Current Perspectives on the Role of TNF in Hematopoiesis Using Mice With Humanization of TNF/LT System

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TNF is a multifunctional cytokine with its key functions attributed to inflammation, secondary lymphoid tissue organogenesis and immune regulation. However, it is also a physiological regulator of hematopoiesis and is involved in development and homeostatic maintenance of various organs and tissues. Somewhat unexpectedly, the most important practical application of TNF biology in medicine is anti-TNF therapy in several autoimmune diseases. With increased number of patients undergoing treatment with TNF inhibitors and concerns regarding possible adverse effects of systemic cytokine blockade, the interest in using humanized mouse models to study the efficacy and safety of TNF-targeting biologics in vivo is justified. This Perspective discusses the main functions of TNF and its two receptors, TNFR1 and TNFR2, in steady state, as well as in emergency hematopoiesis. It also provides a comparative overview of existing mouse lines with humanization of TNF/TNFR system. These genetically engineered mice allow us to study TNF signaling cascades in the hematopoietic compartment in the context of various experimental disease models and for evaluating the effects of various human TNF inhibitors on hematopoiesis and other physiological processes.

Keywords: cytokines, cytokine blockade, steady-state hematopoiesis, emergency hematopoiesis, humanized mouse models

INTRODUCTION TO HEMATOPOIESIS

Hematopoiesis is the process of blood cell development that in vertebrates is initiated early during embryogenesis and may be divided into 3 phases or so-called distinct waves of hematopoiesis. The first (or primitive) wave takes place in the yolk sac starting in mice at embryonic day 7.5 (E7.5) and generates unipotent blood cell types (1). The second (or pro-definitive) wave occurs in
the yolk sac, embryo proper and allantois of the mouse embryo and gives rise to multipotent progenitors (2). The third wave of hematopoiesis represents definitive hematopoiesis and is dependent on the activity of hematopoietic stem cells (HSCs), which are the basic units of the adult hematopoietic system. HSCs generated in the embryonic aorta-gonad-mesonephros region first colonize the fetal liver (E10.5) and then shortly before birth (E16) migrate to the bone marrow (BM), where the majority of HSCs reside to sustain steady state hematopoiesis (3).

HSCs are multipotent, self-renewing cells capable of differentiating into all mature blood cell lineages over the lifespan of the animal. Lineage choice may be directed both intrinsically and extrinsically via activation of transcription factors or extrinsically by cytokines (4). The majority of HSCs are quiescent under steady-state conditions, and few HSCs cycle to sustain hematopoiesis (5). In order to maintain hematopoietic homeostasis and to prevent development of malignancies, the self-renewal and differentiation capacities of HSCs are tightly regulated. This is, at least partly, achieved by the specialized network of interactions between distinct cell types (6, 7) and secreted factors (8, 9) in the BM niche that maintains HSC activity in steady-state conditions.

However, in the case of systemic infections and pathological conditions, such as myeloablation after chemotherapeutic or radiotherapy, some HSCs may respond and exit their quiescent state. These ‘activated’ HSCs contribute to the pool of hematopoietic progenitor cells, which will undergo further differentiation in order to replenish the population of immune cells being in high demand at the sites of inflammation in the process of so-called emergency hematopoiesis. This is possible because hematopoietic stem and progenitor cells (HSPCs) express Toll-like receptors (10) and cytokine receptors (11) and, thus, can respond to inflammatory signals. Activation of TLR signaling in HSPCs not only drives myeloid cell differentiation (10), but also leads to production of cytokines, which regulate myeloid differentiation and HSPC proliferation (12). HSPCs may respond to cytokines released during inflammation either systemically or locally by cells in the hematopoietic microenvironment or BM niche. Indeed, it was shown that HSPCs express various cytokine receptors, including IL-1R (13), IL-6Rα, as well as both TNF receptors, TNFRI and TNFRII (12).

