (**Figure 3A**). Treg stimulated with CD28-SA + Rap + TNFR2 agonist were highly suppressive, as determined in *in vitro* suppression assays. We did not observe significant suppressive advantages as compared to the Treg that were cultured under the other stimulatory culture conditions (**Figure 3B**). Interestingly, depletion of TNFα under triple stimulation with CD28-SA + Rap + TNFR2 agonist significantly downregulated the expression of HLA-DR, TIGIT, Helios, and FOXP3 (**Figure 3C**). These data further support the notion of an autocrine TNFα–TNFR2 feedback loop that promotes a homogeneous Treg population upon activation, whereby Rap enhances TNFR2 expression and TNFR2 agonist stimulation increases TNFα production.

The Addition of Rap and TNFR2 Agonist to CD28 Superagonist-Stimulated Treg Leads to Activation of NFκB Signal Pathway

To test the potential involved downstream signal pathways that were induced by triple stimulation with CD28-SA + Rap + TNFR2 agonist, we focused on NFκB pathway target genes using RT-qPCR analysis. Treg stimulated with CD28-SA or CD28-SA + Rap were also included. As shown in **Figure 4**, the addition of Rap to CD28-SA stimulated Treg led to the enhanced *RELA* (RelA) mRNA expression, whereas the combined addition of Rap and TNFR2 agonist significantly increased the NFκB pathway gene expression including *NFKB1* (NFκB1/p65), *NFKB2* (NFκB2/p50), *NFKBIA* (IkBα), and *RELB* (RelB). The data suggest that the activation of the NFκB pathway underlies the enhanced Treg stability mediated by the autocrine TNFα–TNFR2 loop.

TNFα–TNFR2 Signaling Regulates the Expression of Histone Methyltransferase EZH2

Recently, CD28-dependent induction of histone methyltransferase EZH2 was reported in murine Treg (36). In the same study, EZH2 was shown to be crucial for Treg lineage stability following cell activation. Here, we first performed a time kinetic analysis of EZH2 expression in human Treg stimulated with CD28 superagonist. From day 2 of culture, enhanced EZH2 expression was detected, and the highest frequency of EZH2-positive Treg was observed at day 7 (47.6 ± 5.5 vs. 2.9 ± 1.3% for medium control, $p < 0.001$) (**Figure 5A**). Thereafter, we focused on day 7 to analyze the effect of Rap and/or TNFR2 agonist on the expression of EZH2. As shown in **Figure 5B**, TNFR2 agonist itself slightly enhanced the expression of EZH2 (13.4 ± 2.8 vs. 2.9 ± 1.0% for medium control group, $p = 0.1250$). When Treg were stimulated with CD28-SA, addition of TNFR2 agonist to the culture minimally affected EZH2 expression (51.6 ± 10.3 vs. 51.0 ± 6.6% for CD28-SA condition), whereas addition of Rap decreased EZH2 expression (32.3 ± 5.9%) when compared to CD28-SA condition ($p < 0.05$). Of note, the combined addition of Rap and TNFR2 agonist resulted in a similar frequency of EZH2-positive cells (53.6 ± 6.3%) as compared to CD28-SA condition, suggesting that the presence of TNFR2 agonist could rescue Rap-mediated downregulation of EZH2. Thus, TNFR2 agonist induced signals were positively involved in the regulation of EZH2 expression. Indeed, when TNFα was depleted by adding Etanercept to triple stimulated (CD28-SA + Rap + TNFR2 agonist) Treg, the frequency of EZH2-positive cells was significantly decreased (**Figure 5C**). Altogether, the data indicate that EZH2 expression is modulated by TNFα–TNFR2-mediated pathways.

DISCUSSION

The limited number of circulating Treg and the instability and plasticity of Treg function are main issues that hamper successful application of Treg for clinical cell-based immunotherapy. In the past decades, several interventions have been used to optimize Treg *ex vivo* expansion protocols that not only maximize Treg proliferation but also maintain their potent suppressive function. Standard Treg expansion protocols include anti-CD3 and anti-CD28 mAb together with the exogenous addition of rhIL-2 cytokines (4). In the absence of anti-CD3, single stimulation of human Treg with a CD28 superagonist induces polyclonal

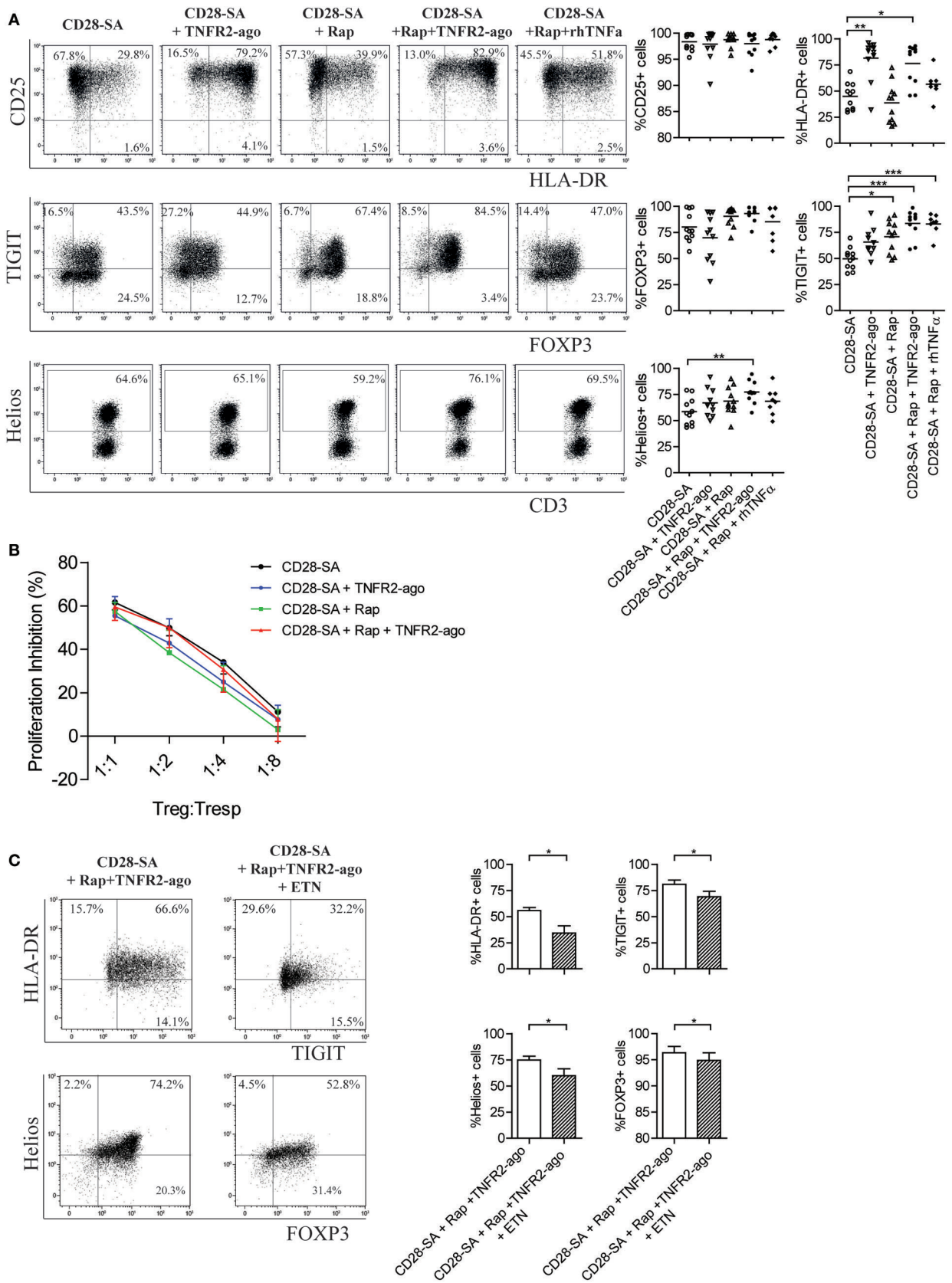

FIGURE 3 | Continued

FIGURE 3 | Addition of rapamycin and TNFR2 agonist to CD28-superagonist stimulated Treg cultures leads to a homogenous Treg phenotype that is dependent on the interaction of TNFα–TNFR2. **(A)** Flow cytometry of CD25, HLA-DR, TIGIT, FOXP3, and Helios expression on CD28-SA stimulated Treg that were additionally cultured with Rapamycin (Rap) with or without TNFR2 agonist or soluble rhTNFα as indicated. Dot plots show representative result of one blood donor. Cumulative data are given in the graphs. *N* = 8–11. Lines show the mean values. **(B)** Treg cultured under the indicated conditions (legend) were harvested at day 7 of culture, washed, allowed to recuperate, and analyzed for their suppressive capacity in a CFSE-based coculture suppression assay. *N* = 4. Friedman with Dunn's *post hoc* test were used for statistical analysis. **(C)** FACS-sorted human Treg were stimulated with CD28-SA + Rap + TNFR2 agonist in the presence or absence of the TNFα-blocking agent Etanercept (ETN). Dot plots showing TIGIT vs. HLA-DR, and Helios vs. FOXP3 expression of one representative experiment. Cumulative data are shown in the graph. *N* = 7. Numbers in dot plots show the percentage of positive cells. All data are shown as mean ± SEM. Friedman with Dunn's *post hoc* test was used for statistical analysis. Asterisks indicate significant differences (**p* < 0.05, ***p* < 0.01, or ****p* < 0.001).

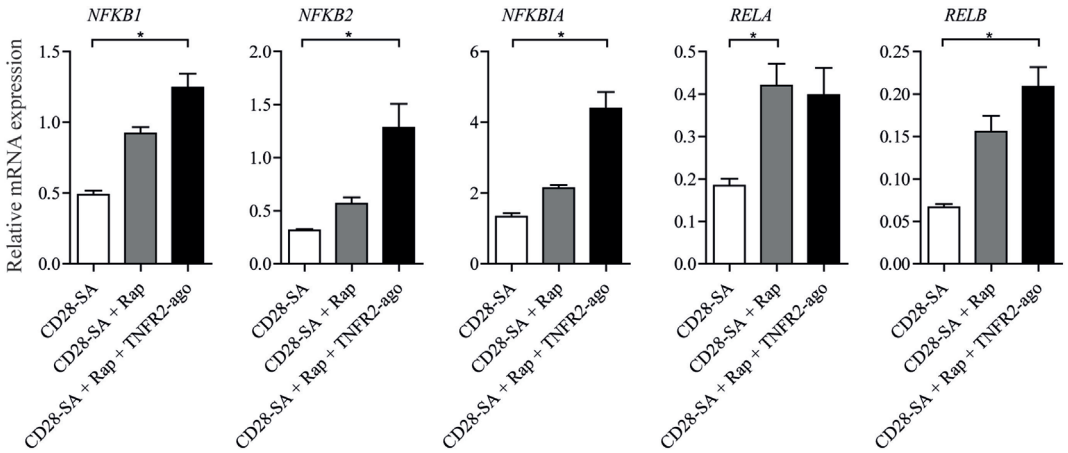

FIGURE 4 | Addition of rapamycin and TNFR2 agonist to CD28-superagonist stimulated Treg cultures results in the activation of the NFκB pathway. FACS-sorted Treg cells were stimulated using CD28-SA, CD28-SA + Rap, or CD28-SA + Rap + TNFR2 agonist for 4 days. The mRNA expression of NFκB family of members was analyzed using RT-qPCR. All data are shown as mean ± SEM. *N* = 4. Friedman with Dunn's *post hoc* test was used for statistical analysis. Asterisks indicate significant differences (**p* < 0.05).

expansion of Treg with enhanced Treg stability (9). The mTOR inhibitor rapamycin enhances FOXP3 expression, preserves Treg stability, and increases Treg suppressor capacity *in vitro* as well as *in vivo*, but rapamycin also inhibited Treg cell proliferation (15, 17, 42, 43). Previously, we showed that the combined addition of rapamycin and TNFR2 agonist to Treg cell culture facilitates *ex vivo* expansion of Treg (20). In this study, we found that rapamycin enhanced the expression of TNFR2 on activated Treg and that the additional supplementation of a TNFR2 agonist enhanced the production of TNFα. This resulted in a positive autocrine feedback loop of TNFα–TNFR2 signaling that promotes Treg stability as indicated by the high expression of FOXP3, Helios, and EZH2, and the low production of the pro-inflammatory cytokine IL-17A. Despite this increased expression of FOXP3, Helios, and EZH2, we did not observe an increase in suppressor potential. This is remarkable, since our group and others have demonstrated before that TNFα–TNFR2 stimulation increases Treg function in both humans and mice (19, 20, 44). Previously, we reported that Treg stimulated with CD3/CD28-microbeads + Rap + TNFR2 agonist hardly produced IL-17A and IFNγ, and these cells revealed superior suppressive activity at a Treg:Tresp ratio of 1:8 as compared to CD3/CD28 or CD3/CD28 + Rap-stimulated Treg (20). In our current study, CD28-SA + Rap + TNFR2 agonist-treated Treg showed similar suppressive capacity as Treg treated with CD28SA, CD28SA + Rap, or CD28SA + TNFR2 agonist. It seems that TNFR2-mediated signals somehow interact with T cell receptor/CD3 induced downstream targets and promote Treg suppressor function. Furthermore, Treg display their immunosuppressive function *via* controlling T cell proliferation and cytokine production, as well as regulating the stimulatory capacity of antigen presenting cells. Especially, inhibition of T cell effector function can occur independently of suppression of proliferation (45, 46). Loss of Treg lineage commitment is often reflected by the decreased expression of Treg markers on their progenies, which mostly occurs following several rounds of stimulation.

Tumor necrosis factor receptor 2 plays a crucial role in Treg cell biology. Both human and murine Treg constitutively express high levels of TNFR2 as opposed to non-Treg cells. The interaction of TNFα–TNFR2 promoted both Treg proliferation and their suppressor capacity (25, 47). Stimulation of TNFR2 using a TNFR2 agonist antibody resulted in a homogenous expansion of human Treg (19, 20). Interestingly, we here demonstrate that rapamycin enhanced the expression of TNFR2 on activated human Treg, whereas it inhibited TNFα cytokine production. When TNFR2 agonist was added to rapamycin-treated Treg cell cultures, we found the preferential stimulation of Treg with high expression levels of HLA-DR, FOXP3, Helios, and TIGIT, as well

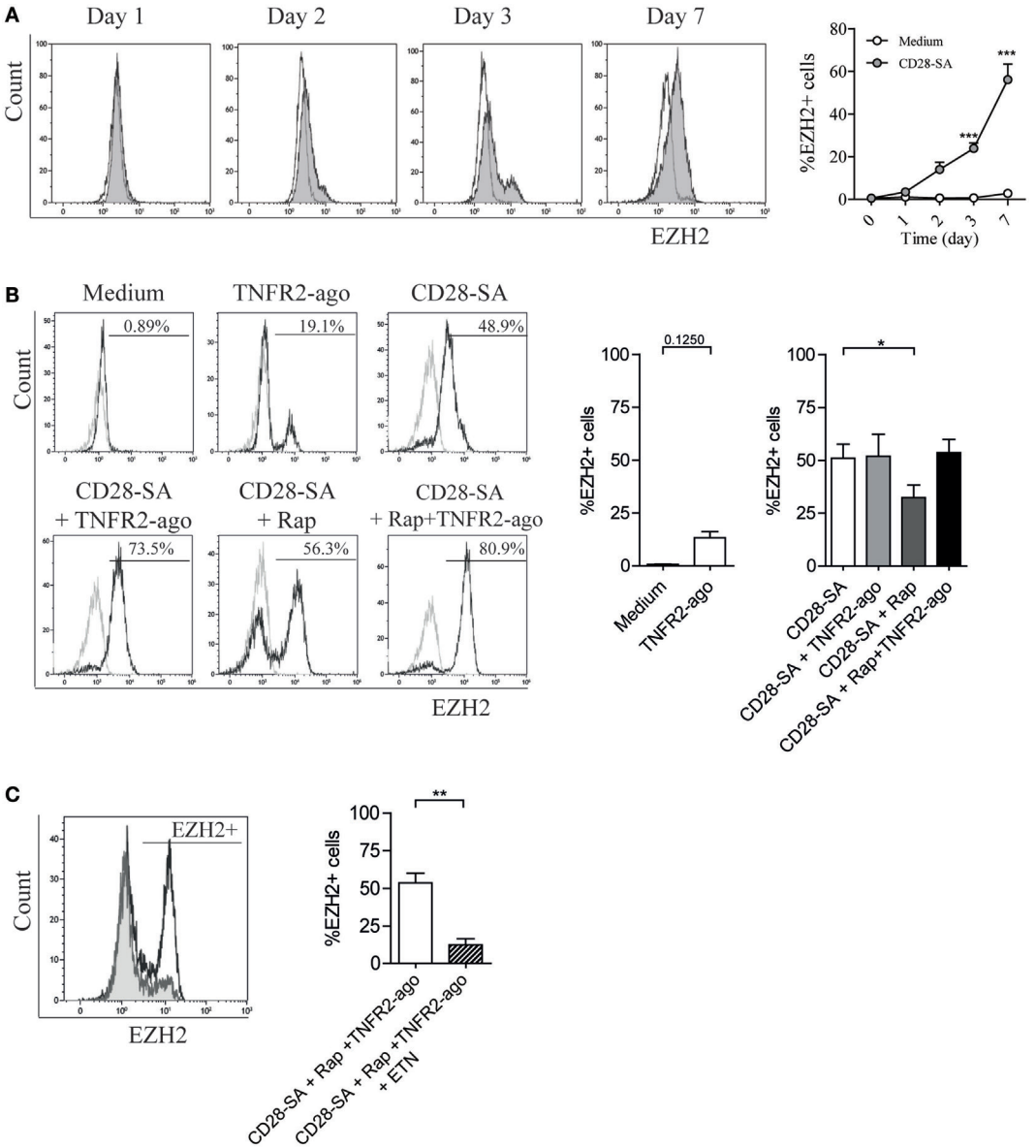

FIGURE 5 | CD28 superagonist induces EZH2 expression in human Treg and addition of rapamycin downregulates EZH2 frequency which can be rescued by TNFR2 agonist in a TNFα-dependent manner. Flow cytometry of EZH2 expression in FACS-sorted human Treg that were cultured for 7 days in medium (Medium) or stimulated with CD28 superagonist mAb (CD28-SA) with or without Rapamycin (Rap) in the absence or presence of TNFR2 agonist mAb, or the TNFα blocker Etanercept (ETN). **(A)** Representative experiment showing EZH2 expression at different days in time (indicated at the top). Graph shows cumulative data. $N = 3$. Open circle: medium control; shaded circle, CD28-SA stimulation. **(B)** Representative overlay histograms showing EZH2 expression at day 7 of the culture. Graph shows cumulative data. $N = 12$. Numbers in the overlay histograms show the percentage of EZH2-positive cells. Gray line: isotype control; black line, Treg expanded under conditions where described at the top. $N = 4$ for the medium control and TNFR2 agonist conditions, $N = 8$ for CD28-SA + TNFR2 agonist condition, and $N = 12$ for other conditions. Kruskal–Wallis with Dunn's *post hoc* test was used for statistical analysis. **(C)** Representative overlay histogram showing EZH2 expression in Treg that were stimulated with CD28-SA + Rap + TNFR2 agonist together with or without ETN. $N = 7$. Graph shows the cumulative data. Wilcoxon matched pairs signed rank test was used for statistical analysis. All data are shown as mean ± SEM. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$).

as a high TNFα-producing potential. That depletion of TNFα using Etanercept led to a reduction of Treg-associated markers including FOXP3, Helios, TIGIT, and EZH2 further supports a role for TNFα–TNFR2 signaling in the FOXP3 expression of Treg and the notion of an autocrine TNFα–TNFR2 feedback loop that promotes Treg stability. Consistent with our data, in an acute graft-versus-host disease (aGvHD) mouse model the treatment with a selective TNFR2 agonist led to the *in vivo* expansion of host Treg and the protection from aGvHD (48). Interestingly, the suppressive activity of Treg to control GvHD seems to depend on TNFα produced by donor T cells and TNFR2 expressed on Treg in allogeneic hematopoietic stem cell transplantation (49).

In response to TCR stimulation, $CD4^+FOXP3^-$ Teff as well as cytotoxic $CD8^+$ cells also upregulates TNFR2 expression. TNFR2-positive CD4 Teff are highly proliferative and more resistant to Treg-mediated inhibition (50). Intriguingly, TNFR2 agonism effectively and selectively induces the apoptosis of insulin-autoreactive $CD8^+$ cells in patients with type 1 diabetes (51). Therefore, specific TNFR2 agonism would have two desired cellular immune effects for treatment of autoimmune diseases: (1) selective death of autoreactive T cells and (2) expansion of beneficial Treg. The positive effect of TNF–TNFR2 activation on Treg numbers is also reported in cancer patients. For example, enhanced abundance of $TNFR2^+$ Treg and high TNFα serum level were reported in patients with ovarian cancer, lung cancer as well as colorectal cancer (33, 52, 53). In a mouse model of colorectal cancer, blockade of TNFα–TNFR2 signaling prevented rapid resurgence of Treg after cyclophosphamide-induced lymphodepletion and inhibited the growth of established tumors (33).

The effect of TNFα on human Treg is not yet fully clear. Oppenheim and colleagues showed TNF-induced Treg (iTreg) proliferation and survival *via* TNFR2 (25, 47) and $TNFR2^+$ Treg exhibited maximal suppressive capacity (29). In the context of autoimmunity, Treg suppressive function is optimized by pathogenic T cells and TNFα is one of factors involved in this optimization (54). Zaragoza et al. reported that TNFα together with IL-2 increased the expression CD25 and FOXP3 and maintained the suppressive activity of human Treg (31). In our cell culture system, we noticed that, upon stimulation with CD28-SA + Rap, the addition of exogenous soluble rhTNFα showed a similar effect as the addition of TNFR2 agonist on the maintenance of a *bona fide* Treg phenotype, whereas blocking TNFα signaling using Etanercept decreased the frequency of FOXP3-positive cells (**Figure 3C**). This supports the positive effect of TNFα on the Treg phenotype. Previously, TNFα was shown to downregulate Treg function since the TNFα-blocking agent Infliximab increased FOXP3 expression and restored their suppressive function in rheumatoid arthritis (RA) patients (32). However, a follow-up study demonstrated that iTreg, but not naturally occurring Treg (nTreg) were increased in RA patients following anti-TNF therapy (55). Of note, nTreg and iTreg differentially require TNFα signals for optimal suppressive function, at least in mice (28). Nie et al. showed that TNFα impaired Treg suppressive function *via* the dephosphorylation of FOXP3 protein (30). They also demonstrated that rhTNFα did not affect FOXP3 expression; instead, TNFα enhanced the expression of protein phosphatase PP1 which mediated FOXP3 dephosphorylation, thus rending the Treg defective. It is worth noting that anti-TNF therapy often results in psoriatic and lupus-like symptoms in patients being treated for other conditions (56); this suggests a direct correlation between TNF and immune suppression.

Metabolic changes directly modify T cell function. Signaling *via* PI3K–Akt–mTOR pathway facilitates the induction of glucose transporter Glut 1 and aerobic glycolysis in Teff (57). Interestingly, proliferative Treg cells have high mTOR activity as well as high glucose uptake together with downregulated FOXP3 expression and impaired suppressive capacity (58). FOXP3 expression is inversely related to Akt activity (59) and promotes mitochondrial oxidative metabolism. It would seem that Treg proliferation and suppressive function is regulated by separate metabolic pathways. Rapamycin induced retardation of Treg growth might be caused by the shifting of glycolysis metabolism to lipid oxidative metabolism *via* the inhibition of the PI3K–Akt–mTOR pathway and enhanced FOXP3 expression. Non-canonical NFκB activation upon TNFα stimulation is involved in T cell survival and differentiation (23). Here, we showed that the combined addition of rapamycin and TNFR2 agonist resulted in high expression of Treg associated marker, and activation of NFκB pathway. The autocrine feedback loop of TNFα and TNFR2 might fine-tune the metabolic balance between glycolysis and oxidative phosphorylation, thereby favoring homogenous Treg proliferation together with the preservation of potent suppressive function. Further experiments on metabolic pathway regulation are required to test this hypothesis.

Epigenetic mechanisms that alter chromatin organization are important to control the differentiation and maintenance of polarized T cell subsets. EZH2 functions primarily within the polycomb repressive complex 2 and catalyzes the trimethylation of lysine 27 on the exposed N-terminal tail of histone H3 (H3K27me3), a histone modification associated with repression of expression of nearby genes. EZH2, *via* the formation of a complex with FOXP3 in activated Treg, is crucial for proper Treg suppressive function since mutant mice bearing Treg-specific deletion of EZH2 developed fatal inflammation associated with massive T cell activation and cytokine production (35, 36). Mice that specifically lack EZH2 expression in Treg develop spontaneous inflammatory bowel disease (37), which further supports the crucial role of EZH2 for Treg function. Recently, human Treg were reported to express *EZH2* mRNA (60). In our current study, we demonstrate that CD28 superagonist stimulation induced EZH2 expression in human Treg, which was decreased by the presence of rapamycin, whereas the combined addition of rapamycin and TNFR2 agonist to Treg cultures maintained expression of EZH2 in a TNFα-dependent manner. Interestingly, the NFkB family of proteins RelA as well as c-Rel were reported to enhance luciferase activity in an *EZH2* reporter system, and c-Rel regulated the induction of *EZH2* gene expression in activated primary murine lymphocytes and human leukemia cell lines (61).

In summary, we showed that stimulation of human Treg using a triple combination of CD28 superagonist, rapamycin, and TNFR2 agonist leads to homogenous expansion of Treg that reveal a stable and suppressive phenotype. Mechanistically, rapamycin enhanced TNFR2 expression of the CD28 superagonist-stimulated Treg; the TNFR2 agonist promotes TNFα production and this supports an autocrine TNFα–TNFR2 feedback loop that favors high expression of TIGIT, FOXP3, Helios, and EZH2.

ETHICS STATEMENT

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) of buffy coats that were purchased from Sanquin blood bank (Region South-East, Netherlands). All donors gave written informed consent for the use of these buffy coats for scientific research purposes and according to Dutch law.

AUTHOR CONTRIBUTIONS

XH, PU, HK, and IJ designed experiments; XH and PU performed experiments and analyzed the data. XH, PU, HK, and IJ wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00573/full#supplementary-material.

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The Significance of Tumor Necrosis Factor Receptor Type II in CD8+ Regulatory T Cells and CD8+ Effector T Cells

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Tumor necrosis factor (TNF) is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory functions. The biological functions of TNF are mediated by two receptors, TNF receptor type I (TNFR1) and TNF receptor type II (TNFR2). TNFR1 is expressed universally on almost all cell types and has been extensively studied, whereas TNFR2 is mainly restricted to immune cells and some tumor cells and its role is far from clarified. Studies have shown that TNFR2 mediates the stimulatory activity of TNF on CD4+Foxp3+ regulatory T cells (Tregs) and CD8+Foxp3+ Tregs, and is involved in the phenotypic stability, proliferation, activation, and suppressive activity of Tregs. TNFR2 can also be expressed on CD8+ effector T cells (Teffs), which delivers an activation signal and cytotoxic ability to CD8+ Teffs during the early immune response, as well as an apoptosis signal to terminate the immune response. TNFR2-induced abolition of TNF receptor-associated factor 2 (TRAF2) degradation may play an important role in these processes. Consequently, due to the distribution of TNFR2 and its pleiotropic effects, TNFR2 appears to be critical to keeping the balance between Tregs and Teffs, and may be an efficient therapeutic target for tumor and autoimmune diseases. In this review, we summarize the biological functions of TNFR2 expressed on CD8+Foxp3+ Tregs and CD8+ Teffs, and highlight how TNF uses TNFR2 to coordinate the complex events that ultimately lead to efficient CD8+ T cell-mediated immune responses.

Keywords: tumor necrosis factor, tumor necrosis factor receptor type II, CD8+ regulatory T cells, CD8+ effector T cells, CD4+ regulatory T cells

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INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic cytokine involved in regulating diverse functions, including cell growth modulation, viral replication, septic shock, tumorigenesis, inflammation, and autoimmunity (1, 2). These functions hinge upon the binding of TNF to two distinct membrane receptors on target cells: TNF receptor (TNFR) 1and TNFR2. TNFR1 is expressed universally on almost all cell types, whereas TNFR2 is restricted to immune cells (2–6) and some tumor cells (7–13). Since TNFR1 and TNFR2 were identified (14), multiple studies have been carried out to characterize their structures and functions. While TNFR1 has been extensively characterized, the biological functions of TNFR2 have remained elusive (15). There is mounting evidence to suggest that TNFR2 is expressed on and has critical roles in immune cells, including CD4+ regulatory

T cells (Tregs) (16), CD4+ effector T cells (Teffs) (4), CD8+ Tregs (17), and CD8+ Teffs (18). This implies that TNFR2 is involved in various T cell-mediated immune responses. TNFR2 expressed on CD4+ T cells has been studied in depth with many studies indicating that TNFR2 mediates the stimulatory activity of TNF on CD4+ Treg cells, resulting in their phenotypic stability, proliferation, and activation (3, 19–22). Furthermore, TNFR2 can be used to identify the maximally suppressive subset of CD4+ Tregs (20). However, studies on TNFR2 expression on CD8+ T cells are relatively deficient. Several studies have identified TNFR2 as a potent costimulatory molecule on CD8+ T cells required to sustain cell survival and protect from apoptosis, while TNFR2 expressing CD8+Foxp3+ Tregs exhibited highly suppressive activity (17, 23, 24).

The restricted distribution of TNFR2 has identified it as a potential target for immunotherapy. Targeting TNFR2 for cancer immunotherapy has seen remarkable success. Treatment of OVVAR3, an ovarian cancer cell line with surface expression of TNFR2, with a TNFR2 antagonist induced significant tumor cell death. Furthermore, the TNFR2 antagonist preferentially suppressed the activity of tumor-associated CD4+ Treg cells, but had little inhibitory effects on peripheral CD4+ Treg cells or cells from healthy donors (25). This result indicates that patients treated with a TNFR2 antagonist can maintain immunological homeostasis and mitigate the collateral damage to healthy tissues (20, 25). While the potential effects of TNFR2 antagonists on tumors have been documented, major questions remain unanswered, including how much the effects of therapeutically targeting TNFR2 *in vivo* are directly related to modulating T cell activity. Better knowledge of the fundamental biological processes, such as signaling pathway activation and the molecular mechanism underlying the T cell response to TNFR2 stimulation, especially in Treg cells, may help design safer and more effective targeted therapeutics. As TNFR2 expression on CD4+ T cells has been documented in detail, in this review, we mainly summarize and discuss the biological effects of TNFR2 expression on CD8+Foxp3+ Tregs and CD8+ Teffs.

TNFR2 EXPRESSED ON CD8+ Tregs

The suppressive effects of CD8+ Tregs on normal and pathologic immune responses are well described (**Figure 1**) (26–28). Previous study demonstrated that human CD8+CD25+ Tregs share many features with CD4+CD25+ Tregs in the thymus, such as phenotype, function, and mechanisms of action (23). Increasing evidence suggests that TNFR2 is a significant biomarker for highly potent suppressive Tregs, because TNFR2 promotes the activation, expansion, and survival of CD4+ Tregs by mediating

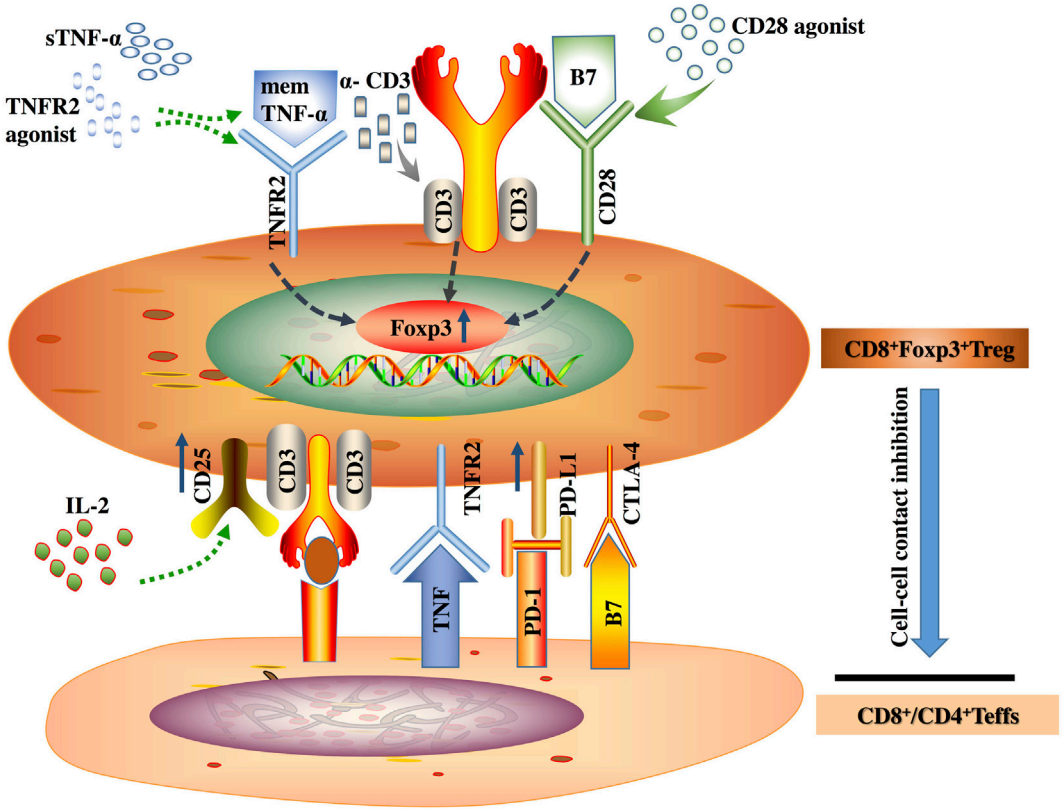

FIGURE 1 | Tumor necrosis factor (TNF) receptor type II (TNFR2) acts as a suppressive marker for CD8+ regulatory T (Tregs) cells. The TNF/TNFR2 interaction, as well as TNFR2 and CD28 agonists, could promote the induction of Foxp3 in the presence of anti-CD3. Additionally, the TNF/TNFR2 interaction could also upregulate CD25 and PD-L1, the negative molecules on the surface of CD8+ Tregs, to mediate a contact-dependent inhibition to CD4+ and CD8+ effector T cells, cooperation with other negative molecules on the surface of CD8+ Tregs, such as CTLA-4.

the effect of TNF (29). However, most studies on TNFR2 expression on Tregs have focused on the CD4+ Tregs population, rather than CD8+ Tregs. Current results suggest that TNFR2 might also be a critical suppressive maker of the functional CD8+Foxp3+ Tregs. However, CD8+ Tregs are not the CD8+ counterpart of CD4+ Tregs. There are multiple subsets of CD8+ Tregs reported in both humans and mice (30), such as CD8+CD122+ Tregs (31), CD8+CD28− Tregs (32, 33), and CD8+CD103+ Tregs (34, 35). Unfortunately, the published studies on TNFR2 expression on CD8+Tregs all focused on CD8+Foxp3+ Tregs. As a consequence, we can only summarize the biological effects of TNFR2 expressed on CD8+Foxp3+ Tregs.

TNFR2 Is a Better Functional Treg Cell Marker Than CD25 for CD8+Foxp3+ Tregs

CD8+Foxp3+ Tregs can be generated *in vitro* with anti-CD3 antibodies (17, 36, 37) or anti-CD3/28 beads (24). These cells expressed CD25, Foxp3, TNFR2, and the negative co-stimulatory receptors CTLA-4, PD-1, PDL-1, and Tim-3 (24). When CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors and cultured with anti-CD3 mAb for 5 days, the TNFR2+CD25+ cells were identified as the main subset that expressed Foxp3 (17). Similarly, human CD25 and TNFR2-coexpressing CD4+ Tregs were identified as a potent subpopulation of Tregs (22, 38–40). Interestingly, when these CD8+Tregs were sorted into four subsets, CD25+TNFR2+, CD25+TNFR2−, CD25−TNFR2+, and CD25−TNFR2−, to identify their respective ability to inhibit proliferation of target CD4+ Teffs, the results identified that both CD8+CD25+ and CD8+CD25− cells were more potent inhibitors of proliferation if they coexpressed TNFR2, suggesting that TNFR2 is a more important marker than CD25 on CD8+Foxp3+ Tregs (17). Additionally, *in vitro*-induced CD8+Foxp3+ Tregs expressed both TNFR2 and PDL-1. When sorting CD8+ T cells into TNFR2+PDL-1+, TNFR2+PDL-1−, TNFR2−PDL-1+, or TNFR2−PDL-1−, it was observed that TNFR2-PDL-1 double positive cells exhibited much stronger suppressive activity than control sham sorted cells. TNFR2 or PDL-1 single positive cells had modest suppressive activity, while the double negative cells had none (24). Once more, these data emphasized that TNFR2 might be a characteristic expression marker for functional CD8+Foxp3+ Tregs and the coexpression of TNFR2 and PDL-1 on CD8+Foxp3+ Tregs may represent cells with stronger suppressive activity.

TNF/TNFR2 Interaction Delivers a Co-Stimulatory Signal to Induce Foxp3 by CD8+Foxp3+ Tregs

It was shown that Foxp3 appears to function as a master regulator of the regulatory pathway in the development and function of Tregs (41–43). Interestingly, the TNF/TNFR2 interaction on the surface of CD8+ T cells could promote the induction of Foxp3 in the presence of anti-CD3/CD28 beads to generate more CD8+Foxp3+ Tregs. Previous studies have shown that when PBMCs from rheumatoid arthritis (RA) patients were cultured with anti-CD3 for 24 h, a greater percentage of CD8+Foxp3+ Tregs were generated and expressed high levels of CD25 and TNFR2 (44). However, when anti-TNF monoclonal antibodies (mAb) were added into the *in vitro* culture system, the percentage of Foxp3 expression on CD8+ Tregs decreased significantly (44). Furthermore, experimental results show that membrane TNF/TNFR2 interactions, in combination with CD80/CD28 interactions between monocytes and CD8+ T cells from RA patients, could also promote the induction of CD8+Foxp3+ Tregs *in vitro*, while combined CD86 and TNF blockade completely ablated the process (44). These data all indicated that the effect mediated by TNFR2 expression on CD8+ T cells played a prominent role for the generation of CD8+Foxp3+ Tregs in the presence of anti-CD3 *in vitro*. However, a defined mechanism remains elusive and the corresponding process *in vivo* remains to be studied.

TNF/TNFR2 Interactions Mediate the Suppressive Activity of CD8+Foxp3+ Tregs

Tumor necrosis factor was also found to be responsible for the induction of CD8+Foxp3+ Tregs, as anti-TNF monoclonal antibodies (mAb) could dramatically abrogate the proliferation of CD8+Foxp3+ Tregs, prevent the upregulation of CD25 in response to anti-CD3 *in vitro* on CD8+ Tregs, and interfere with the suppressive activity of CD8+Foxp3+ Tregs. Furthermore, TNFR2 expression was upregulated significantly after CD8+Foxp3+ Tregs were stimulated with anti-CD3 mAb *in vitro*, whereas the TNFR1 level was relatively low (17), indicating that the effect of TNF was more potent *via* TNFR2 to mediate the downstream signal. Additionally, TNF could upregulate PDL-1 expression on CD8+Foxp3+ Tregs *via* TNFR2 and which was greatly decreased by blocking with soluble TNF receptors (TNFR2-Fc) (17). Therefore, upregulating PDL-1 expressing on CD8+ Tregs might be a specific mechanism for TNF/TNFR2 mediating CD8+ Treg suppressive activation (45). Compared with TNFR2 expressed on CD4+ Tregs, little is known about the significance of TNFR2 on CD8+ Tregs. The available evidence indicates that TNFR2 expression on CD8+Foxp3+ Tregs is beneficial for their function, and defects in their suppressive function occurred when TNFR2 was neutralized. CD8+ Tregs have been shown to exhibit different phenotypes in different diseases, including viral infection (46), autoimmune diseases (47), graft-versus-host disease (GVHD) (48, 49), and cancer (44, 50). However, it is unclear whether TNFR2 can be used as a suppressive marker for all the reported CD8+ Treg subsets.

TNFR2 EXPRESSED ON CD8+ EFFECTOR T CELLS

Studies on TNFR2 expressed on CD8+ Teffs are relatively more sufficient than studies on TNFR2 expressed on CD8+ Tregs (**Figure 2**). Numerous reports have shown that CD8+ Teffs are critical players involved in various immune responses (51–53). Efficient induction of CD8+ Teffs requires coordinated signaling through a number of pathways, including T cell receptor (TCR) ligation with peptide in the context of major histocompatibility complex class I (MHC I), costimulatory molecules, and cytokines(53). TNFR2, but not TNFR1, has been previously shown to be the predominant TNF receptor on activation CD8+

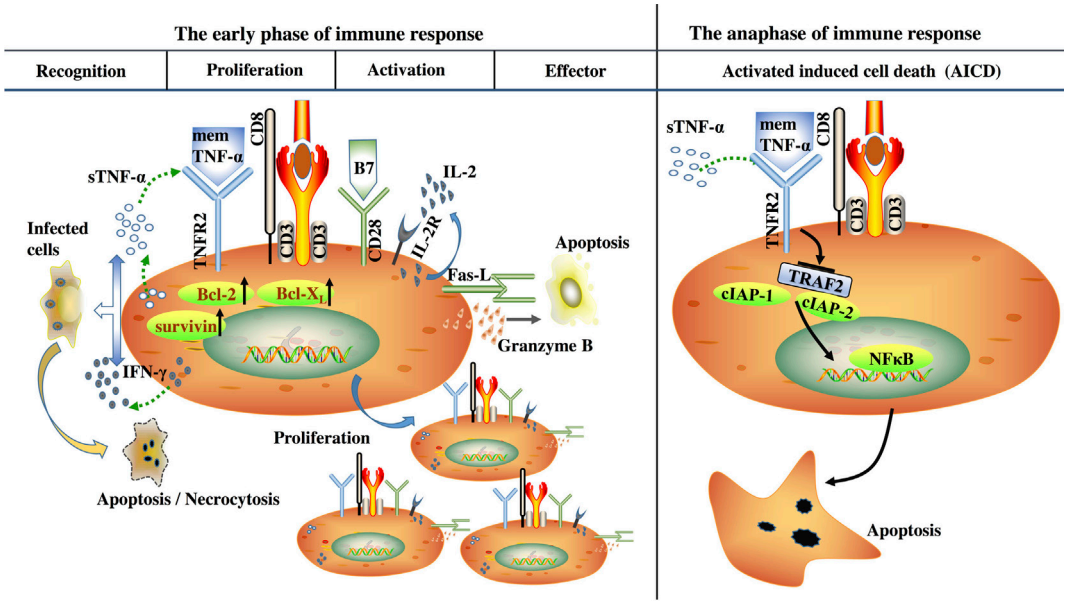

FIGURE 2 | Tumor necrosis factor (TNF) receptor type II (TNFR2) modulates the process of immune response mediated by CD8+ effector T cells (Teffs). In the early phase of immune response, TNFR2 is a CD8+ Teff costimulatory molecule for IL-2, survivin, Bcl-2, and Bcl-x_L induction to promote CD8+ Teffs survival, proliferation, and activation and involve in controlling the cell fate during TCR/CD28-mediated stimulation. TNFR2 is essential for the production of IFN-γ and TNF-α in CD8+ Teffs to promote antigen clearance. Additionally, TNFR2 is also required for CD8+ Teffs to upregulate Fas-L and granzyme B to enhance their cytotoxic activity. However, in the anaphase of immune response, TNFR2 can induce activated CD8+ T cells programmed cell death to terminate the immune response *via* the degradation of a pro-survival signal—TRAF2, which is required for the recruitment of cellular inhibitor of apoptosis proteins cIAP-1 and cIAP-2 to the TNFR2 signaling complex and activates nuclear factor-κB.

Teffs (54, 55). Thus, the direct effects of TNF on CD8+ Teffs are mainly mediated through TNFR2 (54, 55). Typically, T-cell-mediated immune responses can be divided into three parts: (1) antigen recognition; (2) proliferation and differentiation; and (3) activation-induced cell death (AICD). Once an activation signal has been received, primary CD8+ T cells undergo proliferation, expansion, and differentiation. It has been reported that TNFR2 expression was involved in CD8+ Teffs activation in certain phases of an immune response. For instance, it has been found that TNFR2 not only lowered the threshold for T cell activation, but also provided early costimulatory signals during T cell activation (56–58). Additionally, TNFR2 also plays a critical role in regulating AICD in activated CD8+ Teffs (59).

TNFR2 as an Activator Molecule in the Early Phase of Immune Response

TNFR2 Is Required for Primary CD8+ T Cell Survival and Proliferation

CD28 is a key costimulatory molecule for IL-2 induction, based on its ability to substantially augment expression in T cells stimulated *via* the TCR (60). However, the effects mediated by CD28 were found to be insufficient to sustain long-term T cell survival (61). Nevertheless, TNFR2 plays a critical role in promoting activation and survival of naive T cells during a primary response (5, 62). CD8+ T cells deficient in TNFR2 possessed a marked defect in IL-2 production, a critical T cell growth factor (63, 64), resulting in a decreased proliferative response (57), suggesting that TNFR2 is a CD8+ T cell costimulatory molecule involved in controlling the cell fate during TCR/CD28-mediated stimulation (57). Additionally, TNFR2 deficiency in CD8+ T cells increased the requirements for a TCR agonist, approximately fivefold to achieve a proliferative response equivalent to wild-type CD8+ T cells in several infection models (5, 56–58). Additionally, in a mouse tumor model, the proportion of proliferating transgenic tumor-specific CD8+ T cells in TNFR2 deficient mice were significant reduced in tumor-draining lymph nodes (54). These data indicated that TNFR2 sustained the early proliferative phase during CD8+ T cell cells activation. Moreover, during CD8+ T cell activation in response to antigen *in vitro*, TNFR2 deficiency was related to a reduction of anti-apoptotic molecules, such as survivin, Bcl-2, and Bcl-x_L (57, 58), indicating the critical roles of TNFR2 in CD8+ T cell survival.

TNFR2 Is Required for the Secretion of Effector Molecules by CD8+ Teffs

One of the key effector functions of activated CD8+ T cells is the ability to produce antiviral and pro-inflammatory cytokines, including interferon (IFN)-γ and TNF-α (65). Typically, cytokine production by antiviral CD8+ T cells occurs in a hierarchical fashion, with the majority producing IFN-γ, and a subset of those producing TNF-α (66–68). During infection, such as respiratory influenza or *C. muridarum* infection, the production of IFN-γ was significantly decreased in TNFR2$^{-/-}$CD8+ T cell, with significantly delayed antigen clearance in TNFR2$^{-/-}$ mice (69, 70). These results suggest that TNFR2 primarily promotes

the activation of CD8+ T cells and enhances the ability of CD8+ T cells to clear antigen. When tumor-special CD8+ T cells, isolated from TNFR2−/− mice, TNFR1−/−, or wild-type mice, were cultured with specific antigens *in vitro*, IFN-γ levels produced by TNFR2−/−CD8+ T cells was less than TNFR1−/− or wild-type CD8+ T cells (54), indicating that TNFR2 was also necessary for the optimal production of IFN-γ to clear tumor antigens during the T cell activation phase.

Second, TNF-α is increased during CD8+ T cell activation following antigenic stimulation (71, 72). Similar to IFN-γ, TNF-α is critically required for efficient CD8+ T cell-mediated responses from initiation to pathogen clearance. However, TNF-α levels produced by CD8+ T cells were not always in line with INF-γ production. During colitis, CD8+ T cells from TNFR2−/− mice expressed significantly higher levels of TNF-α compared with wild-type mice, which was sufficient to worsen colonic inflammation (73). Similarly, after intranasal challenge with HKx31 influenza A virus, TNF-α production was also increased in TNFR2−/− mice, compared with wild-type mice (73). It is possible that the increased TNF-α in TNFR2 deficient mice may be due to a negative feedback loop in the TNF-TNFR2 signaling (5, 59, 62, 73).

TNFR2 Is Required for CD8+ T Memory Cells Recovery

After encountering with microbial antigen, T cells can differentiate into memory cells to provide long-lasting protection against subsequent pathogens (18, 74, 75). During transplantation, microbe-elicited T memory cells can also cross-react with allogeneic antigen and mediate graft rejection, a process termed allogeneic heterologous immunity. TCR affinity is hypothesized to be critically important in the context of allogeneic heterologous immunity (18, 76, 77). Notably, TNFR2 plays an important role for low-affinity-primed memory CD8+ T cells mediating optimum recall responses. During heterologous rechallenge, low-affinity-primed memory effectors upregulated TNFR2 surface expression to mediated graft rejection, whereas blockade of TNFR2 significantly attenuated graft rejection and prolonged graft survival (18). These data indicated that TNFR2 is required and critical for memory CD8+ T cells recovery in immune responses.

TNFR2 Is Required for Cytotoxic T Lymphocyte (CTL) Activity

Granzyme B is a serine protease expressed by CTL and together with the pore forming protein, perforin, mediates apoptosis in target cells (78). Notably, TNFR2 engagement with TNF-α induces the expression of granzyme B in CD8+ T cells, when costimulation with CD86 is provided simultaneously. TNFR2 was also shown to be upregulated on granzyme B+CD8+ T cells in aging mice and humans (79), indicating that the TNF/TNFR2 signaling pathway in CD8+ T cell could reinforce the cells' cytotoxic activity to induce target cells apoptosis *via* the release of granzyme B.

A second way for CTL to induce apoptosis is *via* cell-surface Fas–Fas ligand (FasL) interactions between CTL and infected cells. FasL is expressed predominantly on activated lymphocytes and is able to induce programmed cell death in most Fas-expressing cells (80, 81). The number of FasL-expressing CD8+ intrahepatic lymphocytes isolated from various strains of hepatic adenovirus-infected TNFR2−/− mice were found to be significantly reduced compared with wild-type mice (82). Furthermore, TNFR2−/− intrahepatic lymphocytes were significantly less efficient in killing adenovirus-infected hepatocyte target cells than intrahepatic lymphocytes obtained from adenovirus-infected wild-type mice (82). These data provide evidence suggesting that TNFR2 can potentiate FasL-mediated cytotoxicity for CD8+ Teffs.

TNFR2 Is as an Apoptosis Signal on Activated CD8+ Teffs

Tumor necrosis factor receptor type II is essential for both optimal proliferation during CD8+ T cell activation and for the induction of AICD that terminates the proliferative response (59). Previous study had shown that TNFR2−/−CD8+ T cells exhibited consistently high resistance to AICD, leading to worsen colonic inflammation (73), indicating that TNFR2 is a critical negative regulator of activated CD8+ T cells by promoting AICD to terminate the immune response. Moreover, TNFR2 signaling was reported to lead to the degradation of TNF receptor-associated factor 2 (TRAF2) (83), which were important in the regulation of the receptor signaling (83–86). Notably, TRAF2 is known as a pro-survival signal (87), which is required for the recruitment of cellular inhibitor of apoptosis proteins (cIAP)-1 and -2 to the TNFR2 signaling complex (88) and activates nuclear factor (NF)-κB to mediate its anti-apoptotic effects (89–91). The overexpression of TRAF2 in wild-type CD8+ T cells did not affect the percentage of apoptotic cells, whereas the silencing of TRAF2 in activated TNFR2−/−CD8+ T cells could render them as sensitive to AICD as activated wild-type CD8+ T cells (59). Collectively, these results provide evidence that the TNFR2 signaling pathway is involved in regulating AICD and that TRAF2 depletion induced by TNFR2 is critical to this process.

CONCLUSION

Tumor necrosis factor receptor type II is an attractive molecular marker to identify both CD8+Foxp3+ Tregs and CD8+ Teffs. For CD8+Foxp3+ Tregs, TNFR2 is necessary for the induction of Foxp3 and regarded as a functional marker of their suppressive ability. For CD8+ Teffs, TNFR2 serves as an activator for proliferation and cytotoxic ability in the early stage of an immune response and as an apoptosis signal for activated CD8+ Teffs to terminate the immune response. Both CD8+Foxp3+ Tregs and CD8+ Teffs could express high levels of TNFR2 and were involved in various diseases. It is noteworthy that there is an antagonistic relationship between CD8+ Tregs and CD8+ Teffs. The TNF-TNFR2 signaling pathway potentially activates both of them, so targeting TNFR2 may impair the function of protective Tregs or Teffs as a side effect in the treatment of diseases (4). Furthermore, studies have shown that TNFR2 is a potential therapeutic target with remarkable success in cancer immunotherapy. A TNFR2 antagonists could specifically inhibit

$CD4^+Foxp3^+$ Tregs expansion in the tumor microenvironment, whereas it had little inhibitory effects on $CD4^+$ Tregs in periphery or from healthy donors, and killed human ovarian tumor cells directly. However, little is known about TNFR2 agonists or antagonists aimed at altering TNFR2 expression on tumor-associated $CD8^+$ Tregs and $CD8^+$ Teffs. Further understanding of TNFR2 expression on $CD8^+$ T cells and the pathways that are active and important in different disease-related microenvironments will provide better understanding of its impacts on TNF-mediated pathology, and may help in the development of more effective targeted therapeutics.

Furthermore, recent evidence indicated that the relationship between TNF/TNFR2 and T cell responses is complex and, at times, paradoxical. There is controversy to the specific effects of TNF on different T cell subsets (92). The explanation for such contradictory outcomes may lay in how downstream signaling pathways are activated and drive disease (92). Consequently, a precise understanding of the level and/or ratio of TNFR2 expressed on different T cell subsets will help in the use of TNFR2 agonists or antagonists as therapies.

AUTHOR CONTRIBUTIONS

L-LY and QZ contributed to the design and writing of this review. X-SW, MZ, and Y-RN contributed to collection of references.

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Modulation of Regulatory T Cell Activity by TNF Receptor Type II-Targeting Pharmacological Agents

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There is now compelling evidence that tumor necrosis factor (TNF)–TNF receptor type II (TNFR2) interaction plays a decisive role in the activation, expansion, and phenotypical stability of suppressive $CD4^+Foxp3^+$ regulatory T cells (Tregs). In an effort to translate this basic research finding into a therapeutic benefit, a number of agonistic or antagonistic TNFR2-targeting biological agents with the capacity to activate or inhibit Treg activity have been developed and studied. Recent studies also show that thalidomide analogs, cyclophosphamide, and other small molecules are able to act on TNFR2, resulting in the elimination of TNFR2-expressing Tregs. In contrast, pharmacological agents, such as vitamin D3 and adalimumab, were reported to induce the expansion of Tregs by promoting the interaction of transmembrane TNF (tmTNF) with TNFR2. These studies clearly show that TNFR2-targeting pharmacological agents represent an effective approach to modulating the function of Tregs and thus may be useful in the treatment of major human diseases such as autoimmune disorders, graft-versus-host disease (GVHD), and cancer. In this review, we will summarize and discuss the latest progress in the study of TNFR2-targeting pharmacological agents and their therapeutic potential based on upregulation or downregulation of Treg activity.

Keywords: TNF receptor type II, regulatory T cells, TNF receptor type II agonists, TNF receptor type II antagonists, immunotherapy

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INTRODUCTION

$CD4^+FoxP3^+$ regulatory T cells (Tregs) play an indispensable role in maintaining immunological homeostasis and inhibiting autoimmune responses, while they also represent a major cellular mechanism in immune evasion of tumors by dampening antitumor immune responses (1, 2). Consequently, Tregs have become important therapeutic target in the treatment of autoimmune diseases, graft-versus-host disease (GVHD), transplantation rejection, and cancer.

We (Xin Chen and Joost J. Oppenheim) previously reported that tumor necrosis factor (TNF)-alpha stimulates the activation and expansion of Tregs, and this effect of TNF is mediated by TNF receptor type II (TNFR2) (3). Moreover, we showed that the expression of TNFR2 correlated with suppressive function and phenotypical stability of Tregs (4–7). Our finding that TNF–TNFR2 interactions play a decisive role in Treg function is now supported by compelling evidence from both human Treg studies (8–24) and mouse Treg studies (25–40) by other groups. Some of these independent studies also clearly show that the Treg-stimulatory effect of TNF–TNFR2 pathway can be therapeutically harnessed for the treatment of major human diseases, including cancer and autoimmune disorders (10, 12, 14, 16, 18, 20, 23, 24).

TABLE 1 | TNF receptor type II (TNFR2)-targeting pharmacological agents.

Category	Class	Agent	Activity	Reference
TNFR2 agonists	Agonistic TNFR2 monoclonal antibodies (mAbs)	"TNFR2 antagonist"	• Binds to and activates human TNFR2 • Stimulates the activation and expansion of homogeneous and highly functional regulatory T cells (Tregs) isolated from normal donors and patients with type 1 diabetes (T1D) (*in vitro* assay)	(10, 18)
		MR2-1 (isotype: IgG1)	• Binds to and activates human TNFR2 • Promotes the expansion of homogenous Foxp3^{+}Helios^{+}CD127^{low} Treg population with highly suppressive capacity (*in vitro* assay and in humanized mouse study)	(20)
	Tumor necrosis factor (TNF) muteins	TNF07	• Binds to and activates human TNFR2 • Expands Foxp3^{+} Treg cells from normal donors (*in vitro* assay) • Selectively induces the death of autoreactive CD8^{+} T cells from T1D patients (*in vitro* assay)	(14)
		STAR2	• Binds to and activates mouse TNFR2 • Stimulates proliferative expansion of Foxp3^{+} Tregs (*in vitro* assay) • Selectively activates and expands Foxp3^{+} Tregs in WT mice (*in vivo* assay) • Markedly prolongs the survival and decreases the severity of graft-versus-host disease (GVHD) (*in vivo* assay)	(38)
		TNC-scTNF(R2)	• Binds to and activates human TNFR2 • Protects TNFR2-expressing oligodendrocyte progenitor cells from death induced by oxidative stress (*in vitro* assay) • Unknown effect on human Tregs	(41)
		EHD2-scTNFR2	• Binds to and activates mouse TNFR2 • Inhibits neuroinflammation and promotes neuronal survival in a mouse model of neurodegeneration in combination with a TNFR1 antagonist (*in vivo* assay) • Unknown effect on mouse Tregs	(42)
	Anti-TNF mAbs	Adalimumab	• A therapeutic humanized mAb binding to both soluble TNF (sTNF) and transmembrane TNF (tmTNF) • Increases expression of tmTNF on monocytes from rheumatoid arthritis (RA) patients (*in vitro* assay) • Promotes the binding of tmTNF (expressed on monocytes) to TNFR2 (expressed by Tregs of RA patients), resulting in selective activation and proliferation of Tregs (*in vitro* assay)	(16)
		Infliximab	• A therapeutic humanized mAb against TNF-α • Increases the suppressive function of Tregs in autoimmune patients, at least partially caused by the elevated levels of TNF (*in vivo* assay)	(4, 15, 22)
	Small molecule compounds	Vitamin D3	• VD3-DCs induces induced Tregs (iTregs) through the interaction of tmTNF expressed by VD3-DCs and TNFR2 expressed by Tregs (*in vitro* assay)	(26)
TNFR2 antagonists	Antagonistic TNFR2 mAbs	"TNFR2 antagonist"	• Blocks the binding of TNF to human TNFR2 • Markedly inhibits the expansion of Tregs and reduces the suppressive capacity of Tregs (*in vitro* assay)	(10)
		Dominant anti-human TNFR2 antagonistic Abs	• Block the binding of TNF to human TNFR2 and hamper TNFR2 signaling activation • Inhibit TNF-induced expansion of human Tregs (*in vitro* assay) • Induce the death of Tregs, especially those isolated from ovarian cancer tissue (*in vitro* assay) • Induce the death of TNFR2-expressing OVCAR3 tumor cells (*in vitro* assay)	(23)

(*Continued*)

TABLE 1 | Continued

Category	Class	Agent	Activity	Reference
	Small molecule compounds	Thalidomide and its analogs	• Inhibit TNF synthesis • Inhibit the surface expression of TNFR2 on T cells (*in vitro* assay) • Reduce the number and function of Tregs and TNFR2 expression on Tregs in patients with leukemia (*in vivo* assay) • Increase the number of Tregs in patients with multiple myeloma (MM) (*in vivo* assay)	(13, 62, 63, 65, 67, 110)
		Panobinostat	• Reduces the expression of Foxp3 and inhibit the suppressive function of Tregs at low doses (*in vitro* assay) • Reduces the proportions of TNFR2^{+} Tregs in the blood and bone marrow of acute myeloid leukemia (AML) patients in combination with azacitidine (*in vivo* assay)	(12, 70)
		Cyclophosphamide	• Selectively depletes TNFR2^{hi} Tregs population in a mouse model of mesothelioma (*in vivo* assay)	(74)
		Triptolide	• Reduces TNF and TNFR2 expression in colon of colitis mice (*in vivo* assay) • Reduces the number of Tregs and inhibits tumor growth in melanoma-bearing mice (*in vivo* assay)	(76, 77)

To translate this basic research finding into therapeutic benefit, a number of agonistic or antagonistic TNFR2-targeting biological agents with the capacity to upregulate or downregulate Treg activity have been developed. Recent study also revealed that some small molecule compounds can suppress TNFR2 expression or eliminate TNFR2-expressing Tregs. Some pharmacological agents were found to induce Tregs by promoting interaction of transmembrane TNF (tmTNF) with TNFR2. In this brief review, recent reports of TNFR2-targeting pharmacological agents with the capacity to upregulate or downregulate Treg activity were reviewed, analyzed, and discussed (**Table 1**).

TNFR2 AGONISTIC BIOLOGICAL AGENTS

Faustman's group has screened a panel of monoclonal antibodies (mAbs) against human TNFR2 generated from her own lab or purchased from commercial sources. They identified a potent agonistic TNFR2 mAb which was designated as "TNFR2 agonist" in their study. In the presence of IL-2, "TNFR2 agonist" potently stimulated the expansion of Foxp3^{+} Tregs present in cultures of CD4 cells, accompanied by the upregulation of TNF, TRAF2, TRAF3, BIRC3 (cIAP2), and Foxp3 mRNA expression (10). Furthermore, this property of the "TNFR2 agonist" was harnessed to generate highly homogenous Foxp3^{+} Tregs. To this end, MACS-purified CD4^{+}CD25^{+} cells were cultured under standard *in vitro* human Treg expansion conditions (anti-CD3 Ab, anti-CD28 Ab, IL-2, and rapamycin), with or without the "TNFR2 agonist." Expanded Tregs in the presence of "TNFR2 agonist" expressed markedly higher levels of Foxp3 and other characteristic Treg markers, and possessed more potent suppressive capacity (10). More recently, Faustman's group examined the effect of such "TNFR2 agonist" on the activation and expansion of Tregs isolated from patients with type 1 diabetes (T1D) (18). The results show that *in vitro* treatment with "TNFR2 agonist" stimulated the activation of T1D Tregs which initially showed a resting phenotype. Furthermore, under the aforementioned standard Treg expansion culture condition, "TNFR2 agonist" promoted the homogenous expansion of Tregs isolated from T1D patients by magnetic beads (18). "TNFR2 agonist"-expanded T1D Tregs were more potent in the inhibition of autologous CD8^{+} T cells (18). A similar result was obtained by using MR2-1, a commercially available agonistic human TNFR2 mAb (mouse IgG_1) by another group (He/Joosten and colleagues) (20). In this study, low purity MACS-isolated human Tregs were expanded with the aforementioned standard protocol. The treatment with MR2-1 resulted in the generation of more homogenous Foxp3^{+}Helios^{+}CD127^{low} Tregs. The phenotype of resultant Treg cells remained stable, even in the pro-inflammatory environment. Importantly, Tregs expanded with MR2-1 maintained highly suppressive activity in a humanized mouse model (20). Thus, TNFR2 agonists can facilitate *ex vivo* expansion of Treg cells from less pure population for Treg-based immunotherapy.

Prompted by the potential therapeutic effect on autoimmune diseases, Faustman's group also generated soluble TNF (sTNF) muteins with TNFR2 agonistic effect, designated S95C/G148C or TNF07 (14). This stable TNF trimer, TNF07 double mutant, functioned as a TNFR2 agonist. It could trigger a strong TNFR2

signaling, with the capacity to expand Foxp3$^+$ Treg cells and to selectively induce the death of autoreactive CD8$^+$ T cells isolated from T1D patients (14).

Chopra/Beilhack and colleagues developed a novel nonameric TNFR2-specific variant of mouse TNF (STAR2), which was a selective agonist of mouse TNFR2 and had no capacity to bind to TNFR1 (38). STAR2 had *in vitro* and *in vivo* activity to stimulate the proliferation of Tregs in a TNFR2-dependent and IL-2-independent manner. Furthermore, pretreatment with STAR2 before allogeneic hematopoietic stem cell transplantation (allo-HCT) markedly prolonged the survival and decreased the severity of GVHD, in TNFR2- and Treg-dependent manner. A human TNFR2-specific STAR2 equivalent agonist also potently stimulated the expansion of Foxp3$^+$ Tregs from healthy donors *in vitro* (38).

A number of TNFR2-targeting agents, such as TNC-scTNF(R2) (a human TNFR2 selective agonist) (41) and EHD2-scTNFR2 (a mouse TNFR2 selective agonist) (42), were developed to examine their protective effect on neurodegeneration. It would be interesting to ask if their neuroprotective effect is attributable to their capacity to activate and expand Tregs, and if they have beneficial effect in the inhibition of autoimmune diseases.

It was shown recently that TROS, a nanobody-based selective inhibitor of TNFR1, was able to inhibit mouse experimental autoimmune encephalomyelitis (EAE) and this effect is attributable to the diversion of TNF to interact with TNFR2 (43). TNFR2 is also expressed by oligodendrocytes or astrocytes, with neuroprotective function through tmTNF–TNFR2 signaling to promote CNS cells differentiation and remyelination, and such effect of TNFR2 signaling was based on its directly action on the cells in CNS (44–46). Therefore, selectively blocking TNFR1, thus favoring TNFR2, may represent another strategy to stimulate TNFR2$^+$ Tregs in the treatment of autoimmune diseases and GVHD.

TNFR2 ANTAGONISTIC BIOLOGICAL AGENTS

In addition to a TNFR2 agonist, Faustman's group also identified a potent mAb antagonist of human TNFR2, designated as "TNFR2 antagonist" in their study (10). In the standard Treg expansion culture condition, this "TNFR2 antagonist" markedly inhibited the expansion of Tregs and reduced the suppressive capacity of Tregs (10). More recently, Torrey/Faustman and colleague developed two potent dominant anti-human TNFR2 antagonistic Abs that outcompeted TNF, the natural agonist of TNFR2, and inhibited TNF-induced *in vitro* expansion of human Tregs (23). These TNFR2 antagonists specifically bound to TNFR2 through F(ab) region, independent of Fc region or crosslinking of antibodies. Through binding to the antiparallel dimers of TNFR2 protein, the TNFR2 antagonists blocked the binding of TNF to TNFR2. Consequently, they inhibited TNF-triggered activation of nuclear factor-κB (NF-κB) pathways in Tregs, and suppressed conversion of tmTNFR2 to sTNFR2. These two TNFR2 antagonists could induce the death of Tregs *in vitro*. Interestingly, Tregs isolated from ovarian cancer tissues were more sensitive to TNFR2 antagonist-induced cell death (23), presumably attributable to the higher levels of TNFR2 expression on tumor-infiltrating Tregs (4). TNFR2 is also expressed on the surface of OVCAR3, an ovarian cancer cell line. Intriguingly, TNFR2 antagonists could also induce the death of OVCAR3 tumor cells (23). Thus, this *in vitro* evidence strongly supports the idea that TNFR2 antagonists may represent novel cancer therapeutics by simultaneously targeting tumor-infiltrating Tregs and tumor cells.

Progranulin (PGRN), a glycosylated protein, has immunosuppressive and anti-inflammatory activity (47–49), presumably due to its capacity to promote the induction of induced Tregs (iTregs), as shown in an *in vitro* study (50). Progranulin was initially reported as an endogenous TNFR2 antagonist (51). However, controversial results were reported (52, 53) and thus further study is needed to clarify its effect on TNFR2.

SMALL MOLECULE TNFR2 INHIBITORS

Thalidomide is a synthetic small molecule glutamic acid derivative (54) that was initially developed for alleviation of morning sickness of pregnant women in Europe several decades ago (55). It was withdrawn from the market because it caused developmental defects in newborns (55). The interest in using this compound as a therapeutic agent reawakened recently, due to its suggested effect in the treatment of erythema nodosum leprosum (ENL) (56, 57). This led to the discovery of immunomodulatory and anti-inflammatory properties of thalidomide and to clinical trials of thalidomide and its analogs in various malignancies (54). Thalidomide and its structural analogs (lenalidomide and pomalidomide) are now classified as immunomodulatory drugs (IMiDs) (54). It has been well established that thalidomide and its analogs are able to inhibit TNF protein synthesis through downregulation of NF-κB, destruction of TNF mRNA, and targeting reactive oxygen species and α1-acid glycoprotein (58–61). Thalidomide and its analogs also have the capacity to inhibit the surface expression of TNFR2 on T cells without reducing the expression of total TNFR2 protein (62), which is associated with the inhibition of intracellular TNFR2 transport to the cell surface (13). Giannopoulos et al. showed that, in patients with chronic lymphocytic leukemia, thalidomide treatment reduced the number and function of Tregs (63, 64), presumably by blockade of TNF–TNFR2 interaction. Moreover, Plebanski's group reported that, in acute myeloid leukemia (AML) patients, combination therapy with lenalidomide and a demethylating agent, azacitidine, downregulated TNFR2 expression on CD4 T cells and reduced the number of TNFR2$^+$ Tregs, resulting in enhanced effector immune function (13). However, it was reported that treatment with thalidomide and its analog actually increased the number of Tregs in patients with multiple myeloma (MM) (65, 66), which may be attributable to the elevated serum levels of TNF after treatment (62, 66). Furthermore, thalidomide was reported to promote *de novo* generation of iTregs (67), which is consistent with current understanding of responses of iTreg to TNF–TNFR2 stimulation (29, 68). Thus, the effect of thalidomide on TNFR2$^+$ Tregs is likely to be disease- and condition-specific, which should be clarified by future study.

Histone deacetylase inhibitor panobinostat is effective in the treatment of MM in combination with bortezomib and dexamethasone (69). A recent study found that low doses of

panobinostat could reduce the expression of Foxp3 and inhibit the suppressive function of Tregs (70). Furthermore, Govindaraj et al. reported that the combination treatment with panobinostat and azacitidine reduced the proportions of TNFR2$^+$ Tregs in the blood and bone marrow of AML patients (12). One of the mechanisms may be the disruption of the AML bone marrow niche by panobinostat and azacitidine, resulting in reduced blast cell levels and preventing Treg induction by blast cells (12). The reduction of TNFR2$^+$ Tregs and consequently increase of IFNγ and IL-2 production by effector T cells (Teffs) is attributable to the clinical beneficial effect of patients with AML (12). This study indicates that epigenetic therapeutics may represent a strategy to eliminate TNFR2$^+$ Treg activity and to enhance antitumor immune responses.

Cyclophosphamide (CY) is a DNA alkylating agent which is commonly used as a cytotoxic chemotherapy in cancer treatment (71). CY at low dosages can inhibit immunosuppressive function of Tregs (72), and a single dose of CY depletes the maximally suppressive Tregs in PROb colon cancer bearing mice, resulting in the activation of antitumor immune responses (73). Moreover, van der Most et al. reported that, in a mouse model of mesothelioma, CY treatment depleted TNFR2^{hi} Tregs (74). This effect of CY was based on its capacity to induce the death of replicating Tregs which co-express TNFR2 and Ki-67 (4, 74). Furthermore, CY in combination with etanercept, a therapeutic TNF antagonist, markedly inhibited the growth of established CT26 tumor in mice, by eliminating TNFR2-expressing Treg activity through blockade of TNF–TNFR2 interaction (75).

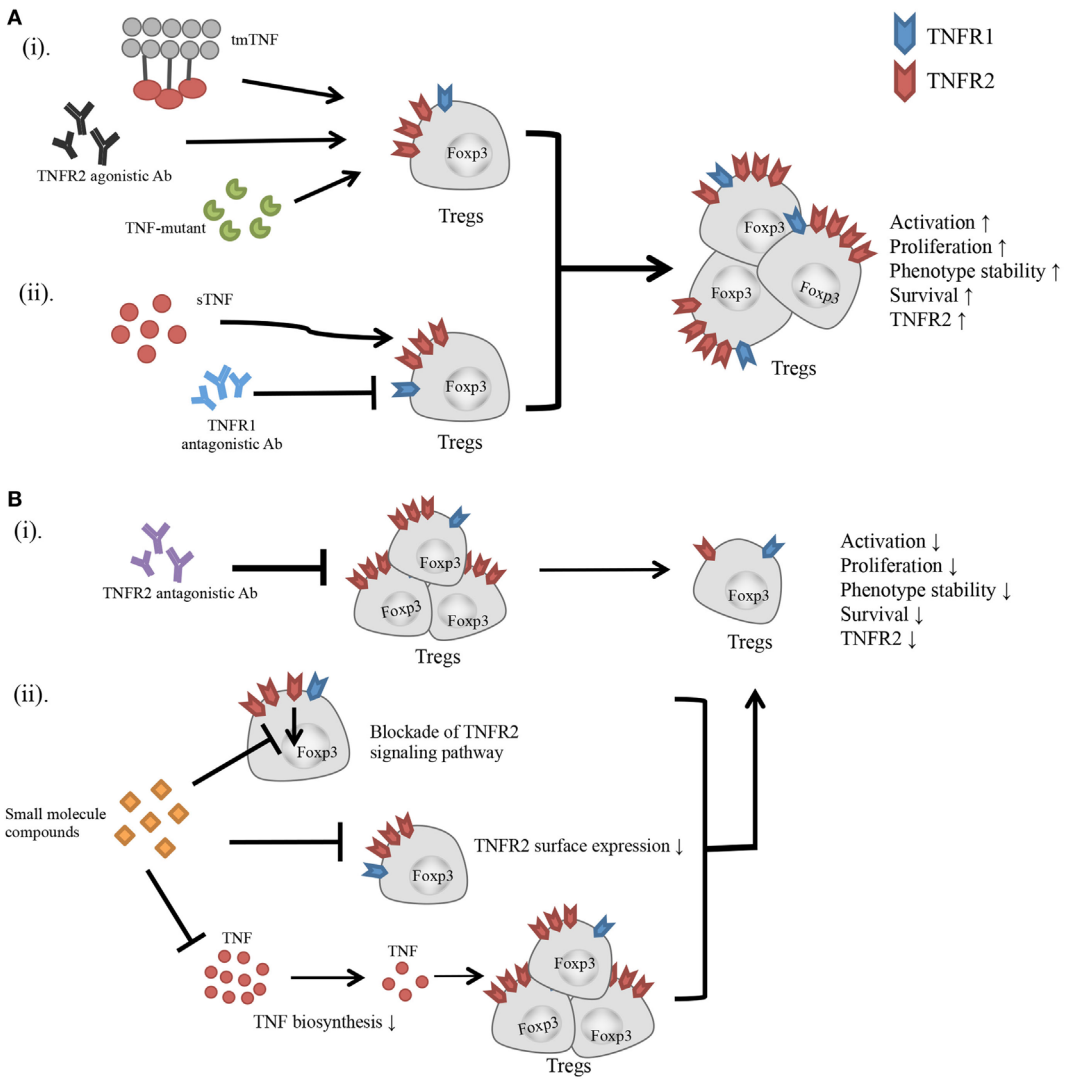

FIGURE 1 | Effect of TNFR2-targeting agents on the activity of Tregs. **(A)** (i) Transmembrane TNF, TNFR2 agonistic Ab, and TNF mutant preferentially bind to and stimulate TNFR2. (ii) Blockade of TNFR1 with antagonistic Ab diverts the stimulatory effect of TNF to TNFR2. All these agents have potential to activate Tregs, and promote the proliferative expansion, phenotypic stability, survival, and TNFR2 expression on Tregs. **(B)** (i) TNFR2 antagonistic Ab blocks TNF–TNFR2 interaction. (ii) Small molecule compounds with the capacity to inhibit TNFR2 signaling pathway, or downregulate TNFR2 surface expression, or suppress TNF biosynthesis. These agents may inhibit the activation and proliferation of Tregs, and reduce the phenotypic stability, survival, and surface TNFR2 expression on Tregs.

Triptolide (TPT), an immunosuppressive compound isolated from Chinese herb *Tripterygium wilfordii* Hook F., was reported to inhibit TNF as well as TNFR2 expression in the colon of mouse colitis model (76). TPT was also reported to decrease the number of Tregs and consequently inhibited the growth of mouse tumor (77). Thus, it would be interesting to investigate if TPT and other naturally occurring compounds have the capacity to downregulate Treg activity by blockade of TNF–TNFR2 interaction.

PHARMACOLOGICAL AGENTS THAT PROMOTE THE INTERACTION OF tmTNF AND TNFR2

TNF binds and signals through two structurally related functionally distinct receptors: TNFR1 and TNFR2 (78). Once synthesized, TNF is expressed initially as a cell surface type II polypeptide consisting of 233 amino acid residues (26 kDa). Transmembrane TNF is then cleaved by TNF-alpha converting enzyme into a sTNF consisting of 157 amino acid residues (17 kDa) (79). Soluble TNF predominantly binds and activates TNFR1, while tmTNF preferentially binds and activates TNFR2 (80). Therefore, agents which have the capacity to enhance the expression of tmTNF or promote the interaction of tmTNF and TNFR2 may also selectively activate and expand Tregs. This is exemplified by a recent study reported by Nguyen/Ehrenstein showing the paradoxical effect of adalimumab in the expansion of Tregs (16). Adalimumab is a therapeutic anti-TNF mAb which is effective in the treatment of rheumatoid arthritis (RA) and other autoimmune diseases (81). This Ab was developed to bind to both sTNF and tmTNF, aiming to block the interaction of TNF with its receptors (82). It was reported that adalimumab treatment increases the number of Tregs in RA patients (83). A recent *in vitro* study found that adalimumab bound to tmTNF expressed by monocytes from RA patients. This resulted in the upregulation of tmTNF expression, consisting with *in vivo* observations that adalimumab treatment enhanced TNF expression by monocytes from RA patients (16). Furthermore, adalimumab promoted the binding of tmTNF expressed by monocytes to TNFR2 expressed by Tregs of RA patients, consequently enhanced the activation and proliferation of Tregs (16). This study suggests that targeting of tmTNF–TNFR2 interaction may represent a novel strategy in the treatment of autoimmune diseases, especially in those patients that do not to respond to conventional anti-TNF treatment, by mobilization of TNFR2$^+$ Tregs (84). Coincidentally, these findings also clarify why adalimumab is more effective in the treatment of Crohn's disease (85), than etanercept which merely inhibits the effect of sTNF without the concomitant stimulation of Tregs (85, 86).

Infliximab (Remicade) is a therapeutic chimeric mAb against TNF used in the treatment of autoimmune diseases (87). A recent study shows that, in patients with sarcoidosis, surface expression of TNFR2 on CD4$^+$CD25^{hi} "Tregs" was higher in responders to therapy, as compared to those non-responders (22). Since TNFR2 expression is associated with suppressive function of Tregs (4, 15), this study suggests that infliximab treatment may also increase the suppressive function of Tregs in autoimmune patients.

It was reported that tolerogenic dendritic cells (DCs), designated as VD3-DCs, were induced by the treatment with 1 alpha, 25-dihydroxyvitamin D3 (VD3). Such DCs expressed high levels of TNF and PD-L1 upon LPS stimulation and were able to induce functionally suppressive Tregs (88). A subsequent study by the same group (Kleijwegt/Roep and colleagues) found that VD3-DCs expressed high levels of tmTNF. Furthermore, induction of Ag-specific Tregs by VD3-DCs depended on the interaction of tmTNF expressed by VD3-DCs and TNFR2 expressed by Tregs, since blockade of binding of tmTNF to TNFR2 abrogated the induction of suppressive function of Tregs (26). In this study, Tregs induced by VD3-DCs were converted from naïve CD4 T cells (26). Thus, the possibility that VD3-DCs can also promote the activation and expansion of naturally occurring Tregs (nTregs) in a tmTNF–TNFR2 dependent manner, especially in the physiologically relevant *in vivo* settings, should be addressed in a future study. Furthermore, since CD8$^+$Foxp3$^+$ Tregs also expressed high levels of TNFR2 on their surface and TNF signaling is required for the generation of CD8$^+$Foxp3$^+$ Tregs (89), it would be interesting to investigate if they can be generated or expanded by tmTNF-expressing VD3-DCs.

CONCLUSION

Although the first of the TNFR2 inhibitors identified was thalidomide (62), recent research actually focused on the development of TNFR2-targeting biological agents. This may be because the difficulty to block TNF–TNFR interaction with a small molecule, due to the large contact surface area (90), and due to the apparent advantage of biological therapeutics, such as high target specificity, well-understood mechanism and minimal toxicity (91, 92). Nevertheless, cell-permeable small molecules may also effectively block TNFR2 signaling pathways, and consequently inhibit Treg activity induced by TNF–TNFR2 interaction. So far, three signaling pathways of TNFR2 in T lymphocytes, e.g., IKK/NFκB, MAPK (Erk1/2, p38, JNK), and PI3K/Akt pathways, have been reported (93–95). The effect of small molecule inhibitors specific for major components of these pathways on Treg activity should be investigated. Thoroughly understanding of TNFR2 signaling pathways in Tregs, especially those different from Teffs, is a key to identify or design selective Treg inhibitors and thus merits future study. Moreover, it has been shown that TNFR2-specific TNF muteins have the capacity to activate and expand Tregs (38). Since LTα homotrimer can also bind to TNFR2 (96), it would be interesting to investigate if TNFR2-specific mutant LTα have the capacity to preferentially activate Tregs.

In addition to being constitutively and predominantly expressed by highly suppressive Tregs (4), TNFR2 can also be induced and upregulated on CD4$^+$Foxp3$^-$ Teffs upon TCR stimulation (28, 97). However, the level of TNFR2 expressed by Teffs is much lower than its expression on Tregs (6, 9, 23, 28). This may explain why TNFR2 antibody mimetics preferentially bind to Tregs (21). Nevertheless, TNFR2-targeting agents on the function of Teffs should be carefully evaluated in the future study. Furthermore, in addition to T cells, TNFR2 is also expressed by other cell types, such as endothelial cells (98), microglia and selected neuronal subtypes (99, 100), oligodendrocytes (101),

cardiac myocytes (102), and thymocytes (103). Since those TNFR2-expressing cells can also respond to TNFR2-targeting therapeutics, the off-target effect and safety of TNFR2 agonist and antagonist should be carefully evaluated.

Current experimental evidence suggest that TNFR2-targeting agents preferentially act on Tregs, and consequently promote or inhibit immune responses by downregulating or upregulating TNFR2$^+$ Treg activity (**Figure 1**). However, this idea has to be confirmed by more physiologically relevant *in vivo* studies. TNFR2 is also reported to play a key role in the accumulation and immunosuppressive function of myeloid-derived suppressive cells (MDSCs) (34, 104, 105) and mesenchymal stem cells (MSCs) (106, 107). Since these cells exert their immunosuppressive function in a collaborative manner with Tregs (108, 109), the effect of a TNFR2 agonist or antagonist may have a greater effect on the modulation of immune responses, by acting on multiple components of the immunosuppressive network.

Taken together, recent studies regarding TNFR2-targeting agents not only further confirmed and substantiated the concept that TNFR2 signaling plays a decisive role in the activation and expansion of Tregs but they also clearly indicate that TNFR2-targeting pharmacological agents have great potential in the treatment of major human diseases and deserve further research and development.

AUTHOR CONTRIBUTIONS

HZ, RL, and XC drafted the manuscript. HZ, RL, HH, YH, and XC approved the final version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Role of TNF–TNF Receptor 2 Signal in Regulatory T Cells and Its Therapeutic Implications

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Tumor necrosis factor α (TNFα) is a pleiotropic cytokine which signals through TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Emerging evidence has demonstrated that TNFR1 is ubiquitously expressed on almost all cells, while TNFR2 exhibits a limited expression, predominantly on regulatory T cells (Tregs). In addition, the signaling pathway by sTNF *via* TNFR1 mainly triggers pro-inflammatory pathways, and mTNF binding to TNFR2 usually initiates immune modulation and tissue regeneration. TNFα plays a critical role in upregulation or downregulation of Treg activity. Deficiency in TNFR2 signaling is significant in various autoimmune diseases. An ideal therapeutic strategy for autoimmune diseases would be to selectively block the sTNF/TNFR1 signal through the administration of sTNF inhibitors, or using TNFR1 antagonists while keeping the TNFR2 signaling pathway intact. Another promising strategy would be to rely on TNFR2 agonists which could drive the expansion of Tregs and promote tissue regeneration. Design of these therapeutic strategies targeting the TNFR1 or TNFR2 signaling pathways holds promise for the treatment of diverse inflammatory and degenerative diseases.

Keywords: tumor necrosis factor α, tumor necrosis factor receptor 1, tumor necrosis factor receptor 2, regulatory T cells, autoimmune diseases

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INTRODUCTION

Tumor necrosis factor α (TNFα) is an essential signaling protein in the innate and adaptive immune systems. It also plays an important role in tissue degeneration and repair (1). It is now recognized that the expression of TNF receptor 2 (TNFR2) is more limited than that of TNF receptor 1 (TNFR1). In addition, new evidence suggests that the sTNF-mediated signaling pathway *via* TNFR1 drives a predominantly pro-inflammatory program whereas mTNF binding to TNFR2 primarily initiates immune modulation and tissue regeneration. These findings suggest that we may selectively target TNFR1 and TNFR2 for therapeutic purposes, providing promise for the context-specific treatment of autoimmune diseases. This review is provided to summarize TNFα and TNFR expression, structure, and signaling pathways, to discuss TNFR1/TNFR2 signaling in autoimmune diseases especially concerning their correlation with Tregs and organ regeneration, as well as to propose treatment strategies aimed at TNFR1/TNFR2 in autoimmune diseases.

THE BASIC BIOLOGY OF TNFα AND TNFR

Expression, Structure, and Function of TNFα

Tumor necrosis factor α plays a vital role in many physiological and pathological conditions. First, TNFα is essential for the regulation of embryonic development, the sleep–wake cycle, lymph node

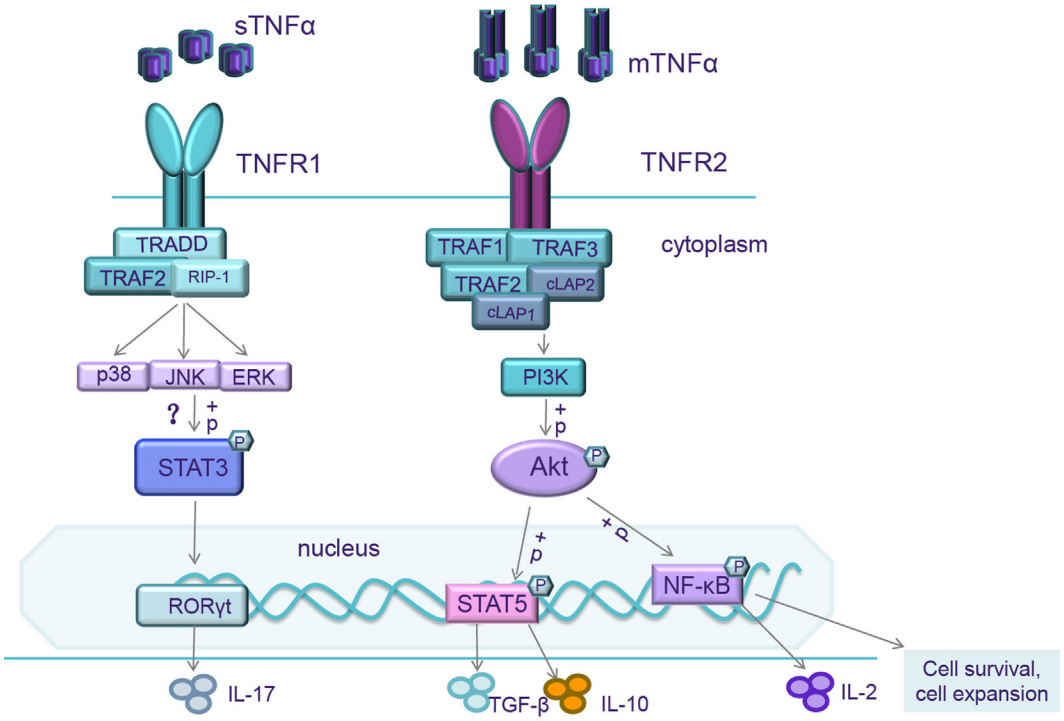

FIGURE 1 | When mTNF/TNF receptor 2 (TNFR2) is activated, the intracellular domains recruit existing cytoplasmic TNF receptor-associated factor-2 (TRAF-2)–cIAP-1–cIAP-2 complexes resulting in the initiation of both canonical and non-canonical NF-κB/Rel and MAPK pathways activation. NF-κB/Rel and MAPK pathways activate IL-2 promoter and trigger IL-2 expression. NF-κB pathways also transcript genes associated with cell survival and cell proliferation. So, mTNF/TNFR2 signaling can enhance expansion and stability of Tregs and increase Treg sensitivity to low level of IL-2. It also activates the reciprocal PI3K/Akt pathway. Activation of Akt signaling impairs Th17 differentiation, correlated with an increased phosphorylation of STAT5 (143). When soluble TNFα (sTNFα)/TNFR1 is activated, the intracellular domains interact with TRADD, which receptor interacting protein-1 (RIP-1) and TRAF-2 to form Signal complex I, then further triggering extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinase (JNK). The mechanisms of TNFR1 on Th17 differentiation are still unclear. These transcription factors might phosphorylate STAT3, upregulate the level of ROR-γt, and increase IL-17 production.

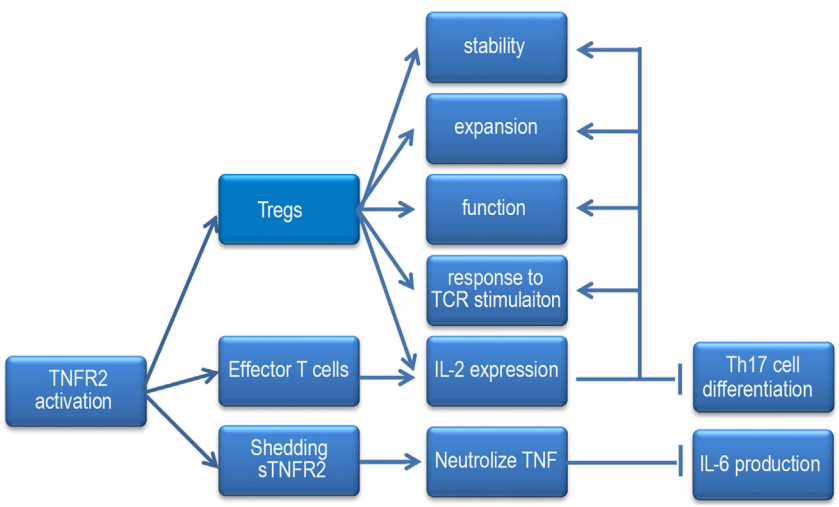

FIGURE 2 | When the TNF receptor 2 (TNFR2) signaling pathway is activated, it increases Tregs stability, responses to TCR stimulation, expansion, and function. It enhances Tregs and effector T cells to produce IL-2 and promotes the sensitivity of Tregs to IL-2. IL-2 can inhibit Th17 cells differentiation and the effect of TNFR2 signaling on Tregs. Under inflammatory condition, mTNFR2 can shed to sTNFR2, sTNFR2 neutralizes TNF and hampers IL-6 expression.

follicle, and germinal center formation. Second, TNFα not only promotes the production of inflammatory cytokines but also enhances the adhesion and permeability of endothelial cells and promotes the recruitment of immune cells such as neutrophils, monocytes, and lymphocytes to sites of inflammation (2, 3). These actions help to mediate both acute and chronic systematic inflammatory reactions under conditions of infection or autoimmunity. In addition, TNFα also causes cell apoptosis and necrosis under specific conditions. Furthermore, high levels of TNFα can also result in cachexia and endotoxin-induced septic shock (4). It has also been identified as an endogenous pyrogen.

Tumor necrosis factor α is primarily generated by macrophages and monocytes. However, other cells such as some subsets of T cells, NK-cells, dendritic cells, B cells, cardiomyocytes, fibroblasts, and astrocytes are also the producers of this cytokine at a low level (5, 6).

Tumor necrosis factor α is a type II transmembrane protein. It exists as a membrane-bound form (mTNFα) with relative molecular weight 26 kDa primarily. mTNFα can be processed into 17 kDa soluble TNFα (sTNFα) through the action of the matrix metalloproteinase known as TNFα converting enzyme (TACE: ADAM17) (7, 8). In addition, mTNFα also has the ability to process external signals as a receptor (9). sTNFα circulates throughout the body and confers TNFα with its potent endocrine function, far away from the site of its synthesis. Both sTNFα and mTNFα are active as non-covalently bonded homotrimers.

While bacterial lipopolysaccharide (LPS) serves as a major stimulant of the innate immune system, microbial antigens, enterotoxins, and cytokines including TNFα itself are also able to trigger TNFα production. TNFα also stimulates the generation of numerous pro-inflammatory cytokines including IL-6, IL-8, TNFα itself, adhesive molecules, chemokines, and metalloproteinases (10, 11), potentially leading to a TNFα-mediated pro-inflammatory autocrine loop (12). On the other hand, TNFα can boost the synthesis of anti-inflammatory factors such as IL-10 and corticosteroids, to limit the inflammatory cytokines secretion. As a whole, TNFα initiates a rapid and vigorous immune reaction, thus limiting the extent and duration of inflammation when the invasion has been resolved (13). Furthermore, serving as a co-stimulator, TNFα enhances the reactions of neutrophils, monocytes, and lymphocytes for defense against microbes.

Expression, Structure, and Signaling Pathways of TNFR

Tumor necrosis factor α exerts its function *via* two different type I transmembrane receptors, TNFR1 and TNFR2. Each has a characteristic extracellular domain, a transmembrane segment, and intracellular domain. The extracellular domains of both receptors have similar a cysteine-rich motif that is repeated two to six times, are active as homodimers but intriguingly do not form TNFR1/TNFR2 heterodimers (14). Nevertheless, the intracellular segments of TNFR1 and TNFR2 do not bear homologous sequences and activate distinct signaling pathways (15).