As mentioned above, proinflammatory cytokines are critical components of inflammation-induced myelopoiesis. However, inflammatory signals may also be implicated in the maintenance of homeostatic hematopoiesis. During embryonic development proinflammatory cytokines control HSPC specification in the pro-definitive wave of hematopoiesis (14). Moreover, proinflammatory cytokines may regulate adult hematopoiesis and maintain the balance between HSC dormancy and lineage commitment (15). This question is important because systemic and long-term anti-cytokine therapy is being applied to treat an increasing number of conditions, including autoimmune disorders. One of the proinflammatory cytokines implicated in hematopoiesis is TNF, which we discuss in the context of humanized mouse models in this Perspective.

## TNF IN STEADY-STATE HEMATOPOIESIS

TNF is a pleiotropic cytokine involved in inflammation, development of secondary lymphoid organs and immune regulation. TNF is produced as a transmembrane protein and can be proteolytically cleaved into a soluble form. TNF exerts its functions via two distinct receptors – TNFRI and TNFRII (16). Both receptors also may interact with soluble (LTα1) and membrane-bound (LTα2β1) lymphotoxins, respectively (17). Interestingly, TNF may play a role both in embryonic and in adult hematopoiesis (Figure 1).

The role of TNF during fetal hematopoiesis was mostly studied in zebrafish. These studies revealed that TNF derived from primitive neutrophils binds to TNFRII on endothelial cells resulting in the upregulation of Notch ligand Jagged1a, which in turn binds to Notch1a receptor on the neighboring hemogenic endothelium triggering HSC fate specification. Moreover, TNF/TNFRII axis also activates canonical NF-κB pathway in hemogenic endothelium, which triggers a transcriptional program to establish HSC generation (18). Of note, not only TNF but also other inflammatory stimuli such as TLR4-MyD88 signaling or G-CSF may lead to NF-κB activation required for HSC development (29). Interestingly, inflammatory signaling represents a highly conserved pathway regulating the HSC development. Studies in E9.5 mouse embryos revealed that hematopoietic cluster cells and endothelial cells respond to IFNγ and to a lesser extent to TNF stimulation. The most likely source of TNF in the mouse embryo is the population of primitive F4/80+ macrophages, similar to the situation in zebrafish embryo (30). However, the precise contribution of TNF to mammalian HSC development is not completely understood. Taken together, TNF signaling may be required for HSC emergence in the developing embryo via activation of evolutionarily conserved signaling pathways, but it might be partially redundant with other inflammatory stimuli.

Many studies have been performed to examine the role of TNF in the adult hematopoiesis; however, most of these have relied on cell culture and/or bone marrow chimeras, which could indirectly affect HSPC phenotype and functions. Another challenge in understanding the role of TNF in steady-state conditions is due to its capacity to induce systemic inflammation when administered in vivo which in turn may activate stress-induced hematopoiesis thus obscuring TNF contribution to hematopoiesis. Therefore, in this section we will focus on in vivo studies using gene-deficient mice. TNF deficiency did not alter the number of Lin−Sca-1+ c-kit+ (LSK) in the BM, consisting mostly of lineage-biased multipotent progenitors (19). In early studies TNFRI−/− deficient bone marrow was characterized by increased number of LSK (22). However, examination of purified LSKFLT3+ HSPCs revealed no differences in the numbers of HSPC isolated from the adult BM of TNFRI/TNFRII double knockout mice (31). Hence, it is likely that TNF does not affect HSPC compartment under steady-state conditions in vivo.

Regarding the differentiation of HSCs to more committed progenitors, TNFRI−/− deficient mice showed a decrease in pre-B cell compartment and an increase in myeloid progenitors (32).
Accordingly, TNF-deficient mice demonstrated an increase in the number of Gr-1+ neutrophils both in the BM and in peripheral blood (19). Transcriptome analysis of monocytes and their BM precursors revealed an increase in TNF expression upon differentiation of Ly6Chi/Ly6Cint monocytes into Ly6Clo monocytes (20). Therefore, TNF may control granulocyte number in the blood and BM and support monocytic differentiation in vivo.

**TNF IN STRESS- AND INFLAMMATION-INDUCED HEMATOPOIESIS**

Numerous studies on the role of TNF in HSPC functions relied on *in vitro* and *in vivo* colony formation assays together with the assessment of reconstitution potential, engraftment and survival abilities of multipotent progenitors upon transplantation into lethally irradiated recipient mice. However, results obtained from these studies should be carefully interpreted since these setups may affect HSPC proliferation, survival, self-renewal and differentiation. We will discuss some experiments and their possible applicability to hematopoietic compartment.

Studies with competitive co-transplantation of TNFR1−/− TNFR2−/− CD45.2+ and wild-type (WT) CD45.1+ BM cells into lethally irradiated congenic CD45.1+CD45.2+ WT recipients showed enhanced activity of TNFR1−/−TNFR2−/− HSCs as determined by long-term reconstitution by TNFR-deficient HSCs following transplantation. Moreover, *in vivo* administration of TNF to WT mice led to a decrease in BM cellularity and to reduction in HSC cycling activity in a competitive transplantation assay (31). These data suggest that TNF blockade may be beneficial for post-transplantation reconstitution and also supports the idea that TNF may suppress HSC activity. In contrast, transplantation of bone marrow cells from 6 months old TNFR1-deficient mice into lethally irradiated recipients showed reduced repopulating ability of TNFR1−/− BM cells as compared to WT cells (32). However, this effect was shown on non-purified HSPCs and under long-term transplantation conditions that may affect the outcome of the experiment. TNF does not inhibit expansion of highly purified Lin−Sca-1−c-kit+Flk2 CD150+CD48− HSCs in medium supplemented with IL-3, SCF, TPO, EPO, GM-CSF, IL-11 and Flt3-L, or so-called cytokine-rich medium, regardless of TNF concentration. Under cytokine-poor conditions (medium supplemented with SCF and G-CSF only) HSC expansion rate upon addition of TNF was significantly decreased (21). Similar findings were reported by Pronk et al. when TNF was added to the culture of LSK cells (31). Importantly, studies by Yamashita et al. revealed that this effect was due to the inhibition of autophagy by TNF, which sensitized HSCs to cell death in...
cytokine-deprived environment (21). Taken together, HSCs appear to be resistant to TNF cytotoxicity but this resistance may be changed by the environmental stress.

Addition of exogenous TNF to bone marrow cultures of LSK resulted in reduced numbers of large-sized colonies, such as CFU-GEMMs (colony-forming unit – granulocyte, erythroid, macrophage, megakaryocyte) and CFU-GMs (granulocyte/macrophage colony forming units), in methylcellulose assay (22). LSK isolated from TNF-deficient mice gave rise to an increased number of splenic colony-forming units in lethally irradiated recipient mice (19). Experiments with TNFR1 and TNFR2 agonists demonstrated that TNFR2 is essential for TNF-mediated inhibition of colony formation of early Lin-Sca-1+ progenitors, while addition of TNFR1 agonist had no significant effect on the number of Lin-Sca-1+ proliferative clones (23). Thus, TNF may inhibit formation of CFU-GEMM and CFU-GM colonies and differentiation potential of multipotent progenitors, presumably via TNFR2.