Both TNFR1 and TNFR2 membrane receptors also can be converted into soluble forms (sTNFR1 and sTNFR2) through the activity of TACE enzymes. Both TNFRs can interact with either mTNFα or sTNFα. TNFR1 is ubiquitously expressed on nearly all cells in the body and can be activated by both mTNFα and sTNFα. TNFR2, conversely, is restricted to thymic T lymphocytes, endothelial cells, microglia, and oligodendrocytes (16), and can only be fully initiated by mTNFα. Once mTNFα binds to TNFR2, the combination is too stable to dissociate (17). This is not the case for sTNFα which induces weak signaling and exhibits a low affinity for TNFR2 (18). Other salient features of TNFR2 are that cellular activation status highly regulates its expression and unlike TNFR1, it does not contain a cytoplasmic death domain.

It is well accepted that TNFα binding to TNFR1 activates two different intricate signal pathways: the maintenance of cell survival and the promotion of inflammatory cytokine expression; cell apoptosis and necrosis. The balance between these two pathways hinges upon many factors such as cell type, cell activation status, an intracellular or extracellular microenvironment, recruitment of adaptor molecules, the concentration of complex inhibitors of apoptosis proteins (cIAP), or the level of NF-κB expression (19). When TNFα binds to TNFR1, the intracellular domains interact with TNFR type 1-associated death domain protein (TRADD), which recruits receptor interacting protein-1 (RIP-1) and TNF receptor-associated factor-2 (TRAF-2) to form Signal complex I (5) (**Figure 1**). Signal complex I can trigger NF-κB, which launches the transcriptions of many different genes including those associated with cell survival, production of inflammatory cytokines, and antiapoptotic gene pathways. Signal complex I is also able to activate extracellular signal-regulated kinases, the stress-activated MAP kinases p38, and c-Jun N-terminal kinase (JNK), which are important for AP-1, the important promoter of inflammation and proliferation, and other transcription factors through MAPK3 signaling pathways (20–22).

Signal complex I formation is temporary and rapidly dissociates from TNFR1, mediating the binding of the Fas-associated death domain protein (FADD) to form Signal complex II which coordinates downstream signaling of the caspase cascade (23). When the kinase activities of RIP-1 and RIP-3 inhibit apoptosis signaling, necrosis is activated (24).

Recently, several studies have demonstrated that TNFR2 promotes a remarkable degree of cell activation, migration, and proliferation (24). When TNFα binds to TNFR2, the intracellular domains recruit existing cytoplasmic TRAF-2–cIAP-1–cIAP-2 complexes (25) (**Figure 1**). cIAP can exert ubiquitin-ligase activity and can inhibit caspases and other apoptosis-inducing factors (5), resulting in the initiation of both canonical and non-canonical NF-κB activation (25–27). The interaction of TNFα with TNFR2 also activates the reciprocal PI3K/Akt pathway. This pathway not only maintains survival and enhances proliferation (28, 29) but also recruits Etk and forms the TNFR2–Etk–VEGFR2 (vascular endothelial growth factor receptor 2) complex which participates in cell adhesion, migration, survival, and proliferation (30, 31).

Although TNFR2 triggers NF-κB in a slower manner than TNFR1, TRFR1 maintains a longer duration of NF-κB activity (25). Even though TNFR2 lacks a death domain, caspase activation and cell apoptosis can be initiated under conditions of stress or when the cIAP pool exhausted *via* interaction of intracellular domains with Signal complex II. Other theories suppose that TNFR2 activation exhausts the cIAP pool, which facilitates a shift of TNFR2 signaling to FADD, triggering the apoptosis pathway.

THE FUNCTION OF TNFR1 AND TNFR2 ON AUTOIMMUNE DISEASES

Disease models using transgenic mice have broadened our horizons concerning the importance of pathogens in triggering or shaping autoimmunity. Compared with wild-type mice, TNFα$^{-/-}$ mice exhibit an enhanced susceptibility to pathogen invasion (2). They also exhibit a deficiency in TNFR1 (32). It is noteworthy that mice expressing non-cleavable TNF (which cannot be processed into sTNF) have a diminished capacity to resist pathogens (33, 34). This demonstrates that many of the pro-inflammatory functions of sTNF are indeed mediated by TNFR1 signaling, while mTNF (predominantly *via* TNFR2) can at least partly provide the immune system with some pathogen protection.

Overexpression of TNF results in a severe chronic inflammatory arthritis in collagen-induced arthritis (CIA) mice, an animal model of rheumatoid arthritis (RA). When the TNFR1 gene is knockout, these arthritic effects are largely diminished, whereas TNFR2 deficiency exacerbates disease (35, 36). Interestingly, the use of TNF inhibitors dramatically improves symptoms in a manner reminiscent of TNFR1 deficiency (36, 37). In addition, central nervous system-specific overexpression of TNF in transgenic mice also resulted in a spontaneous severe demyelination (38). These results confirm the pro-inflammatory role of TNFR1, while leaving the door open for an immune-modulatory role of TNFR2. In the experimental autoimmune encephalomyelitis (EAE) mouse model, TNF knockout delayed disease onset, however, once established, the symptoms were more serious in knockouts than in wild-type mice (39). This raises the suggestion that TNF is essential to the promotion of a potent immune response *via* TNFR1. However, once the immune response is triggered, the absence of TNF may result in the failure to expand and activate Tregs *via* TNFR2 which in turn results in tissue and organ damage. Increasing evidence indicates that TNFR2 plays a vital role in the modulation of the immune system, most likely through its interactions with Tregs.

It has been well studied that polymorphisms in the TNFR2 gene have a strong correlation with a wide variety of autoimmune diseases, e.g., RA (40–42), Crohn's disease (43), systemic lupus erythematosus (44), ankylosing spondylitis (AS) (45), inflammatory bowel diseases (IBD) (46), and ulcerative colitis and scleroderma (47). The consequence of this polymorphism is to hamper TNF binding to TNFR2, which subsequently limits the activation of NF-κB (48), and most likely hampers TNFR2 signaling pathway in Tregs.

TNFR2's RELATIONSHIP WITH TISSUE REGENERATION AND Tregs

The Relationship Between TNFR2 and Tissue Regeneration

TNF receptor 2 provides a critical contribution to neural survival and regeneration. In the mouse model of retinal ischemia, TNFR2 showed a protective function by activating the Akt signaling pathway (49). The cuprizone-induced demyelination and remyelination mouse model gave similar results. In this model, TNF or TNFR2 knockout led to delayed remyelination and a decreased proliferation and maturation of oligodendrocyte progenitors. These findings provide support for the notion that TNF/TNFR2 serves as principal players in oligodendrocyte regeneration (50). A tissue regenerative role for the TNFR2 signaling pathway has also been described in several other disorders (51). Several other studies have also indicated that TNFR2 agonists are active in pancreatic regeneration, cardioprotection, remyelination, and survival of some neuron subtypes and also in stem cell proliferation (51–54).

The Relationship Between TNFR2 and Tregs

The interplay between inflammatory and regulatory pathways orchestrates an effective immune response that provides protection from pathogens while limiting injury to host tissue. Tregs are prototypical immunosuppressive cells that dampen excessive immune responses and maintain immune homeostasis by inhibiting effector T cell proliferation and cytokine production which prevents the development of autoimmune diseases and tissue destruction (55–58). Regulatory T cells can mediate their suppressive function either by secreting cytokines like IL-10, TGF-β, or IL-35 or by direct cell–cell contact (59, 60). These cells can act by suppressing the effector T cells directly at the target site (61), by suppressing DC in the regional lymph nodes and thereby preventing priming of T cells in the regional lymph nodes (62), or by recruiting mast cells to the site (63). Mice deficient in Foxp3$^+$ T cells develop fatal autoimmune disease (64), and continuous expression of Foxp3 throughout life prevents autoimmunity (65). Recent studies have contradictorily demonstrated that TNF upregulates or downregulates the expansion and function of Tregs *via* TNFR2.

One specific study has demonstrated that as with human Tregs, both thymic and peripheral murine CD4$^+$CD25$^+$ Tregs expressed remarkably high levels of TNFR2 relative to CD4$^+$CD25$^-$ effector T cells (66). By contrast, TNFR1 was barely detectable.

When responding to TCR stimulation, TNFR2 expression on Tregs is further increased relative to activated effector T cells (67). In TNFR2 knockout mice, although the numbers and function of Tregs are comparable with wild-type mice, these Tregs failed to expand when stimulated under inflammatory conditions either *in vivo* or *in vitro*. This suggests that under non-inflammatory conditions, TNF is not required for thymic Tregs to maintain immune homeostasis (67, 68). Conversely, TNFR2 can mediate the activation of anergic Tregs in response to TCR stimulation (67), having profound effects on their stabilization (69), proliferation (70), and function (71).

Interestingly, one study about type 1 diabetes model on NOD mice found that TNFR1 deficiency protected the mice from diabetes and showed mild peri-insulitis. The absence of TNF–TNFR1 signaling increased the number and function of Tregs both *in vitro* and *in vivo* (72). They proposed that the primed effector T cells secreting TNF that signals through TNFR2, which is constitutively expressed at a high level on Tregs (67), leads to expansion of Tregs. We also considered the TNFR1 deficiency is consequential to elevated TNFR2 signaling on Tregs, as a result

of increased ligand availability as opposed to a loss of stimulatory TNFR1 signaling. As a consequence, increased Tregs prevent effector T cells migrating into islets. Similar work has been exhibited in EAE model (73).

We and other investigators have reported that IL-2 is essential for the development and maintenance of Foxp3$^+$ Treg cells (56, 57). Neutralization of circulating IL-2 elicits autoimmune gastritis in BALB/c mice and triggers early onset of diabetes by inhibiting physiological proliferation of peripheral CD4$^+$CD25$^+$ cells, but not CD4$^+$CD25$^-$ cells (74). Interestingly, TNF can enhance this effect markedly both in human and mice (67, 75). In mice, it exerts the effect markedly in a time-dependent and dose-dependent manner (67). The initial exposure to TNF transiently abrogates Treg suppressive functions, whereas longer exposures restore their suppressive activity. This means that short-term stimulation of TNF mimics the early phases of the inflammatory reaction, thus allowing effector T cells to escape from the inhibition mediated by Tregs, presumably favoring elimination of the pathogens. However, long-term exposure to TNF may facilitate the activation and expansion of Tregs, restoring their suppressive capabilities, thus limiting excessive inflammation.

Recently, one investigation showed that anti-TNF antibody, adalimumab expanded the pool of Tregs and maintained their function *via* mTNF/TNFR2 both *in vitro* and *in vivo*. Moreover, upregulation of Foxp3 by adalimumab was reliant upon low levels of IL-2 production and subsequently STAT5 activation of Tregs (76). They proposed that TNFR2 is able to increase the sensitivity of IL-2 signaling, thereby amplifying the impact of small changes in IL-2 production (77). Coincidentally, another study demonstrated that IL-2 transcription was directly triggered by TNFR2. It showed that CD4$^+$ T cell intrinsic tmTNF/TNFR2, but not sTNF/TNFR1, promotes Il2 promoter activity and IL-2 mRNA stability in a Foxp3-independent manner both *in vitro* and *in vivo*. When tmTNF/TNFR2 signaling is blocked or impaired, IL-2 production is reduced, and the Th17 differentiation elevated, which was associated with increased STAT3 activity and ROR-γt level, decreased STAT5 activity, while, it can be prevented by adding exogenous IL-2 (78). However, whether TNFR2 regulates IL-2 expression in a Foxp3-independent manner or a Foxp3-dependent manner, whether IL-2 can in turn regulate TNFR2 expression, the precise signaling pathways by which TNFR2 regulates IL-2 expression still remain important areas for future studies (**Figure 2**).

Work in the last decade has established that the subset of Foxp3$^+$ Tregs expressing TNFR2 showed increased suppressive function relative to those that did not express TNFR2. However, these studies suggested that Foxp3 expression may not be the only factor to confer Treg suppressive capacity (79). Indeed, TNFR2 may be needed as a unique activator to maximize their suppressive activity (67, 79). Furthermore, when TNFR2$^{-/-}$ mice are used in the colitis model, some studies have found that nTregs require TNFα *via* TNFR2 as a critical factor for optimizing Treg suppressive function under inflammatory conditions whereas iTregs are fully suppressive without TNF signaling (79). Therefore, it is likely that anti-TNFα therapy for different human autoimmune diseases may have dichotomous effects on the function of nTregs versus iTregs. Whether or not this has any influence on disease expression would depend on whether nTregs or iTregs play the predominant regulatory role in that specific disease. Diseases in which iTregs are functionally predominant would encourage the anti-inflammatory effects of anti-TNFα therapy, with no deleterious effects on iTregs and a favorable response. By contrast, diseases in which nTregs functionally predominate, anti-TNFα therapy might result in a loss of Treg function. This dichotomy offers a novel mechanistic paradigm for the enigma of variable responses to anti-TNF-α therapy in different human diseases.

It is well accepted that Tregs consist of two major identified subtypes: natural or thymic Tregs (nTregs/pTregs), developed in the thymus; induced Tregs or peripheral Tregs (iTregs/pTregs) generated in the periphery from CD4$^+$CD25$^-$ T cells (pTregs) or iTregs induced with anti-CD3/CD28 coated beads, IL-2 and TGF-β from naive CD4$^+$ T cells *in vitro* (iTregs) (80–82). The two populations have subtle difference, such as methylation status of conserved non-coding sequence 2 [known as the Treg cell-specific demethylated region (TSDR) in the Foxp3 locus]. Although the stability of Tregs is still controversial, most researchers generally recognized that nTregs are predominantly more stable and long-lived than iTregs because TSDR in nTregs but not iTregs is hypomethylated, which is great important for Foxp3 stability (83, 84). While, a small percentage of nTregs may become unstable, losing Foxp3 expression and transforming to effector T cells, such as Th1, Th17 cells under some pathogenic conditions. As stated earlier, the suppressive function of Foxp3$^+$ Tregs expressing TNFR2 was superior to those that did not express TNFR2 (79). Okubo et al. found one method for Tregs expansion *ex vivo* using a synthetic TNFR2 agonist which produces Tregs with 14 homogenous cell-surface markers (85). Although it still needs more researches to definite this issue, the relationship between TNF–TNFR2 and nTregs provides a promising way to apply Tregs to autoimmune therapy. Even if some researchers insisted that iTregs induced by IL-2 and TGF-β is less stable, we and others found these iTregs are more stable and resistant to phenotypic plasticity in some autoimmune diseases and acute graft-versus-host disease (58, 86–90). Furthermore, our laboratory recently found that TNF (*via* TNFR2) enhanced the differentiation and suppressive function of iTregs induced *in vitro* (unpublished observation). As such, therapeutic intervention with these iTregs *via* TNFR2 has become a promising strategy for the treatment of autoimmune disorders (91).

Intriguingly, van Mierlo et al. described that CD4$^+$CD25$^+$ Treg cells were able to shed higher amounts of TNFR2 for a longer period of time than CD4$^+$CD25$^-$ T cells. In addition, WT Tregs can suppress IL-6 production when LPS was injected into mice. By contrast, TNFR2-deficient Tregs failed to do this but maintained their suppressive function *in vitro* (92). Thus, shedding of TNFR2 might represent yet another novel mechanism for Tregs to inhibit the pro-inflammatory action of TNF at inflammatory sites. It was presumed that low concentrations of TNFR2, through receptor shedding or other processes, might possibly decrease the concentration of TNF and prevent it from binding to inflammatory cells (93, 94). Unfortunately, this concept highlights a discrepancy. If CD4$^+$CD25$^+$ Tregs shed enough of their TNFR2, TNF will fail to have any effects on Treg activation, expansion, or function.

Despite this question, some laboratories have offered conflicting results concerning the effect of TNF on human Tregs. They

believe that TNF stimulation directly hampers the suppressive capacity of human $CD4^+CD25^{high}$ Tregs both *in vitro* and *ex vivo* (71, 95–98). Moreover, the inhibitory effects of TNF are more apparent under coculture conditions than they are under pretreatment condition (99). This discrepancy may be due to the cross-species differences between murine and human T cells. It also raises the possibility of differences in the sensitivity of the experimental conditions or the differing methodologies employed in each laboratory. Valencia et al. showed that $CD4^+CD25^{high}$ Tregs stimulated with high levels of TNF and IL-2 lost their immune suppressive capabilities, presumably these findings were as a result of only the early stages of TNF stimulation (95).

Indeed, these studies also showed that neutralization of TNF might actually restore Treg suppressive ability and maintain the survival of Tregs in patients with RA and IBD (95, 98, 100, 101). In neonatal NOD mice, treatment with TNF promoted the development of diabetes accompanied with a reduced number and impaired function of Tregs instead (102). By contrast, administration of TNF to young adult NOD mice also ameliorated diabetes but enhanced the proliferation of Tregs (102) instead. We hypothesized that the NOD mouse is a spontaneous animal model of T1DM. The severity of inflammation is deteriorating with age in NOD mice, and the immune microenvironment is changing, which may impact the density of TNFR1 and TNFR2 on the surface of Tregs, the sensitivity to TNF, even the TNF form tending to sTNF or mTNF, and the activation of TNFR1 or TNFR2 signaling pathways. Nonetheless, the precise effect of TNF on Tregs activity remains elusive, and it still needs more in-depth investigations.

TARGETING sTNF/TNFR1 IN AUTOIMMUNE DISEASES

Treatment with TNF inhibitors has been a successful strategy for several diseases such as RA, IBD, psoriasis, and cancer-related cachexia. Recent anti-TNF therapies are all aimed at directly binding the ligand to TNF. Five anti-TNF drugs are currently approved for the therapy of human autoimmune disorders: RA, plaque psoriasis, psoriatic arthritis, AS, and IBD. Trade names for these drugs include infliximab, adalimumab, certolizumab pegol, golimumab, and etanercept (103–107). Notably, it raises a novel mechanism that adalimumab prefers binding to mTNF on monocytes and increased their mTNF expression, followed by enhancement of Treg TNFR2 expression and its binding to mTNF. As a consequence, adalimumab expanded functional Tregs equipped to suppress Th17 cells (76).

Despite the wide use of TNF inhibitors, drawbacks include severe side effects like opportunistic infections, reactivation of tuberculosis, and even development of autoimmune diseases, lymphoma, and many other cancers (108–111). In addition, some patients do not respond well to these anti-TNF treatments (105, 106). Furthermore, in clinical trials on MS patients, treatment with TNF inhibitors resulted in disease exacerbation (112, 113).

Because sTNF/TNFR1 may play a role in promoting inflammation, and because mTNF/TNFR2 can result in immune modulation and tissue regeneration, new therapeutics selectively targeting sTNF/TNFR1 have emerged. Both TNFR1-selective antagonists and sTNF-special antagonists may leave the mTNF/TNFR2 signaling pathway intact, which may minimize or diminish the detrimental effects caused by TNF inhibitors. This provides protective TNF-mediated responses such as neural regeneration, survival, and immune modulation without promoting inflammation. Indeed, mTNF alone may be enough to form TNF-dependent secondary lymphoid organ structure and granulomas (114, 115), and to partially provide resistance to pathogens (116, 117), without causing any autoimmune diseases (114, 116).

sTNF-Selective Dominant-Negative TNF (dnTNF) Derivatives

A novel type of TNF inhibitor called signaling-incompetent dnTNF derivatives was described in 2005 (118). This TNF mutein can rapidly and specifically inactivate sTNF through interaction with endogenous sTNF, followed by formation of mixed TNF heterotrimers, leaving mTNF unaffected. XPro1595, an improved version of this mutein, exhibited a profound ameliorating effect on EAE and inflammatory arthritis. XPro1595-treated animals were also less susceptible to infection (119, 120). In addition, relative to etanercept, XPro1595 treatment significantly delayed onset and more efficiently ameliorated EAE symptoms (121), even when applied at the disease peak period (122). Interestingly, XPro1595 administration increased the level of TNFR2 expression in the lesion area, illustrating that mTNF signaling *via* TNFR2 may indeed play a role in neural regeneration (123).

TNFR1-Selective Antagonistic TNF

Some investigators have identified R1antTNF, a TNFR1-selective antagonistic mutant TNF from a phage display library (124). The affinity of R1antTNF to TNFR1 is comparable to that of the human wild-type TNF, and it does not interfere with TNFR2-mediated bioactivity. In two acute hepatitis models, R1antTNF significantly ameliorated liver injury as demonstrated by reduced serum levels of alanine aminotransferase and pro-inflammatory cytokines. This therapeutic effect of R1antTNF had an advantage over that of current TNF inhibitors (125). PEG-R1antTNF, another TNFR1-selective antagonistic mutant TNF, remarkably decreased morbidity, ameliorated disease symptoms, and improved demyelination in EAE mouse model. Furthermore, it significantly suppressed Th1 and Th17 cell activation and infiltration in the spinal cord (126).

TNFR1-Specific Antibodies

Recently, one study compared the therapeutic effects of the TNFR1-specific antibody, DMS5540 with that of etanercept in the CIA model. Both reagents comparably suppressed disease progression. One difference noted was that etanercept administration increased effector T-cell activity, specifically in joints undergoing remission. This was not observed in mice treated with DMS5540 (127). These findings suggest that the immune regulatory function of TNFR2 is masked by traditional TNF inhibitors, like etanercept. It proves the hypothesis that selective targeting of TNFR1 not only inhibits autoimmune responses but also enhances Treg activity, making it a better choice for TNF therapy over standard TNF inhibitors (127).

TARGETING TNFR2 IN AUTOIMMUNE DISEASES

It remains to be verified whether selective inhibition of sTNF/TNFR1 proves enough to redirect the available TNF to TNFR2 for improving immune regulation and tissue regeneration. In support of this, mTNF/TNFR1 plays a role in neuron cell survival in under certain circumstances (128). On the other hand, sTNF probably activates TNFR2 in high TNFR2-expressing cells such as Tregs (128). It still remains to be determined whether mTNF signaling *via* TNFR2 is enough to activate Tregs and if additional activation of sTNF *via* TNFR2 can promote Treg activity. Most importantly, it is likely to have a narrow range of safe and effective dose since TNFR1 is expressed ubiquitously almost all types of cells throughout the body. Thus, selective TNFR2 agonists can provide yet another tissue-specific or cell-specific therapy for autoimmune disorders.

TNF receptor 2 agonists were engineered using point mutation (129). Treatment with these TNFR2 agonists has been successfully used for cancer therapy and also for research in immunology (130). As mentioned earlier, mTNF binds much greater affinity to TNFR2 than to sTNF itself (130). The investigators undertaking this line of research also found another small synthetic molecule that acted as trimer ligands which were similar to TNFR2 (131). Over time, additional literature indicates that TNF and TNFR2 agonists exert a tremendous effect on heart regeneration, bone marrow stem cells, and even neuron regeneration in murine models of Parkinson's disease (132–134).

TL1A-Ig, a natural TNF-receptor superfamily member agonist is used as a novel method for the *in vivo* expansion of Tregs (135). Okubo et al. found another method for Treg expansion *ex vivo* using a synthetic TNFR2 agonist which produces Tregs with 14 homogenous cell surface markers (85). This method provides an optimal way to obtain sufficient quantities of Tregs use in autoimmune therapy.

TNF Inducers

One well-known TNF inducer is the mycobacterium bacillus Calmette–Guerin (BCG) vaccine. Another one is the BCG equivalent, complete Freund adjuvant. Although BCG induction of TNF can interact with both TNFR1 and TNFR2, it may induce TNF production at low levels, thereby possibly boosting the expansion of Tregs (85), which can provide benefits for the treatment of autoimmune diseases. Newly synthesized TNF inducers improve the specificity for the TNFR2 receptor and may hold the promise for the treatment of type 1 diabetes (136).

CD3-Specific Antibodies

As a distinctive cell-surface marker of T cells, CD3 antagonists are targeted to be mainly applied as immunosuppressive agents to protect against organ transplant rejection. While a CD3-specific antibody generally seen as an immunosuppressive agent, it may promote TNF generation and TNFR2 expression (137), and thus exert the similar effects as the TNFR2 agonist.

It is particularly noteworthy that the safety profile of TNFR2 agonists has not been well defined. Not all of TNFR2 agonists exert their effects using the same mechanism. Some can function as agonists while some TNFR2 antibodies can be antagonists and still others may induce anergy. It is clear that many different factors come into play concerning the activation and regulation of the balance between TNFR1 versus TNFR2 signaling. These subtle differences in TNF stimulation can result in the generation of divergent intracellular signaling pathways (138). More studies with these new agents are needed to determine if their use results in any changes in TNF signaling and what effects these changes have on physiological consequences.

To avoid the systemic toxicity, injection of TNFR2 agonists directly into sites of inflammation or lymphoid organs might be a promising approach. Their local application would be attractive for diseases such as autoimmune Sjogren's syndrome or skin diseases because the skin is an easily accessible organ that is amenable to agonist delivery at desired concentrations, thus reducing systemic toxicity. However, not all lesions are as accessible. Pancreatic injection might potentially trigger pancreatitis.

In spite of their non-specificity, several alternative strategies are also in progresses which act *via* immune modulation of TNFR2 signaling pathways. However, high concentrations of TNFR2 agonists could potentially overwhelm TNFR2 signaling and might shift their activities to the pro-apoptotic pathway *via* TNFR1. This would result in apoptosis of both autoreactive and bystander cells. In spite of their lower relative toxicity to TNFR1 agonists, TNFR2 agonists might still have some degree of toxicity, particularly toward CNS cells (139). Overexpression of TNFR2 in a transgenic mouse model resulted in systemic toxicity (140) and also elicited several autoimmune diseases as mentioned earlier (141). It is noteworthy that one specific TNFR2 agonist enhanced thymocyte proliferation *in vitro* and *in vivo*, caused a febrile reaction and a transient inflammatory reaction (142). Thus, the potential toxicity of TNFR2 agonist therapy still needs to be investigated carefully.

CONCLUSION

Taken together, as with anti-cytokine or immunosuppressive drug therapies which must be used continuously to keep steady blood concentration, any of the absolute TNF inhibitors, sTNF inhibitors, TNFR1 antagonists, or TNFR2 agonists, could potentially be given at a low dose discontinuously and intermittently. However, sTNF/TNFR1-special antagonists can specifically block TNFR1 signaling pathway and leave the protective effect, e.g., neural regeneration and immune modulation *via* TNFR2. Furthermore, TNFR2-special agonists avoid the detrimental effects initiated by total TNF inhibitors and sTNF/TNFR1-special antagonists due to their limited tissue expression. sTNF inhibitors, TNFR1 antagonists, and TNFR2 agonists, when used alone or in combination therapy, may provide a superior therapeutic strategy for the treatment of various autoimmune and degenerative diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Lack of Evidence for a Direct Interaction of Progranulin and Tumor Necrosis Factor Receptor-1 and Tumor Necrosis Factor Receptor-2 From Cellular Binding Studies

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Progranulin (PGRN) is a secreted anti-inflammatory protein which can be processed by neutrophil proteases to various granulins. It has been reported that at least a significant portion of the anti-inflammatory effects of PGRN is due to direct high affinity binding to tumor necrosis factor receptor-1 (TNFR1) and TNFR2 and inhibition of tumor necrosis factor (TNF)-induced TNFR1/2 signaling. Two studies failed to reproduce the interaction of TNFR1 and TNFR2 with PGRN, but follow up reports speculated that this was due to varying experimental circumstances and/or the use of PGRN from different sources. However, even under consideration of these speculations, there is still a striking discrepancy in the literature between the concentrations of PGRN needed to inhibit TNF signaling and the concentrations required to block TNF binding to TNFR1 and TNFR2. While signaling events induced by 0.2–2 nM of TNF have been efficiently inhibited by low, near to equimolar concentrations (0.5–2.5 nM) of PGRN in various studies, the reported inhibitory effects of PGRN on TNF-binding to TNFR1/2 required a huge excess of PGRN (100–1,000-fold). Therefore, we investigated the effect of PGRN on TNF binding to TNFR1 and TNFR2 in highly sensitive cellular binding studies. Unlabeled TNF inhibited >95% of the specific binding of a *Gaussia princeps* luciferase (GpL) fusion protein of TNF to TNFR1 and TNFR2 and blocked binding of soluble GpL fusion proteins of TNFR1 and TNFR2 to membrane TNF expressing cells to >95%, too. Purified PGRN, however, showed in both assays no effect on TNF–TNFR1/2 interaction even when applied in huge excess. To rule out that tags and purification- or storage-related effects compromise the potential ability of PGRN to bind TNF receptors, we directly co-expressed PGRN, and as control TNF, in TNFR1- and TNFR2-expressing cells and looked for binding of GpL-TNF. While expression of TNF strongly inhibited binding of GpL-TNF to TNFR1/2, co-expression of PGRN had not effect on the ability of the TNFR1/2-expressing cells to bind TNF.

Keywords: binding studies, *Gaussia princeps* luciferase fusion protein, progranulin, tumor necrosis factor, tumor necrosis factor receptor-1, tumor necrosis factor receptor-2

Abbreviations: ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorting; GpL, *Gaussia princeps* luciferase; HEK293, human embryonal kidney cells 293; KD, equilibrium dissociation constant; PGRN, progranulin; SPR, surface plasmon resonance; TNF, tumor necrosis factor; TNFR1/2, tumor necrosis factor receptor-1/2.

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INTRODUCTION

Tumor necrosis factor-alpha (TNF) is a pleiotropic cytokine which has not only crucially implicated in a variety of immunoregulatory processes in innate and adaptive immunity, but has also manifold roles in the control of tissue homeostasis (1). TNF is initially expressed as a trimeric type II transmembrane protein (memTNF) from which a soluble trimeric molecule (sTNF) is released by cleavage by the protease TNF converting enzyme (TACE) (1). Both sTNF and memTNF bind with high affinity to two types of receptors, tumor necrosis factor receptor-1 (TNFR1) and TNF receptor-2 (TNFR2). While memTNF binding results in strong activation of both TNFR1 and TNFR2, sTNF binding triggers efficient TNFR1 signaling but has no or only a modest effect on TNFR2 activity (1). TNFR1 and TNFR2 interact furthermore with high affinity with lymphotoxin-α (LTα), also named TNFβ, a soluble ligand trimer which is structurally closely related to TNF (1). TNFR1 and TNFR2 share a similar extracellular domain architecture comprising four cysteine-rich domains (CRDs) defining their affiliation to the TNF receptor superfamily (TNFRSF) (2). The crystallographic structures of TNF in complex with the ectodomain of TNFR2 and of LTα in complex with the ectodomain of TNFR1 have been solved. Both structures show that three molecules of TNFR1 or TNFR2 bind into the three grooves formed by three protomers of a ligand trimer (3, 4). Since the two TNF receptors have different types of intracellular domains with basically different binding partners, TNFR1 and TNFR2 elicit significantly different cellular responses upon activation. Excessive and/or chronic TNF activity has a pivotal role in various immune diseases and can contribute to various aspects of cancer development. TNF and its receptors are, therefore, considered as promising targets in a variety of diseases. Indeed, TNF inhibitors are already in clinically practice, since almost two decades and are powerful drugs in the therapy of Crohn's disease, ulcerative colitis, psoriasis, and several arthritic diseases, including rheumatoid arthritis, psoriatic arthritis, and juvenile idiopathic arthritis. Although, five TNF-neutralizing biologicals have been approved, there are still enormous preclinical and clinical efforts to develop new drugs (antibodies, ligand mutants, small-molecules) inhibiting TNF, TNFR1, or TNFR2.

Progranulin (PGRN) or granulin precursor (Gene ID GRN) is a phylogenetically conserved unique protein without stringent homology to other proteins (5, 6). PGRN is a secretory glycoprotein expressed by a variety of cell types and present in blood and cerebrospinal fluid (5, 6). PGRN is composed and characterized by cysteine-rich so-called granulin domains. Human PGRN comprises N-terminal a truncated version of this domain type which is followed by seven complete granulin domains. PGRN can be proteolytically processed in most of the linker regions connecting the granulin domains resulting in the release of various granulin peptides covering one or more granulin domains (5, 6). Both PGRN and PGRN-derived peptides display complex biological activities, including stimulation of cellular proliferation, immune regulation, modulation of synaptic activity and neurogenesis (5, 6). In accordance with the latter, mutations in the GRN gene have been identified as cause of a familiar form of the neurodegenerative disease frontotemporal lobar degeneration (5, 6).

There is growing *in vitro* and animal model evidence that the immune modulatory effects of PGRN are based, at least in part, on the modulation of TNF signaling (7–19). The basis of this crosstalk, however, appears to be complex, since inhibitory and stimulatory effects of PGRN on TNF signaling have been reported. Tang et al. noteworthily identified PGRN as a TNF antagonist and reported, based on cell-free binding studies, high-affinities of PGRN for TNFR1 and TNFR2 which even exceeded those of TNF (7). This could explain the inhibitory effects of PGRN on TNF signaling. Two other groups, however, failed to demonstrate direct inhibition of TNF–TNF receptor interaction (20, 21). A third group reported that PGRN is unable to inhibit TNF-induced cell death (22). The researchers identifying PGRN as a competitive inhibitor of TNF binding suggested that these contradictory findings could result from different chip types used to analyze the PGRN–TNFR interaction in cell-free assays by help of the surface plasmon resonance (SPR) method and/or the use of PGRN from distinct sources/suppliers or different quality [**Table 1** and Ref. (23, 24)]. Nevertheless, even under consideration of these unverified speculations, the available literature is still inconsistent with respect to the PGRN concentrations reported to inhibit TNF binding and TNF signaling (**Table 2**). The inhibitory effects of PGRN on TNF signaling in cellular assays have been observed at low, roughly equimolar nanomolar concentrations of sTNF and PGRN. In contrast, the demonstration of the inhibitory effect of PGRN on TNF-binding to cell expressed TNFR1/2 required a huge excess of 2–3 orders of magnitude of PGRN (7, 10, 14). The use of cell-free systems as well as the use of tagged and/or purified proteins can lead to misleading results in binding studies. We, therefore, performed cell-based competitive binding studies with TNFR1- and TNFR2-expressing cells and various PGRN variants, including non-tagged non-purified and thus maximally authentic PGRN derived from human embryonal kidney cells 293 (HEK293) cells. From these experiments, we gained no evidence for inhibitory effects of high concentrations (20–500 nM) of PGRN on the interaction of TNF with TNFR1 and TNFR2.

RESULTS

In SPR experiments, in which PGRN binding to the soluble monomeric extracellular domains of TNFR1 and TNFR2 monomers adsorbed to a sensor chip has been investigated, Tang et al. identified PGRN as a high affinity ligand of TNFR1 and TNFR2 with K_D-values of 1.77 and 1.52 nM (7). These K_D-values were close or even much lower than those of sTNF (7.94 nM for TNFR1; 910 nM for TNFR2) measured in the same study with the same methodology (7). It is, however, important to note in this context that on intact cells the affinities of sTNF for its two receptors are much higher and are in the range of 0.02–0.65 nM for TNFR1 and 0.08–0.4 nM for TNFR2 [e.g., Ref. (25–32)]. Since PGRN was furthermore reported to act as a competitive inhibitor of sTNF binding, we evaluated the ability of recombinant purified PGRN to inhibit binding of a *Gaussia princeps* luciferase (GpL) fusion protein of soluble TNF (GpL-TNF) to TNFR1 and TNFR2. The luciferase from the mesopelagic copepod *Gp* is a secreted, small (19 kDa), monomeric luciferase with superior brightness. GpL fusion

TABLE 1 | Progranulin (PGRN) variants used to study the PGRN-tumor necrosis factor (TNF) crosstalk.

Variant and purification	Effect	Reference
PGRN-myc-6xHis, Ni-NTA purified	Inhibition of TNF signaling and TNF–TNFR1/2 interaction	(7)
PGRN-myc-6xHis, Ni-NTA purified	Inhibition of TNF-induced chemokine production	(11)
PGRN-myc-6xHis, Ni-NTA purified	Inhibition of TNF-induced chemokine production	(10)
PGRN-myc-6xHis, Ni-NTA purified	Inhibition of TNF-induced chemokine production	(12)
PGRN-myc-6xHis, Ni-NTA purified	Inhibition of TNF binding to Jurkat cells	(23)
PGRN-6xHis, purified (R&D Systems)	Inhibition of TNF signaling and TNF–TNF receptor interaction	(14)
PGRN-6xHis, purified (R&D Systems)	No effect on TNF signaling and TNF–TNFR1/2 interaction	(20)
PGRN-6xHis, purified (Sino Biologicals)	Anti-TNFR2 blocks PGRN-induced Akt signaling	(15)
PGRN-6xHis, purified (Sino Biologicals)	Neutralizing anti-TNFR2 blocks PGRN-induced signaling	(18)
mPGRN-6xHis, purified (R&D Systems)	Inhibition of TNF-induced osteoclastogenesis	(17)
PGRN-myc-6xHis	Inhibition of TNF-triggered ICAM1/VCAM1 induction	(13)
PGRN, untagged purified (Adipogen)	Enhancement of TNF-induced proliferation of Tregs	(9)
PGRN, untagged purified (Adipogen)	Inhibition of TNF-induced cytotoxicity	(19)
PGRN, untagged purified (Adipogen)	TNFR1 and TNFR2 binding in surface plasmon resonance	(23)
PGRN, untagged purified (Adipogen) mPGRN, untagged purified (Adipogen)	No effect on TNF signaling and TNF–TNFR1/2 interaction	(20)
mPGRN-6xHis, purified (R&D Systems) PGRN, purified (five prime therapeutics) N-TAP-PGRN,[a] Strep-Tactin purified PGRN-C-TAP,[b] Strep-Tactin purified PGRN-3xFlag, anti-Flag purified mPGRN-Fc, protein A purified	No effect on TNF signaling and TNF–TNFR1/2 interaction, all PGRN variants tested for their capacity to induce pERK in H4 glioma cells	(21)

Inhibitory effects of PGRN on TNF-induced signaling or TNF–tumor necrosis factor receptor (TNFR)1/2 interaction are shown with white background, lack of effect(s) of PGRN on TNF signaling/TNF receptor binding are shaded in blue, and studies indicating that PGRN effects are mediated by TNFR2 activation are shaded in red.

[a]*N-TAP, tandem Strep-II tag followed by the V5 epitope.*

[b]*C-TAP, tandem Strep-II tag followed by the Flag epitope.*

proteins offer, therefore, an exquisite sensitivity and a for several orders of magnitude linear signal strength (33). In particular, we have demonstrated that fusion of a GpL domain to sTNF neither affects sTNF activity nor sTNF receptor binding (32, 34). TNF-GpL is, therefore, ideally suited to evaluate competitive inhibitors of TNF–TNFR1 and TNF–TNFR2 interaction in cell-free and cellular binding studies. In a prototypical cell-free competition assay with plastic-bound Fc fusion proteins of the TNFR1 and TNFR2 ectodomains (TNFR1ed-Fc and TNFR2ed-Fc), we obtained no evidence for a significant inhibition of GpL-TNF binding to the two TNF receptors by a >1,000 fold excess of commercially available PGRN samples (**Figure 1A**). Since it has been argued that PGRN of some suppliers does not interact with TNFR1 and TNFR2, we used PGRNs from Adipogen ($PGRN_{Adi}$) and R&D Systems ($PGRN_{RD}$) which have been cited in the studies reporting direct TNFR1/2–PGRN interaction (**Table 1**). Next, we analyzed the effect of PGRN on GpL-TNF binding to cells transfected with expression plasmids encoding TNFR1 and TNFR2. To prevent disturbance by signaling related effects of the overexpressed receptors (in the case of TNFR1 there is, for example, apoptosis induction after transient expression!), we used a cytosolic deletion mutant of TNFR1 in which the death domain of the molecule has been replaced by the yellow fluorescence protein (YFP) and a TNFR2 variant in which the intracellular binding site for the signaling molecule TNF receptor associated factor-2 (TRAF2) has been substituted by YFP, too. Specific binding of 20 ng/ml (=200 pM) GpL-TNF to the transiently expressed TNFR1 and TNFR2 molecules was >800- and >2,400-fold over the unspecific background of mock-transfected control cells pretreated with a 50,000 ng/ml of unlabeled sTNF. Preincubation of the TNF receptor transfectants with 50,000, 5,000, and 500 ng/ml of sTNF diminished specific binding of GpL-TNF to both receptors completely, for >99 or >90% (**Figure 1B**). Preincubation of the TNFR1/2 transfectants with 50,000 ng/ml PGRN from Adipogen, however, showed no significant inhibition of GpL-TNF binding (**Figure 1B**). We obtained similar negative results with two other batches of PGRN from the same supplier and with PGRN from R&D systems (Figure S1 in Supplementary Material). Reciprocal binding studies with membrane TNF expressing cells and GpL fusion proteins of soluble TNFR1 and TNFR2 variants containing the ectodomains (ed) of these receptors (TNFR1ed-GpL and TNFR2ed-GpL) yielded comparable results. Specific binding of TNFR1ed-GpL and TNFR2ed-GpL to membrane TNF expressing HEK293 cells was >2,000- and >200-fold over background (**Figure 1C**). Pretreatment of the soluble GpL-receptor molecules with an excess of sTNF again reduced specific binding for more than 95% while preincubation with $PGRN_{Adi}$ showed again no significant inhibitory effect on TNF–TNFR1/2 interaction (**Figure 1C**).

To minimize possible unknown negative effects of the purification process and storage conditions of the commercially obtained PGRN samples on their ability to bind to TNFR1 and TNFR2, we used next cultures supernatants (SNs) and lysates of HEK293 cells transiently transfected with an expression plasmid encoding human PGRN. The human cell line HEK293 has been used here because HEK293 cells not only ensure high transfection efficiency, but has also used for PGRN production by groups reporting PGRN–TNFR interaction (7, 10, 23). Western blotting with a PGRN-specific antibody and $PGRN_{Adi}$ as mass standard showed that PGRN production in the SN ($PGRN_{SN}$) reached up to

TABLE 2 | Inhibitory effects of progranulin (PGRN) on tumor necrosis factor (TNF) activity and TNF receptor binding of TNF in intact cells.

TNF activity or binding assay	TNF conc. (ng/ml)[a]	PGRN conc. (ng/ml)[b]	Effect	Reference
NFκB signaling	10	225	Complete inhibition	Figures 6A,C of ref. (7)
NFκB reporter	10	9, 45, 225	IC50: approximately 45 ng/ml	Figure 6E of ref. (7)
NFκB regulated genes	10	225	Approximately 90% inhibition	Figure 6F of ref. (7)
p38/JNK activation	10	225	Complete inhibition	Figure 6G of ref. (7)
TNF inhibition of Treg activity	50	10, 50, 250	IC50: approximately 10 ng/ml	Figure S3A of ref. (7)
TNF toxicity	0.08	0–90	IC50: approximately 0.09 ng/ml	Figure S14D of ref. (7)
Treg proliferation	50	2, 20, 200	No inhibitory effect of PGRN, but enhancement at 2 and 20 ng/ml	Figure 1 of ref. (9)
Treg proliferation	20	2, 20, 200	No inhibitory effect of PGRN, but enhancement at 2 and 20 ng/ml	Figure 4 of ref. (9)
Gene induction	20	500, 2,500	80% to complete inhibition at 2,500 ng/ml	Figure 1 of ref. (10)
Gene induction	20	200	Approximately 50% to near complete inhibition	Figures 2 and 3 of ref. (11)
Gene induction	10	200	Approximately 50–90% inhibition	(12)
Gene induction	5	10, 50, 100	Approximately 50% inhibition with 100 ng/ml	(13)
Migration	100	250	Approximately 30%	(14)
Inhibition of osteoclastogenesis	10	5, 50	Strong inhibition	(17)
Cell death	0.1	250	Strong inhibition	(19)
Fluorescence-activated cell sorting (FACS)	250	75,000[c]	Reduction of mean fluorescence intensity approximately 30%	Figure 1D of ref. (7)
		375,000[c]	Reduction of mean fluorescence intensity approximately 90%	
FACS	Not indicated	5,000[d]	Reduction of mean fluorescence intensity approximately 30%	Figure 1B of ref. (23)
		25,000[d]	Reduction of mean fluorescence intensity approximately 90%	
		50,000[d]	Reduction of mean fluorescence intensity approximately 95%	
FACS	250	25,000[e]	Reduction of mean fluorescence intensity, quantification not possible due to missing indication of background staining	Figure 1 of ref. (10)
^{125}I-TNF cell binding	0.05	0–250	Reduction of bound ^{125}I-TNF approximately 50% with 250 ng/ml	(14)

Inhibitory effects of PGRN on TNF-induced cellular responses are shown with white background, inhibitory effects on TNF–TNFR1/2 interaction are shaded in blue.

[a]*TNF (MW 50,000) concentrations were converted as follows: 1 nM = 50 ng/ml.*

[b]*PGRN (MW 90,000) concentrations were converted as follows: 1 nM = 90 ng/ml.*

[c]*In a volume not indicated in the manuscript, cells were preincubated with 15,000 or 75,000 ng PGRN followed by addition of 50 ng biotinylated TNF. Finally, cell-bound TNF was detected using avidin-FITC in 200 µl. Indicated concentrations are based on the assumption that the latter volume has also been used in all other incubation steps.*

[d]*In a volume not indicated in the manuscript, cells were preincubated with 1000, 5,000 or 10,000 ng PGRN followed by addition of a not indicated amount of biotinylated TNF followed by detection of cell-bound TNF using streptavidin-FITC. Indicated concentrations are based on the assumption of a volume of 200 µl which is typical for this type of assay.*

[e]*In a volume not indicated in the manuscript, cells were preincubated with 5,000 ng PGRN followed by addition of 50 ng biotinylated TNF. Cell-bound TNF was detected using streptavidin-FITC. Indicated concentration is based on the assumption of a volume of 200 µl which is typical for this type of assay.*

approximately 10,000 ng/ml and lysates of PGRN expressing cells ($PGRN_{lys}$) contained approximately 30,000 ng/ml of the protein (**Figure 2A**). Noteworthy, $PGRN_{lys}$ somewhat faster in the gel than $PGRN_{SN}$ and both $PGRN_{lys}$ and $PGRN_{SN}$ migrated slower (approximately 85–90 kDa) compared to the $PGRN_{Adi}$ standard (70–80 kDa, Adipogen data sheet indicates 74 kDa) which was derived of HEK293 cells, too. Thus, PGRN of different sources appears to be differentially modified (e.g., by glycosylation). The sizes of $PGRN_{lys}$ and $PGRN_{SN}$ are in accordance with the literature typically indicating a size of 88 kDa for PGRN. Next, we subjected $PGRN_{lys}$ and $PGRN_{SN}$ along with corresponding samples of empty vector (EV)-transfected cells to competitive binding studies with plate-bound TNFR2-Fc and GpL-TNF. There was again no evidence for an interference of PGRN with the interaction of GpL-TNF and TNFR2. Neither pretreatment with $PGRN_{lys}$ nor with $PGRN_{SN}$ showed a significant inhibitory effect on GpL-TNF binding to TNFR2-Fc (**Figures 2B,C**). In contrast, lysates and SNs of EV-transfected cells (EV_{lys} and EV_{SN}) supplemented with 2,000 ng/ml soluble TNF showed efficient inhibition of binding of GpL-TNF (**Figures 2B,C**). Similar results were obtained with plastic-bound TNFR1-Fc instead of TNFR2-Fc (Figure S2 in Supplementary Material). There was also no significant inhibitory effect of $PGRN_{SN}$ on TNF–TNFR2 interaction on intact cells (**Figure 2D**; Figure S3 in Supplementary Material). Please note, cellular binding studies with the PGRN containing cell lysates ($PGRN_{lys}$) were not possible due to the cell lytic effects of the lysis buffer. It should also be stressed that the lysis buffer used was prepared according to Tang et al. reporting PGRN-TNFR2 co-immunoprecipitation in this buffer (7). To evaluate the effect of PGRN on TNF–TNFR1/2 interaction in a second independent cellular model, we performed competitive binding studies with HeLa-TNFR2 cells. HeLa-TNFR2 is a stable HeLa transfectant expressing in addition to endogenous TNFR1 also TNFR2 due to stable transfection (35). Despite their obvious different degree of modification (**Figure 2A**), the various PGRN variants ($PGRN_{Adi}$, $PGRN_{RD}$, and $PGRN_{SN}$) had no effect on GpL-binding. In contrast, pretreatment with sTNF or Flag-LTα (F-LTα) inhibited GpL-binding for >99 and >98% indicating efficient blockade of TNFR1 and TNFR2 (Figure S4 in Supplementary Material).