Evaluation of TNF effects on myeloid lineage commitment revealed that addition of TNF or TNFR1 agonists resulted in a decrease in G-CSF-induced colony formation and in inhibition of G-CSF expression by BM cultures (23). This was further supported in vitro in TNF-deficient long-term bone marrow cultures, which were characterized by increased proliferative potential of granulocytic progenitors and increased numbers of CFU-GMs within sorted LSK population, as compared to WT BM cultures (19). Consistent with in vitro data, CFU-GM formation was reduced in WT-recipient mice reconstituted with TNF-deficient BM, suggesting a possible role of TNF, expressed by hematopoietic and not by stromal cells, in inducing cell death at the GMP stage (33). This is also in agreement with data showing elimination of GMPs by TNF in a dose-dependent manner (21). Next, TNF was demonstrated to block granulocytic differentiation and IL-3-dependent proliferation of granulocyte-committed cells (24). At the same time, an autonomous effect of TNF on monocytes was proposed. Specifically, TNF is required for monocyte survival in vivo (20). Thus, TNF stands at the crossroad of myeloid lineage commitment via negatively affecting granulocytic cell differentiation and driving the survival of monocytes. Interestingly, these findings suggest possibilities to control the differentiation of myeloid progenitors. For example, TNF was shown to directly upregulate a central transcription factor for myeloid lineage commitment, PU.1, in HSPC in vivo during acute inflammation (34). Moreover, in some abnormal hematopoiesis conditions, such as clonal hematopoiesis associated with aging, TNF blockade may help to overcome TET2-mutant HSPC skewing toward myeloid lineage and the formation of CFU-GMs (35).

Other studies revealed that addition of TNF to the LSK cultures negatively regulated both long-term and short-term reconstituting activity after transplantation of LSK into lethally irradiated recipient mice (36). However, TNF produced by BM microenvironment is required for long-term engraftment and survival of purified LSK in allogeneic and syngeneic recipients (37). This is in line with the fact that the engraftment of Lin- BM cells from TNFR1- and TNFRs-deficient donors in wild-type recipients was defective suggesting a stimulatory role of TNF in successful progenitor engraftment. Interestingly, homing of the engrafted progenitors to the BM was primarily mediated by TNFR1 (25). On the other hand, total body irradiation and inflammation within BM was associated with elevated levels of TNF. Subsequently, TNF induced ROS accumulation in LSK leading to impairment of their reconstitution ability. Addition of TNFR1 antagonistic peptide to LSK cultures inhibited ROS accumulation suggesting that TNFR1 blockade prior to transplantation may lead to improved reconstitution capability (26). Altogether, more precise in vivo studies with defined protocols of total body irradiation and transplantation are needed to identify the role of TNF in the engraftment and survival of HSPCs. For example, TNF may contribute to regeneration of BM niche after HSC transplantation, since TNF-deficient mice displayed reduced number of BM endothelial cells upon myeloablation with a single injection of 5-fluorouracil. Furthermore, Gr1+CD115- granulocyte-derived TNF promoted vascular regeneration following transplantation (27). In the context of hematological malignancies, such as myeloproliferation, inflammation may be implicated in the disruption of BM microenvironment. Flt3ITD/ITD mice, harboring the most common somatic mutation in patients with acute myeloid leukemia, were shown to upregulate TNF production by endothelial cells in the BM niche, which subsequently may lead to suppression of HSC activity. Treatment of Flt3-/- and Flt3ITD/ITD mice with Etanercept resulted in partial rescue of LSKCD150+CD48+ engraftment capacities (38).

As a consequence of inflammation, TNF may mobilize B cell progenitors from BM to peripheral tissues by suppressing their CXCL12-induced retention in the BM. This mobilization establishes extramedullary lymphopoiesis possibly needed for resolution of inflammation (28). Contribution of TNF to emergency myelopoiesis was clearly demonstrated by Yamashita et al. (21). In a model of poly(I:C)-induced inflammation, TNF, on one hand, induced NF-kB activity, which protected HSPCs from inflammation-induced necroptosis and, on the other hand, promoted myelopoiesis and induced apoptosis of GMPs (21). This effect may be protective in pathogenesis of some viral infections, in which case GMPs may act as the latent reservoirs for viruses in the BM and should be eliminated (39).

Defects in hematopoiesis may lead to the development of hematologic disorders. Although the exact role of TNF in different hematological diseases remains incompletely understood, elevated levels of TNF were found in patients suffering from myeloid leukemia (40) and myelodysplastic syndromes (41, 42), Fanconi anemia (43), Hodgkin’s disease (44) and Non-Hodgkin lymphoma (45).