To further minimize possible influencing variables affecting PGRN–TNF receptor interaction, we secondarily transfected TNFR2 transfectants with expression plasmids encoding PGRN (PGRN), soluble TNF (sTNF), or membrane TNF (memTNF), and analyzed the transfected cells finally again for GpL-TNF binding. As expected transfection of plasmids encoding sTNF or memTNF resulted in strong reduction of GpL-TNF binding

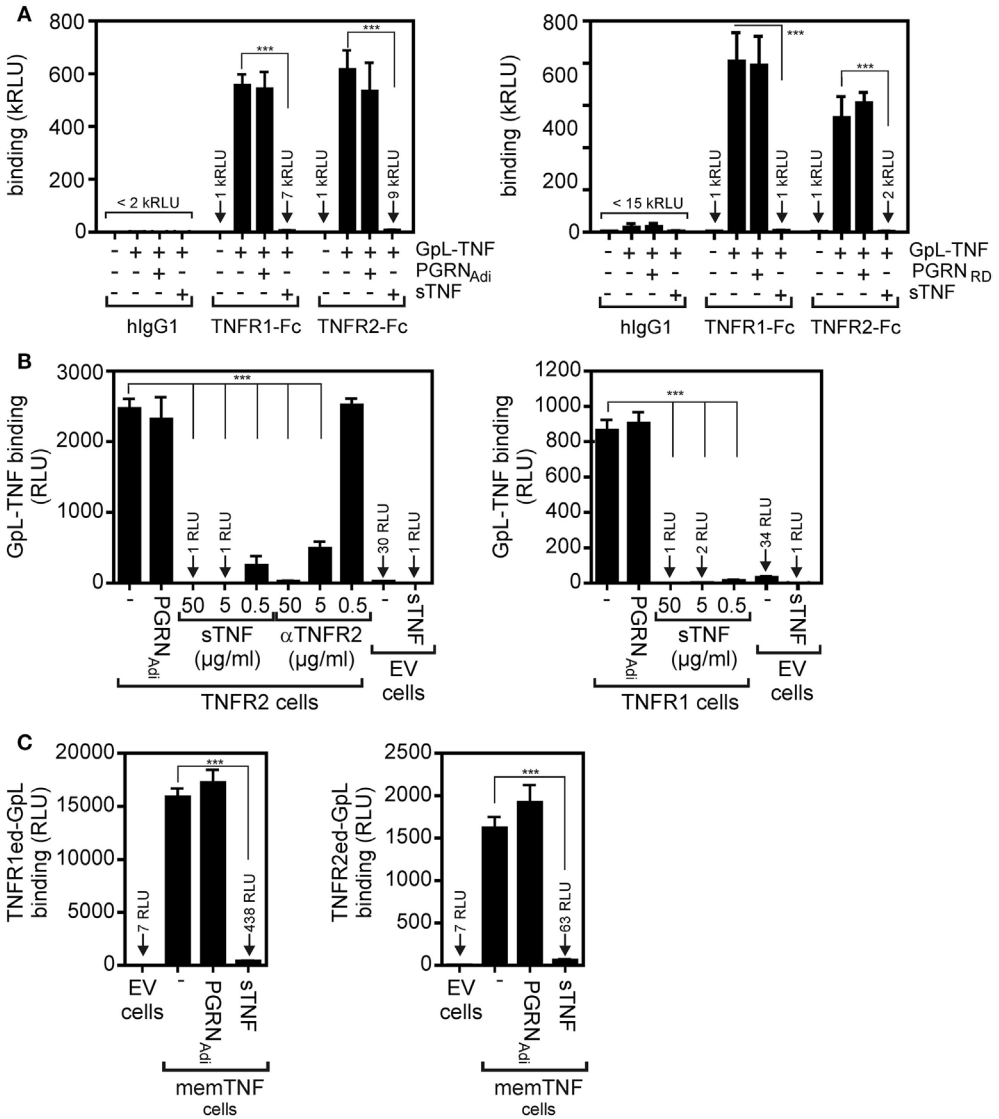

FIGURE 1 | Preincubation with purified progranulin (PGRN) samples does not interfere with tumor necrosis factor (TNF)-tumor necrosis factor receptor-1 (TNFR1) and TNF–TNFR2 interaction in cell-free and cellular binding studies. **(A)** TNFR1-Fc and TNFR2-Fc or an irrelevant human IgG1 (Rituximab) were immobilized to black enzyme-linked immunosorbent assay (ELISA) plates. Where indicated wells were preincubated with 25 µg/ml untagged PGRN from Adipogen ($PGRN_{Adi}$) or 25 µg/ml myc-6xHis-tagged PGRN from R&D Systems ($PGRN_{RD}$) for 1 h. GpL-TNF was then added to reach a concentration of 10 ng/ml and finally bound GpL-TNF was quantified by measuring its GpL activity. As positive control for successful competitive binding inhibition groups were included, where 10 µg/ml of soluble TNF (sTNF) have been added instead of PGRN. **(B)** Human embryonal kidney cells 293 (HEK293) cells were transfected with empty vector (EV) or expression plasmids encoding a deletion mutant of TNFR1, where the death domain has been replaced by yellow fluorescence protein (YFP) (TNFR1) or deletion mutant of TNFR2, where the TRAF2 binding site has been replaced again by YFP (TNFR2). Next day, aliquots of cells (1×10^6) were preincubated with 500, 5,000, or 50,000 ng/ml of sTNF or 50,000 ng/ml $PGRN_{Adi}$ for 2 h at 37°C or remained untreated. Binding studies were performed in technical triplicates with 20 ng/ml GpL-TNF. In the experiment with TNFR2-transfected cells, a group was pretreated with 20 µg/ml of a blocking TNFR2-specific antibody (αTNFR2). Please note, GpL-TNF binding of EV-transfected cells in the presence and absence of an excess of sTNF defines the low endogenous expression of TNF receptors which was about 1–3% of the ectopically expressed receptors. **(C)** EV-transfected control cells and membrane TNF (memTNF) expressing transfectants were incubated with 100 ng/ml of TNFR1ed-GpL or TNFR2ed-GpL and mixtures of these GpL variants with 2,000 ng/ml sTNF or 2,000 ng/ml $PGRN_{Adi}$. After 90 min, unbound molecules were removed and specific binding was again obtained by subtracting non-specific binding (EV transfectants) from total binding (memTNF transfectants). Please be aware, the fact that specific binding of TNFR1ed-GpL is app. Tenfold higher than those of TNFR2ed-GpL reflects the fact that soluble monomeric TNFR1 has much higher affinity for TNF than soluble TNFR2 molecules and that non-saturating soluble receptor concentrations have been used in this competition assays. ***$p < 0.0001$.

to TNFR2 (**Figures 3A,B**). Once again PGRN expression failed to have an effect on TNF–TNFR2 interaction despite robust expression yielding approximately 3,000 ng/ml PGRN in the cell culture SN and cell-associated expression comparable to those of memTNF (**Figure 3A**). Coexpression of PGRN neither showed an effect on the number of binding sites for GpL-TNF nor on the K_D-value of the interaction of GpL-TNF and TNFR2 (**Figure 3B**). In this simplified and highly sensitive experimental

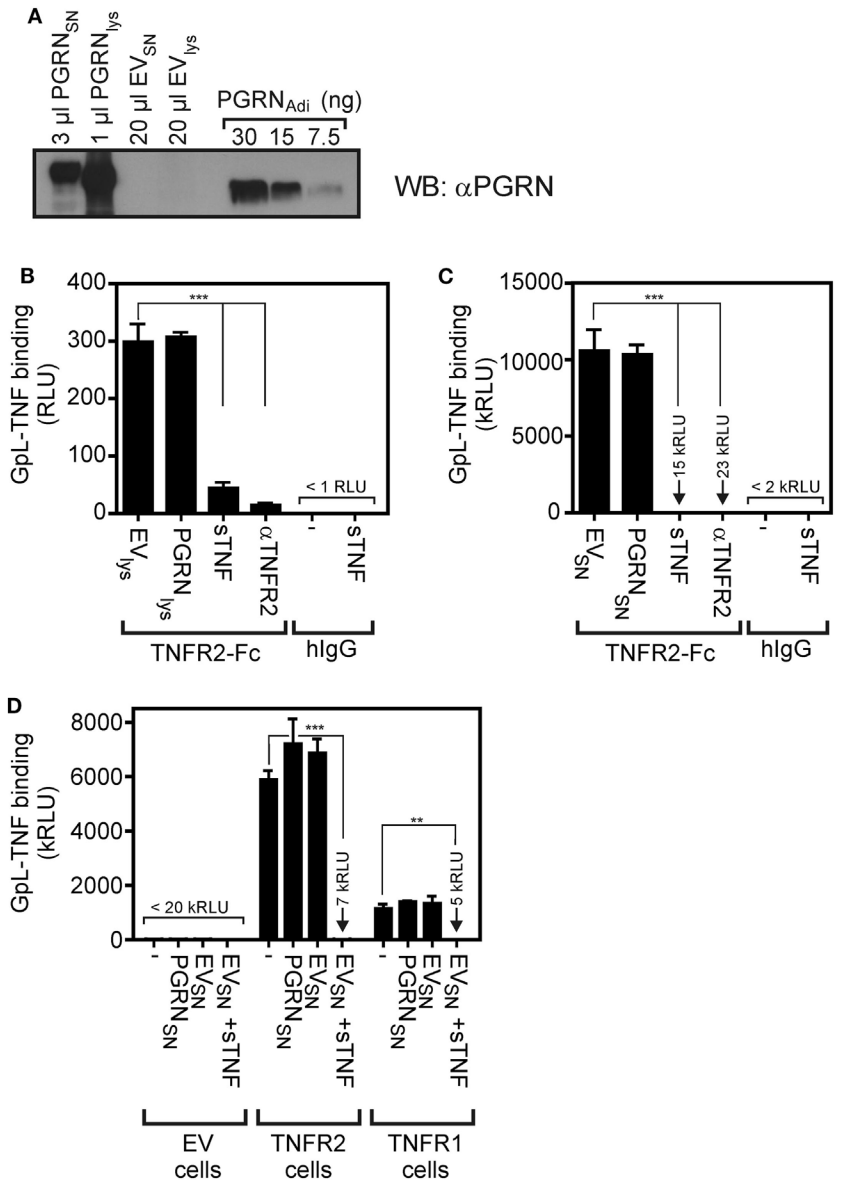

FIGURE 2 | Preincubation with untagged human embryonal kidney cells 293 (HEK293) cell-derived progranulin (PGRN) does not interfere with tumor necrosis factor (TNF)-tumor necrosis factor receptor-1 (TNFR1) and TNF–TNFR2 interaction in cell-free and cellular binding studies. **(A)** HEK293 cells were transfected with empty vector (EV) or an expression plasmid encoding non-tagged PGRN. The indicated volume of supernatants (SNs) and cell lysates derived from these transfectants along with recombinant $PGRN_{Adi}$ (30, 15, and 7.5 ng) as standard were analyzed by Western blotting for the presence of PGRN and estimation of PGRN concentration. PGRN concentrations reached approximately 10,000 ng/ml in the SN of PGRN transfected cells ($PGRN_{SN}$) and approximately 30,000 ng/ml in the corresponding cell lysate ($PGRN_{lys}$). There was no detectable endogenous PGRN expression neither in the SN (EV_{SN}) nor the lysate (EV_{lys}) of EV-transfected cells. **(B,C)** TNFR2-Fc or, as a control for unspecific binding, IgG1 was immobilized to black enzyme-linked immunosorbent assay plates. Lysates **(B)** and SNs **(C)** of PGRN and EV-transfected cells [$PGRN_{lys}$ and EV_{lys} **(B)**, $PGRN_{SN}$ and EV_{SN} **(C)**] were added for 1 h before the specific binding of 50 ng/ml and GpL-TNF was determined in triplicates. Where indicated immobilized TNFR2-Fc was pretreated for 1 h with 2,000 ng/ml sTNF or 20 µg/ml of a neutralizing TNFR2-specific antibody (αTNFR2). **(D)** HEK293 transfectants expressing TNFR1 or TNFR2 along with control HEK293 cells transfected with EV were preincubated for 1 h with pure $PGRN_{SN}$, pure EV_{SN}, and pure EV_{SN} with and without supplementation with 10,000 ng/ml sTNF. After preincubation, cells were incubated in triplicates with 10 ng/ml GpL-TNF at 37°C for 1 h and finally cell-bound GpL activity was determined. ***$p < 0.0001$; **$p < 0.001$.

setting, both PGRN and its potential binding partner TNFR2 were directly expressed by the cells in the assay. This maximally rules out that experimental handling of the two possible binding partners or their purification can affect or change their interaction. In comparable experiments where TNFR1 has been transiently expressed along with the sTNF and PGRN encoding expression plasmids, similar results were obtained (Figure S5 in Supplementary Material).

Finally, we generated PGRN fusion proteins with an N- and a C-terminal GpL domain (GpL-PGRN and PGRN-GpL)

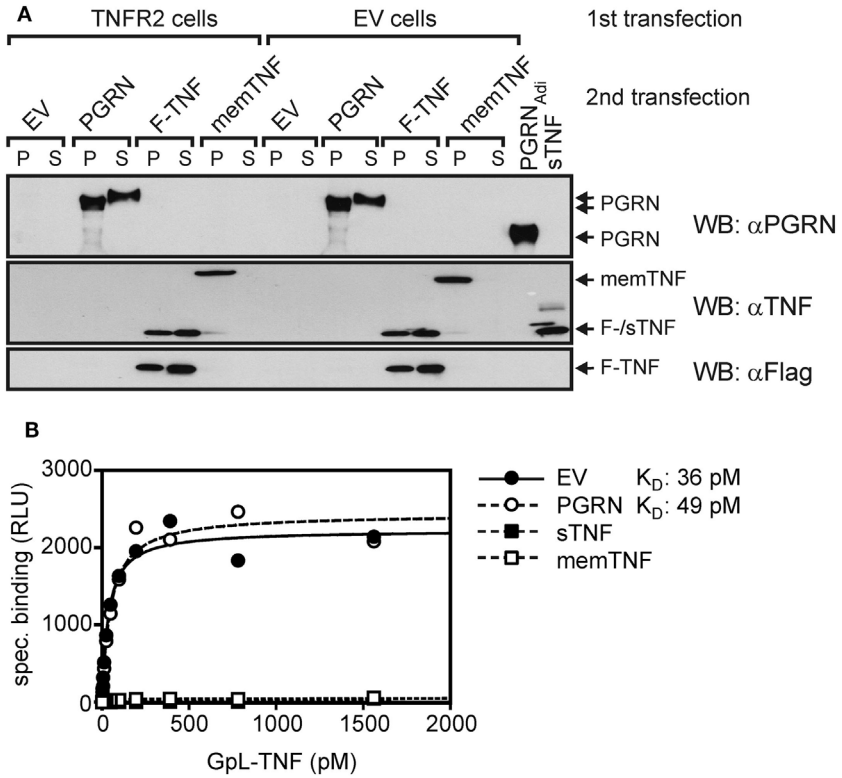

FIGURE 3 | *Gaussia princeps* luciferase (GpL)-tumor necrosis factor (TNF) binding to cells with endogenous coexpression of tumor necrosis factor receptor-2 (TNFR2) and progranulin (PGRN). **(A)** Human embryonal kidney cells 293 (HEK293) cells were transfected (first transfection) with empty vector (EV) or an expression vector encoding TNFR2, where the TNF receptor associated factor 2 (TRAF2) binding site has been replaced by yellow fluorescence protein (YFP) (TNFR2). The following day, transfecfed cells were split into four aliquots which were transfected a second time (second transfection) with expression plasmids encoding PGRN, membrane TNF (memTNF), soluble Flag-tagged TNF (F-TNF), or EV. After an additional day, aliquots of 30,000 cells (P) and 15 µl SN (S) were analyzed by Western blotting with anti-PGRN, anti-TNF, and anti-Flag along with 100 ng $PGRN_{Adi}$ and 100 ng purified untagged soluble TNF (sTNF). **(B)** Equilibrium binding studies were performed with the indicated concentrations of GpL-TNF. Specific binding of GpL-TNF in the presence of PGRN (second transfection PGRN), membrane TNF (second transfection memTNF), and Flag-TNF (second transfection F-TNF) or the absence of an potential modulator (second transfection EV) was obtained by subtracting unspecific binding values (first transfection EV) from the corresponding total binding values (first transfection TNFR2). Specific binding values were fitted by non-linear regression analysis to a single binding site type of interaction by help of the GraphPad Prism 5 software.

and investigated their binding to TNFR1 and TNFR2. While 4–250 ng/ml GpL-TNF showed significant binding to plastic-bound TNFR1-Fc and TNFR2-Fc, there was no significant binding with lysates and SNs of GpL-PGRN (GpL-$PGRN_{lys}$ and GpL-$PGRN_{SN}$) and PGRN-GpL (PGRN-GPL_{lys} and PGRN-GpL_{SN}) expressing HEK293 cells despite using concentration of up to 5,000–30,000 ng/ml (**Figures 4A,B**). Likewise, there was no relevant specific binding of GpL-$PGRN_{SN}$ and PGRN-GpL_{SN} to TNFR1 and TNFR2 transfected cells (**Figure 4C**). Although, one cannot fully rule out that an authentic N- or C-terminus of PGRN is important for its putative interaction with TNF receptors, this appears unlikely because PGRN activity has been reported with various N- and C-terminally tagged variants (**Table 1**).

DISCUSSION

A variety of studies demonstrated that PGRN can inhibit TNF-induced cellular activities. The identification of PGRN as a protein that "*directly binds to TNFR*" and causes "*dose-dependent inhibition of TNFα binding to TNFR1 and TNFR2*" (7) offered a simple and straightforward explanation of the inhibitory effects of PGRN on TNF activity at the molecular level. However, two independent groups failed to reproduce PGRN binding to TNFR1 (20, 21). Two other groups found furthermore no evidence for an inhibitory action of PGRN on TNF-induced signaling or even reported enhanced TNF activity (9, 22). It has been suggested that this was due to "problematic" PGRN preparations and technical differences in the cell-free analysis of PGRN–TNFR1/2 interaction by SPR (24). Indeed, the PGRN variants used by the various groups differed with respect to the position and nature of tags or were from different suppliers (**Table 1**). To avoid the possible impact of the commercial source, purification procedures or tagging and to maximally reduce the relevance of "technical" factors, we analyzed the inhibitory effect of PGRN on receptor binding of TNF in cellular binding studies at 37°C in normal culture medium not only with PGRN from commercial sources, but also with fresh,

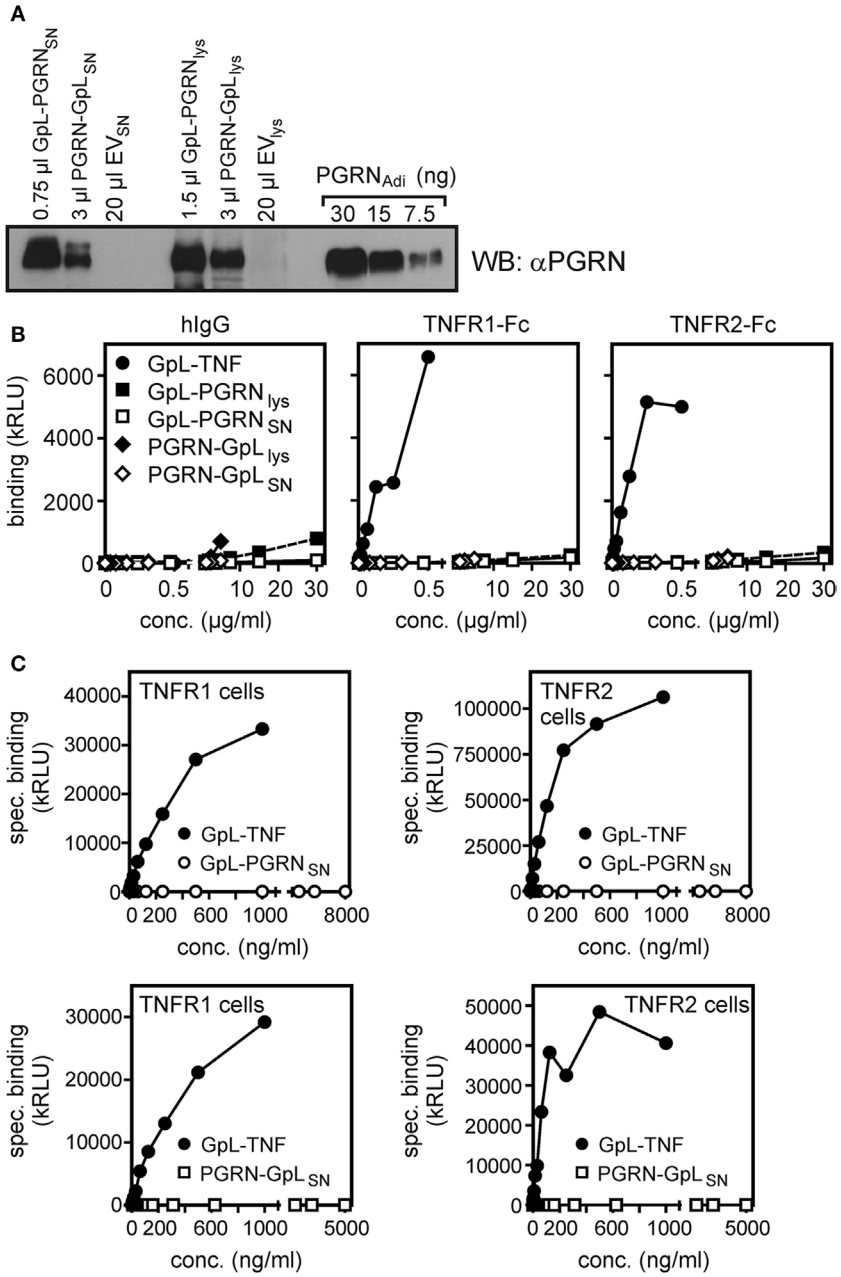

FIGURE 4 | *Gaussia princeps* luciferase (GpL) fusion proteins of progranulin (PGRN) show no relevant binding to tumor necrosis factor receptor-1 (TNFR1) or TNFR2. **(A)** Human embryonal kidney cells 293 (HEK293) cells were transiently transfected with expression plasmids encoding GpL-PGRN (GpL-PGRN), PGRN-GpL (PGRN-GpL), or empty vector (EV). GpL-PGRN concentrations in supernatants (SNs) and cell lysates were determined by help of a GpL fusion protein of known concentration. SNs and cell lysates, containing approximately 100 ng PGRN-GpL or GpL-PGRN along with 100 ng $PGRN_{Adi}$, were subjected to Western blotting with a PGRN-specific antibody to verify the integrity of the PGRN GpL fusion proteins. **(B)** TNFR1-Fc, TNFR2-Fc or, as a control for unspecific binding, hIgG1 were immobilized to black enzyme-linked immunosorbent assay plates. Lysates and SN of the GpL-PGRN (GpL-$PGRN_{lys}$ and GpL-$PGRN_{SN}$) and PGRN-GpL (PGRN-GpL_{lys} and PGRN-GpL_{SN}) transfected cells and GpL-tumor necrosis factor (TNF) were added for 1 h and binding was determined in triplicates. **(C)** TNFR1 and TNFR2 expressing transfectants (total binding) and EV-transfected HEK293 cells (non-specific binding) were subjected to equilibrium binding studies with the indicated GpL fusion proteins. Specific binding (= total − non-specific binding) values were fitted by non-linear regression analysis to a single binding site type of interaction by help of the GraphPad Prism 5 software.

non-purified untagged, and thus fully authentic PGRN released from transiently transfected HEK293 cells. Neither, PGRN samples from Adipogen and R&D Systems, which has been used in reports demonstrating PGRN–TNF receptor interaction, nor HEK293-derived SNs containing untagged PGRN showed an inhibitory effect on binding of a GpL-TNF fusion protein

to plastic-bound or cell-expressed TNFR1/2 (**Figures 1** and **2**; Figures S1, S2, and S4 in Supplementary Material). In the experiments with commercially available purified PGRN samples, we used concentrations up to 25 and 50 μg/ml (approximately 280 and 560 nM) and the HEK293-derived PGRN containing SNs reached concentrations of around 30 μg/ml, too (**Figure 2A**). This was not only a huge excess over GpL-TNF (MW 100,000), which was applied with 2–50 ng/ml (approximately 0.02–0.5 nM), but also far higher than the PGRN concentrations used in the literature to modulate TNF signaling, or than the PGRN levels in the synovial fluid of patients suffering on rheumatoid arthritis (68 ng/ml) or malignant lymphomas (91.3 ng/ml) (36, 37). Intriguingly, expression of PGRN in TNFR2 expressing (**Figure 3**) or in TNFR1 expressing cells (Figure S5 in Supplementary Material) showed no effect on TNF binding. In an independent approach, we looked also for direct binding of PGRN to plastic-bound and cell-expressed TNFR1 and TNFR2. For this purpose, we used non-purified HEK293-derived variants of PGRN with an N- or C-terminal GpL-flag reporter domain. With none of these two variants we found evidence for significant TNFR1 or TNFR2 binding (**Figure 4**). Since various PGRN variants (**Table 1**) successfully used in the literature to study the PGRN-TNF crosstalk also carried N- and/or C-terminal tags including a His-tag which has the potential to interfere with the numerous Cys residues in PGRN, it appears unlikely that these negative data have been caused by the sole use of a tag.

Our studies are mainly based on the use of a GpL-fusion protein of TNF. One possibility for the failure of PGRN to block GpL-TNF binding to TNFR1 and TNFR2, was that the GpL domain might, instead, directly interact with PGRN, and artifactually prevent it from interacting with the TNF receptors. This can, however, be ruled out because we used up to >1,000-fold molar excess of PGRN in our PGRN/GpL-TNF competition experiments. Therefore, even if the GpL domain of GpL-TNF did bind irreversibly to PGRN, a huge surplus of "GpL-free" PGRN would have been available in our experiments to block TNFR1/TNFR2 binding by GpL-TNF (or by GpL-TNF in complex with PGRN).

In sum, we found no evidence for a direct and TNF binding-competing interaction of PGRN and TNF receptors even not in experimental settings, where PGRN and TNF receptors were expressed directly by the cells in the assay and where their potential interaction can thus not be affected by unknown factors related to experimental processing (**Figure 3**; Figure S5 in Supplementary Material). Our results convincingly argue against a direct generally occurring prototypic PGRN–TNFR1/2 interaction, but of course cannot rule out complex interaction scenarios, requiring for yet unknown additional factors or chemical or biological modification of PGRN. We want to stress in this context that already the studies reporting direct PGRN–TNFR1/2 interaction give indications which challenge the idea that PGRN acts as a prototypic ligand binding-blocking interaction partner of TNFR1 and TNFR2.

First, in the initial study describing PGRN as a high-affinity ligand for TNFR1 and TNFR2, Tang et al. reported affinities of 1.77 and 1.52 nM for these receptors (7). However, despite the strong affinities of the PGRN–TNFR1 and PGRN–TNFR2 interactions, excessive high concentrations of PGRN (75,000 ng/ml = 833 nM) were required to see inhibitory effects on binding of sTNF to TNFR1 and TNFR2 and this although TNF receptor activities were inhibited at much lower PGRN concentrations (**Table 2**). Likewise, in various follow up studies the fluorescence-activated cell sorting (FACS)- and enzyme-linked immunosorbent assays (ELISA)-based demonstration of PGRN binding to TNFR1/2 in competition experiments with TNF required again concentrations in the μM instead of nM range while modulation of TNF signaling was evident at >two orders of magnitude lower PGRN concentrations (**Table 2**). Of course, the huge discrepancy in the reported PGRN concentrations required to inhibit TNF signaling and to block TNF binding is not compatible with the mode of action of a simple competitive inhibitor. Second, Tian et al. analyzed TNFR2-expressing Raw264.7 and THP-1 cells for TNF binding (50 ng/ml) by FACS and reported that the ability of a high excess of PGRN (5,000 ng) to reduce sTNF binding was diminished at higher cell densities (10). Such a cell density/receptor number dependency of the ability of PGRN to interfere with TNF binding is again not straightforwardly compatible with competitive binding inhibition. Third, PGRN enhances TNF-induced TNFR2-mediated proliferation and suppressive activity of regulatory T cells (9) and PGRN-induced Akt signaling has found to be inhibited by neutralizing TNFR2 antibodies (15, 18). Both observations again argue against competitive inhibition of TNF binding by PGRN.

CONCLUSION

Two independent studies failed to demonstrate inhibition of TNF binding to TNF receptors by PGRN (20, 21). Our results obtained in highly sensitive cellular binding studies with two commercially available PGRN samples and GpL-tagged and untagged PGRN containing cell culture SNs also gave no evidence for high affinity and/or competitive PGRN binding to TNFR1 and TNFR2. Thus, it is obvious that the putative direct and competitive interactions of PGRN with the two TNF receptors are not robust and straightforwardly reproducible. Future studies must identify the factors or modifications which enable PGRN to bind TNF receptors. Till then we recommend to be careful in assigning inhibitory effects of PGRN on TNF function to competitive inhibition of TNF–TNFR1/2 interaction without direct concomitant experimental evaluation.

MATERIALS AND METHODS

Reagents and Cell Lines

Progranulin was purchased from Adipogen, Liestal, Switzerland (untagged protein, #AG-40A-0188Y) and R&D Systems, Wiesbaden, Germany (C-terminally myc-6xHis-tagged, #). The expression vector (pCMV6-XL5) encoding untagged human PGRN (Ac. No.: NM_002087) was from Origene, Rockville, MD, USA (#SC118822). The anti-PGRN mouse monoclonal antibody C-11 and the TNF-specific goat IgG N-19 were from Santa Cruz Biotechnology, Dallas, USA (C-11: #sc-377036; N-19 #sc-1350). The pEF-BOS-based expression vector encoding membrane TNF

have been described elsewhere (31). TNFR1-Fc was from R&E Systems, Wiesbaden, Germany; TNFR2-Fc (Enbrel) was from Pfizer, the TNF-specific antibody Humira was from AbbVie (Wiesbaden, Germany), and soluble TNF was a kind gift of Prof. Daniela Männel (University of Regensburg). The TNFR1-specific antibody H398 was kindly provided by Prof. Klaus Pfizenmaier (University of Stuttgart). Production, characterization, and use of GpL-Flag-TNC-TNF (abbreviated in the study as GpL-TNF) and Flag-LTα have been described in detail elsewhere (32, 34). The Flag tag in GpL-Flag-TNC-TNF was introduced for affinity purification and the tenascin-C trimerization domain stabilizes the trimeric nature of the TNF molecule. HEK293 cells (ATCC) were cultivated in RPMI1640 medium (Sigma-Aldrich, Schnelldorf, Germany) supplemented with 10% fetal calf serum (Gibco—Thermo Fisher Scientific, Darmstadt, Germany). The Flag tag-specific antibody was again from Sigma-Aldrich (Schnelldorf, Germany). HeLa cells stably transfected with TNFR2 (HeLa-TNFR2) have been described elsewhere (35). Typical FACS results of TNFR2 and TNFR1 expression of HeLa-TNFR2, HEK293, and HEK293 cells transiently expressing TNFR1/2 variants are shown in Figure S6 in Supplementary Material.

Molecular Cloning and Expression of Recombinant Proteins

The expression vector encoding soluble Flag-TNF was generated by replacement of the TRAILR2 encoding part in PS435 (kind gift of Prof. Pascal Schneider, University of Lausanne), a pCR3.1-based expression vector (Invitrogen—Thermo Fisher Scientific, Darmstadt, Germany) encoding the human Ig leader followed by a Flag-tag and TRAILR2, with a DNA amplicon encoding aa 85–223 of human TNF (ac. no.: NP000585). The TNFR1ed-GpL and TNFR2ed-GpL encoding expression plasmids are also based on pCR3.1 and encode expression cassettes comprising the ectodomain of TNFR1 (aa 1–211 of ac. no.: M58286.1) or TNFR2 (aa 1–257 of ac. no.: M55994.1) followed by the Flag epitope and aa 18–185 of ac. no.: GM037681 encoding mature, thus leader free GpL. Two aa insertions (GSAGEF and LE) resulting from molecular cloning furthermore separate the Flag tag from the receptor and GpL parts, respectively. The GpL-PGRN encoding expression plasmid is also a pCR3.1 derivative and encodes GpL including its leader sequence followed by a Flag tag and aa 21–593 of human PGRN whereby the Flag epitope is connected with the GpL and the PGRN domain by a five aa (SGAGS) and a two aa (EF) insertion.

Recombinant proteins were produced in HEK293 cells by transient transfection of the expression plasmids described above. For this purpose, the medium of tissue culture dishes with confluent HEK293 cells was replaced by 15 ml of serum-free RPMI 1640 medium containing penicillin–streptomycin. For each culture, 2 ml of RPMI 1640 medium containing 12 µg of the expression plasmid of interest were prepared and supplemented dropwise and under vortexing with 36 µl of a 1 mg/ml polyethylenimine (PEI, Polysciences Europe, Hirschberg, Germany). After 15 min at room temperature, the plasmid/PEI solution was added and transferred to the HEK293 cells. Next day, the serum-free plasmid/PEI-containing medium was replaced by fresh RPMI 1640 medium containing 2% FCS and penicillin–streptomycin. After 4–6 days, SNs were collected and cellular debris was removed by centrifugation (10 min, 4,630 *g*). The resulting PGRN containing SN ($PGRN_{SN}$) was directly used for experiments or after dilution in cell culture medium. To obtain cell-associated PGRN ($PGRN_{lys}$), correspondingly transfected cells were harvested 48 or 72 h post transfection and lysed in RIPA buffer (Cell Signaling, Leiden, Netherlands). Finally, concentration of the protein of interest was evaluated by Western blotting and an appropriate protein mass standard and/or by measuring the activity of the GpL domain. The complete Western blots of the cuttings shown in **Figures 2A**, **3A** and **4A** are documented in Figure S7 in Supplementary Material.

Binding Studies

For cell-free binding studies with plastic surface-immobilized protein, solutions (2 µg/ml in PBS or 0.1 M carbonate buffer) of the purified protein of interest [TNFR1-Fc, TNFR2-Fc, PGRN, Rituximab (Roche, Basel, Switzerland), a CD20-specific human IgG1 molecule, as a negative control for TNFR1/2-Fc] were subjected to black high bind ELISA plates (Greiner, Frickenhausen, Germany). After overnight incubation at 4°C and three washes with PBS Tween, remaining free binding sites were saturated by incubation (1 h, room temperature) with blocking buffer (10% FCS in PBS). After three washing cycles with PBS Tween, the actual binding studies were performed. In the case of equilibrium binding studies wells were incubated for 2 h with increasing concentrations of the GpL fusion protein of interest at room temperature. Unbound protein was then removed by five wash cycles with PBS Tween and finally well-associated luciferase activity was determined (see below). Values for non-specific binding were derived from wells coated with control protein or with coating buffer only and were subtracted from the corresponding total binding values obtained from the wells coated with the protein of interest to obtain specific binding values. In the case of competition binding studies, wells were treated for 0.5–1 h with increasing concentrations of the potential inhibitor (PGRN variants, sTNF, Flag-LTα) or remained untreated before the GpL fusion protein was added for an additional hour. Finally, wells were again washed five times and used for quantification of bound luciferase activity.

For cellular binding studies, HEK293 cells were transiently transfected with expression plasmids encoding the protein of interest and EV. Next day, cells were divided into the required number of aliquots of 0.5–1 × 10^6 cells in 150 µl RPMI 1640 medium with 10% FCS. Where indicated, cells were then pretreated at 37°C for 1 h with potential antagonistic proteins (sTNF, Flag-LTα, and PGRN variants), otherwise cells remained untreated. Cells were then supplemented with the GpL fusion protein of interest and after an additional incubation period of 1 h, unbound proteins were removed (five washes with PBS). Finally, cells were collected in 50 µl of RPMI 1640 medium with 0.5% FCS to quantify the remaining cell-bound GpL fusion protein molecules. Binding values derived of EV-transfected cells were considered as non-specific binding and binding values obtained from the transfectants expressing the protein of interest were considered as total binding. Please note, the expression levels observed after transfection of expression plasmids encoding

TNFR1 and TNFR2 were regularly >100-fold higher than those of endogenously expressed TNFR1. There was no evidence for endogenous expression of TNF in the HEK293 cells.

Gaussia princeps luciferase activity was measured with the *Gaussia* luciferase Assay Kit (New England Biolabs, Frankfurt, Germany) essentially as described by the supplier. After starting the reaction by adding substrate-buffer solution, light emission was immediately (<10 s) quantified (Lucy 2 or a LUmo Luminometer; both Anthos Labtec Instruments) to minimize errors due to the decay of GpL activity. Please note, the LUmo Luminometer has a much higher sensitivity compared to the Lucy 2 luminometer. Data are reported as mean ± SEM and were analyzed by Bonferroni's test or were analyzed with the "nonlinear regression to a one-site specific binding curve" or the "nonlinear regression to a one-site competitive binding curve" function of the GraphPad Prism5 software.

AUTHOR CONTRIBUTIONS

HW designed the study and prepared the manuscript. IL and SF performed the experiments and contributed to study design. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00793/full#supplementary-material.

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MINI REVIEW
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Selective Modulation of TNF–TNFRs Signaling: Insights for Multiple Sclerosis Treatment

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Autoimmunity develops when self-tolerance mechanisms are failing to protect healthy tissue. A sustained reaction to self is generated, which includes the generation of effector cells and molecules that destroy tissues. A way to restore this intrinsic tolerance is through immune modulation that aims at refurbishing this immunologically naïve or unresponsive state, thereby decreasing the aberrant immune reaction taking place. One major cytokine has been shown to play a pivotal role in several autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS): tumor necrosis factor alpha (TNFα) modulates the induction and maintenance of an inflammatory process and it comes in two variants, soluble TNF (solTNF) and transmembrane bound TNF (tmTNF). tmTNF signals *via* TNFR1 and TNFR2, whereas solTNF signals mainly *via* TNFR1. TNFR1 is widely expressed and promotes mainly inflammation and apoptosis. Conversely, TNFR2 is restricted mainly to immune and endothelial cells and it is known to activate the pro-survival PI3K-Akt/PKB signaling pathway and to sustain regulatory T cells function. Anti-TNFα therapies are successfully used to treat diseases such as RA, colitis, and psoriasis. However, clinical studies with a non-selective inhibitor of TNFα in MS patients had to be halted due to exacerbation of clinical symptoms. One possible explanation for this failure is the non-selectivity of the treatment, which avoids TNFR2 stimulation and its immune and tissue protective properties. Thus, a receptor-selective modulation of TNFα signal pathways provides a novel therapeutic concept that might lead to new insights in MS pathology with major implications for its effective treatment.

Keywords: tumor necrosis factor alpha, TNFR2, TNFR1, immune tolerance, multiple sclerosis, neurodegeneration

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INTRODUCTION

Multiple sclerosis (MS) affects approximately 2.5 million people worldwide. It is considered as an autoimmune disease characterized by white and gray matter lesions in the central nervous system (CNS) caused by autoreactive T cells that escaped from central and peripheral tolerance patrolling mechanisms. These cells travel along with activated B cells and monocytes to the CNS where they infiltrate, starting a synergistic attack against myelin (1). As demyelination is a key feature of MS pathology, several myelin proteins have been investigated as targets of these autoreactive lymphocytes. It has been shown that myelin basic protein and myelin oligodendrocyte glycoprotein are recognized by mature autoreactive T-helper cells in MS patients but also in healthy individuals (2). The identification of a major T cell autoantigen in MS is still a matter of controversy. It may be due

to technical limitations in autoantibodies' detection or epitope spreading (3). Anyway, the search of pathological anti-myelin immune responses is still open-ended.

Currently, the etiology of MS has been investigated from another angle that favors the idea that initial pathology occurs within the CNS, similarly to other neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (4). This theory argues that degeneration of oligodendrocytes and/or myelin initiates pathology by releasing autoantigen, which in turn are responsible for the autoimmune, inflammatory response in the organism. Of importance, mitochondrial dysfunction (5), ROS production (6), misfolding of proteins (7), and release of proapoptotic signals (8) are just few of the consequences (9). Myelin-loaded microglia/macrophages are also largely involved in this pathological process (10). They are constantly producing ROS through oxidative burst giving rise to mitochondrial dysfunction and proapoptotic signals release causing oligodendrocytes death and demyelination.

Being it autoimmune or neurodegenerative, the study of the nature of this disease is yet mostly descriptive than causative. With this limited understanding, the animal models currently available seem to mimic only few and separated features of the disease, which further restrict our view of the underlying mechanisms causing MS. Even though it seems very difficult to achieve a solid and unifying explanation, great efforts have been made to develop treatments to reduce the symptomatic incidences in MS patients. The available therapeutic strategies are primarily focused on suppressing or modulating certain immune functions thereby leading to a partial and temporary recovery sometimes with major side effects (11). There is still a strong urge for an effective treatment that slows down MS disease progression or to prevent its development.

This review will focus on the potential value for MS treatment of tumor necrosis factor alpha (TNFα), a major cytokine involved in several biological functions. Furthermore, the different and dual functions of TNFα are specified by the two receptors (TNFR1 and TNFR2) that it activates. Current research highlights a great potential of selectively targeting TNF–TNFRs signaling with promising immune protective, tissue regenerative, and neuroprotective therapeutic properties.

ANTI-TNF THERAPIES IN AUTOIMMUNITY

Self-recognition is an essential biological process that gives rise to immune tolerance: a state of indifference or non-reactivity toward a substance that would normally be expected to excite an immunological response (12). Yet, when the immune system erroneously identifies a self-antigen as a danger, it initiates an inflammatory response against it. The latter mechanism is defined as *autoimmunity*, which encompasses tissue damage, caused by T-cell or antibody reactivity to self. Many inflammatory diseases are autoimmune diseases, including rheumatoid arthritis (RA), MS, Graves' disease, type 1 diabetes mellitus, Crohns' disease, and others. Nowadays, they affect 12.5% of the world's population (13) and they can be distinguished based on their primary target organ (joints, skin—psoriasis; CNS—MS; intestine—inflammatory bowel disease (IBD); pancreas—type 1 diabetes mellitus). For many years, the standard treatment relied on diminishing autoimmune pathology with general immunosuppressive agents, anti-proliferative drugs, and corticosteroids. Halting the immune system has always major and diffuse side effects that increase the toxicity of the intervention thereby decreasing its therapeutic value. Immunosuppressant drugs are widely used by clinicians to reduce inflammatory attacks on tissues but, due to their low efficacy, disease-modifying drugs with greater specificity and lower toxicity were implemented. Monoclonal antibodies and engineered biological products have become now standard interventions for several autoimmune diseases, including MS. A long standing class of biologics used for many autoimmune diseases is TNF blockers which includes infliximab, etanercept, adalimumab, PEGylated certolizumab, and golimumab. These are the FDA-approved anti-TNF biologics for the treatment of Crohn's disease, ulcerative colitis, RA, ankylosing spondylitis (AS), psoriatic arthritis, and plaque psoriasis (14). Even though it has been extensively studied, the potential therapeutic value of blocking TNF is limited by its partial efficacy in different diseases. Anti-TNF treatment is discontinued in 1/3 of RA patients within the first year of treatment (15). Around 10–30% of IBD patients do not respond to initial treatment while 23–46% lose response over time (16). Similarly, 27% of patients with psoriasis discontinue anti-TNF treatment after a year or lose its efficacy over time (17). So far, there is little evidence explaining the reasons and risk factors for primary or secondary non-response. Therefore, other strategies are implemented by clinicians to maintain efficacy with acceptable tolerability such as using a different TNF blocker, switching class of biologic, dose adjustments, and change in route of administration, when possible. Moreover, failure of anti-TNF therapies can also be due to development of adverse effects such as infections, malignancies, acute infusion and injection reactions, autoimmunity, and cardiovascular effects (18, 19).

In 1999, a clinical trial testing the efficacy of a TNF inhibitor, Lenercept, for MS treatment had to be halted due to exacerbations of symptoms when compared to placebo-treated MS patients (20). Likewise, there are several clinical reports of RA and AS patients treated with TNF blockers that developed CNS demyelination after treatment (21, 22).

Although partially effective in other autoimmune diseases, anti-TNF therapies in MS patients seem to worsen pathology and clinical symptoms. A possible explanation for this failure is the inability of the drug to grant access to the CNS (23). In other tissues is rather easy to penetrate and exert a local effect, the brain is a privileged site that instead restricts entry to macromolecules such as biologics. Furthermore, non-selective TNF inhibitors dampen down the active inflammatory response ongoing in certain diseases such as RA and IBD. While for these diseases the anti-inflammatory effects could be enough for (at least partial) recovery, MS treatment requires a more profound reestablishment of homeostasis that includes tissue protective and regenerative properties. Interestingly, the last two decades of research on TNFα signaling showed that the soluble form of TNF (solTNF) triggers apoptotic and proinflammatory signals to the cell *via* TNFR1 while the transmembrane form (tmTNF) is able to promote cell survival through TNFR2 activation (see **Figure 1**). The following chapter recapitulates the current studies trying to specify and optimize selectively targeting TNF–TNFRs within an MS therapeutic frame.

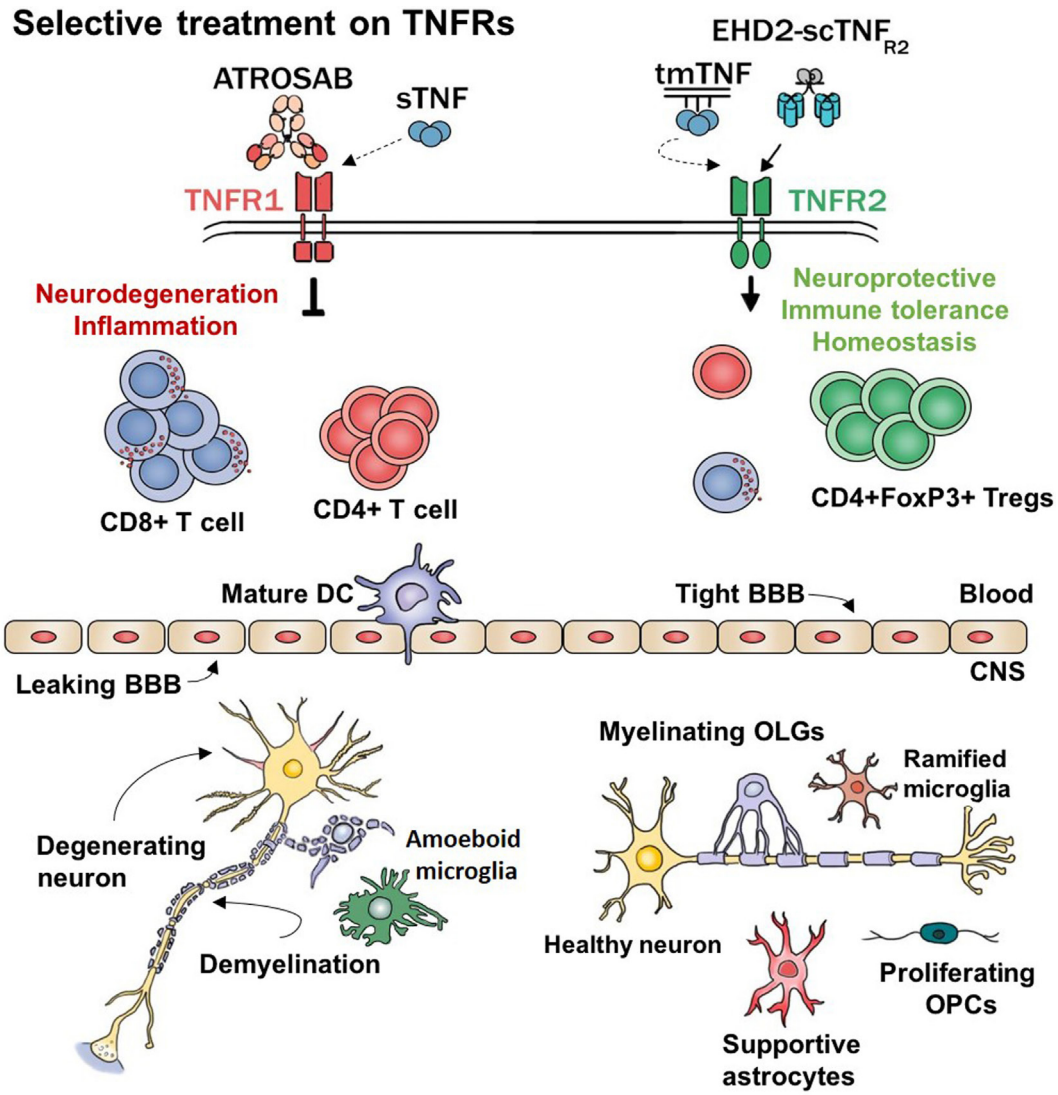

FIGURE 1 | Hypothetical working model. TNFα and its receptors regulate major functions of several cell types. This model represents the expected effects of selectively modulating TNF–TNFRs signaling. sTNF, soluble TNF; tmTNF, transmembrane TNF; ATROSAB, TNFR1 antagonistic antibody; EHD2-scTNFR2, TNFR2 agonist; CD8$^+$ T cell, cytotoxic T cells; CD4$^+$ T cells, helper T cells; DC, dendritic cell; BBB, blood–brain barrier; Tregs, regulatory T cells; CNS, central nervous system; OPCs, oligodendrocyte's precursor cells; OLGs, oligodendrocytes; TNFα, tumor necrosis factor alpha.

TNF–TNFR SIGNALING: THERAPEUTIC IMPLICATIONS FOR MS

Tumor necrosis factor alpha is a pleiotropic cytokine regulating many physiological and pathological functions such as cell survival (24), apoptosis (25), inflammation (26–28), autoimmunity (29), demyelination (30), and cancer (31). TNFα is synthesized as a transmembrane protein of 26 kDa and forms a stable homo-trimeric molecule (tmTNF). Proteolytic cleavage of the protein *via* TNFα converting enzyme (TACE/ADAM17) produces a 17-kDa monomeric protein, a soluble homo-trimeric molecule of 51 kDa (solTNF). TNFα signaling is then generated through the interaction with two distinct transmembrane receptors, the 55-kDa TNF receptor type I (TNFR1) and the 75-kDa TNF receptor type II (TNFR2). The two TNFα variants display different affinities for the two receptors. TNFR1 is activated by both soluble and transmembrane forms with higher affinity for solTNF while activation of TNFR2 is solely due to tmTNF. Furthermore, the two receptors differ in the intracellular pathways that they trigger leading to various cellular responses (32–34). TNFR1 has been described as stimulator of effector caspase-mediated apoptosis (35, 36), while TNFR2 promotes cell survival through PI3K-Akt/PKB signaling pathway (37, 38). However, TNFR1 activation may also prevent TNF-induced apoptosis by activating the classical NF-κB pathway (39) and receptor-interacting protein 1 (RIP1) ubiquitination (40). Upon TNFR1 stimulation, the intracellular death domain (DD) recruits RIP1 and TNFR-associated death domain (TRADD). TRADD engages TNFR-associated factor 2

(TRAF2), inhibitor of apoptosis protein 1 (cIAP1) and inhibitor of apoptosis protein 2 (cIAP2) thereby leading to the formation of complex I (41). RIP1 ubiquitination and complex I activation later stimulates catalytic IκB kinase (IKK) complex, which favors the activation of NF-κB pathway (42). If this signaling fails, complex II will trigger caspase 8-mediated apoptosis upon TNFR1 ligand binding (43). Importantly, the initiation of this apoptotic process heavily relies on the levels of the inhibitory protein (cFLIP). The more NF-κB is activated by complex I, the more cFLIP will be available to inhibit caspase-mediated apoptosis (44).

In addition, it was shown that TNFRs cross talk intracellularly giving rise to TNFR1-induced cell survival and TNFR2-induced apoptosis (33). In contrast with TNFR1, TNFR2 does not contain a DD but it is still capable of inducing apoptosis upon its activation (45). A common intracellular molecule family in the TNFα signaling cascade is TRAFs, which are recruited by both TNFRs complexes. In CD30 and CD40 cells, TNFR2 stimulation might lead to TRAF2 degradation, which results in caspase 8 activation and eventually apoptosis (46). TRAF2 is also an important regulator of cell survival through TNFR1-mediated activation of C-Jun and NF-κB. *In vitro* studies showed that NF-κB activation leads to production of TRAF1, which blocks TNFR2-mediated degradation of TRAF2 (47).

Another distinctive feature of these two receptors is their differential expression in different tissues. While TNFR1 is ubiquitously expressed, TNFR2 can be found mainly on endothelial cells, various immune cells, and certain CNS cells (48).

All these peculiar features enable such a complex cytokine to have major, sometimes conflicting, effects depending on its form, the receptor that it triggers and the cell type on which it may act (see **Figure 1**). Because of this pleiotropic effect, the function of TNFα will depend on the ratio of co-expression of its receptors which will shift the balance between cellular survival and apoptosis.

The following sections will highlight the beneficial properties of targeting selectively TNFRs' signaling pathway found in *in vitro* and *in vivo* models of MS (see **Table 1**). This may help to further elucidate the therapeutic value of TNFα in the treatment of MS and other autoimmune diseases.

SELECTIVE TARGETING OF TNFRs: IMMUNE PROTECTION PROPERTIES

Growing evidence suggest that proinflammatory factors are intertwined with a complex resolution program of inflammation after few hours an inflammatory response begins (54–56). In a coculture experiment with murine $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and $CD4^+CD25^-$ effector T cells (Teffs), short-term exposure to TNFα promotes Teffs expansion while a more prolonged treatment favors proliferation and activation of Tregs (57). Moreover, $TNFR2^{-/-}$ mice show normal pool of Tregs but, when stimulated with septic challenge, they fail to expand. This suggests that TNFα might have a proinflammatory action in the early phases of inflammation through Teffs to leave then Tregs to re-establish homeostatic balance through TNFR2 signaling. Similar evidence comes from leukocytes isolated from RA patients on anti-TNF medications (58). Adalimumab, an anti-TNF antibody, but not the solTNFR2 Etanercept, promotes the interaction between monocytes and Tregs leading to expansion of $FoxP3^+$ Tregs and suppression of Th17 cells through IL-2/STAT5 pathway. This effect is caused by adalimumab binding to tmTNF on monocytes, which is able to enhance both expression of tmTNF and its binding to TNFR2 on Tregs. Additionally, increased IL-17 production in TNFR2-deficient T cells is prevented by exogenous IL-2 showing that tmTNF–TNFR2 signaling suppresses Th17 differentiation by promoting IL-2 expression (59). Furthermore, an *in vivo* EAE study with $TNFRs^{-/-}$ mice showed a reduction of clinical symptoms, demyelination score, $CD3^+$ T cell infiltrates, and activated microglia/macrophages in $TNFR1^{-/-}$ mice. On the contrary, lacking TNFR2 seems to worsen EAE disease course. In the same study, EAE was induced in normal C57BL/6 to investigate the effect of antibody-mediated TNFR1 inhibition. These results show attenuated EAE severity and delayed the onset of disease in the treated group mainly through decreased demyelination score and neuronal loss while there is only a mild reduction in immune infiltrates into the CNS (60). If silencing TNFR1 is not enough, activation of TNFR2 in mouse microglia culture promotes expression of anti-inflammatory and neuroprotective genes as granulocyte colony-stimulating factor, adrenomedullin, IL-10, and IFN-γ (61). Specifically, conditional knockout of microglial TNFR2 reveals earlier EAE onset by means of increased number of infiltrates, T cell activation, and demyelination scores. On the other hand, ablation of microglial/macrophage TNFR2 leads to EAE suppression (62). This experiment further expands our knowledge of TNFα functions on its receptors: TNFR2 has dual roles depending on its location in central or peripheral myeloid cells as much as solTNF and tmTNF have detrimental or protective properties, respectively.

SELECTIVE TARGETING OF TNFRs: TISSUE REGENERATION PROPERTIES

A crucial pathological hallmark of MS is white matter lesions caused by axonal demyelination. An effective pharmacological intervention for this disease requires tissue regenerative properties to counteract tissue damage at the lesion site. *In vitro* studies reveal that TNFR2 activation protects oligodendrocyte's progenitor cells (OPCs) from oxidative stress (63). OPCs are increasingly being studied in MS research as they are shown to be essential to the remyelination process (64). The beneficial effect of TNFR2 seems to continue in later stages of development of these critical cells. In a primary coculture setup, maturation of oligodendrocytes into myelinating cells appears to be boosted through astrocyte-specific TNFR2 stimulation (65). In 2001, an important *in vivo* study investigated the different role of TNFRs in the cuprizone model for demyelination (see **Table 1**) in mice lacking either TNFα or one its receptors (66). In this study, the absence of TNFα delays the remyelination process due to reduction of proliferating OPCs and mature oligodendrocytes when compared to wild type mice. Interestingly, similar effects are found in mice lacking TNFR2, but not TNFR1, underling a substantial role of TNFR2 in promoting oligodendrocytes proliferation and

TABLE 1 | Animal models to investigate pharmacological interventions for multiple sclerosis (MS).

Models	Species	Induction	Mechanism of action	Effect on physiology	Relevance	Relevance for TNFRs selective approach
EAE model (49)	Rodents, rabbit, primate	Immunization	Autoimmune reaction vs. myelin protein	T cell dysfunction	Autoimmunity	To study the anti-inflammatory effects against T cell autoreactivity and immune protection through Tregs
Cuprizone model (50)	Rodents	Toxic	Unknown. Iron chelator causing mt dysfunction in OLGs	Degeneration of OLGs, mainly in CC	Myelin degeneration and regeneration	To study the (re)generative properties on myelin components, OPCs and OLGs
NBM lesion model (51)	Rodents	IC injections of NMDA	Neuronal excitotoxicity	Neuronal degeneration in NBM	Neurodegeneration and/or protection	To study the neuroprotective properties on axonal loss, apoptosis, mt dysfunction and signal transmission

Selectively targeting TNFR1 and/or TNFR2 has promising therapeutic potential. The animal models available nowadays have several limitations (52, 53) that require the study of different pathological hallmarks of disease in different models. Here, we show three widely used animal models that give an almost exhaustive overview of MS pathology when taken altogether.

EAE, experimental autoimmune encephalomyelitis; NBM, nucleus basalis of Meynert; IC, intracerebral; NMDA, N-methyl-D-aspartate acid; MT, mitochondrial; OLG, oligodendrocyte; CC, corpus callosum; OPC, oligodendrocytes precursors cell; Tregs, regulatory T cell.

regeneration. In this vein, inhibition of solTNF shows that tmTNF increases axon preservation and improves myelin compaction in an EAE mouse model for MS (67). In the same study, myelin-specific genes and increased number of OPCs are found upon tmTNF treatment. A recent EAE study with conditional knockout mice highlights that TNFR2 specifically on oligodendrocytes drives their differentiation and remyelination (68). Furthermore, treatment with XPro1595, a selective solTNF inhibitor, in a cuprizone mouse model (see **Table 1**) shows faster remyelination due to improved myelin phagocytosis by microglia (69).

SELECTIVE TARGETING OF TNFRs: NEUROPROTECTION PROPERTIES

In the progressive stages of MS, axonal loss and neurodegeneration seem to take over, at least partially, inflammation as main pathological hallmarks (70). Several *in vitro* studies underline the potential neuroprotective effect of selective targeting of TNFRs. In a human dopaminergic neuronal cell line (LUHMES), TNFR2 stimulation of the PI3K-PKB/Akt pro-survival pathway rescues neurons from oxidative stress-induced cell death (71). Furthermore, similar results were found in an *in vitro* model of glutamate-induced excitotoxicity in primary cortical neurons. TNFR2, and not TNFR1, induces persistent PI3K PKB/Akt-mediated NF-κB activation leading to neuroprotection, which is enhanced by *N*-methyl-D-aspartate receptor co-stimulation (37). Using the same *in vitro* model, another study shows that activation of TNFR2 signaling pathway mediated lovastatin-induced neuroprotection against glutamate excitotoxicity (72). Statins are widely prescribed in clinical practice for lowering cholesterol levels. Nonetheless, a specific statin, called Simvastatin, has been shown to be effective in decreasing whole-brain atrophy in patients with secondary progressive MS in a phase II trial (73). The neuroprotective effect of TNFR2 was also found in an *in vivo* model using TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. After retinal ischemia, TNFR1 deficiency leads to strong decrease in neuronal death while absence of TNFR2 leads to enhanced neurodegeneration (36). Another *in vivo* model using genetic ablation of solTNF shows neuroprotection against focal cerebral ischemia (74). Interestingly, a recent study reveals that the neuroprotective and anti-inflammatory effects found by antagonizing TNFR1 in the nucleus basalis lesion (NBM) model (see **Table 1**) is enhanced through TNFR2 signaling (75).

FUTURE PERSPECTIVES

Due to contradicting results concerning TNFα and TNFR signaling in neurodegenerative diseases in the late 90s, major advances have been made in recent years in understanding the biology of TNF–TNFRs signaling in health and disease. This review highlighted the potential therapeutic value of this target, specifically within MS pathology. Of importance, the available MS treatments are focused on limiting the burden and occurrence of autoreactive peripheral immune cells. We can see them as drugs boosting the immune system's resistance toward an insult against self-tissue. Obviously, this leads to a temporary effect of the treatment, which is followed by a partial decrease in symptoms, mainly in patients with relapsing-remitting MS. Moreover, most of these drugs are not able to slow down disease progression. Recently, several immunologists and evolutionary ecologists introduced the concept of *disease tolerance* as a defense mechanism against infectious agents (76–78). In flies (79), rodents (80), and humans (81) studies, modulating disease tolerance resulted in protection against several types of infection and restored homeostasis (82). As in different patrolling mechanisms, attack is not always the best defense mechanism: damage control is as important as pathogen control. Within autoimmunity, dysregulated disease tolerance can be seen as a failure of the immune system to control tissue damage caused by autoreactive immune cells. Interestingly, selective modulation of TNFRs triggers a variety of protective and pro-survival properties, which in turn are positively affecting the pathological milieu derived from autoreactive lymphocytes. Breaking the vicious circle of chronic inflammation and protect tissue against further damage are essential features for a therapeutic agent that aims at restoring proper immune functions and general homeostasis.

Nevertheless, some challenges need to be addressed to further elucidate the potential of this treatment target. As briefly

mentioned above, activating TNFR2 in peripheral or central myeloid cells resulted in opposing therapeutic effects (62) underling the need for a pharmacological approach that minimizes peripheral immune activation. However, blood–brain barrier (BBB) permeability of these compounds might be an obstacle to overcome in order to reach all beneficial effects of this target. In the past decade, great progress has been made in developing nanoparticles (83, 84) and cell-specific drug carriers (85) through the BBB, giving a promising perspective for CNS diseases (86).

To conclude, selective modulation of TNFRs through TNFR2 activation and/or TNFR1 silencing has great therapeutic potential in terms of immune, tissue, and neuroprotective properties, especially for MS treatment.

AUTHOR CONTRIBUTIONS

The authors contributed equally to this work.

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REVIEW
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TNF Receptor 2 Makes Tumor Necrosis Factor a Friend of Tumors

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Tumor necrosis factor (TNF) is widely accepted as a tumor-suppressive cytokine *via* its ubiquitous receptor TNF receptor 1 (TNFR1). The other receptor, TNFR2, is not only expressed on some tumor cells but also on suppressive immune cells, including regulatory T cells and myeloid-derived suppressor cells. In contrast to TNFR1, TNFR2 diverts the tumor-inhibiting TNF into a tumor-advocating factor. TNFR2 directly promotes the proliferation of some kinds of tumor cells. Also activating immunosuppressive cells, it supports immune escape and tumor development. Hence, TNFR2 may represent a potential target of cancer therapy. Here, we focus on expression and role of TNFR2 in the tumor microenvironment. We summarize the recent progress in understanding how TNFR2-dependent mechanisms promote carcinogenesis and tumor growth and discuss the potential value of TNFR2 in cancer treatment.

Keywords: tumor necrosis factor, TNF receptor 2, tumor, myeloid-derived suppressor cells, regulatory T cells, macrophages

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INTRODUCTION

The 34 kDa pleiotropic cytokine tumor necrosis factor (TNF) is a type II transmembrane protein important in carcinogenesis, cancer progression, and metastasis, as well as in immunity (1–3). Connecting a wide variety of cell types, TNF constitutes itself a central player in the multi-faceted tumor microenvironment. TNF can exert both tumor-promoting and -suppressing roles, and those distinct effects are transmitted by two receptors, TNF receptor 1 (TNFR1) and TNFR2 (4–8). Although the role of TNFR2 is less well understood, many reports indicated it as crucial in tumors (**Table 1**). This review does not aim to give all details of TNFR2-mediated cellular and molecular mechanisms. We specifically emphasize how tumor progression is accelerated after TNFR2 activation on tumor and immune cells within a tumor and briefly discuss the outcome of treatments targeting TNFR2.

TNF RECEPTOR 1 AND TNF RECEPTOR 2

TNF receptor 1 (p55 or CD120a) and TNFR2 (p75 or CD120b) are type I transmembrane receptors. TNFR1 shows extensive expression, whereas TNFR2 expression is limited to immune cells and a few other cell types (7, 9, 10). TNFR1 and TNFR2 have similar extracellular TNF-binding structures characterized by four repeated cysteine-rich domains (CRDs) (CRD1 also called pre-ligand binding assembly domain, CRD2, CRD3, and CRD4) but have different intracellular domains (11, 12). Most critical for the diverse biological effects of the two receptor subtypes is the lack of the intracellular death domain in TNFR2. Hence, TNF promotes apoptosis *via* binding to TNFR1 but exerts pro-survival effects *via* TNFR2 (4, 5, 13). After being engaged by extracellular TNF, TNFR1 recruits and clusters the adaptor protein TNFR1-associated death domain protein (TRADD) and the

TABLE 1 | Tumor development promoted by TNFR2-mediated signaling in tumor or tumor-associated cells.

Cancer type	Impacts of TNF receptor 2 (TNFR2) expression on various cells
Breast cancer	Promoting tumor cell growth (49); inhibiting programmed death of tumor cells (48); stabilizing myeloid-derived suppressor cells (MDSC) (91); and relating to suppressive function of regulatory T (T_{reg}) cells (82)
Colon cancer	Advancing carcinogenesis of epithelia cells (59–61); promoting tumor cell proliferation (55, 56); enhancing angiogenesis by upregulating VEGF-A in tumor cells (56); inducing cancer-associated fibroblasts (100); activating T_{reg} cells (78, 105); and supporting metastasis (92)
Cervical cancer	Facilitating tumorigenesis (58)
Fibrosarcoma	Promoting MDSC accumulation (85)
Liver cancer	Expanding T_{reg} cells (78) and promoting tumoral accumulation of MDSC by inducing specific chemokine receptor (87)
Lung cancer	Helping to form metastasis niche by stabilizing MDSC (92); promoting VEGF release and anti-apoptotic ability of tumor cells (50, 51); and associating with inhibitory effects of T_{reg} cells (67, 82)
Melanoma	Maintaining T_{reg} cells (71); contributing to T cell exhaustion (80)
Ovarian cancer	Acting as oncogene in tumor cells (104); expanding T_{reg} cells (104); and promoting T_{reg} cells to impair T-helper 1 immunity (77)
Renal cancer	Driving proliferation of tumor stem cells (54) and accelerating tumor cell division (53, 79)
Skin cancer	Advancing malignant transformation of epidermal cells (40)
Plasmacytoma	Driving MDSC expansion (85)
Lymphoma	Enhancing angiogenesis by inducing interleukin-6 secretion from malignant cells (52) and augmenting activation-induced death of cytotoxic T cells (79)
Leukemia	Relating to T_{reg} cell expansion (102, 103)

References exploring biological functions of TNFR2 in tumorigenesis, tumor progression, anti-apoptosis, or non-specified other processes in human cancer and murine tumor models within the last 10 years.

downstream caspases (14–16). This finally leads to programmed cell death. In contrast, activated TNFR2 results in recruitment of the TNF receptor-associated factor (TRAF) 2 and stimulates the pro-survival nuclear factor (NF)-κB pathway (17). TNFR2 has a high affinity to membrane-bound TNF and can deliver TNF to TNFR1 (18–21). Only by this cooperation, TNFR2 can feed a cell to its death (22).

TNF RECEPTORS AND THE COMMON NUCLEAR FACTOR-κB (NF-κB) PATHWAY

Nuclear factor-κB is activated by both TNF receptor subtypes. Upon stimulation by its ligands including TNFα or lymphotoxin, TNFR1 forms a complex with the adaptor TRADD at the plasma membrane (23, 24). TRAF2 is transported and clustered into the complex that recruits the cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) proteins (25–27). Together with TRAF2, cIAP1/2 proteins degrade the TRADD-bound ubiquitinated receptor interacting protein (RIP) 1. Multiple ubiquitination of RIP1 and the NF-κB essential modulator [NEMO; also called IκB kinase (IKK)γ] engages the kinase TAK1 to the NEMO-containing IKK complex (5). IKKβ in the IKK complex becomes phosphorylated and phosphorylates the NF-κB inhibitor IκBα that is subsequently cleaved. Released NF-κB translocates into the nucleus and induces target gene expression.

The detailed mechanisms of how TNFR2 induces NF-κB remain more elusive. So far, only TRAF2 is clear as the key component. Different from TNFR1, TNFR2 directly interacts with TRAF2 (28). Activated TNFR2 binds to TNFR2 through two conserved intracellular domains, the TRAF2-binding motif SKEE (amino acid residues 402–405) and the C-terminal motif (amino acid residues 425–439) (29, 30). TRAF1 and TRAF3 also associate with TNFR2 directly or *via* TRAF2 at the two conserved domains (31–33). Genetic manipulation confirmed those two domains as most critical for TNFR2-induced NF-κB activation.

As mentioned above, TNFR1 and TNFR2 have distinct impacts on individual cell fates although they both regulate the outcomes through NF-κB. It is reported that the receptor crosstalk depends on the relative expression of each receptor. At high expression of TNFR1, low amounts of TNFR2 enhance TNFR1-induced NF-κB activation (34). In contrast, TNFR2 at high levels effectively competes for TRAF2. Consequently, recruitment to the classical TNFR1 pathway and downstream NF-κB activation is compromised. Concentration and conformation of the ligand TNF are also related with the balance between TNFR1 and TNFR2 signals (20, 35). Interestingly, a crosstalk of TNFR1 with TNFR2 strongly affects the cell fate decision. When both TNF receptor isotypes are co-expressed, specific activation of TNFR1 leads to continuous expression of anti-apoptotic factors and barely induced apoptotic pathways. Here, cell death is due to the loss of anti-apoptotic factor expression after TNFR2-dependent TRAF2 degradation and abrogated recruitment of cIAP1/2 to TNFR1 (36–38). If both receptors are activated at same time, the balanced signal transduction of TNFR1 and TNFR2 leads to cell survival (39, 40). The TNFR1–TNFR2 crosstalk is context- and time-dependent, and their intricacy clearly needs further exploration.

TNFR2 PROMOTING TUMORIGENESIS AND PROGRESSION

TNF receptor 2 is implicated in the occurrence and growth of tumors, therapeutic responses, and patients' prognosis (41–43). In direct and indirect manners, TNFR2 plays important roles in multiple aspects of tumor progression, including the proliferation of tumor cells, the evasion of immune surveillance, the activation of endothelia cells and angiogenesis, and the formation of a pre-metastasis milieu (**Figure 1**).

TNFR2 on Tumor Cells and Non-Immune Cells in the Tumor Microenvironment

Several studies have indicated that TNFR2 expression in tumor tissues relates to advanced disease progression and poor clinical outcomes (44–46). TNFR2 is aberrantly expressed on several types of tumor cells (47) and induces tumor progression through several signal transduction cascades (**Figure 2**). In breast cancer, TNFR2 protects malignant cells from DNA damage *via* the AKT

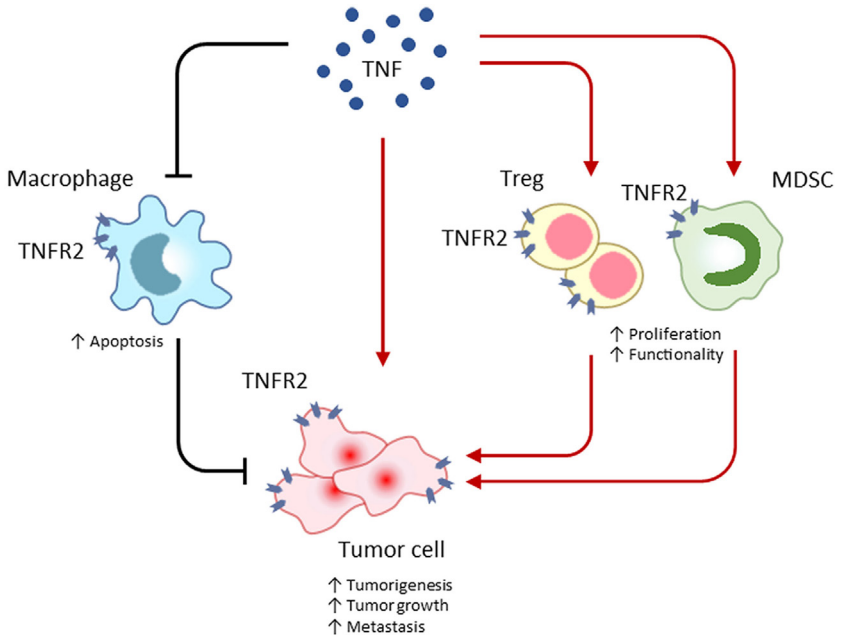

FIGURE 1 | TNF receptor 2 (TNFR2) promotes tumor progression by maintaining a tumor-favoring immune-microenvironment or by facilitating malignant cell proliferation and survival. In the tumor microenvironment, TNFR2 is extensively expressed on many types of cells, including immune cells and malignant cells. TNFR2 often accelerates the malignant transformation and growth of tumor cells, instead of inducing cell death by apoptosis. Similar to tumor cells, TNFR2 protects immunosuppressive regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSC) from the death-inducing TNF and consequently enhances proliferation and function of those tumor-promoting cells. Even worse, TNFR2 deteriorates the programmed death of phagocytic macrophages responsible for clearing of tumor cells. Mediating those direct and indirect effects, TNFR2 exacerbates cancer progression.

signaling pathway (48) and induces NF-κB *via* p42/p44 mitogen-activated protein kinase (MAPK) to accelerate tumor cell growth (49). Interestingly, blocking TNFR2 is sufficient to diminish TNF-evoked cell growth (49), indicating TNFR2 as more important for tumor progression than the activation of signal kinases including p42/p44 MAPK, JNK, and AKT *via* the ubiquitous TNFR1. Underlining this, loss of TNFR2 results in a large increase of TNF-associated tumor cell death and a significant halt of tumor growth in lung cancer (50).

TNF receptor 2 deficiency in a mouse model of lung cancer not only enhances tumor cell apoptosis but also leads to downregulated pro-angiogenic factors, like vascular endothelial, hepatocyte, and placental growth factors from endothelial progenitor cells (51). Pointing to a general role for TNFR2 in tumor development, TNFR2-deficient mice decrease melanoma cell growth in the same way (51). Additionally, TNFR2 signaling indirectly promotes angiogenesis by inducing interleukin (IL)-6 secretion (52). In renal carcinoma, TNFR2 on endothelial and tubular epithelial cells activates the endothelial/epithelial tyrosine kinase and then upregulates vascular endothelial growth factor receptor-2 (53) or directly promotes cell division (54). Consequently, expression of TNFR2, but not of TNFR1, correlates with the grading of malignancy. In colorectal carcinoma, TNFR2 promotes tumor cell proliferation through the PI3K-AKT pathway (55, 56) or *via* NF-κB activation (57). These studies imply that TNFR2 directly enhances tumor growth, but TNFR2 is also involved in malignant transformation (58). In animal models of chronic inflammation, TNFR2 induces NF-κB activation in epithelial cells that subsequently leads to carcinogensis (59–61).

TNFR2 on T Cells

TNF receptor 2 not only affects tumor cells but also regulates tumor-infiltrating immune cells. The resulting immunosuppressive microenvironment supports tumor development. Regulatory T (T_{reg}) cells are the central player in regulating tumor-specific immune responses (62–64). Hence, T_{reg} cells also represent the most important tumor-promoting cell type and are most extensively studied. TNFR2 expressed on T_{reg} cells indicates the maximally suppressive subset (47, 65–68) and relates to poor prognosis of patients (69). TNFR2 mediates the effects of TNF on $CD4^+$ forkhead box (Fox)$P3^+$ T_{reg} cells (70, 71). TNFR2 promotes the development of T_{reg} cells in thymus (72), the expansion of differentiated T_{reg} cells (73), and mediates the activation effects of TNF on T_{reg} cells (70, 71). It leads to activation, expansion, and phenotypic stability of the strongly suppressive T cells (74), partially through an epigenetic mechanism that demethylates the *Foxp3* gene (75). TNFR2 is highly expressed on resting and activated T_{reg} cells compared to their $FoxP3^-$ counterparts (65). TNF expands the $TNFR2^+$ T_{reg} subset and augments the IL-2-induced induction of signal transducer and activator of transcription-5 to increase the suppressive function. Consistently, $TNFR2^+$ T_{reg} cells comprise the most highly suppressive subset of T_{reg} cells (67). T_{reg} cells within the tumor microenvironment show higher expression of TNFR2 than T_{reg} cells from normal tissues or the periphery (76).

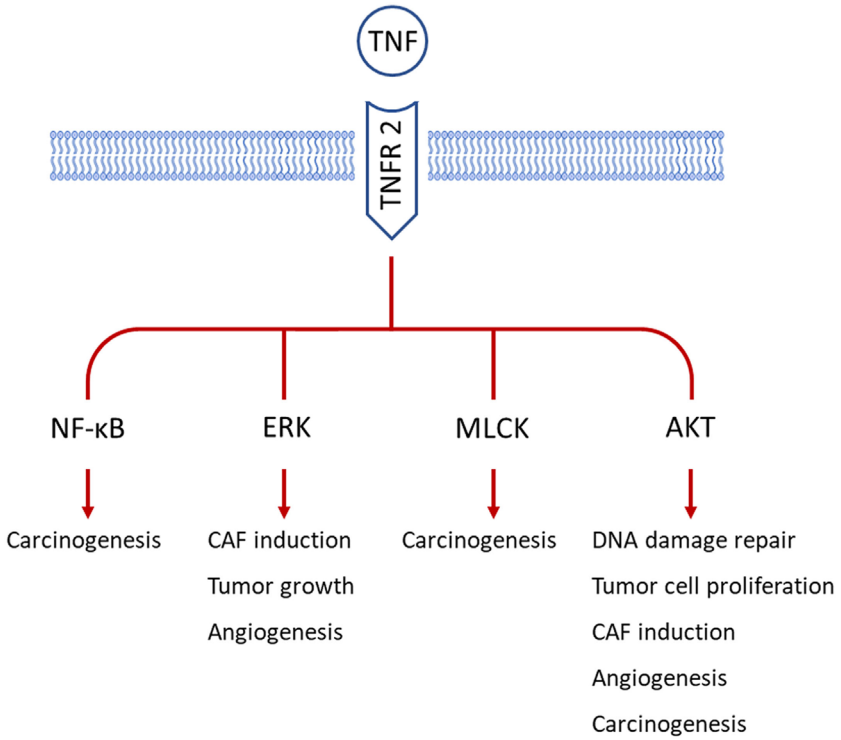

FIGURE 2 | TNF receptor 2 (TNFR2) participates in various processes of tumor development by employing different signal pathways in tumor cells. So far, TNFR2 is reported to be expressed on tumor cells from breast cancer, cervical cancer, colon cancer, and renal cancer. AKT signaling is the major mediator of TNFR2 in carcinogenesis, tumor growth, angiogenesis, and cancer-associated fibroblast (CAF) induction. Besides, myosin light-chain kinase (MLCK) and nuclear factor-κB (NF-κB) are involved in TNFR2-related malignant transformation of epithelial cells. Extracellular signal-regulated kinase (ERK) is also important for the above-mentioned functions of TNFR2.

Emphasizing the clinical relevance of those findings in mouse models, T_{reg} cells infiltrating human tumors have high levels of TNFR2 expression and maximal suppressive capacity (77). Increased TNFR2 in T_{reg} cells enhances TNF-dependent T_{reg}-cell proliferation and suppressive effects in tumors susceptible to anti-TNF treatment (78). In lung cancer patients, expression of TNFR2 strongly correlates with the transcription factor FoxP3 than the expression of CD25 (69). TNFR2 expression levels on T_{reg} cells closely associate with lymphatic invasion, distant metastasis, and advanced clinical stage (69). This not only underlines the functional importance of effective T_{reg} cell-mediated control of tumor-specific immune responses but also suggests TNFR2 as a more appropriate marker for tumor-resident T_{reg} cells compared to integrin-αE (CD103) (67).

Taken together, the abundance and strong immunosuppressive capacity point to TNFR2$^+$ T_{reg} cells as critical in promoting tumor progression and metastasis.

Although TNFR2 is preferentially expressed on T_{reg} cells, the expression of TNFR2 can be induced or up-regulated on CD8$^+$ T cells and conventional CD4$^+$ FoxP3$^-$ (T_{con}) T cells.

In CD8$^+$ T cells, TNFR2 can elicit activation-induced cell death (79). It also may upregulate the expression of the inhibitory receptor Tim3 (80). Both direct mechanisms further hamper the efficacy of cytotoxic T cells.

Activation of TNFR2 on T_{con} cells can lead to enhanced tumoricidal effects (81). TNFR2 on T_{con} cells also activates those effector T cells making them resistant to T cell-mediated suppression (82). However, T_{reg} cells within the tumor microenvironment demonstrate much higher expression levels of TNFR2 that overcome this resistance and maintain the local dominance of immunosuppression (77).

TNFR2 on Myeloid-Derived Suppressor Cells (MDSC)

Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cells mainly characterized by their strong immunosuppressive capacity. In healthy individuals, myeloid cells outside the bone marrow are mainly matured into granulocytes or monocytes (83, 84). Lineage-specific differentiation fails in chronic inflammation or malignancy, and this associates with a potent immunosuppressive function in the resulting myeloid cells (21, 85). These cells appear in peripheral tissues and also represent an important subset of cells in the tumor microenvironment that promotes tumor growth (83). TNF signaling is believed to be critical for MDSC to accumulate and perpetuate their immature state (86, 87). Elevated TNF in an inflammatory milieu augments MDSC accumulation and immunosuppression, whereas TNF antagonists reduce the inhibitory function of MDSC and support differentiation into dendritic cells or macrophages (88).

The feature of TNF to promote immunosuppression is intimately related to TNFR2. TNFR1 and TNFR2 double-negative mice spontaneously reject implanted tumors. This correlates with decreased accumulation of MDSC, which is mainly mediated by TNFR2, but not by TNFR1 (85). TNFR2-mediated signaling increases the induction of MDSC from bone marrow cells and inhibits the apoptosis of MDSC through c-FLIP upregulation and caspase-8 inhibition. TNFR2 also directs the suppressive functions of MDSC (89). In monocytic MDSC, TNFR2 deficiency compromises the development of MDSC and reduces the production of immunosuppressive factors, like NO and IL-6 (89). Additionally, TNFR2 is important for the production of the immunosuppressive factors, IL-10 and transforming growth factor-β (90). The p38 MAPK-NF-κB axis is indispensable for the process of TNFR2-transmitted signals in MDSC. Inhibition of this axis in TNFR2$^+$ MDSC stimulated with TNF could reverse T-cell suppression (91). The induction of suppression-related markers, including arginase-1, inducible NO synthase, NO, reactive oxygen species, IL-10, and transforming growth factor-β clearly correlates with the activation of the p38MAPK-NF-κB pathway *via* TNFR2 (91). All reports support the assumption that TNFR2 promotes primary tumor growth by maintaining the naïve state and enhancing the suppressive character of MDSC to control tumor-specific T-cell responses.

TNF receptor 2-expressing MDSC also contribute to metastasis. TNFR2 deficiency reduces the liver metastasis of lung cancer (92). In a mouse model, TNFR2$^{-/-}$ MDSC fail to accumulate in pre-metastatic lesions and show reduced expression of the suppressive arginase-1. Of note, the loss of TNFR2 also alleviates T_{reg}-cell infiltration into metastasis sites of human lung cancer (92). We conclude that TNFR2 coordinates T_{reg} cells and MDSC in original tumor growth as well as in metastasis.

TNFR2 on Macrophages

Macrophages are the most dominant innate immune cell type in tumor control, and they are the main sources of TNF (93). Macrophages also simultaneously express TNFR1 and TNFR2, although the effects of TNFR2 on macrophages remain unclear. Similar to the immature MDSC, activation of TNFR2 on macrophages induces the p38 MAPK-NF-κB pathway (94). TNFR2 on tumor-associated macrophages correlates with malignancy grades in human triple-negative breast cancer and is thought to participate in metastasis (95).

Prompting to the significance of TNF receptor crosstalk discussed above, the TNFR2 also takes part in inducing macrophage death by necroptosis upon TNF-induced TNFR1 activation upon contact with pathogen (22). Without TNFR2 signaling, the induced necroptosis is reversed. Although not shown so far for tumors, TNF-related macrophage death may represent an alternative way of how TNFR2 signaling in macrophages might contribute to tumor progression.

TARGETING TNFR2 FOR TUMOR THERAPY

TNF receptor 2 is mainly expressed on malignant cells and in the immunosuppressive cell compartment within the tumor microenvironment. It is involved in promoted tumor development and facilitated metastasis. Hence, TNFR2 represents an attractive target for tumor treatment.

Specifically, blocking the ligand TNF is one option. Due to the higher expression of TNFR2 relative to TNFR1 in tumor and tumor-associated cells, TNF is likely to have a tumor-promoting function instead of an inhibitory impact. TNF ablation effectively reduces tumor growth (96).

Of note, activating TNFR2 on tumor-promoting cell types, such as fibroblasts might limit tumor cell invasion and metastasis and improve tumor therapy (97–100).

Depleting TNFR2$^+$ T_{reg} cells augmented the efficacy of chemotherapy in preclinical studies (101). In a clinical trial with acute myeloid leukemia patients, patients received the demethylating agent, azacitidine, and the histone deacetylase inhibitor, panobinostat, which effectively eliminated TNFR2$^+$ T_{reg} cells in peripheral blood and bone marrow (102). These TNFR2$^+$ T_{reg} cells were earlier found as potent suppressive immune cell subset with enhanced migratory ability that promote disease progression and hamper tumor therapy (65, 102). Beneficial clinical responses came from more active effector T cells as determined from increased production of interferon-γ and IL-2. A combination of azacitidine with lenalidomide decreasing TNFR2 expression and activity in T_{reg} cells may improve clinical outcomes in hematological malignancies (103).

More recently, antibodies specifically blocking TNFR2 were developed for tumor therapy. TNFR2 is abundant on tumor cells and tumor-infiltrating T_{reg} cells in ovarian cancer (104). Here, antagonistic antibodies to TNFR2 suppress TNF-induced T_{reg}-cell activation and reduce amount as well as immunosuppressive function of T_{reg} cells (104). They inhibit NF-κB activation, hence the T_{reg} cell expansion and immunosuppression but synergistically directly induce tumor-cell death (104). This study showed that targeting TNFR2 on T_{reg} cells was well tolerated. It mostly affected the tumor-infiltrating T_{reg} cells that express much higher levels of TNFR2 than normal T_{reg} cells. A concomitant administration of TNFR2-neutralizing antibody and a toll-like receptor agonist has the potential to further improve the therapeutic effectiveness (105).

CONCLUDING REMARKS

A strongly immunosuppressive microenvironment is a major obstacle in tumor therapy. Over the last decade, immunotherapies using checkpoint blockade and engineered T cells have gained great success. However, many patients fail to benefit from these therapies. One important reason for the ineffectiveness is the focus on evoking cytotoxic T-cell responses that overlooks the impact of the immunosuppressive cell compartment. Therapy-related changes in the tumor environment often enhance immunosuppressive effects and finally result in a failure of therapy. Considering this, we need to emphasize the immunosuppressive cells and factors in tumor treatment. Here, TNF and its diverse effects mediated by TNFR1 or TNFR2 provide a clue.

Tumor necrosis factor is abundant in any tumor microenvironment. This cytokine is usually involved in anti-tumor

responses. However, TNFR2 may convert the anti-tumor effect into tumor-promoting function. TNFR2 expression is limited to several cell types that include tumor and immune cells (**Figure 1**). Tumor cells highly expressing TNFR2 resist TNF-induced cell death *via* binding of the ligand to the TNFR2. TNFR2 is not only highly expressed on tumor cells but also on immunosuppressive cells, including T_{reg} cells and MDSC. Thus, TNFR2 is tightly related with the immunoinhibitory capacities of tumor-promoting cells.

All these specific properties of TNFR2 make it an ideal candidate for targeted tumor therapy. Several studies targeting TNFR2 already proved its great potential in treating tumor. Future investigations will provide more detailed knowledge on all facets and on the cell-type dependency of TNFR2's immunosuppressive effects that we need to translate it into the treatment of malignant diseases.

AUTHOR CONTRIBUTIONS

YS, FL, and ZQ wrote the manuscript; ZQ critically revised the manuscript.

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TNF Receptor Type II as an Emerging Drug Target for the Treatment of Cancer, Autoimmune Diseases, and Graft-Versus-Host Disease: Current Perspectives and *In Silico* Search for Small Molecule Binders

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There is now compelling evidence that TNF receptor type II (TNFR2) is predominantly expressed on $CD4^+Foxp3^+$ regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), and plays a major role in the expansion and function of Tregs and MDSCs. Consequently, targeting of TNFR2 by either antagonists or agonists may represent a novel strategy in the treatment of cancer and autoimmune diseases, by downregulating or upregulating suppressor cell activity. The advance in the understanding of complex structure of TNFR2 and its binding with TNF at molecular levels offers opportunity for structure-guided drug discovery. This article reviews the current evidences regarding the decisive role of TNFR2 in immunosuppressive function of Tregs and MDSCs, and the current effort to develop novel TNFR2-targeting therapeutic agents in the treatment of cancer, autoimmune diseases, and graft-versus-host disease. To shed light on the potential TNFR2-targeting small molecules, we for the first time performed virtual screening of 400,000 natural compounds against the two TNF-binding sites, regions 3 and 4, of TNFR2. Our result showed that the top hits at region 4 had slightly higher docking energies than those at region 3. Nevertheless, free energy calculation from the TNF–TNFR2 molecular dynamics simulation revealed that the binding strength of TNF in region 3 is only one-tenth of that in region 4. This suggests that region 3 is a potentially more viable binding site to be targeted by small molecules than region 4. Therefore, the effectiveness in targeting region 3 of TNFR2 deserves further investigation.

Keywords: TNF receptor type II, TNF, regulatory T cells, virtual screening, drug discovery, MM-PBSA

INTRODUCTION

Tumor necrosis factor-alpha (TNF) is a pleiotropic cytokine that plays a major role in immune and inflammatory responses through two distinct receptors: TNF receptor type I (TNFR1, also known as p55 and TNFRSF1A) and TNF receptor type II (TNFR2, also known as p75 and TNFRSF1B). TNFR1 is ubiquitously expressed on almost all cell types, and TNF–TNFR1 signaling has various functions

such as activation of nuclear factor kappa B (NF-κB) and induction of cell death, which depends on its cellular environment (1). By contrast, TNFR2 is more restrictedly expressed on certain cell types, such as minor subsets of lymphocytes (2, 3), endothelial cells (4), and human mesenchymal stem cells (5). Importantly, TNFR2 is predominantly expressed on the mouse and human $CD4^+Foxp3^+$ regulatory T cells (Tregs) (2), which are professional immunosuppressive cells in mammals (6). There is compelling evidence that TNFR2 expression not only defines the maximally suppressive Treg subset (2, 7) but also plays a decisive role in the proliferative expansion, suppressive function, and phenotypical stability of Tregs (8–14). TNFR2 agonist has been approved to be a novel approach for the treatment of autoimmune diseases and graft-versus-host disease (GvHD) (15), while TNFR2 antagonist has the potential to enhance antitumor immune responses (16), by upregulating or downregulating Treg activity.

Virtual screening (or *in silico* screening), the search for potential drug leads to specific target receptor by computer programs, is of central importance in early-stage drug discovery (17). In structure-based virtual screening, each compound from a large library of small molecules is docked to the ligand-binding site of the target and its binding affinity is estimated based on the predicted optimal-binding pose using an empirical scoring function. High-quality docking predictions not only reduce the time and cost for experiment but also offer in-depth structural details about the interactions of the target with ligands useful for further optimization.

Unlike TNFR1, no *in silico* studies about TNFR2 has been reported so far, and no small molecules targeting TNFR2 have been identified. Here, we aim to provide an *in silico* perspective on the potential binders to the two TNF-binding regions of TNFR2, namely, region 3 and region 4, identified from the TNF–TNFR2 structure (18). Moreover, molecular dynamics (MD) simulation combined with Molecular Mechanics-Poisson Boltzmann Surface Area (MM/PBSA) method was used to assess the per-residue energy contribution in the complex binding of key residues important to target TNFR2.

TNFR2 AGONISTS STIMULATE THE EXPANSION AND ACTIVATION OF Tregs

Immunosuppressive Tregs are a subset of Foxp3-expressing CD4 T cells which play an indispensable role in the maintenance of immune homeostasis and prevention of autoimmune reactions (19, 20). Defect in Tregs is attributable to the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, type 1 diabetes (T1D), rheumatoid arthritis (RA), autoimmune thyroid disease, psoriasis, inflammatory bowel disease, and autoimmune liver disease (21). Therefore, restoring the function or increasing number of Tregs has become a therapeutic strategy and the goal of treatment for patients with autoimmune diseases and GvHD (22).