Moreover, since TNF inhibitors are widely used to treat autoimmune disorders, it is important to address possible side effects of anti-TNF therapy. Indeed, hematological complications were reported in patients on TNF blockade (Table 1). For example, a case report was published showing pancytopenia...
| Adverse effect | Diagnosis | Treatment | Brief report | Onset upon anti-TNF treatment | Reference |
|---------------|-----------|-----------|--------------|-------------------------------|-----------|
| Pancytopenia  | Juvenile idiopathic arthritis | Etanercept | 2/61 cases | After 0.5-12 months | (46) |
|   | Scleroderma | Infliximab | 45F case | After 2 weeks | (47) |
|   | RA | Infliximab + MTX | 66M case | After 10 days | (48) |
|   | (Previously leflunomide) | BM hypoplasia |

| | Indeterminate colitis | Infliximab + antibodies | 32F case | After 6 days | (49) |
| | RA | Etanercept | 1/1073 case | NR | (50) |
| | RA | Etanercept + MTX | 68F case | After 3 weeks | (51) |
| | RA | Etanercept | 78M case | After 16 weeks | (52) |
| Aplastic anemia | Psoriatic arthritis | Infliximab | 1/16 case | After 12 weeks | (53) |
| | Crohn’s disease | Infliximab | 1/21 case | After 13 months | (56) |
| | Scleroderma overlap/rheumatoid arthritis | MTX + Predisone + Infliximab | 44F case | Anticardiolipin antibodies positive | (59) |
| Thrombocytopenia | Crohn’s disease | Infliximab then Adalimumab | 42F case | 30 weeks after Infliximab treatment | (57) |
|   | (Previously metronidazole and azathioprine) | Platelet associated antiplatelet antibodies positive | 1 week after Adalimumab treatment |

| | Psoriatic arthritis | Etanercept | 61M case | After 2 months | (58) |
| | Psoriasis | Etanercept | 1/39 case | After 9 weeks | (59) |
| | Psoriatic arthritis | Infliximab | 1/26 case | After 29 weeks | (60) |
| | Psoriasis | Infliximab | 2/26 cases | After 30 weeks | (61) |
| | Crohn’s disease | Infliximab | 79M case | After 14 weeks | (60) |
| | Ulcerative colitis | Adalimumab + azathioprine + mesalazine | 54F case | After 4 years | (61) |
| | Juvenile idiopathic arthritis | Etanercept | 2/95 cases | NR | (62) |
| Thrombocytopenia and leucopenia | RA | MTX + Infliximab | 60F case | After 7 weeks | (63) |
| Thrombocytopenia and neutropenia | RA | Etanercept (Previously MTX + folic acid + Hydroxychloroquine; Leflunomide and Adalimumab discontinued months before Etanercept) | 62F case | After 23 days | (64) |