We for the first time showed that TNF has the capacity to induce the activation and proliferation of Tregs (14). This effect of TNF is mediated by TNFR2, one of the TNF receptors that is predominately expressed by Tregs (2, 7, 23–25). $TNFR2^+$ Tregs are the most potent suppressors, while $TNFR2^-$ Tregs, even $Foxp3^+$, have minimal or no suppressive activity (2, 7, 23). Furthermore, TNFR2 is also critical for the stabilization of phenotype of Tregs, in term of Foxp3 expression, and survival in the inflammatory environment (4, 9). It was shown recently that TNF priming induces the proliferation and activation of Tregs *in vivo via* TNFR2 that prolongs animal survival when compared with unprimed Tregs in acute mouse GvHD model, and TNF–TNFR2 interaction represents a novel therapy to prevent GvHD after allogeneic hematopoietic stem cell transplantation (allo-HCT) (12, 13). In a mouse model of autoimmune diabetes, TNF produced by pathogenic Teffs stimulates the expansion and suppressive function of Tregs through TNFR2 (8). In RA patients, anti-TNF therapy drives the expansion of Tregs by enhancing the binding of membrane-bounded TNF (mTNF) expressed by monocytes to TNFR2 (26). Taken together, these studies indicate that TNFR2 is an emerging target to expand functional Tregs for the treatment of autoimmune diseases and GvHD. Several agonistic TNFR2-recognizing monoclonal antibodies have been developed to expand functional Treg populations *in vitro* or *ex vivo* and showed therapeutic effects in T1D and skin inflammation (27–29). STAR2 protein, a selective mouse TNF-based agonist of TNFR2, has been shown to expand host-type radiation-resistant Tregs and improve the outcome after allo-HCT, prolong the survival without compromising the anti-leukemia or anti-infective effects in a mouse model of GvHD (11). These findings shed a light on the therapeutic potential of novel TNFR2-targeting agents in the treatment of autoimmune and inflammatory diseases. However, small molecule agonist of TNFR2 has not been identified so far.

TNFR2 ANTAGONISTS INHIBIT THE SUPPRESSIVE ACTIVITY OF Tregs

TNFR2-expressing Tregs accumulate in the tumor microenvironment and presumably represent a major cellular mechanism of tumor immune evasion. In mouse Lewis lung carcinoma and the 4T1 breast tumor model, the majority of tumor-infiltrating Tregs have abundant surface TNFR2 expression and they are highly immunosuppressive (2, 30). In lung cancer patients and ovarian cancer patients, the proportion of $TNFR2^+$ Tregs is increased in the peripheral blood or in the tumor-associated ascites (31, 32). Single-cell RNA-Seq shows that TNFR2 is one of the most markedly increased genes expressed by Tregs, when compared with $CD4^+$ effector T cells (Teffs) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) in metastatic melanoma patients, and the expression of TNFR2 is associated with $CD8^+$ CTLs exhaustion (33). Furthermore, the expression of TNFR2 on Tregs is associated with greater lymphatic invasion, a higher incidence of tumor metastasis, a higher clinical stage, and poorer response to the treatment in patients with lung cancer and acute myeloid leukemia (31, 34, 35).

In addition to Tregs, TNFR2 is also expressed on myeloid-derived suppressor cells (MDSCs) and some tumor cells. It has been shown that mTNF, by interacting with TNFR2, activates MDSCs and enhances their suppressive activities, including upregulating arginase-1 and inducible NO synthase

transcription, promoting secretion of NO, reactive oxygen species, interleukin (IL)-10, and transforming growth factor beta (21, 36). TNFR2$^+$ MDSCs have the capacity to promote liver and lung metastasis of tumor (37). The signaling of TNFR2 is responsible for the accumulation and survival of MDSCs through upregulation of cellular FLICE-inhibitory protein and inhibition of caspase-8 activity (3). Moreover, TNFR2 is also expressed by tumor cells, including colon cancer (38), Hodgkin lymphoma (39), myeloma (40), renal carcinoma (41), and ovarian cancer (42). Therefore, TNFR2 is considered as an oncogene and targeting of TNFR2 with antagonistic antibodies as a novel strategy in cancer immunotherapy have been studied recently. For example, it was reported that antagonistic antibody targeting TNFR2 induces the death of both Tregs and OVCAR3 ovarian cancer cells, which have abundant surface TNFR2 expression (42). Our group found that TNFR2-blocking antibody markedly enhanced the efficacy of immunotherapy with CpG in mouse colon cancer model (43). This combination therapy resulted in the marked reduction of TNFR2 expression on tumor-infiltrating Tregs and consequently increases tumor infiltration of interferon-gamma-producing CD8$^+$ CTL (44). Thus, novel antagonists against TNFR2 are potential drug candidates for cancer immunotherapy.

VIRTUAL SCREENING OF SMALL MOLECULES TARGETING TNFR2

Despite the important roles of TNFR2 in cancer, autoimmune diseases, and GvHD, to the best of our knowledge, no small molecule agonists or antagonists against TNFR2 have been successfully identified. With the recently available TNF–TNFR2 crystal structure (18), the specific binding pattern between TNF and TNFR2 has been revealed. This information is crucial for successful design of molecules that can directly compete against TNF to bind with TNFR2 by means of virtual screening. In virtual screening, a library of compounds is examined to predict their binding poses and binding affinities at the potential binding site of the target protein. Compounds that resemble the binding pose to the native ligand with better binding affinity will be selected as candidates for further research and development in the drug discovery pipeline. Several previous studies on virtual screening of small molecules against TNF and TNFR1 are exemplary. For example, Choi et al. screened 240,000 compounds *in silico* against the TNF dimer, and 3 compounds with a common derivative of the pyrimidine-2,4,6-trione moiety were found to be the top binders to TNF and all of them showed marked inhibitory activities in *in vitro* experiment (45). In another study, Chan et al. identified two natural product-like TNF inhibitors—quinuclidine and indoloquinolizidine—from over 20,000 compounds by virtual screening. Their activities to inhibit the binding of TNF to TNFR1 were experimentally validated. The result showed that indoloquinolizidine had the similar potency (IC_{50} = ~10 μM) as SPD304 (IC_{50} = ~3 μM), the most potent TNF binder known at that time (46). To date, the most potent small molecule antagonist targeting TNF is C87 (K_d = 0.11 μM). It was again found by virtual screening from a library of 90,000 compounds (47). In addition to virtual screening, the molecular structure of the protein–ligand complex can be used to guide the design of larger molecules such as peptides. Using the critical binding sites of TNFR1 by TNF as a template, Takasaki et al. successfully designed the first exocyclic peptidomimetics which act as TNF antagonists (48). Regarding TNFR1, using a homology model of TNF–TNFR1 complex, Chen et al. successfully found one ligand that binds to TNFR1 out of 20 hits from virtual screening of ~213,000 compounds (49); though these ligands do not show improved affinity to TNFR1 than the antagonist physcion-8-*O*-β-D-monoglucoside (K_d = 0.376 μM) identified by Cao et al. in high-throughput screening experiments (50).

The crystal structure of TNF–TNFR2 suggests that major interactions between TNF and TNFR2 occur in two regions, namely, regions 3 and 4. In region 3 of TNFR2, it contains three acidic residues, such as Asp54, Glu57, and Glu70, which together create a highly negatively charged molecular surface. On the other hand, region 4 contains three basic residues, such as Arg77, Lys108, and Arg113, which form a highly positively charged surface. Since the two centers of the binding regions are separated by a distance of at least ~20 Å, TNF binding resembles two short arms holding onto regions 3 and 4 simultaneously. To gain an insight into the relative contribution of the two regions to the overall binding, we performed a MD simulation of the TNF–TNFR2 complex [PDB 3ALQ (18)] and MM/PBSA calculation using the GROMACS simulation package (51) and the *g_mmpbsa* tool (52) to assess the free energy of protein–ligand binding. Our result shows that all three key basic residues, such as Arg77, Lys108, and Arg113, in region 4 contribute significantly to the binding energy with a total of ca. −153 kcal/mol. By contrast, the two acidic residues in region 3, such as Glu57 and Glu70, together contribute only ca. −14 kcal/mol and Asp54 did not show strong interaction with TNF. As the binding strength of TNF in region 3 is only one-tenth of that in region 4, this suggests that ligand binds in region 3 may be more competitive against TNF than in region 4. On the other hand, since region 4 is the stronger binding site for TNF, targeting region 4 with small molecules would be highly challenging, although the inhibitory effect should be greater if succeed.

As a first attempt to identify potential TNFR2 binders, we performed virtual screening of 400,000 natural compounds from the Traditional Chinese Medicine Database (53). This comprehensive natural compound library was successfully used to find potent inhibitors for EGFR (54), SIRT1 (55), and H1 (56), etc. After preparation of the TNFR2 structure by the *Preparation wizard* of Schrödinger software (57), the Glide docking box was defined to include both regions 3 and 4. The virtual screening workflow included the ligand preparation step and a pre-filtering step to screen out compounds neither satisfying the Lipinski's rule of five nor the criteria of Absorption, Distribution, Metabolism, Elimination and Toxicity using the *QikProp* module. Filtered compounds were subjected to *Glide* high-throughput virtual screening, followed by standard precision docking and Extra Precision (XP) docking. Candidates with high XP scores and Glide energies were analyzed for their residual binding patterns. Selected compounds were further subjected to QM-polarized ligand docking (QPLD) available in the *Glide* module. We also docked a model tripeptide *RRA* which contains the same three

residues of TNF that interact with TNFR2 at region 3 when bound. This is to provide a baseline energy value for compound selection.

As listed in **Table 1**, five top-scoring compounds for region 3 and three compounds for region 4 were obtained through this virtual screening workflow. All of them exhibited better QPLD scores (−4.624 to −6.952 kcal/mol) than the baseline molecule *RRA* (−4.404 kcal/mol). Compounds targeting region 3, the negatively charged pocket, contain amine groups that can interact with the key residues, such as Asp54, Glu57, and Glu70. However, top hits in region 4 have only slightly higher QPLD scores than top hits at region 3. Since TNF binds much stronger to region 4 than to region 3, region 4 compounds are very unlikely to be able to compete with TNF.

To assess the stability of top virtual hits in region 3, compounds 1 and 2 were subjected to 15-ns MD simulations. Binding poses of these compounds are depicted in **Figure 1**. Compound 1 (ID ZINC72321887) is stable in the binding pocket with four hydrogen bonds. Two hydrogen bonds contributed by the hydroxyl group of the ligand that binds with Glu57 and Asp54, and the amino group with Cys71. Compound 2 (ID ZINC20465842) which contains 4 amino and 2 hydroxy groups forms 4 hydrogen bonds with Glu57 and Asp54. Our MM/PBSA analysis on the MD trajectories reveals that in the compound 1–TNFR2 complex, Asp54, Glu57, and Glu70 together contribute binding energy of ca. −70 kcal/mol in the ligand-bound state versus −13 kcal/mol in the TNF-bound state (i.e., $\Delta\Delta G = -57$ kcal/mol). The enhanced binding is due to the closer contact of the ligand with Asp54 and

TABLE 1 | Top-ranked compounds targeting regions 3 and 4 of TNF receptor type II from *in silico* screening.

No.	Region 3	QPLD score	Glide energy	E_{vdw}	E_{coul}	$E_{internal}$	E_{HB}	HB_{acc}	HB_{don}	Mol. weight	Rot
1	ZINC72321887	−5.366	−38.217	−10.770	−27.447	0	−3.427	7	3	316.36	10
2	ZINC67911837	−5.131	−45.119	−15.823	−29.296	14.86	−2.588	6	4	326.35	7
3	ZINC01611597	−4.518	−34.228	−4.504	−29.724	4.317	−2.700	2	4	229.31	5
4	ZINC77265363	−4.624	−44.233	−13.455	−30.778	10.036	−2.802	6	3	298.36	7
5	ZINC20465842	−4.521	−45.128	−11.514	−33.614	12.067	−2.830	4	4	281.36	8
ref	*RRA (baseline)*	*−4.404*	*−41.591*	*−15.455*	*−26.136*	*8.553*	*−2.924*	*14*	*12*	*456.54*	*20*
No.	**Region 4**	**QPLD energy**	**Glide energy**	E_{vdw}	E_{coul}	$E_{internal}$	E_{HB}	HB_{acc}	HB_{don}	**Mol. weight**	**Rot**
6	ZINC71316232	−6.952	−50.896	−27.661	−23.235	6.061	−4.911	9	5	368.34	11
7	ZINC01532677	−5.92	−31.731	−12.547	−19.184	0.000	−3.645	5	4	164.16	4
8	ZINC00281472	−5.494	−23.339	−8.581	−14.758	2.054	−1.822	6	3	222.20	5

QPLD: QM-polarized ligand docking score; glide energy from XP docking and their energetic components: van der Waals (E_{vdw}), electrostatics (E_{coul}), and ligand internal energy ($E_{internal}$); HB: energy of the hydrogen bonding term in Glide XP scoring function of the whole complex or individual key receptor residues. All energies are in kcal/mol. HB_{acc}: number of hydrogen bond acceptors; HB_{acc}: number of hydrogen bond donors; Mol. weight: molecular weight; Rot: number of rotatable bonds. Molecular structures of these compounds can be found in Figure S1 in Supplementary Material.

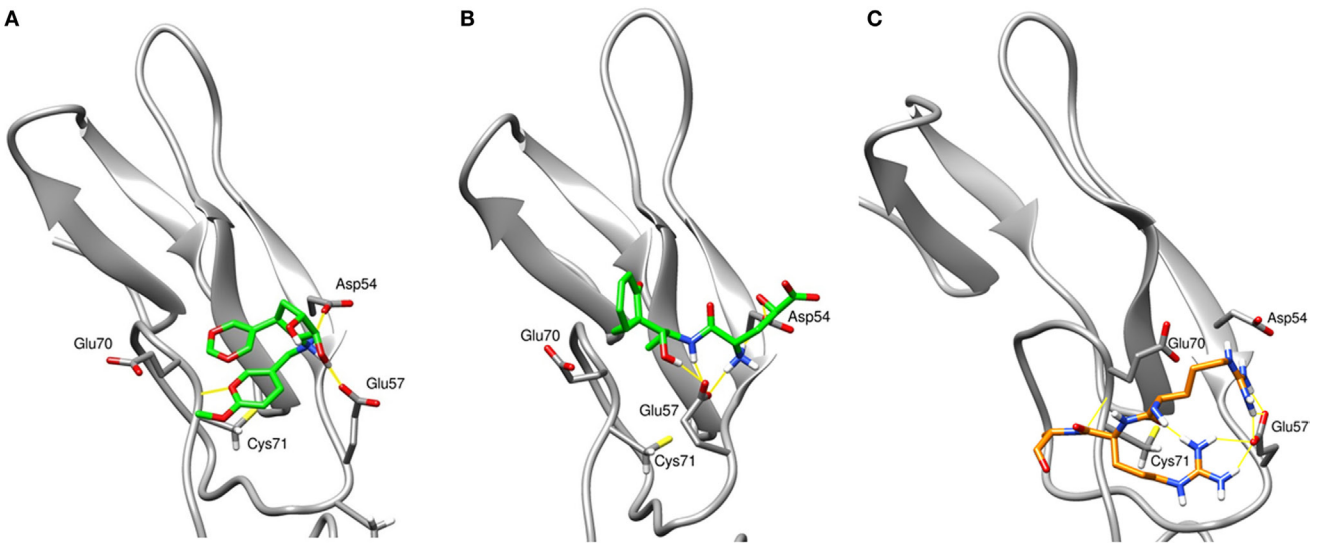

FIGURE 1 | The final snapshots of 15-ns molecular dynamics (MD) simulations of the TNF receptor type II (TNFR2)–ligand complexes: **(A)** compound **ZINC72321887** and **(B)** compound **ZINC67911837** at region 3 of TNFR2. **(C)** The binding pattern of TNF–TNFR2 at the 20-ns MD snapshot. Only contacting residues, such as Arg31, Arg32, and Ala33, of TNF are displayed. The TNFR2 protein is drawn with cartoon style in gray and the ligand or TNF with sticks in green or orange. Hydrogen bonds are indicated with yellow lines.

Glu57 resulting in highly favorable electrostatic interactions and a tight network of hydrogen bonds. By contrast, in TNF–TNFR2 complex, the weak interaction of TNF with Glu70 and Asp54 is presumably caused by the flipped Glu70 side chain which pulls Arg31 of TNF to stay away from the two negative charged receptor residues. Other compounds will be further subjected to the same analysis and validated of their efficiency on inhibiting TNF-induced activation, expansion of Tregs, and enhancing antitumor immune responses in *in vitro* and *in vivo* experiments.

FUTURE PERSPECTIVES

Targeting TNF–TNFR2 with small molecules is a challenging task. Here, we demonstrated the use of virtual screening, MD, and MM/PBSA methods to identify promising hits from a screening of 400,000 natural compounds to target the major TNF–TNFR2 binding regions. Combined MD and MM/PBSA method can provide detail picture of the protein–protein and protein–ligand interactions which helps to identify and compare key receptor residues that contribute to the binding. Our analysis indicates that region 3 is potentially more druggable by small molecules due to its relatively much weaker but essential binding to TNF than region 4 (58). Also, TNF is not able to optimally position itself at the acidic pocket of region 3 presumably due to the physical restriction imposed by the strong binding of itself in region 4. Indeed, our top screened compound for region 3 achieved significantly better affinity ($\Delta\Delta G = -57$ kcal/mol) to TNFR2 than TNF. Altogether, our study shows that the hit list targeting region 3 might serve as a good starting point to further investigate the effect of small molecules binding to TNFR2, and their efficiency on inhibiting TNF-induced activation, expansion of Tregs, and enhancing antitumor immune responses.

AUTHOR CONTRIBUTIONS

FS, JH, XC, and SS designed the overall project. XC supervised JH, and SS supervised FS and PB. FS performed virtual screening, FS and PB performed simulations and analyzed data. FS, JH, XC, and SS wrote and edited the manuscript. XC and SS provided funding for the project. FS and JH contributed equally.

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SUPPLEMENTARY MATERIAL

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The p38 MAPK Inhibitor SB203580 Abrogates Tumor Necrosis Factor-Induced Proliferative Expansion of Mouse CD4$^+$Foxp3$^+$ Regulatory T Cells

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There is now compelling evidence that tumor necrosis factor (TNF) preferentially activates and expands CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) through TNF receptor type II (TNFR2). However, it remains unclear which signaling transduction pathway(s) of TNFR2 is required for the stimulation of Tregs. Previously, it was shown that the interaction of TNF–TNFR2 resulted in the activation of a number of signaling pathways, including p38 MAPK, NF-κB, in T cells. We thus examined the role of p38 MAPK and NF-κB in TNF-mediated activation of Tregs, by using specific small molecule inhibitors. The results show that treatment with specific p38 MAPK inhibitor SB203580, rather than NF-κB inhibitors (Sulfasalazine and Bay 11-7082), abrogated TNF-induced expansion of Tregs *in vitro*. Furthermore, upregulation of TNFR2 and Foxp3 expression in Tregs by TNF was also markedly inhibited by SB203580. The proliferative expansion and the upregulation of TNFR2 expression on Tregs in LPS-treated mice were mediated by TNF–TNFR2 interaction, as shown by our previous study. The expansion of Tregs in LPS-treated mice were also markedly inhibited by *in vivo* treatment with SB203580. Taken together, our data clearly indicate that the activation of p38 MAPK is attributable to TNF/TNFR2-mediated activation and proliferative expansion of Tregs. Our results also suggest that targeting of p38 MAPK by pharmacological agent may represent a novel strategy to up- or downregulation of Treg activity for therapeutic purposes.

Keywords: tumor necrosis factor, TNF receptor type II, p38 MAPK, CD4$^+$Foxp3$^+$ regulatory T cells, proliferation

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INTRODUCTION

CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) are crucial for the maintenance of immune homeostasis and for the prevention of autoimmune responses (1). They also play a major role in immune evasion of cancer by dampening immune responses against tumor (2). Targeting Tregs has become a strategy in the treatment of major human diseases, such as cancer, allergic and autoimmune diseases, transplantation rejection, and GVHD (3). A thorough understanding of biological pathways that regulate Treg function is a prerequisite for the up- or downregulation of Treg activity for therapeutic purposes.

We (Xin Chen and Joost J. Oppenheim) for the first time report that tumor necrosis factor-alpha (TNF) can activate Tregs through TNF receptor type II (TNFR2), one of TNF receptors, which is

preferentially expressed by Tregs (4). Furthermore, we found that expression of TNFR2 identifies the maximally potent suppressive human and mouse Treg subsets (5, 6). In contrast, Tregs without TNFR2 expression only had minimal or no suppressive activity (5, 7, 8). Moreover, TNF–TNFR2 signaling is important for the phenotypical stability of Tregs, including Foxp3 expression (4, 8, 9). The notion that TNF–TNFR2 signaling plays a decisive role in the activation, expansion, and phenotypical stability of Tregs is now supported by compelling evidences from other groups (10–21). Nevertheless, which signaling transduction pathway(s) of TNFR2 is required for Treg-stimulatory effect of TNF remains unknown.

The biological functions of TNF are transduced by two receptors, TNFR1 (p55) and TNFR2 (p75) (22). In contrast to the ubiquitous expression of TNF receptor type I (TNFR1), TNFR2 is mainly expressed by lymphocytes (23). Signal transduction by TNFR1 has been intensively investigated and well defined, while the TNFR2 signaling pathway is less well understood (24). So far, three signaling pathways of TNFR2 in T lymphocytes have been documented, including IKK/NF-κB, MAPK (Erk1/2, p38, JNK), and PI3K/Akt pathways (25, 26). Previously, p38 MAPK signaling pathway has been shown to play a key role in the immunosuppressive function of induced Tregs (iTregs) in both *in vitro* and *in vivo* studies (27–29). It was also reported that inhibition of p38 MAPK signaling was able to reduce immunosuppression of iTregs on Teffs, and consequently enhanced antitumor immune responses (29, 30). It has been shown that TNF stimulation resulted in the activation of p38 MAPK, in addition to the activation of NF-κB, in Tregs (31, 32). Thus, we hypothesized that p38 MAPK signaling pathway may be also attributable to the activation and proliferation of Foxp3$^+$ naturally occurring Tregs (nTregs) by TNF–TNFR2 interaction.

In this study, we investigated the effect of SB203580, a p38 MAPK-specific inhibitor, on the expansion of Tregs induced by the interaction of TNF–TNFR2 in both *in vitro* and *in vivo* experimental settings. The results showed that SB203580 potently inhibited TNF-induced proliferative expansion of Tregs. Furthermore, other stimulatory effects of TNF on Tregs, such as upregulation of TNFR2 and Foxp3 expression were also abrogated by SB203580. Therefore, p38 MAPK represents a major component of signaling pathway of TNFR2 in the activation of Tregs.

RESULTS

SB203580 Inhibits TNF-Induced Proliferation of Tregs *In Vitro*

We firstly examined the *in vitro* effect of p38 MAPK-specific inhibitor SB203580 (33) on the expansive proliferation of Tregs induced by TNF. To this end, CD4$^+$ T cells were purified by MACS from spleen and LNs of normal mice. The cells were cultured with IL-2 to maintain their survival (34). Consistent with our previous report (4, 17), addition of TNF preferentially stimulated the proliferation of Tregs, resulting in proliferation of greater than 60% of Tregs (**Figure 1A**). Consequently, the absolute number of Tregs in the cultured CD4$^+$ T cells was increased twofold by TNF stimulation (**Figure 1E**). As shown in **Figures 1B–C**, in a concentration range of 1–25 μM, SB203580 inhibited the TNF-induced proliferation of Tregs in a dose-dependent manner, with a percent inhibition of 32.0–73.2% ($p < 0.05$–0.001). The proportion of Foxp3$^+$ Tregs in the cultured CD4$^+$ T cells was also markedly reduced by SB203580 treatment, with a percent inhibition of 24.9–47.05% (**Figure 1D**, $p < 0.05$–0.01). Furthermore, the absolute number of Tregs in each well was markedly reduced (**Figure 1E**, $p < 0.05$). In contrast, treatment with two NF-κB inhibitors [Sulfasalazine (35) and Bay 11-7082 (36)] failed to inhibit TNF-induced proliferative expansion of Tregs in the cultured CD4$^+$ T cells (**Figures 2A–F**). These results suggest that the activation of p38 MAPK, rather than the activation of NF-κB, is required for the proliferative expansion of Tregs triggered by TNFR2 signaling. Treatment with SB203580 in the concentration range used in our *in vitro* study did not induce cell death (Figure S1 in Supplementary Material). Furthermore, SB203580 treatment did not reduce the number of Tregs in CD4 T cells cultured with IL-2 alone (Figure S2 in Supplementary Material). These data exclude the possibility that the inhibitory effect of SB203580 was based on the cytotoxic effect.

SB203580 Downregulates TNFR2 Surface Expression on TNF-Stimulated Tregs

The surface expression levels of TNFR2 are correlated with immunosuppressive function of Tregs (5, 6). Previously, we showed that treatment with TNF preferentially upregulates TNFR2 expression on Tregs (37). To determine if p38 MAPK pathway plays a role in the upregulation of TNFR2 expression on Tregs, MACS-purified CD4$^+$ T cells were cultured with IL-2, with or without TNF. The cells were treated with SB203580 (1–25 μM). As shown in **Figure 3A**, the treatment with TNF upregulated TNFR2 expression on Tregs by >2-folds, as compared with IL-2 cultured alone. TNF-induced upregulation of TNFR2 expression was inhibited by SB203580 in a dose-dependent manner (**Figures 3A,B**, $p < 0.01$–0.001), with a percent inhibition of 32.3–62.6% (**Figure 3C**, $p < 0.01$–0.001). Thus, inhibition of p38 MAPK with SB203580 can inhibit surface expression of TNFR2 on TNF-treated Tregs.

SB203580 Abrogates TNF-Induced Upregulation of Foxp3 Expression in Tregs

TNF–TNFR2 interaction is also crucial for the phenotype stability of Tregs, in term of Foxp3 expression, in both *in vitro* and *in vivo* settings (8). We thus examined the effect of SB203580 on Foxp3 expression by TCR-stimulated Tregs. To this end, mouse CD4$^+$CD25$^+$ T cells were flow-sorted and stimulated with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab for 3 days, a known condition, which can downregulate Foxp3 expression (8). Treatment with the exogenous TNF could partially maintain Foxp3 expression (**Figures 4A–C**), consistent with our previous report (8). The levels of Foxp3 expression on per cell basis (MFI) and the proportion of Foxp3-expessing cells were increased by twofold after TNF treatment. These effects of TNF were largely abrogated by the treatment of SB203580 (**Figures 4A–C**). It is worth noting that SB203580, in the absence of TNF, did

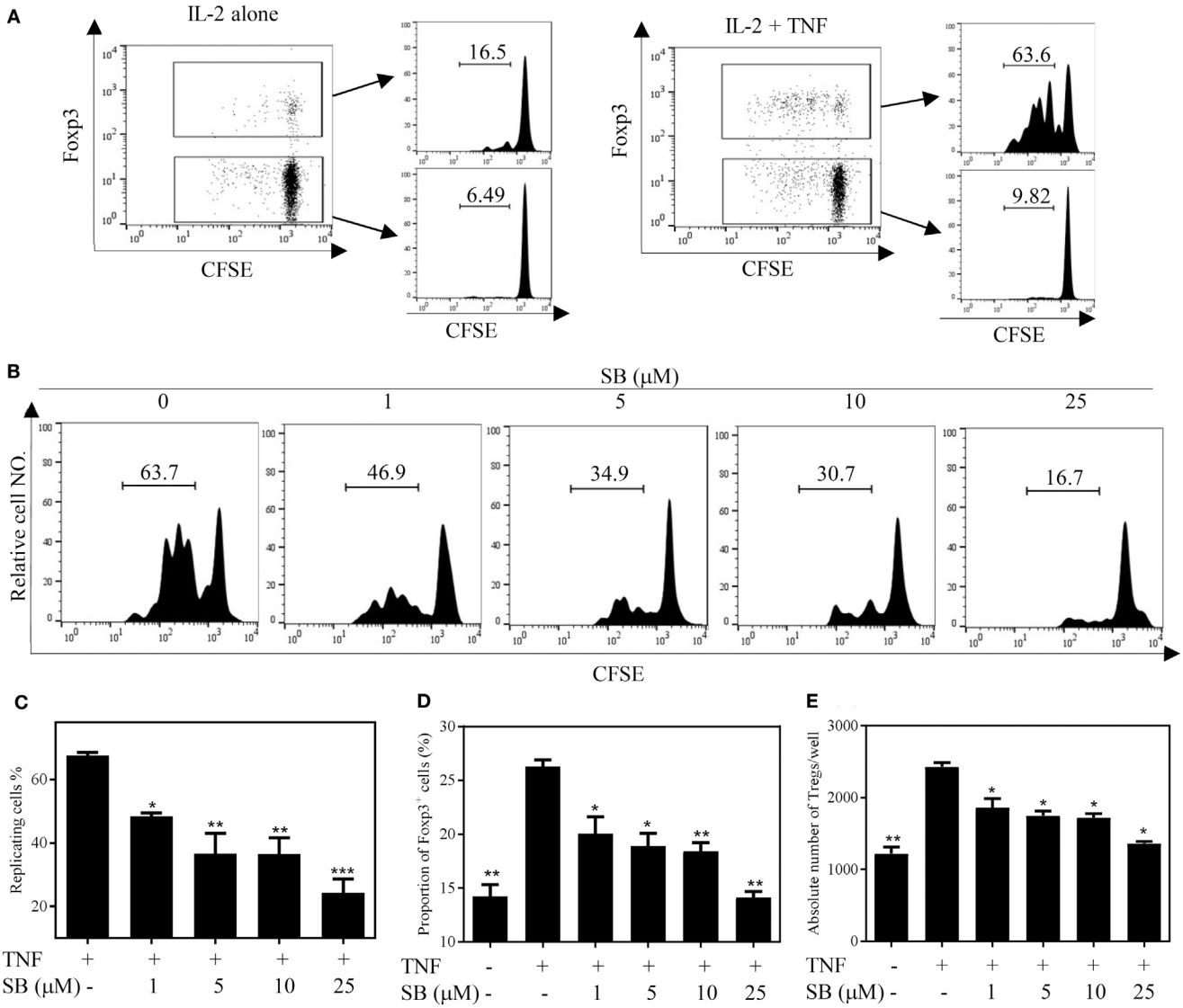

FIGURE 1 | SB203580 (SB) inhibits tumor necrosis factor (TNF)-mediated expansion of regulatory T cells (Tregs) *in vitro*. $CD4^+$ T cells were purified from LNs and spleen of normal C57BL/6J mice by MACS. The cells were labeled with CFSE and cultured in the presence of IL-2 (10 ng/mL), or IL-2 + TNF (10 ng/mL, each), with medium alone or with different concentrations of SB203580 (SB, 1, 5, 10, and 25 μM). After 72 h, the proliferation of Tregs and the proportion of $Foxp3^+$ cells were analyzed by FACS, based on CFSE expression and Foxp3 expression. The absolute number of Foxp3-expressing Tregs was calculated. **(A)** In the presence of IL-2, TNF preferentially stimulated the proliferation of Tregs. **(B,C)** SB203580 blocked TNF-mediated proliferation of Tregs. Analysis was gated on $Foxp3^+$ Tregs. **(D)** SB203580 decreased the proportion of $Foxp3^+$ Tregs in the cultured $CD4^+$ T cells. **(E)** SB203580 reduced the absolute number of Tregs in the cultured $CD4^+$ T cells. **(A,B)** Show the typical FACS plots. The number in the histogram indicates the proportion of gated cells (%). **(C,D)** Show the summary of results ($N = 3$, means ± SEM). By comparison with "TNF + IL-2" group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data shown are representatives of at least three separate experiments with similar results.

not downregulate Foxp3 expression in Tregs (Figure S2 in Supplementary Material).

SB203580 Inhibits *In Vivo* Expansion of Tregs in LPS-Treated Mice

Previously, we showed that TNF–TNFR2 interaction is responsible for LPS-induced proliferation of Tregs in mice (37). More recently, we observed that LPS treatment was able to markedly upregulate the expression of transmembrane TNF on dendritic cells (DCs), and such DCs potently stimulated the proliferation of Tregs (data not shown). Therefore, LPS-treated mice were used to examine if SB203580 had the *in vivo* activity to inhibit TNF-induced expansion of Tregs. As shown in **Figures 5A,C**, the proportion of $Foxp3^+$ cells in splenic $CD4^+$ T cells was increased from 14.6% in control mice to 18.6% in mice 24 h after LPS treatment ($p < 0.01$). Similarly, the proportion of $Foxp3^+$ cells in CD4 T cells present in peripheral blood and

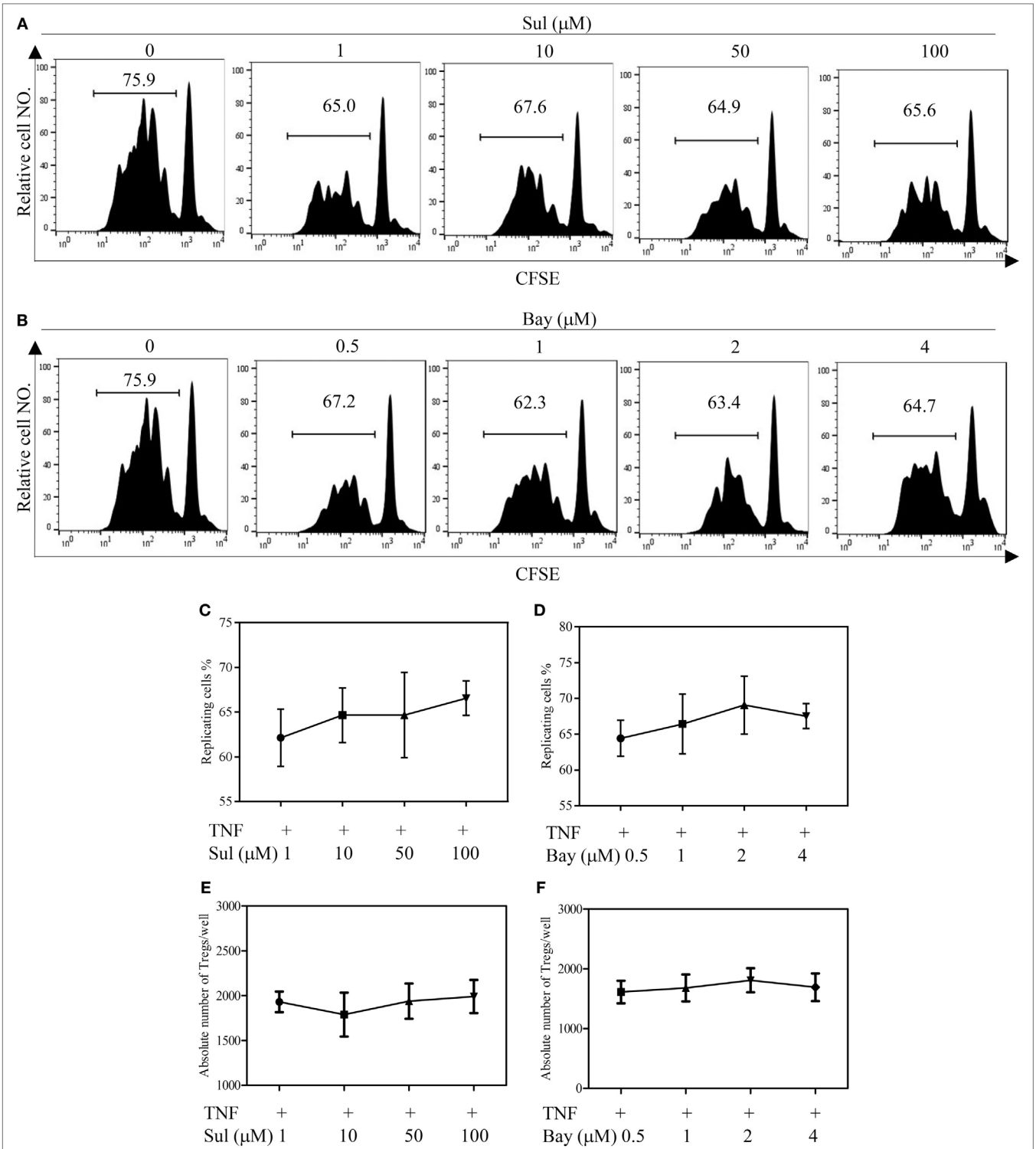

FIGURE 2 | Effect of NF-κB inhibitors on tumor necrosis factor (TNF)-mediated proliferative expansion of regulatory T cells (Tregs). $CD4^+$ cells were purified from LNs and spleen of normal C57BL/6J mice by MACS. The cells were labeled with CFSE and cultured in the presence of IL-2 (10 ng/mL), or IL-2 + TNF (10 ng/mL, each), with medium alone or with different concentrations of Sulfasalazine (Sul, 1, 10, 50, 100 μM) or Bay 11-7082 (Bay, 0.5, 1, 2, and 4 μM). After 72 h, the proliferation of Tregs and the absolute number of $Foxp3^+$ cells were analyzed by FACS, based on CFSE expression and Foxp3 expression. **(A,B)** Typical FACS analysis of Treg proliferation, as shown by dilution of CFSE expression (gating on $Foxp3^+$ cells). The number in the histogram indicates the proportion of gated cells, e.g., replicating cells (%). **(C,D)** The summary of proportion of replicating Tregs. **(E,F)** The absolute number of Treg cells per well. Data shown in **(C–F)** are representatives of at least three separate experiments with similar results ($N = 3$, means ± SEM).

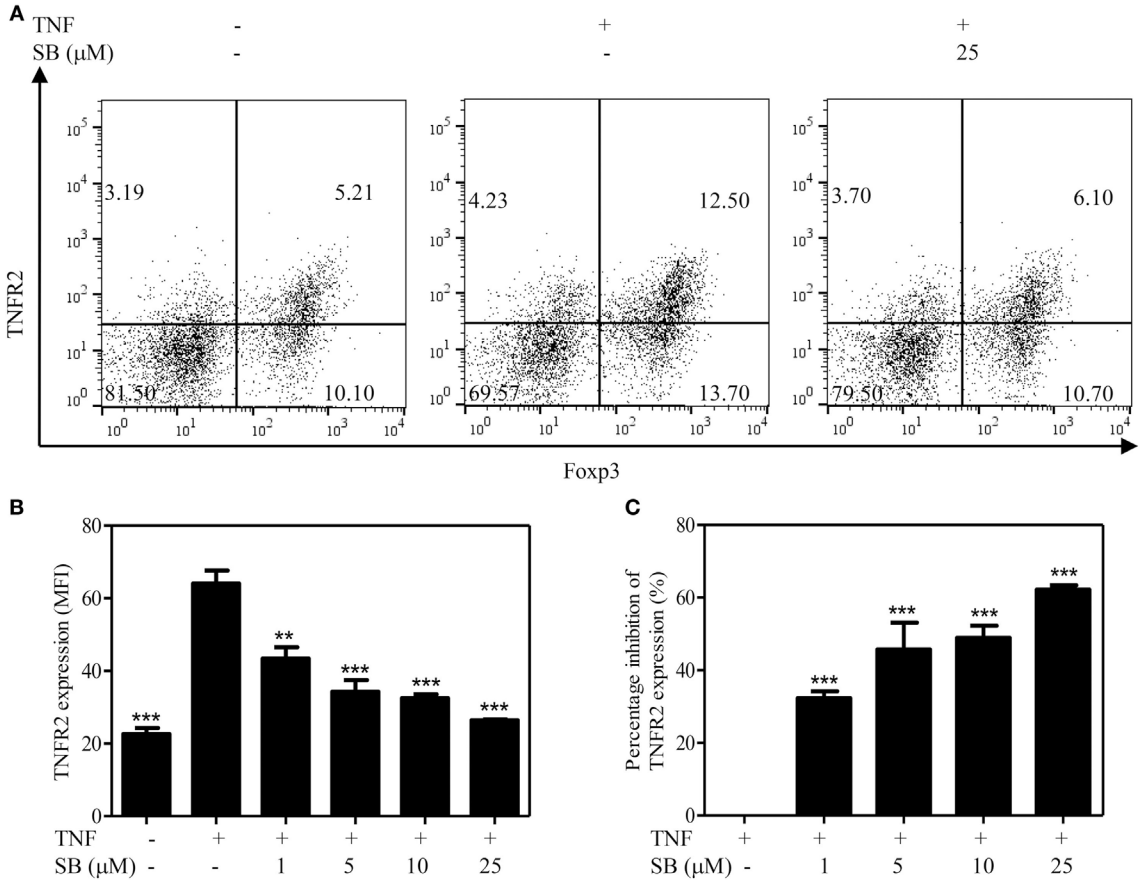

FIGURE 3 | Upregulation of TNFR2 expression on regulatory T cells (Tregs) induced by tumor necrosis factor (TNF) is abrogated by SB203580. MACS-purified CD4$^+$ T cells were cultured in the presence of IL-2 (10 ng/mL), or IL-2 + TNF (10 ng/mL, each), with medium alone or with SB203580 (1–25 μM). The cells were cultured for 72 h. The surface expression of TNFR2 and intracellular expression of Foxp3 were analyzed with FACS. **(A)** Typical FACS dot plot of TNFR2 and Foxp3 expression. Data shown are representatives of at least three separate experiments with similar results. Number in the FACS plot shows the proportion of cells in the respective quadrants. **(B)** Summary of mean fluorescence intensity (MFI) of TNFR2 expression on Tregs (by gating on Foxp3$^+$ cells. $N = 3$, means ± SEM). **(C)** Percent inhibition of TNFR2 expression on Foxp3$^+$ Tregs ($N = 3$, means ± SEM). The formula used to calculate percent inhibition is: (A − B)/A × 100%, A is MFI of TNFR2 expression treated with TNF/IL-2, B is MFI of TNFR2 expression treated with SB203580 (1–25 μM) + TNF/IL-2. By comparison with "TNF + IL-2" group, $^{**}p < 0.01$, $^{***}p < 0.001$. Data shown are representatives of at least three separate experiments with similar results.

lymph nodes following intraperitoneal LPS injection was also increased compared with control mice (**Figure 5C**). The expressions of Ki-67, an indicator of replicating cells, and TNFR2 were markedly increased in the splenic Tregs (**Figures 5B,E** and **6A,C**. $p < 0.01$–0.05), which is consistent with our previous report (7). Since the proportion of Tregs were increased in all observed tissues, which was accompanied by the upregulation of Ki-67, we concluded that the increased number of Tregs in LPS-treated mice was resulted from the proliferative expansion through the interaction of TNF–TNFR2, rather than resulted from the redistribution or alteration of trafficking pattern of Tregs (37). LPS treatment also increased the absolute number of Tregs in spleen by ~1.5-fold (**Figure 5D**, $p < 0.01$). Treatment with single dose of SB203580 (25 mg/kg/day, i.p.) immediately after LPS treatment completely inhibited LPS-induced expansion of Tregs (**Figure 5A**). Moreover, LPS-induced upregulation of Ki-67 and TNFR2 expression on Tregs was also completely abrogated by the treatment of SB203580 (**Figures 5B,E** and **6A,C**). The inhibitory effect of SB203580 on the proliferative expansion of Tregs, as indicated by the proportion of Foxp3$^+$ Tregs and their Ki-67 expression, in LPS-treated mice could last for at least 72 h (Figure S3 in Supplementary Material). CD152 (CTLA4) is a characteristic marker and an effector molecule of Tregs. Expression of CD152 in Tregs was upregulated by LPS-treatment (**Figures 6B,D**, $p < 0.001$), and the elevation of CD152 expression in LPS-treated mice was completely abrogated by SB203580 treatment (**Figures 6B,D**). Therefore, SB203580 has both *in vitro* and *in vivo* activity in the inhibition of TNFR2-mediated activation and expansion of Tregs.

DISCUSSION

The p38 MAPK signaling pathway is known to play a key role in mediating the responses of mammalian cells to LPS stimulation (38), including production of TNF by LPS-treated macrophages (39). The activation of p38 MAPK contributes to

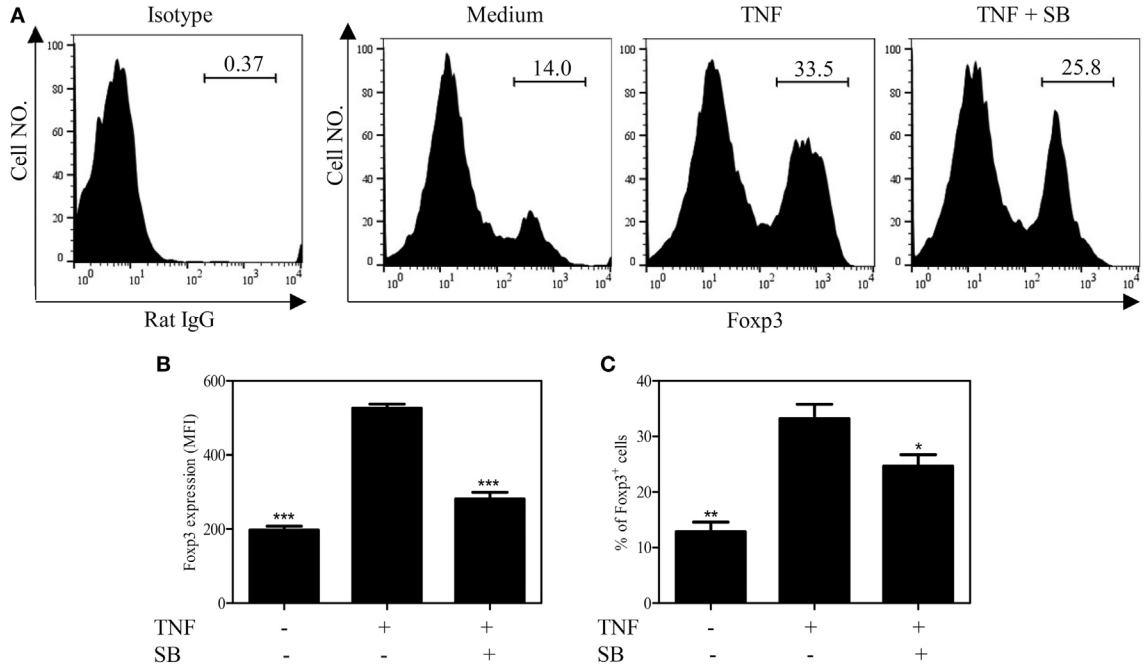

FIGURE 4 | SB203580 inhibits Foxp3 expression in tumor necrosis factor (TNF)-treated regulatory T cells. FACS-sorted $CD4^+CD25^+$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs, in the presence or absence of TNF (10 ng/mL), with or without 25 μM SB203580 for 3 days. Foxp3 expression and ratio of $Foxp3^+$ cells were analyzed by FACS. **(A)** Typical histograms of Foxp3 expression. Number in the histogram indicates the proportion of gated cells. **(B)** Summary of Foxp3 expression (MFI. $N = 3$, means ± SEM). **(C)** Summary of proportion of Foxp3-expressing cells ($N = 3$, means ± SEM). By comparison with TNF group (without SB203580), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data shown are representatives of at least three separate experiments with similar results.

the pathogenesis of autoimmune diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease; however, the results from clinical trials failed to show the therapeutic effect of p38 MAPK inhibitors on these inflammatory diseases (40). The p38 MAPK has a multifaceted role in $CD4^+$ T cells (41), including the activation, cytokine expression, the responses to TCR/co-stimulation, and effector function of Th1 and Th2 cells (42). It was shown that inhibition of p38 MAPK with SB203580 induced immune tolerance in (NZB × NZW)F1 lupus-prone mice, which was purportedly attributable to the increased Treg activity (43). However, more evidence indicates that inactivation or inhibition of p38 MAPK dampens the suppressive function of induced Tregs (iTregs). For example, the number of Tregs was increased in mice with T cells deficient in p38α and p38β (44). Inhibition of p38 MAPK with SB203580 significantly abrogated chronic stress-induced differentiation of $Foxp3^+$ iTregs (45). Furthermore, treatment with SB203580 inhibits the induction and function of human and mouse iTregs (27, 46, 47) and mouse IL-10-producing $CD25^-$ suppressive CD4 T cells (29). To date, the effect of inhibition of p38 MAPK with SB203580 on naturally occurring Tregs (nTregs), especially in an *in vivo* experimental setting, remains unknown.