(Continued)
| Adverse effect | Diagnosis                     | Treatment                  | Brief report                                                                 | Onset upon anti-TNF treatment | Reference |
|----------------|-------------------------------|----------------------------|-------------------------------------------------------------------------------|-------------------------------|-----------|
| Neutropenia    | RA                            | Etanercept                 | 1/208 case                                                                    | NR                            | (53)      |
|                | RA                            | Etanercept                 | 2/207 cases                                                                   | After 4 weeks                 | (65)      |
|                | Spondyloarthropathy/ Crohn's disease | Infliximab            | 20M case Neutrophil-associated antibodies                                          |                               |           |
|                | RA                            | Adalimumab                 | 3/21 cases Adalimumab                                                          | After 1 week – 26 months      | (66)      |
|                | Etanercept                    | Infliximab                 | 13/75 cases Etanercept                                                         |                               |           |
|                |                                |                            | 3/23 cases Infliximab                                                          |                               |           |
|                |                                |                            | 7/65 cases ANA positive                                                        |                               |           |
|                | RA                            | Adalimumab + MTX           | 53F case T-cell lymphocytosis (large granular lymphocytes)                     | After 13 months               | (67)      |
| Sacroiliitis   | Salazopyrine + MTX + Etanercept|                           | 37F case                                                                       | After 6 months                | (68)      |
|                | RA                            | Etanercept (Previously MTX)| 57F case Asymptomatic neutropenia during MTX Increased BM immature granulocyte production ANA positive Neutrophil-associated antibodies negative | After 7 weeks                 | (69)      |
| Psoriatic arthritis | Etanercept (Previously MTX) |                           | 61M case Persistent leucopenia Neutropenia during MTX Normal BM hematopoiesis ANA positive Neutrophil-associated antibodies negative | NR                            |           |
|                | RA                            | Etanercept                 | 50F case Previous asymptomatic neutropenia Normal BM hematopoiesis ANA positive Neutrophil-associated antibodies negative | After 17 days                 |           |
|                | RA                            | Adalimumab + MTX + Prednisone | 55F case ANA negative dsDNA antibodies negative                               | After 1 month                 | (70)      |
|                | RA                            | Adalimumab 6/31            | 56/298 cases                                                                   | NR                            | (71)      |
| Psoriatic arthritis | Etanercept 49/267            | Infliximab 14/69          | 7/31 case                                                                      |                               |           |
|                | Ankylosing spondylitis        | Etanercept + MTX           | 6/38 cases                                                                     |                               |           |
|                | RA                            | Etanercept Etanercept      | 64F case                                                                       | After 2 weeks                 | (72)      |
|                | then Etanercept re-challenge  | then Etanercept re-challenge | 36M case                                                                      | 3 months after Etanercept onset |           |
|                | then Adalimumab               | then 2nd Etanercept re-challenge | 65M case                                                                      | 2nd neutrophil drop after Etanercept re-challenge                        |           |
|                | RA                            | Etanercept then Etanercept then 2nd Etanercept re-challenge | 71F case                                                                      | 6 months after Etanercept treatment                                        |           |
|                | then Etanercept re-challenge  | then Etanercept re-challenge | 16 months after Etanercept re-challenge                                        |                               |           |
|                | then Adalimumab               | then Adalimumab            | 8 weeks after 2nd Etanercept re-challenge                                       |                               |           |
|                | RA                            | Etanercept (Previously hydroxychloroquine + prednisolone) | 42F case                                                                      | After 4 injections             |           |

(Continued)
after treatment with Infliximab (47). Furthermore, side effects of Etanercept [which also neutralizes both LTα3 and LTα2β1 (17)] treatment on hematopoietic cells have been reported including aplastic anemia (52), thrombocytopenia (55), and bone marrow aplasia with pancytopenia (64). Interestingly, a common phenomenon in patients receiving anti-TNF therapy is the development of neutropenia (81). Apart from that, some lymphoproliferative disorders were reported. For example, treatment of rheumatoid arthritis and inflammatory bowel disease (IBD) patients with Etanercept or Infliximab, respectively, led to formation of cutaneous and systemic T-cell lymphomas (75). A case of Hodgkin-type lymphoproliferative lesions was reported for an IBD patient treated with Infliximab for a 6-month period (79).

Altogether, TNF may play a crucial role in inflammation-induced hematopoiesis and may be implicated in the pathogenesis of some hematologic disorders. Anti-TNF therapy may lead to rare but severe side effects affecting hematopoietic compartment and resulting in the development of hematological complications and even malignancies. The exact mechanisms of these side effects are not well understood and should be addressed in the future using humanized mouse models.
HUMANIZED MICE AS THE TOOLS TO STUDY HEMATOPOIESIS AND TO EVALUATE THE CONSEQUENCES OF SYSTEMIC CYTOKINE ABLATION

To identify the effects and to evaluate the efficacy and safety of clinically available or novel human TNF (hTNF) inhibitors proper animal models should be generated and validated. Importantly, in spite of a conservative nature of TNF family of cytokines and their corresponding genes, most hTNF inhibitors do not block mouse TNF (82). Therefore, various panels of cytokines and their corresponding genes, most hTNF inhibitors and/or TNFRs are required to facilitate the research (Table 2). Such preclinical models were first generated in 1991 by G. Kollias group, when the first mice with overexpression of human TNF were reported (83).