It has been shown that TNF–TNFR2 interaction was able to activate p38 MAPK pathway in T cells through activation of Syk protein tyrosine kinase (48). Nagar/Goldstein and colleagues examined TNF-induced gene transcription in flow-sorted human Tregs (31). GCBI analysis of GSE18893 file uploaded by Nagar/Goldstein and colleagues (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18893) indicated that both p38 MAPK pathway and NF-κB pathway in Tregs were markedly activated after TNF stimulation (Tables S1 and S2 in Supplementary Material). Recent evidence also showed that TNFR2-specific TNF-variant scTNF(143N/145R) treatment markedly activated p38 MAPK and NF-κB in purified human Tregs (32). In our study, small molecule inhibitors of p38 MAPK and NF-κB pathways, namely SB203580, Sulfasalazine, and Bay 11-7082, were employed to determine which TNFR2 signaling pathway is required for Treg expansion induced by TNF–TNFR2 interaction. Previously, SB203580 was well characterized as a specific p38 MAPK inhibitor (33), and Sulfasalazine was a specific inhibitor of NF-κB activation (35), while Bay 11-7082 was a direct inhibitor of IKK and thus inhibits the signal-induced nuclear translocation of NF-κB (36). These three compounds have been frequently used by investigators to study the effect of inhibition of p38 MAPK and NF-κB in T cells, including Tregs (27, 46, 47). We confirmed that p38 MAPK and canonical NF-κB pathways in Treg cells were activated by TNF stimulation. Furthermore, such upregulation of p38 MAPK and NF-κB activity could be potently inhibited by SB203580, Sulfasalazine, and Bay 11-7082, respectively (Figure S4 in Supplementary Material). Our study clearly shows that p38 MAPK-specific inhibitor SB203580, but not sulfasalazine nor Bay 11-7082, potently inhibited TNF-induced expansion, expression of TNFR2 and Foxp3 on Tregs in both *in vitro* and *in vivo* experiments. Our results thus provide

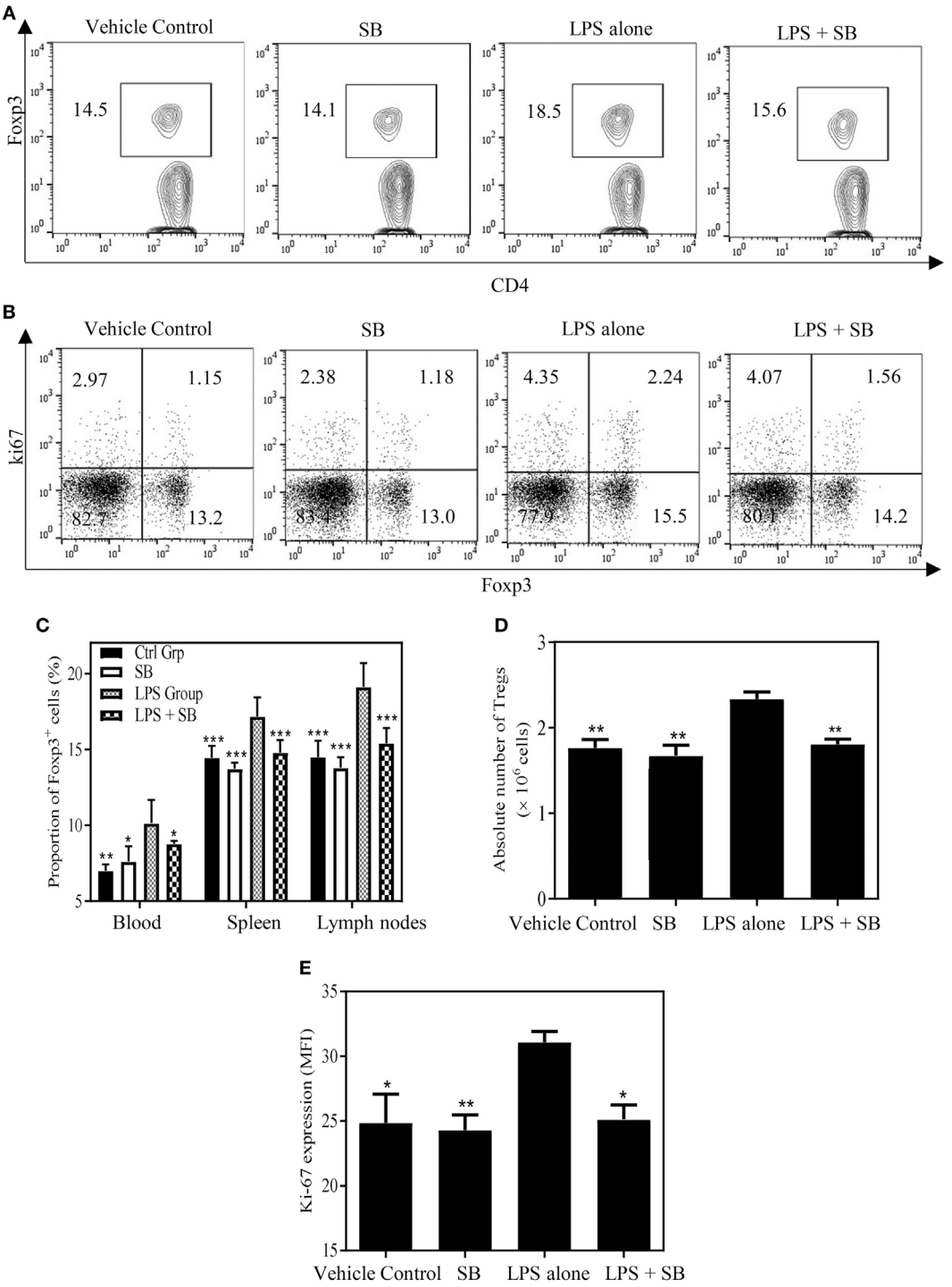

FIGURE 5 | SB203580 inhibits expansion of regulatory T cells (Tregs) in LPS-treated mice. C57BL/6J mice were injected with 200 μg of LPS (i.p.) or PBS, and treated with or without SB203580 (25 mg/kg/day, i.p.) immediately after LPS challenge. All mice were sacrificed 24 h after LPS treatment. Blood, spleen, and lymph nodes were harvested. The proportion of Foxp3$^+$ Tregs in CD4$^+$ T cells and expression of Ki-67 by Tregs were analyzed by FACS, gating on Foxp3$^+$ cells. The absolute number of Tregs was calculated. **(A)** Expression of Foxp3 by CD4$^+$ T cells. Number shows the proportion of gated cells. **(B)** Expression of Ki-67 by Foxp3$^-$ and Foxp3$^+$ cells. Number shows the proportion of positive cells in the respective quadrants. **(A,B)** Typical FACS plots were shown. **(C)** Summary of proportion of Tregs in CD4$^+$ T cells in the peripheral blood, spleen and LNs. **(D)** Summary of absolute number of Tregs in the spleen. **(E)** Ki-67 expression (MFI) by Foxp3$^+$ Tregs. Data [means ± SEM) in **(C)** were pooled from three separate experiments (spleen and lymph nodes: $N = 9$, peripheral blood: $N = 6$), and in **(D,E)** ($N = 3$) were representatives of at least three separate experiments with similar results. By comparison with LPS alone group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

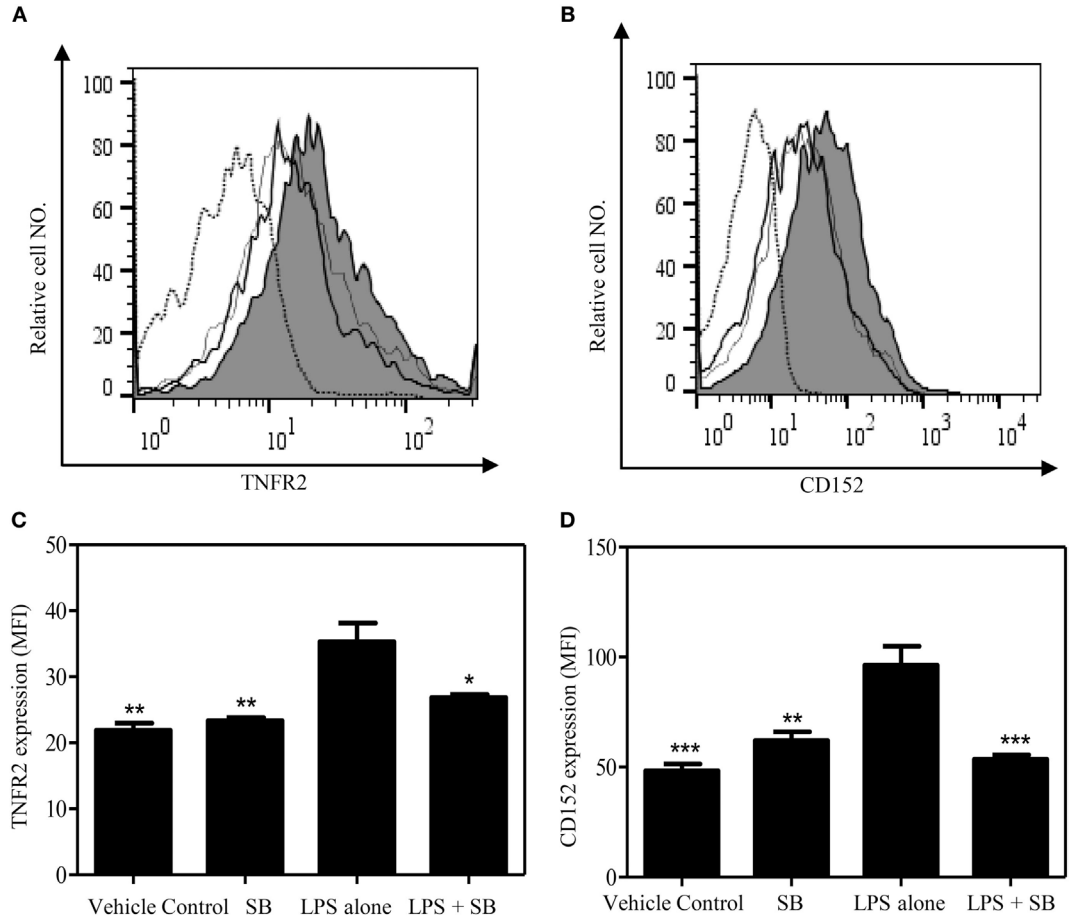

FIGURE 6 | SB203580 inhibits the upregulation of TNFR2 expression and CD152 expression on regulatory T cells (Tregs) in LPS-treated mice. C57BL/6J mice were injected with 200 μg of LPS (i.p.) or PBS, and treated with or without SB203580 (25 mg/kg/day, i.p.). Mouse spleen were harvested at 24 h after injection for the FACS analysis of CD152 and TNFR2 expression, gating on Foxp3$^+$ cells. **(A,B)** Typical FACS histograms were shown. Black solid line: vehicle control; gray-filled histogram: LPS treatment; hair line: LPS + SB203580; Dot histogram: isotype control. Summary TNFR2 expression [MFI. **(C)**] and CD152 expression [MFI, **(D)**] by Foxp3$^+$ Tregs ($N = 3$, means ± SEM). Data shown are representatives of at least three separate experiments with similar results. By comparison with LPS alone group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

clear evidence that p38 MAPK may represent an important component of TNFR2 signaling pathway in the activation and expansion of Tregs induced by TNF.

In our *in vitro* studies, IL-2 was used to maintain the survival of cultured T cells. Previously, we showed that in this *in vitro* culture system, TNF-induced proliferation of Tregs was independent of IL-2 (37). This conclusion was further substantiated by the studies from other groups (12, 49). Thus, inhibition of Treg proliferation by SB203580 is mainly achieved by blockade of p38 MAPK activity triggered by TNF–TNFR2 signaling. This idea is supported by the observation that SB203580 did not reduce the number of Tregs in CD4 T cells cultured with IL-2 alone (Figure S2 in Supplementary Material). Nevertheless, IL-2 and TCR/CD28 co-stimulation can also induce the activation of p38 MAPK pathway (50, 51) and can also stimulate the activation and expansion of Tregs (52, 53). Such effect of IL-2 and TCR/CD28 may also contribute to *in vivo* expansion of Tregs in the inflammatory condition, such as in mice treated with LPS. If this is the case, targeting of p38 MAPK may be able to block Tregs expansion induced by multiple signaling pathways.

Elimination of Treg activity, by either reducing their number or downregulating their immunosuppressive function, has become a strategy to enhance the efficacy of cancer therapy (54). Since TNFR2 signaling plays a crucial role in the activation and expansion of Tregs, the major component of TNFR2 signaling pathway responsible for Treg-stimulatory effect may be harnessed to modulate Treg activity. Recent study indicates that TNFR2 is an emerging target of cancer immunotherapy (15, 55). As suggested by our study, inhibition of p38 MAPK may enhance the efficacy of tumor immunotherapy by eliminating Treg activity. Interestingly, it was shown that inhibition of p38 MAPK with SB203580 markedly enhances DC's capacity to activate Teffs and overcome Treg-mediated suppression, and consequently promote antitumor immune response (30, 56, 57). Thus, p38 MAPK inhibitors may be useful as an immune adjuvant to enhance the

efficacy of tumor immunotherapy by simultaneously acting on both Tregs and DCs.

The rationale of development of p38 MAPK inhibitor as therapeutic agent is largely based on the idea that inhibition of p38 MAPK would inhibit the production of TNF (39), since anti-TNF biologics have been shown great success in the treatment of autoimmune inflammatory diseases (58). Although preclinical studies suggest that p38 MAPK inhibitors had therapeutic potential in the treatment of inflammatory diseases in animal model, such as collagen-induced arthritis (59) and experimental allergic encephalomyelitis (60); however, the subsequent clinical trials have generally failed (40). Moreover, treatment with p38 MAPK inhibitors has the potential to induce additional inflammatory responses in RA patients (61). One possibility raised by our studies is that attenuation of Treg activity through interruption of TNF–TNFR2 interaction might be related to the failure of clinical trials designed to examine the effect of p38 MAPK inhibitors in the treatment of chronic inflammatory diseases.

Taken together, our data clearly show that p38 MAPK inhibitor SB203580 has the capacity to abrogate TNF-induced proliferative expansion, expression of TNFR2 and Foxp3 on Tregs. The results suggest that p38 MAPK may represent a key component of TNFR2 signaling pathway, which is required for the activation and expansion of Tregs. Thus, p38 MAPK pathway may be a therapeutic target to enhance the efficacy of cancer immunotherapy by eliminating Treg activity and other immunosuppressive mechanisms, and this possibility should be addressed in the future study.

MATERIALS AND METHODS

Mice and Reagents

Female wildtype (WT) C57BL/6J (8–12 weeks old) were provided by the Animal Facility of University of Macau. The animal study protocol was approved by Animal Research Ethics Committee of University of Macau. Antibodies purchased from BD Pharmingen (San Diego, CA, USA) consisted of PerCP-Cy5.5 anti-mouse CD3 (145-2C11), PE anti-mouse CD4 (GK1.5), PE anti-mouse CD120b/TNFR2 (TR75-89), PerCP-Cy5.5 anti-mouse CD25 (PC61), PE anti-mouse CD152 (UC10-4F10-11). Antibodies purchased from eBioscience include PE-Cy7 anti-mouse CD4 (GK1.5) and APC anti-mouse/rat Foxp3 staining set (FJK-16s). Functional grade purified hamster anti-mouse CD3ε (145-2C11), Functional grade purified hamster anti-mouse CD28 (37.51), recombinant mouse IL-2 and TNF were obtained from BD Pharmingen. Bay 11-7082 (Cat#: B5556), and Lipopolysaccharides (rough strains) from Salmonella (LPS) (Cat#: L9764) was purchased from Sigma-Aldrich. Sulfasalazine (Cat#: S1576) and SB203580 (Cat#: S1076) was obtained from Selleckchem. LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (for 633 or 635 nm, L10119) was ordered from Thermo Fisher Scientific.

Cell Purification and *In Vitro* Cell Culture

Mouse lymphocytes were harvested from spleens, axillary lymph nodes, inguinal lymph nodes, and mesenteric lymph nodes. $CD4^+$ T cells were purified from lymphocytes by using CD4 (L3T4) microbeads (Miltenyi Biotec, 130-097-145) and MS column (Miltenyi Biotec). MACS-Purified $CD4^+$ cells were labeled with CFSE and cells (5×10^4 cells/well) were cultured in a 96-well plate, then stimulated with IL-2 or IL-2 plus TNF, in the presence or absence of SB203580 (1–25 μM) for 3 days. Proliferation of Tregs was assessed by CFSE dilution assay, and the proportion of $Foxp3^+$ cells in $CD4^+$ subset and TNFR2 expression on Tregs were analyzed with FACS. In some experiments, FACS-sorted $CD4^+CD25^+$ cells (cells purity: 98%, 5×10^4 cells/well) were stimulated with plate-bound anti-CD3ε Ab (10 μg/mL) and soluble anti-CD28 Ab (2 μg/mL) in the presence of TNF (10 ng/mL) or medium alone, with or without 25 μM SB203580, for 3 days. Expression of Foxp3 and TNFR2 were analyzed by FACS.

In Vivo Administration of LPS and SB203580

C57BL/6J mice were injected intraperitoneally (i.p.) with 200 μg of LPS in 0.2 mL PBS. Some mice were treated with SB203580 (25 mg/kg, i.p.) immediately after LPS treatment. SB203580 were dissolved in a stable solvent system (4% DMSO, 30% PEG 300, 5% Tween 80, and 61% ddH_2O). After 24 and 72 h, mice were sacrificed. The spleens, lymph nodes at axillary, inguinal, and mesenteric regions, and blood were harvested for FACS analysis.

Flow Cytometry

After blocking FcR, cells were incubated with appropriately diluted antibodies and finally suspended in FACS buffer for cytometric analysis. Acquisition was performed by BD FACSCanto II and BD FACSAria™ Fusion flow cytometer. Data analysis was conducted by using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western Blot

MACS-purified $CD4^+CD25^+$ T cells were stimulated with TNF (100 ng/mL), with or without selected inhibitors [SB203580 (SB), Bay 11-7082 (Bay), Sulfasalazine (Sul)] for 30 min. The cells were homogenized in RIPA buffer containing a cocktail of proteinase and phosphatase inhibitors. Protein samples were separated on a SDS-PAGE gradient gel (4–12% Bis-Tris protein gel; Thermo Fisher Scientific) and transferred to PVDF membranes. The blots were blocked with 5% BSA for 1 h and incubated with phospho-p38 antibody (1:1,000; Cell Signaling Technology) and phospho-NF-κB p65 antibody (1:1,000; Cell Signaling Technology) overnight at 4°C. The blots were then incubated in HRP-conjugated secondary antibody (1:3,000) for 1 h at room temperature, developed in ECL solution (Thermo Fisher Scientific) for 1 min, and exposed by G-Box imager. The blots were then incubated in stripping buffer (Thermo Fisher Scientific) at 37°C for 15 min and reprobing with IκBα antibody (1:1,000; Cell Signaling Technology) or p38 antibody (1:1,000; Cell Signaling Technology) or NF-κB p65 antibody (1:1,000; Cell Signaling Technology) or GAPDH antibody (1:3,000; Cell Signaling Technology).

Statistical Analysis

Comparisons of two groups of data were analyzed by *t* test using GraphPad Prism 6.0. Comparisons of more than two groups of data were analyzed by one-way ANOVA by using GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of approved guidelines of Animal Research Ethics Committee, University of Macau. The protocol was approved by the Animal Research Ethics Committee of University of Macau.

AUTHOR CONTRIBUTIONS

TH, SL, SC, JY, and XW performed the experiments. TH, ZB, and XC designed the experiments and wrote the manuscript. All authors agree to the submission of the manuscript.

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SUPPLEMENATRY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01556/full#supplementary-material.

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REVIEW
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Critical Role of Tumor Necrosis Factor Signaling in Mesenchymal Stem Cell-Based Therapy for Autoimmune and Inflammatory Diseases

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Mesenchymal stem cells (MSCs) have been broadly used as a therapy for autoimmune disease in both animal models and clinical trials. MSCs inhibit T effector cells and many other immune cells, while activating regulatory T cells, thus reducing the production of pro-inflammatory cytokines, including tumor necrosis factor (TNF), and repressing inflammation. TNF can modify the MSC effects *via* two TNF receptors, i.e., TNFR1 in general mediates pro-inflammatory effects and TNFR2 mediates anti-inflammatory effects. In the central nervous system, TNF signaling plays a dual role, which enhances inflammation *via* TNFR1 on immune cells while providing cytoprotection *via* TNFR2 on neural cells. In addition, the soluble form of TNFR1 and membrane-bound TNF also participate in the regulation to fine-tune the functions of target cells. Other factors that impact TNF signaling and MSC functions include the gender of the host, disease course, cytokine concentrations, and the length of treatment time. This review will introduce the fascinating progress in this aspect of research and discuss remaining questions and future perspectives.

Keywords: mesenchymal stem cells, tumor necrosis factor, TNFR, regulatory T, autoimmune and inflammatory diseases

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INTRODUCTION

Among many multipotent stem cell types, mesenchymal stem cells (MSCs) are a unique cell type that possesses not only stem cell properties but also immunomodulatory capabilities. MSCs refer to multipotent cells derived from the mesenchyme—the embryonic connective tissue that originates from the mesoderm. MSCs can differentiate into a wide variety of cells from the mesoderm, including osteocytes, chondrocytes, adipocytes, and smooth muscle cells (1, 2), and some cell types from the other germ layers, such as neurons from the ectoderm (3, 4) and hepatocytes from the endoderm (5, 6). Recently, neural crest cells were identified as another source giving rise to mesenchymal progenitors, which, similar to MSCs, have a high potential to differentiate into osteocytes and chondrocytes (7, 8). MSCs can be isolated from many fetal and adult tissues or differentiated from human pluripotent stem cells (hPSCs). *In vitro* and *in vivo* studies have demonstrated that MSCs modulate immune responses and inflammation and execute cytoprotective and reparative effects mainly through cell–cell contact and paracrine mechanisms. Thus, MSCs have been used as a cell therapy for an increasing number of autoimmune, inflammatory, and degenerative diseases (1, 2).

Autoimmunity and chronic inflammation are known to share numerous factors, and thus, frequently coexist in the same patients. Autoimmune disease occurs when the immune system abnormally attacks a part of a normal body. Approximately 80 types of autoimmune diseases have been identified, and these diseases can involve almost any part of the body. The abnormal immune response is often associated with complicated genetic factors and the environment. Autoimmune disease is a common and often serious clinical problem due to the chronic nature, high incidence in human populations, especially in women, and rising cost of healthcare. Among the list of common autoimmune diseases, rheumatoid arthritis (RA) (9), inflammatory bowel disease (IBD) (10), and type-1 diabetes (T1D) (11) are on the top. Approximately 7% of people in the United States are affected by autoimmune disease. Tumor necrosis factor (TNF or TNFα), which is involved in a wide range of biological functions, is considered the master mediator of the pathogenesis of chronic inflammation and autoimmune diseases. Therefore, anti-TNF therapies have become mainstay treatments for autoimmune and inflammatory diseases.

Mesenchymal stem cells are susceptible to environmental changes, and their immunosuppressive functions can be modulated when exposed to an inflammatory milieu (12). TNF and other pro-inflammatory cytokines, such as interferon γ (IFNγ) and interleukin 1 (IL-1), determine the disease onset, severity, and relapse of autoimmune diseases and affect the efficacy of treatment, including MSC-based therapy. IFNγ, TNF, and IL-1 present in inflammatory tissues can augment the immunosuppressive functions of MSCs (13–15). Priming of MSCs with IFNγ can yield an augmented immunosuppressive population with a higher efficacy for anti-inflammatory treatment than non-primed MSCs (16). Primed MSCs have been broadly applied in both basic and clinical research (17). However, no focused review has discussed the role of TNF signaling in MSC-based therapy of autoimmune and inflammatory diseases, given the great progress in this area of research. TNF exerts its functions by binding to two receptors (TNFR1 and TNFR2) to regulate the survival, proliferation, migration, and differentiation of target cells, especially immune cells. This molecule also interacts with MSCs to modify or mediate their therapeutic effects. This review, aimed to introduce the progress in this area, will specifically discuss how TNF/TNFR and MSCs converge on the immune system to prevent autoimmune and inflammatory diseases.

MSC EFFICACY ON AUTOIMMUNE AND INFLAMMATORY DISEASES

Mesenchymal stem cells have tremendous potential as a cellular therapy for autoimmune and inflammatory diseases because of their strong immunomodulatory effects and tissue regenerative capability. A growing number of translational studies have been carried out on MSCs for the treatment of many autoimmune and inflammatory diseases, including T1D (18), RA (19), IBD (20), ulcerative colitis (21), systemic lupus erythematosus (SLE) (22), autoimmune uveitis (23, 24), and Sjogren's syndrome (25). So far, over 5,000 MSC-related clinical trials have been registered at ClinicalTrials of the National Institutes of Health in the U.S. (https://clinicaltrials.gov/), of which over 1,900 trials have been completed. Both autologous and allogenic MSCs were used in these trials, in which bone marrow (BM), adipose tissue, umbilical cord, placenta, and dental pulp were the most common sources for MSCs. In addition, MSCs differentiated from hPSCs, including embryonic stem cells and induced pluripotent stem cells (iPSCs), have also been examined and demonstrated efficacy on a variety of animal disease models and may become new options for future clinical applications (21, 26–28).

Mesenchymal stem cells regulate the adaptive immune system by promoting the generation of regulatory T cells (Tregs) and repressing the functions of T effector (Teff) and B effector cells (29–31). These effects are mainly triggered by exposure to pro-inflammatory cytokines, such as TNF, IFNγ, and IL-1β, which are widely present in tissues affected by inflammatory and autoimmune diseases. For instance, TNF deregulates the balance between Tregs and pathogenic Th17 and Th1 cells in the synovium of RA patients and impairs Treg functions in RA and MS patients (32, 33). Systemically transplanting MSCs into patients leads to a decrease in the number of Teff cells and restoration of Treg functions (22, 34). Moreover, IFNγ-primed MSCs inhibit B cell differentiation by arresting the cell cycle and inducing apoptosis (35).

As for innate immunity, MSCs can inhibit natural killer (NK) cell cytotoxicity and block the differentiation and/or maturation of macrophages and dendritic cells (DCs). MSCs skew the polarization of macrophages from M1 to M2 in wound healing (36) and inhibit DC generation and migration to lymph nodes *in vivo* (37). Studies of the molecular mechanisms for the therapeutic effects of MSCs have revealed that MSCs modulate immune responses and promote tissue repair *via* secretion of soluble factors and direct cell–cell contact (29). MSCs exert immunosuppressive effect by secreting soluble factors, such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), transforming growth factor-β1, insulin-like growth factor-1 (IGF-1), nitric oxide, and human leukocyte antigen-G5 (38, 39). Inhibition of IDO or PGE2 synthesis results in reduction of MSC-mediated immunosuppression, and priming MSCs with pro-inflammatory cytokines, such as IFNγ and TNF, enhances the immunosuppressive effects by elevating the secretion of IDO, CXCR4, and PGE2 (29, 39–42). MSCs mixed with activated T cells have the strongest inhibition on the T cells *via* direct cell contact (43), and upregulated expression of intercellular adhesion molecule-1 and vascular adhesion molecule-1 in MSCs strengthens their interaction with T cells (44).

Although promising results have been obtained from MSC-based therapy, the outcomes are not always consistent and sometimes even contradictory, depending on the delivery strategies, MSC sources, and disease course (45–49). A phase I study reported that 7/10 patients with Crohn's disease did not respond to autologous BM-MSC infusion, and three of them even manifested worsened symptoms (50). Site-specific administration of MSCs to patients with Crohn's disease and mice with collagen-induced arthritis (CIA) appeared to be more effective than systemic injection (51, 52). It has been well documented that the functions of

MSCs depend on the microenvironment. MSCs often manifest immunosuppressive effects in a strong inflammatory milieu, and this ability is reduced or lost and the immunogenicity of the cells increased in a weak inflammatory environment (2). Long-term exposure to IFNγ or TNF even converts MSCs from an immunosuppressive to pro-inflammatory status (53–55). Moreover, MSCs are effective at disease onset or when the symptoms reach peaks but fail to alleviate the symptoms after the disease stabilizes or during disease progression (46, 56).

In addition, the origin of MSCs also influences their immunomodulatory effects. For example, autologous BM-MSCs from patients with SLE or synovial-derived MSCs from patients with RA failed to improve the symptoms of the same donor patients (47, 57). Adipose-derived MSCs from mice with experimental autoimmune encephalomyelitis (EAE) had no therapeutic effect on the donor animals (58). MSCs isolated from obese mice or non-obese diabetic mice failed to alleviate the symptoms in EAE and T1D mice (18, 59). Thus, choosing MSCs from the right source and determining the immunomodulatory effects of MSCs are necessary before therapeutic applications.

TNF SIGNALING

Currently, 19 members have been identified in the TNF superfamily (TNFSF), including TNF, TNFβ, CD40L, FasL, and TRAIL, which participate in diverse cellular activities, including inflammation, cell proliferation, apoptosis, and morphogenesis (60). In particular, TNF is abundant in the serum and many other body fluids in patients with autoimmune disease. TNF is a trimeric type-II transmembrane protein that shares a TNF homology domain with the other TNFSF members and is produced mainly by activated macrophages, T, B, and NK cells. TNF is present in two different forms, the membrane-bound TNF (mTNF) and soluble TNF (sTNF or TNF), and TNF is cleaved from mTNF *via* metalloproteinases, such as TNF-converting enzyme (TACE) (61–63).

Tumor necrosis factor and sTNF bind to two structurally distinct transmembrane receptors, TNFR1 and TNFR2, both belonging to the TNFR superfamily, which comprises trimeric type-I transmembrane proteins with repeated extracellular cysteine-rich domains for ligand binding; the two receptors regulate gene expression *via* different signaling pathways (61). TNFR1 can be activated by both mTNF and TNF, whereas TNFR2 preferentially binds to mTNF to initiate the activation of the receptor (64). Moreover, TNFR1 is expressed on almost all cells of the body, whereas TNFR2 is expressed only on limited cells, e.g., immune cells, endothelial cells, nerve cells, and MSCs. TNFR also includes membrane-bound (mTNFR or TNFR) and soluble (sTNFR) forms, and sTNFR is cleaved from TNFR by TACE (63).

In general, TNF induces cell apoptosis or survival through at least five different signals, including caspase, NFκB, ERK, JNK, and P38 MAPK pathways, *via* TNFR1 and -R2 (60). TNFR1 contains 434 amino acids, and its intracellular region contains a death domain (DD), which recruits the TNF-associated death domain (TRADD), and the latter then recruits Fas-associated death domain to trigger the caspase cascades and apoptosis. In addition, TNFR1 also induces reactive oxygen species release from mitochondria to activate apoptotic events. Paradoxically, TRADD can also recruit the TNFR-associated factor (TRAF2) to initiate the NFκB, ERK, JNK, and p38 MAPK signaling pathways to regulate the cell survival and proliferation. By contrast, TNFR2 consists of 439 amino acids and does not include a cytoplasmic DD, which binds to TRAF2 directly and activates pro-survival genes through the NFκB, ERK, JNK, and p38 MAPK pathways (60). There is some degree of cross talk between the TNFR1 and -R2 signaling pathways.

Another key feature of TNF signaling is the phenomenon called "reverse signaling," in which the signal transmits from the TNFRs (including their membrane-bound and soluble forms) to mTNF-bearing cells (outside to inside). Reverse signaling of TNF has been shown to be functional in macrophages and T, B, and NK cells in humans. For example, activation of mTNF reverse signaling enhances the cytotoxicity of CD8 T cells and NK cells and the survival of B cells (65–67). In addition, soluble TNFR1 (sTNFR1)-stimulated monocytes manifest pro-inflammatory effects without TNF treatment and anti-inflammatory effects after TNF treatment, as reflected by regulation of the pro-inflammatory cytokines IL1β and IL8 (68). Moreover, the mTNF reverse signaling renders macrophage resistant to LPS-induced effects by inducing *TGFβ* expression (69, 70). It has been shown that the cytoplasmic domain of mTNF contains a casein consensus sequence, which is dephosphorylated during activation of the mTNF reverse signal. mTNF then triggers the p38 MAPK and JNK pathways *via* interaction with protein kinases. Alternatively, a 10-kDa cytoplasmic domain of mTNF can be cleaved and translocated into the nucleus to regulate the expression of various cytokines, such as *IL1β* and *IL12* (71). However, how the mTNF reverse signal works has not yet been fully understood.

TNF IN AUTOIMMUNE AND INFLAMMATORY DISEASES

The important role of TNF in autoimmune and inflammatory disease has been supported by large amounts of evidence from clinical studies. TNF and sTNFR1 are recognized as useful indicators for assessing disease activity. For example, they are often at high levels in patients with RA and ankylosing spondylitis (72). In SLE patients, TNF is also elevated, and circulating sTNFR is significantly higher than in patients with RA and spondyloarthropathies (73). Chronic progressive MS patients manifest elevated TNF in CSF and active lesions compared with serum (74). The TNF level correlates with the manifestation and degree of disability in patients.

A vast number of animal studies have uncovered much more knowledge than clinical trials about the pathogenesis mediated by TNF. Transgenic mice overproducing TNF develop severe inflammatory arthritis, and the disease onset depends on IL1 production (75). IL17 promotes osteoclastogenesis by stimulating TNF production (76). In IBD patients, TNF disrupts the intestinal epithelial barrier, which makes the intestines vulnerable to infections, thus promoting inflammation (77). Mice overexpressing

TNF develop chronic inflammation resembling IBD (77). However, TNF/lymphotoxin knockout or ectopic expression of mTNF delays the disease onset of EAE in mice (78, 79).

TNFR1 in Autoimmune and Inflammatory Diseases

Activation of TNF/TNFR1 signaling predominantly promotes inflammation and tissue degeneration. Interaction of TNF with TNFR1 activates Teff cells and guides the migration of Teff cells to inflammatory sites (80); for example, $CD4^+$ Teff cells are preferably accumulated in synovial joints in RA patients (81). Meanwhile, TNFR1 knockout prevents the development of arthritis and IBD in mice (82) and shortens the disease course of EAE and T1D in mice (78, 83), indicating a pro-inflammatory role of TNFR1 signaling. Furthermore, TNFR1 signaling likely impairs Treg functions *via* induction of the dephosphorylation of FoxP3 by protein phosphatase 1 in the inflamed synovium of RA, accompanied by increased numbers of Th17 and IFN^+ CD4 T cells (84). Thus, TNF and TNFR1 have been used as therapeutic targets for the treatment of autoimmune and inflammatory diseases. An anti-TNFR1 nanobody protects against EAE development in mice (85), and sTNFR1 has been used as a natural inhibitor of TNFR1 signaling by binding and saturating TNF to repress its signaling (64).

TNFR2 in Autoimmune and Inflammatory Diseases

In contrast to the pro-inflammatory effects of TNFR1, the TNF/TNFR2 interaction preferentially mediates immunosuppressive effects (86–89). In mice with dextran sulfate sodium-induced colitis, TNFR1 ablation exacerbated the severity of the disease, while TNFR2 deficiency led to the opposite results (90). TNFR2 knockout in EAE mice accelerated the disease progression accompanied by severe demyelination (78), suggesting a repressive role of TNFR2 in the disease development. Similarly, polymorphisms in TNFR2 have been found in various autoimmune diseases, which might lead to deregulation of TNF signaling *via* upregulation or shedding of TNFR2 (91).

TNFR2 has been identified as a marker for activated Tregs. TNFR2 and its ligands can activate and stabilize Tregs in an inflammatory environment (92–94). A subset of Tregs with high TNFR2 expression exhibits maximally suppressive activities in both mouse and human, which makes them the most desirable cells for the treatment of autoimmune and inflammatory diseases (95, 96). Furthermore, TNFR2 agonists have proved effective for the treatment of autoimmune disease (91, 97). Upon stimulation, TNFR2 is rapidly upregulated in Tregs, which are empowered to exert stronger immunosuppressive effects on Teff cells than non-stimulated Tregs (93).

However, stimulation of TNFR2 on Teff (e.g., Th1, Th17, and $CD8^+$) cells promotes the cells to proliferate, secrete cytokines, and develop resistance to Treg-mediated suppression (95, 98–100). For example, the $CD25^{hi}/TNFR2^+$ Treg subset induced upon TCR stimulation allows the identification of maximal cytokine-producing effectors (101). These lines of evidence indicate the complex effects of TNFR2 on T cells, which help balance between Treg and Teff cells and partially explain the reasons for the controversial responses of some patients to TNFR2 agonists.

DUAL EFFECTS OF TNF ON AUTOIMMUNE AND INFLAMMATORY DISEASES IN THE CENTRAL NERVOUS SYSTEM (CNS)

Although beneficial effects of TNF therapies have been observed in patients with RA, Crohn's disease, SLE, and psoriasis, clinical trials on MS patients showed the opposite effects, with worsening of their symptoms (102). Adverse effects have also been found in trials on patients with optic neuritis, MS, and other demyelinating diseases following anti-TNF medications (103, 104). The adverse effects occurred in 0.05–0.2% of patients treated with three licensed anti-TNF agents. The opposing outcomes of TNF therapies may result from the dual effects of TNF on inflammation in the CNS.

Circulating TNF in the periphery can cross the blood–brain barrier (BBB) and enter the CNS. Infiltrating immune cells such as macrophages as well as activated microglia in the CNS can produce TNF (105). Generally, binding of TNF to TNFR1 predominantly mediates pro-inflammatory effects of TNF accompanied by activation of the target cells. In murine models of ischemia and EAE, TNFR1-ablation reduced neuronal loss and demyelination (105, 106). In addition, TNFR1 signaling activates microglia to promote neural inflammation due to increased production of pro-inflammatory factors including TNF, IL-1β, and IL-6 (107). TNF also induces apoptosis of human adult oligodendrocytes by causing mitochondrial dysfunction *via* TNFR1/JNK-3 signaling pathway and inhibits differentiation of oligodendrocyte progenitor cells (OPC) *via* AMPK activation and mitochondrial impairment (108, 109). These results indicate the adverse effects of TNFR1 signaling on multiple cell types in the CNS during the disease progression.

By contrast, upregulation of TNFR2 in OPC, microglia, and astrocytes promotes neuroprotection and remyelination, as observed in TNFR1-ablated mice with cerebral ischemia and EAE (105). TNFR2 ablation impairs OPC differentiation and causes dysfunction of oligodendrocytes (110). TNFR2 signaling promotes OPC differentiation and remyelination by inducing secretion of CXCL12 and leukemia inhibitory factor from astrocytes (111) and protects oligodendrocytes from oxidative stress-induced damage (112).

In addition, TNFR2 ablation in microglia in the CNS accelerates the onset of EAE, whereas disruption of TNFR2 in monocytes/macrophages suppresses the disease progression accompanied by reduction of T cell activation and infiltration, and attenuated demyelination (113), indicating that TNFR2 plays opposite roles even in microglia and macrophages during development of EAE. Activated microglia enhance the myelin debris clearance and remyelination, which is likely mediated by TNFR2 signaling (113, 114). These findings are instrumental for developing tissue- and receptor-specific medications to target TNF signaling in the treatment of different autoimmune and inflammatory diseases.

TNF REGULATION OF MSC EFFICACY ON AUTOIMMUNE AND INFLAMMATORY DISEASES

Interferon γ affects MSC efficacy in a dose-dependent manner. At low concentrations, it completely abolishes the therapeutic effect of MSCs on EAE, accompanied by increased secretion of the pro-inflammatory chemokine CCL2 and elevated expression of major histocompatibility complex molecules (115). At higher concentrations, IFNγ strengthens the MSC efficacy to reduce the severity of induced colitis in mice (27, 41). Similarly, TNF also dose dependently alters MSC functions. For example, osteogenic differentiation from murine ST2 MSCs is promoted by TNF at lower concentrations as indicated by elevated expression of the osteogenic genes *Runx2*, *Osx*, *OC*, and *ALP* but inhibited by TNF at higher concentrations, which depends on NFκB signaling (116). Compared with non-primed controls, TNF-primed MSCs have stronger immunomodulatory and tissue-repair capacity, evidenced by increased secretion of immunosuppressive molecules, such as PGE2, sTNFR, and TSG-6 (42, 117–123); chemokines, such as IL-8, CXCL5, and CXCL6 (124, 125); growth factors, such as HGF, IGF1, and VEGF (126–128); and increased tunneling nanotube (TNT) formation (129) through the TNFR1 or TNFR2 signaling pathway. The important effects of MSC through TNF signaling are listed in **Table 1**.

TNFR1-Mediated Regulation of MSC Efficacy

Generally, TNFR1-mediated signaling reduces the MSC efficacy. For example, BM-MSCs derived from mice with TNFR1 knockout caused greater recovery of myocardial functions in a rat model of acute ischemia than wild-type MSCs, which was associated with increased production of VEGF and decreased production of the pro-inflammatory factors TNF, IL-1β, IL-6, etc., in the myocardium (136, 138). Interestingly, another study found that TNFR1 knockout only increased the cardioprotective effect of male, but not female, MSCs in a murine ischemic injury model (137), indicating that the effect of TNFR1 signaling is gender dependent.

TNFR1 signaling reduces MSC efficacy by inhibiting the production of immunosuppressive molecules and growth factors. For example, TNF-priming reversed the immunosuppressive effect of mouse MSCs on T cell proliferation, accompanied by increased secretion of the pro-inflammatory cytokine IL-6 and failure of the MSCs in the treatment of murine CIA (54). In addition, ablation of TNFR1 remarkably increased TNF-stimulated HGF production from human BM-MSCs (142), indicating the inhibitory effect of TNFR1 signaling in HGF production. Similar effects have been observed on MSCs derived from patients with autoimmune diseases. For instance, it has been shown that TNF treatment decreased the HGF production by BM-MSCs derived from SLE patients *via* the TNFR1/IKK-β pathway (80) and induced apoptosis in BM-MSCs from ankylosing spondylitis patients *via* TNFR1-mediated upregulation of *TRAIL-R2* (133).

Interestingly, in some scenarios, TNFR1 signaling can enhance MSC efficacy by inducing production of immunomodulatory molecules. For example, TNFR1 knockdown in mouse skin-derived MSCs abrogated their therapeutic effects on EAE accompanied by reduced inhibition on the polarization of Th17 cells (121), which might be partially explained by the loss of beneficial effects of sTNFR1 produced by MSC under the inflammatory situation. In addition, in dilative cardiomyopathy, acute lung injury, and LPS-induced intoxication, both murine and human BM-MSCs primed by TNF or inflammatory serum secreted more sTNFR1 than the non-primed controls, which promotes disease recovery (119, 120). In addition, human adipose-derived MSCs engineered to express sTNFR1-Fc improved the survival of porcine islets and reversed the hyperglycemia in a mouse model of streptozotocin-induced diabetes (140). sTNFR1 may act by neutralizing circulating TNF and activating mTNF-mediated reverse signaling in immune cells during diseases progression.

TNFR1 signaling can also increase PGE2 secretion by inducing *COX2* expression in mouse or human BM-MSCs, which in turn reprograms host macrophages to increase IL-10 production thus inhibiting inflammation in a mouse sepsis model and experimental allergic conjunctivitis (117, 118). In addition, it has been shown that other immunosuppressive molecules, growth factors, and chemokines such as TSG-6, TGFβ, and IL-8 were produced by TNF-primed MSCs to attenuate the symptoms in diseases including EAE, myocardial infarction, ischemic hind limb, and cutaneous wound probably *via* TNFR1 signaling pathway (122, 135, 139, 141). TNF can also induce TNT formation between iPSC-derived MSC and cardiomyocytes for mitochondria transfer to attenuate the damage in mouse anthracycline-induced cardiomyopathy, which is regulated by TNF/NFκB/TNF-IP2 signaling pathway (129). Thus, TNFR1 signaling can exert dual effects on MSC-based therapy in autoimmune and inflammatory diseases, depending on the type and stage of the diseases.

TNFR2-Mediated Regulation of MSC Efficacy

In contrast to the dual effects of TNFR1, TNFR2-mediated signaling enhances MSC efficacy in general. For example, compared with wild-type controls, both male and female murine BM-MSCs with TNFR2 knockout showed less or no myocardial functional recovery in a rat model of acute ischemia accompanied by increased production of pro-inflammatory factors and a reduced level of VEGF in the myocardium (136, 138). These results are consistent with the *in vitro* observations that production of VEGF, IGF-1, and HGF by TNF-primed human BM-MSCs is mediated through the TNFR2 signaling (126–128). Consistently, TNFR2 knockout reduced the secretion of VEGF and IGF-1 by TNF-primed BM-MSCs, but this only happened on BM-MSC from female mice. By contrast, secretion of these growth factors increased in TNF-primed TNFR2$^{-/-}$ BM-MSCs from male mice (143, 144), and TNFR2$^{-/-}$ BM-MSCs from male mice failed to promote myocardial functional recovery (136, 138). The opposite outcomes implicate that the effects of TNFR2 signaling, like TNFR1 signaling, on MSC functions are also gender dependent. In support of

TABLE 1 | Tumor necrosis factor (TNF) regulation of mesenchymal stem cell (MSC) efficacy on autoimmune and inflammatory diseases.

Disease	MSCs	Findings	Reference
Experimental autoimmune encephalomyelitis (mouse)	Mouse skin MSCs	Secrete soluble TNFR1 (sTNFR1) Inhibit differentiation of Th17 *via* sTNFR1-mediated TNF neutralization	(121)
	Human placental MSCs (TNF primed)	Express TSG-6 Attenuate disease severity	(122)
Systemic lupus erythematosus (SLE) (human)	BM-MSC (TNF primed) from SLE patients	Inhibit *in vitro* migration and *in vivo* homing capacity of BMSC Decrease hepatocyte growth factor production *via* the TNFR1/IKK-β pathway	(80)
Th1 cell induced pre-eclampsia (mouse)	Human decidual MSCs	Reverse abnormal TNF expression in uterine and splenic lymphocytes	(130)
Collagen-induced arthritis (CIA) (mouse)	Human BM-MSCs (expressing sTNFR2-Fc)	Secrete sTNFR2-Fc Decrease Th17 cell population Suppress osteoclastogenesis	(131)
	Mouse MSC line (TNF primed)	Secrete interleukin (IL)-6 Accentuate Th1 response No benefit on disease	(54)
Collagen II antibody-induced arthritis (mouse) or CIA (rat)	Human BM-MSCs (expressing sTNFR2-Fc)	Secrete sTNFR2-Fc Reduce joint inflammation	(132)
Ankylosing spondylitis (AS) (human)	Human BM-MSCs from AS patients (TNF primed)	Express TRAIL-R2 Induce MSC apoptosis *via* TRAIL-R2 and TNFR1 signal	(133)
Myocardial infarction (rat)	Rat BM-MSCs (overexpressing TNFR2)	Secrete sTNFR2 Attenuate expression of TNF, IL-1β, and IL-6	(134)
	Rat BM-MSCs (TNF primed)	Express TGFβ, FGF2, angiopoietin-2, and VEGF-1 Increase BM-MSC migration *in vitro*	(135)
	Mouse BM-MSCs	TNFR1 knockout Increases cardiac protection Decreases TNF, IL-1β, and IL-6 Increases VEGF in myocardium TNFR2 or TNFR1/2 knockout Reduces cardiac protection Increases TNF, IL-1β, and IL-6 Decreases VEGF in myocardium	(136)
Myocardial infarction (mouse)	Human BM-MSCs (TNF primed)	Express TSG-6 Decrease inflammatory responses Reduce infarct size Improve cardiac function	(123)
Myocardial ischemia–reperfusion injury (rat)	Mouse BM-MSCs	TNFR1 knockout increases the cardioprotective effect in male but not in female MSCs	(137)
	Mouse BM-MSCs	TNFR1 (but not TNFR2 or TNFR1/2) knockout MSCs increase the cardioprotective effect	(138)
Anthracycline-induced cardiomyopathy (mouse)	Human induced pluripotent stem cell-MSCs/human BM-MSCs (TNF primed)	Express MCP-1, IL-6, IL-8, and VEGF Form tunneling nanotubes for mitochondria transfer *via* TNF/NFκB/TNFαIP2 signal	(129)
Inflammatory dilative cardiomyopathy or LPS-induced acute lung injury (mouse)	Mouse BM-MSCs	Secrete sTNFR1 to neutralize TNF and LTα Suppress NFκB pathway in cardiomyocytes	(120)
Ischemic hindlimb (mouse)	Human ASCs (TNF primed)	Secrete IL-6 and IL-8 Promote angiogenesis, chemotactic migration of human cord blood-derived endothelial progenitor cell	(139)
Sepsis (mouse)	Mouse BM-MSCs (TNF primed)	Express COX2 to synthesize PGE2, which increases *IL10* expression in macrophages *via* TNF/TNFR1 signaling	(118)
LPS intoxication (systemic inflammation) (rat)	Human BM-MSCs (LPS intoxication serum primed)	Promote sTNFR1 secretion *via* NF-κB signaling Decrease TNF, interferon γ, and IL-6 Decrease infiltration of macrophages and neutrophils	(119)
Pig islet xenotransplantation in streptozotocin-induced diabetes model (humanized mouse)	Human ASCs (sTNFR1-Fc)	Improve survival of porcine islets Reverse hyperglycemia	(140)
Cutaneous wound (rat)	Human ASCs (TNF primed)	Express IL-6 and IL-8 Enhance macrophage infiltration Enhance cell proliferation and angiogenesis	(141)
Experimental allergic conjunctivitis (mouse)	Human BM-MSCs (TNF primed)	Express COX-2 to synthesize PGE2 Decrease IgE production and histamine release Decrease conjunctival vascular hyperpermeability	(117)

this, the male sex hormone testosterone has been reported to exert deleterious effect on myocardial recovery in a rat model (145, 146).

Furthermore, overexpression of *sTNFR2* or *TNFR2* in human or rat BM-MSCs enhanced their therapeutic effects in mice and rats with RA (131, 132) and rats with cardiac ischemia (134, 147), which was associated with reduced TNF level and attenuated expression of *IL1β* and *IL6*. Macrophages are a major cell type that secretes TNF. Treating activated macrophages with culture supernatant of human sTNFR2-expressing MSCs reduced osteoclast formation *in vitro* (131). Similar to sTNFR1, sTNFR2 may also execute cytoprotective effect *via* neutralization of circulating TNF or induction of mTNF-mediated reverse signaling in immune cells.

The expression of *TNFR2* is highly upregulated in oligodendrocytes, microglia, astrocytes, and several subsets of neurons in neurological diseases (105, 148). TNFR2 on astrocytes mediates beneficial activities to protect oligodendrocytes in co-culture (111). Upregulated TNFR2 on activated microglia promotes the clearance of myelin debris and remyelination (149). In addition, MSCs that infiltrate into the CNS can exert immunomodulatory effects by regulating the local microglia and astrocytes as well as infiltrating immune cells, e.g., suppressing the functions of Teff cells and macrophages and promoting the proliferation of Tregs (150). Moreover, TNF in inflamed CNS induces MSC to secrete immunomodulatory factors and neural tropic factors such as BDNF and HGF (151), which exert pleiotropic effects to attenuate the brain inflammation, reduce brain damage, and promote neural regeneration.

TNF SIGNALING INTERACTING WITH MSCs ON Tregs

Regulatory T cells play a central role in the maintenance of the immune balance to tolerate self-antigens and prevent autoimmunity (152). In general, they refer to $CD4^+/FOXP3^+$ T cells, including two major subtypes: natural Treg (nTreg) cells and induced adaptive Treg (iTreg) cells. nTreg cells are generated and selected in the thymus and then migrate to peripheral tissues (153), while iTreg cells acquire CD25 (IL-2Rα) expression outside of the thymus and are typically induced by inflammation and during disease processes, such as autoimmunity and cancer (152). T cell receptor stimulation and the cytokines TGFβ and IL-2 are required for iTreg cell generation *in vitro* and *in vivo* (95, 154, 155). In contrast to the pro-inflammatory effects of TNF/TNFR1 signaling (156), TNF/TNFR2 signaling preferentially activates, stabilizes, and expands Tregs to mediate their immunosuppressive effects and contribute to the treatment of autoimmune disease (86–89). TNFR2 is an expression marker relevant to Treg functions. TNFR2 agonists have been shown to be effective for the treatment of autoimmune and inflammatory diseases (91, 97).

Mesenchymal stem cells regulate both innate and adaptive immune systems partially by promoting the generation of Tregs (29–31). In the presence of high levels of inflammatory cytokines, e.g., TNF and IFNγ, MSCs produce various soluble factors, such as IDO, TGFβ, PGE2, and IGF, to inhibit Teff cells and increase the expression of *FOXP3*, *CTLA4*, and *GITR* in Tregs to enhance their immunosuppressive effects (53). Cell-to-cell contact also mediates the induction of Tregs by cytokine-primed MSCs (53).

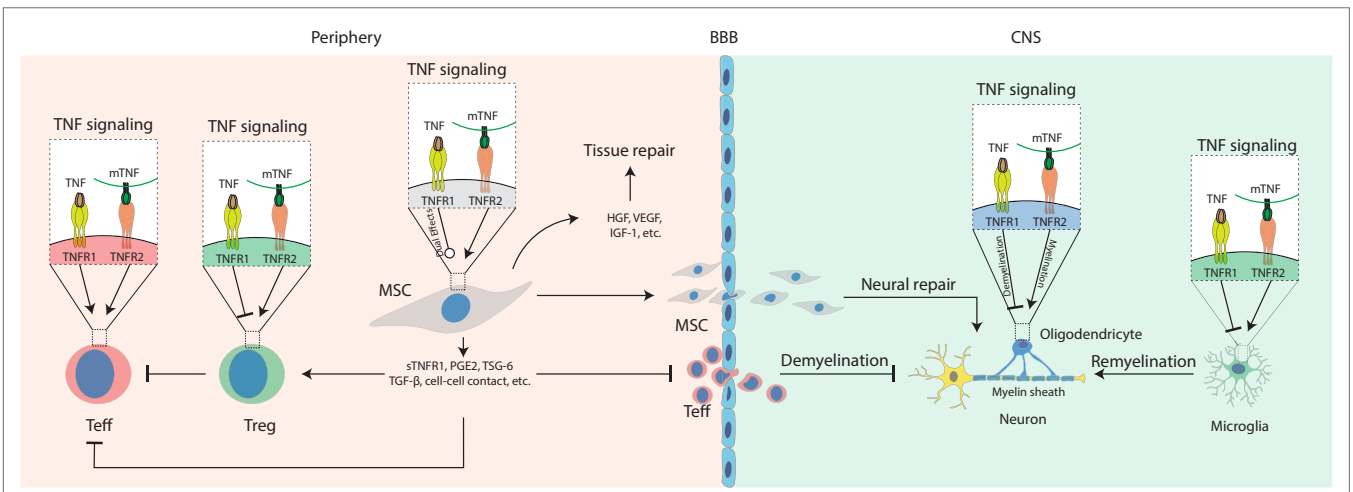

FIGURE 1 | Schematic diagram for the role of tumor necrosis factor (TNF) signaling in mesenchymal stem cell (MSC)-based therapy on autoimmune and inflammatory diseases. Under inflammatory conditions, TNF binds TNFR1 to activate T effector (Teff) cells while impairing regulatory T cells (Tregs); mTNF mostly binds TNFR2 to activate Teff cells and also activate Tregs to mediate their immunosuppressive effects in the periphery. In the central nervous system (CNS), TNFR1 signaling induces cytotoxic effects on oligodendrocytes resulting in neural demyelination and activates microglia to produce pro-inflammatory molecules such as TNF. TNFR2 signaling protects the survival of oligodendrocyte and microglia and promotes myelin clearance and remyelination mediated by microglia. In addition, TNFR1 or -R2 signaling can enhance the immunosuppressive effects of MSCs to alleviate autoimmune and inflammatory diseases. Compared to non-primed controls, TNF-primed MSC produce more soluble TNFR1 (sTNFR1), PGE2, TSG-6, and TGF-β, enhance Treg functions, neutralize TNF *via* sTNFR, prevent Teff cell infiltration into the CNS, release growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and VEGF to promote tissue or neural repair, infiltrate the CNS to mediate neural protection by regulating oligodendrocytes and microglia, suppressing Teff cells that have infiltrated the CNS.

Overexpression of inducible co-stimulator ligands in MSCs promotes the induction of functional Tregs (157).

In addition, MSCs also modulate antigen-presenting cells, such as DCs and macrophages, by converting them to anti-inflammatory phenotypes (M2), which then promote Treg expansion and suppress Teff cell functions (30). Recently, Miyagawa et al. reported that MSCs control Treg proliferation by releasing IGFBP4, an inhibitor of IGF (53). Moreover, some studies have shown that low levels of IFNγ and TNF or long-term exposure to these cytokines converts MSC from an immunosuppressive to pro-inflammatory status (53–55). Thus, these pro-inflammatory cytokines can modify MSC effects on Tregs, altering their efficacy on autoimmune and inflammatory diseases.

STRATEGY AND PERSPECTIVE

Mesenchymal stem cells have demonstrated immunosuppressive effects against various autoimmune and inflammatory diseases. However, the efficacy of MSC on many of the diseases remains controversial, which can be attributed to many reasons. The first is the challenge MSCs encounter when adapting to a new microenvironment following delivery into the body. They have to first survive in the new and often harsh conditions, during which the MSC effects can be reduced or even lost. Thus, improvement of the MSC efficacy should focus on achieving high delivery efficiency, long-term retention, and specific modification to target different inflammatory diseases.

Genetically modified MSCs can gain remarkably enhanced therapeutic capability, in which MSCs serve as a carrier to deliver cytokines or verified biological drugs for target-oriented therapies. For example, compared with unmodified MSCs, MSCs transduced with TGFβ suppressed CIA in a mouse model (158). MSCs expressing IL-12p40 alleviate murine colitis more effectively than a wild-type control (159). Overexpressing IL-10 in MSCs suppressed the development of graft-versus-host disease (160), and MSCs overexpressing TNFR2 treat CIA in mouse more effectively than controls (131). MSCs can also be engineered to release abundant amounts of sTNFR1 to neutralize TNF in the circulation (121, 140). In addition, since MSCs promote activation and proliferation of Tregs, combined therapy of MSCs and Tregs further enhances the number and functions of Tregs and achieves much stronger efficacy than each alone, which has been observed in GVHD (161, 162) and ischemic myocardium (163).

CONCLUDING REMARKS

In this review, we describe the progress in research on how TNF signaling interacts with MSCs in the treatment of autoimmune and inflammatory diseases (**Figure 1**). At appropriate concentrations and timing, TNF promotes secretion of immunosuppressive molecules from MSCs, which inhibit Teff cells and activate Tregs. In the periphery, TNFR2 signaling also stimulates Tregs; thus, it may synergize with MSCs to repress inflammation. In the CNS, TNFR2 signaling protects the survival of astrocytes, OPC, microglia, and neurons. Activated MSCs secrete immunosuppressive molecules to inhibit inflammation and neurotropic molecules to protect neural cells and promote remyelination. Some of the TNF functions mediated by either TNFR1 or -R2 in MSCs can vary in different genders. Together, these findings suggest that TNF signaling plays a pivotal role in MSC-based therapy of autoimmune disease, which is highly dependent on the context, timing, concentration, gender, etc.

Despite these interesting findings, many more questions remain to be addressed than have been solved. For example, how do transplanted MSCs respond to TNF, function in the periphery and infiltrate the inflamed CNS in patients. Why does gender affect TNF functions? Would genetic variations among different individuals affect TNF functions? Can inflammatory factors also epigenetically modify and alter the expression of the genes involved in TNF signaling? Future studies are needed to address these and many new challenging questions. Continuous progress in this field will most likely lead to the identification of new targets for more precise and effective therapies of autoimmune and inflammatory diseases.

AUTHOR CONTRIBUTIONS

LY, DZ, and R-HX conceived, designed, and wrote the manuscript. R-HX gave the final approval of the manuscript.

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REVIEW
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The Key Role of TNF-TNFR2 Interactions in the Modulation of Allergic Inflammation: A Review

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Tumor necrosis factor-alpha (TNF) is a pleiotropic cytokine, which is thought to play a major role in the pathogenesis of inflammatory diseases, including allergy. TNF is produced at the early stage of allergen sensitization, and then continues to promote the inflammation cascade in the effector phase of allergic reactions. Consequently, anti-TNF treatment has been proposed as a potential therapeutic option. However, recent studies reveal anti-intuitive effects of TNF in the activation and proliferative expansion of immunosuppressive Tregs, tolerogenic DCs and MDSCs. This immunosuppressive effect of TNF is mediated by TNFR2, which is preferentially expressed by immunosuppressive cells. These findings redefine the role of TNF in allergic reaction, and suggest that targeting TNF-TNFR2 interaction itself may represent a novel strategy in the treatment of allergy.

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HIGHLIGHTS

- Pleiotropic function of TNF in allergy is likely mediated by its two receptors, TNFR1 and TNFR2
- Activation by TNFR1 results in the allergic inflammatory responses while TNFR2 plays a role in the immune tolerance to allergens
- TNFR2 is preferentially expressed by highly suppressive and replicating Tregs and TNFR2 signaling leads to the activation and proliferation of Tregs
- Targeting of TNFR2 to boost Treg activity may represent a novel strategy for treating patients with allergy.

Keywords: allergy, TNF, TNFR2, regulatory T cells, tolerogenic dendritic cells

INTRODUCTION

Allergy is an immune-mediated hypersensitivity to allergens. Exposure of allergens through inhalation, ingestion or skin contact leads to diseases such as asthma, allergic rhinitis, food allergy, and atopic dermatitis. Allergy is a complex disease, and both genetic and environmental factors contribute to its pathogenesis. It affects 30–35% of the population at some point in their life,

with incidence continuing to rapidly grow each year. The past decades have exhibited large-scale anthropogenic changes which are currently considered the leading causes of the increasing burden of allergic diseases.

EFFECTOR MECHANISMS IN ALLERGIC REACTIONS

The development of allergy can be divided into two phases: (1) the sensitization and memory phase, and (2) the effector phase, which can be further staged into immediate and late responses (1) (**Figure 1**). Sensitization occurs upon the first encounter with allergen that leads to the production of pro-inflammatory cytokines [Interleukin (IL) 33 (IL-33), thymic stromal lymphopoietin (TSLP), tumor necrosis factor (TNF), IL-1β] by epithelial cells. This allergic sensitization is determined by both genetic polymorphisms (2) and environmental risk factors (3). Studies have identified several risk alleles, including cadherin-related protein 3 (CDHR3) and protocadherin 1 (PCDH1), which are both thought to be involved in facilitating allergic sensitization (4). The ubiquitous presence of lipopolysaccharide (LPS) in the environment may also contribute to the exacerbation of allergic responses. Exposure to LPS triggers signaling of toll-like receptor 4 (TLR4) on epithelial cells and can either promote or suppress the sensitization to an allergen in a dose-dependent manner (5, 6). Allergy is primarily a T helper 2 (Th2)-driven disease (7) in which dendritic cells (DCs) stimulated with cytokines released by sensitized epithelial cells have the capacity to induce Th2 responses. This cascade of events drives IgE synthesis and promotes the generation of memory allergen-specific T and B cells (8).

The effector phase of allergic responses is initiated when the allergen cross-links IgE-FcεRI complexes on sensitized mast cells. Subsequently, mediators of the allergic responses such as histamine, leukotrienes, cytokines, chemokines and proteases, largely responsible for type 1 hypersensitivity are released (9). The release of these mediators causes the acute signs and symptoms of allergy, such as vasodilation and airway constriction. While the continuous exposure to allergen activates T cells and consequently triggers the late phase response, it is the allergen-specific Th2 cells that produce IL-4, IL-5, IL-9, and IL-13, which are responsible for the maintenance of allergen-specific IgE levels and activation of tissue eosinophilia, mucus overproduction, and tissue remodeling (10–13).

Furthermore, Th17 cells were shown to be associated with a more severe asthma, which is less responsive to corticosteroid (14), induces neutrophils recruitment (15) and increases airway inflammation and remodeling (16). In addition to Th1, Th2, and Th17 cells, other cell types are also involved in allergy. For example, Th9 and Th22 cells have been shown to play a crucial role in both early and chronic allergic inflammation. The Th9 cells are vital for the recruitment and activation of mast cells during early allergic response and their increased number in allergic patients is correlated with elevated IgE levels (17). On the other hand, Th22 cells are increased in children with asthma and atopic dermatitis. These cells act by increasing the recruitment of leukocytes and disrupting the epithelial integrity on the skin and in the lungs (18, 19).

Another subset of CD4 T cells, which play an indispensable role in the induction and maintenance of tolerance to allergens, are the immunosuppressive $CD4^+Foxp3^+$ regulatory T cells (Tregs) (20). In one study, the $CD4^+CD25^+FoxP3^+$ Treg numbers were found to be normal but the expression of FoxP3 protein, a critical marker for Tregs (21) was diminished. However, the authors did not investigate whether there are functional consequences related to a reduced FoxP3 expression in these cells (22). In another study, Fontenot et al., found that FoxP3 displayed reduced expression in patients with allergic rhinitis (23). Because in general, patients with FoxP3 mutations exhibit excessive autoimmunity with high levels of IgE, peripheral eosinophilia and Th2 skewing, the reduced FoxP3 expression may be a contributing factor to the development of allergic diseases in humans (24). Furthermore, in addition to Th2 responses, other types of effector T cells (Teff) are also attributable to the pathogenesis of allergy (25). In the stage of allergy sensitization, Th1 responses are inhibited through reduction of IL-12. However, in the later stage of allergic response, Th1 cells have been shown to coexist with Th2 cells. Such Th1 cells can exacerbate the symptoms and lead to severe allergy manifestation (26).

Overall a proper maturity and homeostasis of immune system of neonates during the first years of life is fundamental to minimize allergic development. Maternal allergy correlates well with impaired frequency and function of Tregs in neonates and consequently the increased susceptibility for the development of allergy in early childhood (27–29). A study found that Th2 cells were increased in newborns' cord blood with maternal allergy, accompanied by a decreased Tregs/Th2 ratio, indicative of an increased risk for developing atopic dermatitis (29). Furthermore, prenatal environmental exposure such as smoking and the usage of harsh chemicals like disinfectants were found to be associated with reduced Tregs number in newborns' cord blood, which further increases the risk of development of allergy later in life (28). Therefore, as shown by these and other extensive studies [reviewed by (30)], Tregs play a fundamental role in the pathogenesis of allergy and their modulation may harness great potential for treatment of allergic diseases.

Abbreviations: CDHR3, Cadherin-related protein 3; DCs, Dendritic cells; Interleukin, IL; IL-17RD, Interleukin 17 receptor D; MAPK, Mitogen-activated protein kinases; MDSC, Myeloid-derived suppressor cell; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NK, Natural killer cells; NO, nitric oxide; PCDH1, Protocadherin 1; RCT, Randomized controlled trial; SIT, Specific immunotherapy; Teff, Effector T cells; Th, T helper cells; TNF, Tumor necrosis factor; TNFR, Tumor necrosis factor receptor; TRADD, TNFR-associated death domain protein; TRAF, TNFR-associated factor; Tregs, Regulatory T cells; TSLP, Thymic stromal lymphopoietin.

GENERAL BIOLOGY OF TNF

TNF is a pleiotropic cytokine that plays important dual roles in maintaining immune homeostasis and in promoting the development of diseases. TNF is required for host defense against pathogens, immune surveillance against

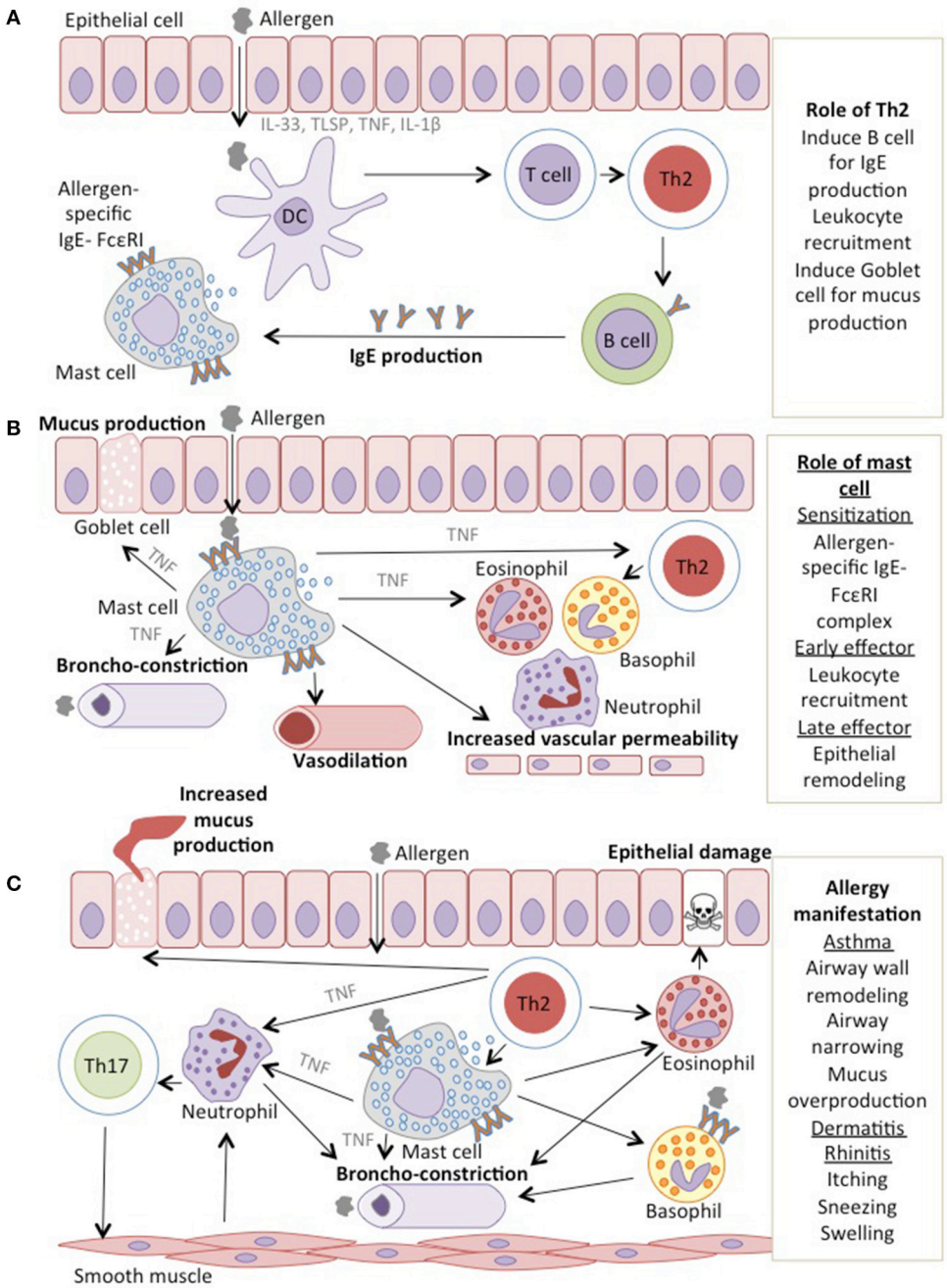

FIGURE 1 | Mechanisms of allergic reactions **(A)**. In sensitization phase, the environmental allergen sensitized epithelial cells and release pro-inflammatory cytokines including TNF. Allergen is taken up by DCs, which are regulated by and produced TNF, induce Th2 cells and drive IgE synthesis and later produced a memory pool of allergen-specific T and B cells **(B)**. Next encounter with the allergen induces assembly with antigen-specific IgE-FcεRI complex on mast cells that secretes TNF and later recruits leukocytes. The leukocytes; basophils, neutrophils, eosinophils and mast cells interact with each other to produce more mucus **(C)**. In the late effector phase, the epithelial remodeling will exhibit the allergic manifestation as in asthmatic and rhinitis patients, the airway wall are narrowed and mucus overproduced while in dermatitis patients, the vasodilation resulted in the itching and swelling of the skin.

malignancies, as well as cell proliferation and survival (31). This cytokine is widely considered as an important inflammatory mediator of several diseases such as autoimmune diseases, cancer, hypernociception, cardiovascular disease and fibrosis (32).

Dual biological functions of TNF are likely to be transduced through its two distinct receptors, TNFR1 and TNFR2. TNF and its receptors have both a membrane-bound form and a soluble form. TNFR1 shows high affinity toward both forms of TNF, while TNFR2 is only fully activated by membrane-bound

TNF (33). Once activated, TNF elicits its biological functions through the activation of two major signaling pathways, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinases (MAPK). These two signaling pathways mediate various cellular activities including proliferation and survival as well as apoptosis and cell death, depending on which receptor is bound and activated by TNF (32).

PATHOGENIC ROLE OF TNF IN ALLERGIC MANIFESTATION

TNF is reported to play significant role in the pathogenesis of allergy and contributes to both early and late stages of allergy development. This is evident when allergy manifestations are inhibited in TNF-knockout (KO) mice as well as with anti-TNF treatments (34–37). Furthermore, upon allergen exposure, TNF is produced by sensitized epithelial barriers and immune cells (such as macrophages, mast cells, DCs). It has the capacity to promote Th2 responses (38), resulting in high levels of IL-4, IL-5, and IL-13, which further activate eosinophils, mast cells and basophils (7). In allergic rhinitis, TNF is essential for the recruitment of eosinophils to the site of allergic inflammation through the induction of adhesion molecules (34). In asthma, TNF is shown to synergize with IL-17 by promoting neutrophil recruitment (36), whereas in atopic dermatitis, TNF is responsible for the production of other cytokines, including IL-32 which induces keratinocyte apoptosis (39). Furthermore, TNF, both induced and are secreted by Th2 cells which promote the production of antigen-specific IgE isoforms from B cells (34). A meta-analysis study suggested that TNF polymorphisms were significantly associated with asthma susceptibility (40). Elevated TNF levels in severe allergy have been shown to contribute to the epithelial barrier dysfunction by upregulating the adhesion molecules (p120, E-cadherin) and increasing the endothelial permeability to allergens (41).

Proinflammatory TNF-TNFR1 Signaling in Allergy

Although binding of TNF to its distinct receptors generally activates the same major signaling pathways (NFκB and MAPK), the distinct structure and motifs of TNFR1 and TNFR2 result in entirely different functional consequences. TNFR1 bears the death domain, which recruits several death signaling proteins such as TNFR1-associated death domain protein (TRADD), Fas associated protein with death domain (FADD) and the TNFR-associated factor (TRAF)-1 to induce inflammation and apoptosis (42).

In the context of apoptosis, TNF-TNFR1 axis signals through caspase 3 and 8, and is mainly responsible for the host defense of pathogen (43) and anti-tumoral activities (44). The impaired regulation of this TNF receptor can cause autoimmune diseases, cancer, chronic infections and allergy (45). In allergic diseases, elevated levels of TNF reportedly trigger inflammatory cascade through TNFR1 (46, 47). For example, Maillet et al. have shown that in a murine model, soluble TNF is a primary driver of allergic airway inflammation, which can be effectively attenuated by neutralization of soluble TNF (48). Increased expression of TNFR1 is shown to play a crucial role in allergic inflammation through the recruitment of eosinophils, neutrophils and other lymphocytes (47). Surprisingly, one study demonstrates anti-apoptotic effects of TNFR1 by enhanced eosinophil survival in asthma, which is contradictory to its known regulation, given the existing cross talk between NFκb and another pathway called c-jun-N-terminal kinase (JNK) pathway (49).

Immunosuppressive TNF-TNFR2 Signaling in Allergy

Unlike TNFR1, TNFR2 lacks the death domain where the activation of TNFR2 recruits TNFR-associated factor 2 (TRAF2) that mainly promotes cell proliferation and survival (50). In allergy, impaired TNF-TNFR2 signaling promotes the polarization of Th2 and Th17 cells, thus aggravating the allergy manifestations (51). This notion was further supported by a study showing that TNFR2-KO mice displayed an increased eosinophilic inflammation, one of the most common allergic manifestations, in comparison with wild type mice, whereas TNFR1-KO mice had a weaker response (47). Nevertheless, as TNFR2 promotes cell survival, this receptor signaling also protect inflammatory cells in diseases including eosinophils (52), thus maintain disease progression. Furthermore, TNFR2 signaling on natural killer (NK) cells help to induce Th2 sensitization toward inhaled allergen (53). To add, in certain inflammatory conditions, TNFR2 can also induce apoptosis when it cross-talks with TNFR1 (54). In the cross-talk, TNFR2 induces TRAF2 to deplete cIAP1/2, the apoptosis inhibitor, thus accelerating the TNFR1-dependent apoptosis (54). In addition, with Fas and Fas ligand, TNFR2 signaling induces apoptosis in IFN-γ-Th1 cells, resulting in a predominance of Th2 in atopic individuals (55). Under certain conditions including prolonged cell stress in disease condition, shift of TNFR2 to TNFR1 apoptotic signaling can occur, leading to opposite known function of TNFR2.

Distinct functions of TNF and its receptor led to the prospective of selectively targeting TNFR1 to inhibit apoptosis, and TNFR2 to induce cell survival. This approach intends to achieve homeostasis in various autoimmune and inflammatory diseases including allergy. These disorders are primarily associated with defects in TNF signaling through TNFR2. Therefore, this particular receptor is of interest and important roles of TNF-TNFR2 interaction on various cell types are further discussed in the next section (**Figure 2**, **Table 1**).

TNF-TNFR2 on Tregs

Due to its important role in allergy manifestations, TNF has been evaluated as a target for therapy and findings have led to the discovery of a more prominent role of TNFR2 in the pathogenesis of allergy. Only a few effects of the exclusive TNF signaling via TNFR2 have been characterized, since its expression is limited to certain cell populations and is only fully activated by membrane-bound TNF. In T cell biology, TNFR2 is directly associated with proliferation and maintenance of function, both in Tregs (67, 68) and Teff (69, 70). What is more interesting is the restricted

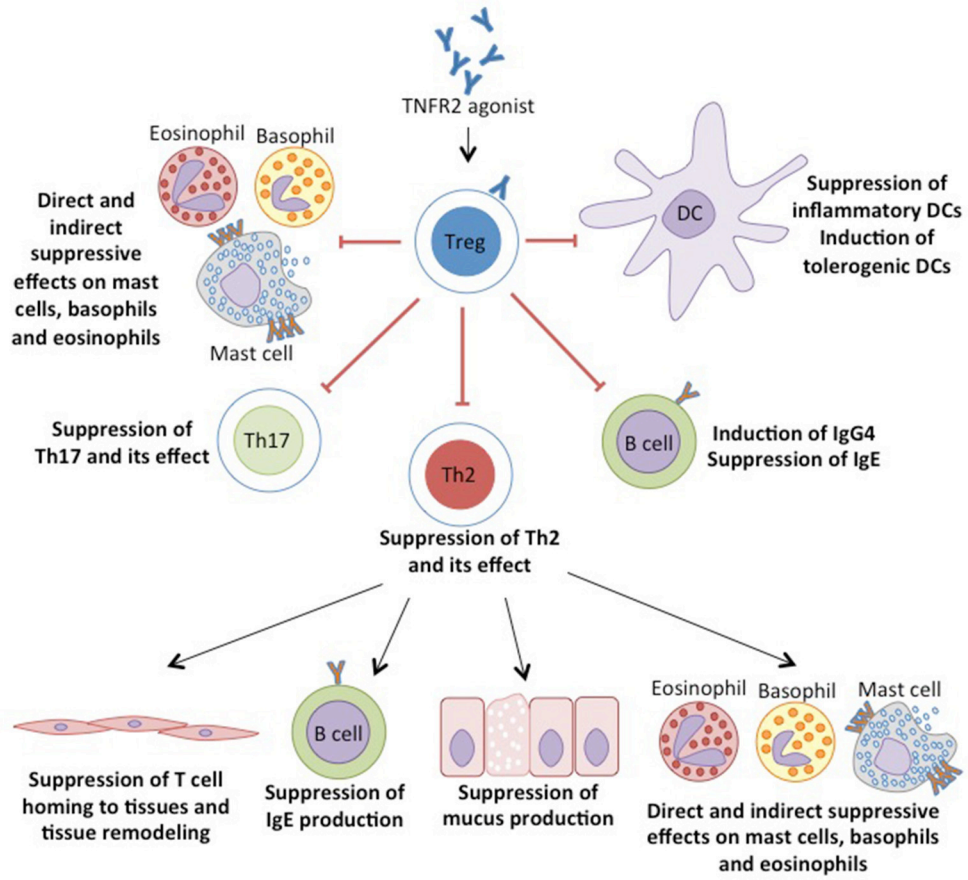

FIGURE 2 | TNFR2 agonist target to enhance the proliferation and suppressive capacity of Tregs. The suppressive Tregs inhibits the inflammation by suppressing the inflammatory cells and induces the tolerogenic DCs by the suppressive MDSCs, hence enabling the suppression of allergic manifestations.

TABLE 1 | Regulation of TNF on Tregs, DCs and MDSCs for protective immune response against allergic reactions.

Target cells	Protective mechanisms	References
Tregs	Increased suppressive capacity	(56, 57)
	Increased proliferative response	(58, 59)
	Inhibited excessive Th2 and Th17 polarization via inhibition on NF-kB signaling	(51)
DCs	Regulate maturation and survival	(60, 61)
	Tolerogenic DCs	(62–64)
MDSCs	Inhibit activation of Th2 cells	(65, 66)

abundance of TNFR2 on Tregs, leading to strong activity of this receptor on the mediator of tolerance.

TNFR2, which is preferentially expressed on human and mouse Tregs, is associated with both phenotypic and functional properties of Tregs (67). Tregs are shown to inhibit inflammatory responses by TNF through TNFR2 signaling (71). In addition, in diseases, downregulation of Foxp3 has been associated with TNF- TNFR2 interaction and is later restored by blockade of TNF with TNF antagonist (59, 72, 73). Unlike Foxp3 of which the forced expression can convert $CD4^+CD25^-$ Teff into functional Tregs (74), TNFR2, does not induce $CD4^+Foxp3^-$ T cells to become suppressive (70). Instead, TNFR2 expressing $CD4^+Foxp3^-$ T cells show greater resistance to suppression by $TNFR2^-$ Tregs. In addition, TNFR2 is also shown to be responsible for a more potent suppressive capacity of Tregs when $TNFR2^+$ Tregs preferentially accumulated intratumorally than in periphery (68). Previously, Tregs were described as $CD4^+$ T cells expressing CD25, the IL-2R α chain, and CD45RB with Foxp3 as functional transcription marker (75). Co-expression of TNFR2 with CD25 has been suggested to identify more functional suppressive $Foxp3^+$ Tregs in human (76). Although TNFR2 induces proliferation of both Tregs and Teff, $TNFR2^+$ Tregs are able to overcome the inhibition of suppression of $TNFR2^+$ Teff (70). A reduction of Tregs in TNFR2 deficient mice indicates the role of TNFR2 in promoting the generation and homeostasis of this cell subtype (56). Both *in vivo* and *ex vivo* expansions of Tregs while maintaining their suppressive capacity have been demonstrated by utilizing TNFR2 signaling (77, 78).

Furthermore, a subset of Treg, $CD8^+$ Tregs, which can be rapidly generated in the presence of IL-4 and IL-12, can both

block activation of naïve or Teff and suppress IgG/IgE antibody response (79). Expansion of this Treg subset is induced in the presence of activated $CD8^+$ T cells via TNFR2 signaling in which TNFR2 is usually identified on a more potent subpopulation (80, 81). However, there are studies that experimentally demonstrate the inhibition of Tregs suppressive function through TNF-TNFR2 axis (57, 73, 82). These inhibitory effects of TNF-TNFR2 axis in Tregs have been associated with several mechanisms including activation of NFκB cascade, preferentially activated by pro-inflammatory TNFR1, (82) and activation of Akt with Smad3 that reduced Foxp3 transcription (57). This discrepancy of TNFR2 signaling on the function of Tregs is hypothesized to be related with the crosstalk of TNFR2 with TNFR1. Under certain conditions including prolonged cell stress in disease condition, shift of TNFR2 to TNFR1 apoptotic signaling can occur, leading to opposite primary function of TNFR2 (50).

TNF-TNFR2 on DCs

The biological function of DCs, the professional APCs, can be regulated by TNF. Conversely, TNF is produced by DCs upon exposure to exogenous antigen or allergen. Subsequently, depending on the cytokine milieu including TNF, DCs would activate distinct T cell responses, which in allergy corresponds primarily to a Th2 response (83). Furthermore, TNF differentially regulates maturation and survival of DCs through interaction with its two receptors, TNFR1 and TNFR2. (60). Interaction between TNFR2 and membrane-bound TNF expressed by tolerogenic DCs induces generation of suppressive Tregs (84). In addition, TNFR2 also regulates survival of DCs, which is crucial in maintaining immunity (60, 61). These studies showed that TNFR2-deficient mice failed to develop matured myeloid cells and DCs thus reducing T cell activation including Tregs. However, the maturation state of DCs when regulating the immune tolerance has been subject of debates (85). Previously, tolerance in terms of T cell anergy and deletion is induced by immature DCs (86). Currently, mature DCs which are characterized by the typical phenotypic expression of CD103, are capable of promoting antigen specific Tregs (87). $CD103^+$ DCs have been shown to selectively prime Th2 responses to inhaled allergens and impaired allergic airway inflammation in mice lacking $CD103^+$ DCs (88). Alternately, $CD103^+$ DCs in lymphoid organs have the capacity to promote generation of $Foxp3^+$ Tregs by metabolizing dietary vitamin A (89). Also, $CD103^+$ DCs in the airway has been shown to restrain airway inflammation, for example through the induction of IL-10 (62) and production of IL-12 that control Th1 (90). Previously, it was shown that TNFR2 maintains adequate IL-12 production by DCs in inflammatory responses by regulating endogenous TNF level (91). In addition, selective expansion of maximally suppressive $TNFR2^+Foxp3^+$ Tregs in pulmonary inflammation can be induced by engineered nanoparticles through the activation of $CD103^+$ DCs (92).

TNF-TNFR2 on MDSCs

Furthermore, TNFR2 also acts as a co-stimulatory receptor, crucial for the development and regulation of myeloid suppressor cells (61). Myeloid-derived suppressor cells (MDSCs) are an innate heterogeneous cell population that plays a crucial role in dampening inflammatory responses. The accumulation and expansion of MDSCs has been observed in several diseases like tumors, infectious diseases, trauma, autoimmune diseases and asthma as well. The suppressive MDSCs regulate the adaptive immunity by inhibiting the activation of T cells, especially Th2 cells in allergy (65, 66), although the upregulation of MDSCs, synergistically with Th2 polarization in asthma has also been observed (65). Similar to Tregs, the TNF-TNFR2 axis also promotes the activation and suppression of MDSCs in tumor progression by several mechanisms such as promoting secretion of nitric oxide (NO), IL-10 and TGF-β as well as enhancing the inhibition of lymphocyte proliferation (93, 94). However, it is unclear whether the interaction of TNFR2 with Tregs and MDSCs have the same signaling pathway of the suppressive mechanism in tumor development and immune evasion. For example the activation of TNFR2 on Tregs enhances immune suppression *in vivo* and stimulates proliferation *in vitro* (61) while MDSCs rely on TNFR2 activation for their maturation and to reach their optimal suppressive function (95).

THERAPEUTIC TARGETING OF TNF-TNFR2 IN ALLERGY

Currently, treatment for allergy such as antihistamine and glucocorticoid only temporarily relieve symptoms in terms of inflammation, hence a disease-modifying treatment is fundamental. In allergic models, TNF antagonist have been shown to induce anti-allergic effects by reducing IgE level, Th2 cytokines, and eosinophils infiltration (35, 48, 96). Moreover, preferential expression of TNFR2 on Tregs makes it a more attractive molecular target for drug development. Studies showing the efficacy of targeting TNF-TNFR2 in allergies such as atopic dermatitis (97) and asthma (46, 59) have made this as a promising treatment option. Although such molecules are capable of neutralizing TNF, their affinity and avidity toward both soluble and transmembrane TNF is highly variable as well as the effects they exert on TNF-producing cells. The big differences can specifically be found in their distinct pharmalogical properties, which explains their variable efficacy in allergy treatment modalities (98) (**Table 2**).

In clinical settings, five TNF antagonists (three human monoclonal antibodies; infliximab, adalimumab, and golimumab, a TNFR2 receptor etanercept and certolizumab, the PEGylated Fab antibodies) have been established as therapeutic options in inflammatory diseases. Etanercept, a genetically engineered recombinant protein that comprises of TNFR2 and Fc portion of human IgG1, specifically binds to TNF and blocks its interaction with cell surface receptors (99). Studies have shown that in an allergy model, etanercept attenuates allergic lung inflammation (48) while in allergic asthma it can even reverse the inhibitory activity of TNF onto Tregs (59). However, in a randomized, phase II controlled trial (RCT), in comparison to placebo, etanercept only showed to be a well-tolerated therapy in moderate-to-severe asthma but with no significant clinical efficacy (100). Targeting TNF with etanercept in mild-to-moderate allergic asthma increased the TNFR2 levels but failed to attenuate disease pathologies (101). Moreover, treatment

TABLE 2 | TNF antagonist and its efficacy in allergy as well as their effects on Tregs.

Biologic name (trade name)	Type of agent	Approved indication	Efficacy in allergy		Effects on Tregs	References
			in vitro	*in vivo*		
Etanercept (Enbrel)	Human TNFR2-Fc fusion protein	RA, JIA, PA, AS, PP	+++	+	Decrease of Foxp3+ cells *in vitro*.	(66, 96–100)
Infliximab (Remicade)	Chimeric anti-TNF mAb	RA, CD, UC, AS, PS, PP	+++	+	Expansion of Tregs in RA	(34, 70, 93, 94, 101)
Adalimumab (Humira)	Human anti-TNF mAb	RA, PA, CD, PP	nd	Nd	Expansion of Tregs in RA	(99, 102)
Golimumab (Simponi)	Human anti-TNF mAb	RA, AS, PA	nd	–	nd	(103, 104)
Certolizumab Pegol (Cimzia)	Human PEGylated Fab anti-TNF mAb	RA, CD, PA, AS	nd	nd	nd	(105)

RA, rheumatoid arthritis; AS, ankylosing spondylitis; CD, Crohn's disease; PP, plaque psoriasis; PA, psoriatic arthritis; UC, ulcerative colitis; JIA, juvenile idiopathic arthritis; +++, very strong; +, weak; -, no effect; nd; no data.

with etanercept in severe atopic dermatitis only showed modest effects (102). Infliximab, the chimeric monoclonal anti-TNF, showed significantly, reduced pathological inflammation in allergy model (35, 96). In addition, treatment with infliximab improved clinical outcome in both moderate and severe atopic dermatitis, but failed to respond in the maintenance therapy (97). Another anti-TNF monoclonal antibodies, golimumab, demonstrated to be unsuitable for treatment in severe asthma when it shows unfavorable risk-benefit profile in a RCT (103). Although it is well established that TNF plays a prominent role in establishment and maintenance of allergy, treatment with its antagonist in allergy population shows to be inefficient although they successfully attenuate the symptoms in allergy model. The severe side effects (immunosuppression, risk of infection, hematological malignancies, demyelinating events and neuropathies, impact on cardiovascular), and unsuccessful trials, are a limitation to their use as a general treatment in allergy (42).

Unlike in allergy, TNF-TNFR2 axis in autoimmune diseases and cancer has been well established. Adalimumab has shown to expand functional Tregs through TNF-TNFR2 axis on monocytes in rheumatoid arthritis (104). Another study in a rheumatoid arthritis model exhibited selective blockade of TNFR1 while sparing TNFR2 ameliorated inflammation and enhanced number and function of Tregs (105). Nguyen and Ehrenstein (104) have demonstrated a mechanism where TNF antagonism selectively neutralized the pro-inflammatory soluble TNF while it enhanced the immunosuppressive function of membrane TNF (104). The aforementioned studies may suggest that targeting TNF through TNFR2 not only neutralizes its pro-inflammatory activities but also induces tolerance by activation and expansion of Tregs (**Table 2**). In allergy perspective, although TNF antagonism does increase the TNFR2 levels (101), but other study demonstrated a functional insufficiency by TNF via TNFR2 signaling pathway (59). Interestingly, effectiveness toward anti-TNF treatment has also been associated with polymorphism of TNF receptor superfamily member 1B that code for TNFR2 protein (106). This may explain the effectiveness of TNF antagonism in only certain allergy population.

Due to its restricted cellular expression and prominent role in immune regulation, TNFR2 is still a more attractive target for treatment in diseases, including allergy. Faustman and Davis (50) suggested both short and long-term strategies to refine TNFR2 as a target in therapy. These include a better TNFR2 agonist by mean to improve specificity, binding duration, and affinity, as well as TNF inducers, modulation in NF-kB pathway and CD3-specific antibodies (107). Furthermore, utilization of nanoparticles to modulate immune response has been widely investigated (108). Nanoparticles, with their various immunological effects in the lung (109), can be utilized to selectively block TNFR1 and/or activate TNFR2. A synthetic nanoparticle has been used to imprint innate immunity when pre-exposed mice preferentially expanded $TNFR2^{+}Foxp3^{+}$ Tregs after allergen challenge, partly via the activation of $CD103^{+}$ DCs (92, 110). This non-toxic engineered nanoparticle evidenced the selective modulation of Tregs homeostasis through mechanisms such as maintenance of TNF-TNFR2 interaction, targeting $CD103^{+}$ DCs, and expanding the proliferative rate of Tregs, thus decreasing the susceptibility to allergic disease. This strategy to use engineered targeting can also be adopted in specific immunotherapy (SIT). It can be considered as an achievable long-term cure for allergy as basic principle in SIT, which involves inducing immune tolerance toward allergens by specifically and repeatedly administrating the causative allergen (111).

CONCLUSIVE REMARKS

In allergy, TNF may have dual pro-inflammatory and anti-inflammatory activities, which are likely mediated by its two distinct receptors. TNFR2 signaling is attributable to the immunosuppressive effects of TNF and thus is protective against allergy. Consequently, targeting TNF-TNFR2 pathway may represent future direction to develop new therapies in allergy. However, as a pleiotropic cytokine, TNF and its effects on TNFR signaling is diverse and can either regulate allergic manifestations or to control disease pathogenesis. Implications of TNFR2 in health and diseases requires additional investigation to further

elucidate their exact mechanisms and provide more insight for future strategies in manipulating TNFR2 for therapeutics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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