Mice With the Overexpression of Human TNF

The very first TNF humanized model was a transgenic mouse with the overexpression of TNF due to intentional dysregulation of TNF mRNA half-life (83). In general, overexpression of cytokines in animal models is a powerful tool to study molecular mechanisms associated with increased cytokine production (98). It is well established that dysregulated TNF production is detrimental in various autoimmune diseases including rheumatoid arthritis, psoriasis and IBD (99). Mice with human TNF overexpression (hTNF Tg mice with a high transgene copy number and dysregulated control) start to develop severe polyarthritis as early as 3-4 weeks after birth with similar characteristics observed in rheumatoid arthritis patients. Administration of antibodies to hTNF Tg mice led to the suppression of arthritis (83). Moreover, TNF overexpression in hTNF Tg mice led to increased incidence of spontaneous spinal disc herniation, which is involved in the development of acute radicular pain (84). These hTNF Tg mice displayed a decrease in hemoglobin associated with mild microcytic hypochromic anemia at the age of 9 weeks. Furthermore, TNF overexpression was associated with a decrease in the frequency of Sca-1⁺ progenitor cells and granulocytes with concurrent increase in the frequency of cells of both lymphoid and monocytic origin in the bone marrow (85). In summary, constitutive TNF overexpression is associated with the development of spontaneous autoimmune conditions. hTNF Tg mice served as an excellent model for associated disorders, such as progressive rheumatoid arthritis, although physiological relevance of using these mice in order to delineate the in vivo effects of systemic TNF inhibition on other functions was limited. To overcome the limitations of non-physiological levels of systemic TNF overproduction, other mouse models, such as tissue-specific, inducible and low copy number hTNF transgenic mice were developed.

Approaches to investigate tissue-restricted overexpression of human TNF in mice were also pioneered by G. Kollias group (88). For example, hTNF overexpression by T cells led to severe systemic effects, for example, CD2-hTNF Tg mice developed lethal progressive weight loss but no arthritis. hTNF overexpression by T cells also resulted in vascular thrombosis, tissue necrosis and lymphoid tissue abnormalities. Particularly, mice were characterized by reduced thymic cellularity and enlarged mesenteric lymph nodes that contained almost no lymphocytes. Overexpression of hTNF in astrocytes or neurons resulted in severe neurologic disease characterized by ataxia, seizures and relapsing hind limb paralysis (89). To delineate the contribution of soluble versus transmembrane TNF, mice that overexpress transmembrane human TNF in astrocytes (GFAP-tmTNF) or neurons (NFL-tmTNF) were engineered. Surprisingly, only astrocyte-specific overexpression of tmTNF was sufficient to trigger the development of neurologic disease (89). Thus, astrocytes appeared to be the source of pathogenic hTNF in a model of neuroinflammation.

| Humanized mouse line | Expression specificity | Hematopoiesis-unrelated phenotype | Hematopoiesis-related phenotype | References |
|----------------------|-----------------------|----------------------------------|-------------------------------|------------|
| hTNF Tg number       | "High copy number"    | Systemic                         | Severe polyarthritis as early as 3-4 weeks after birth | (83–85) |
| (Tg197)              |                       | Spontaneous spinal disc herniation | Mid microcytic hypochromic anemia |            |
|                      |                       |                                  | Decrease in frequency of Sca-1⁺ progenitor cells and granulocytes |            |
|                      |                       |                                  | Increased frequency of lymphoid and monocytic origin in the bone marrow |            |
|                      | "Low copy number"     | Systemic                         | Progressive arthritis at a later age | (86)      |
|                      |                       |                                  | Reduced body weight |            |
|                      |                       |                                  | Increased metabolic rate |            |
|                      |                       |                                  | Restricted motor activity |            |
|                      |                       |                                  | Psoriatic arthritis |            |
|                      |                       |                                  | Keratinocyte activation | (87)      |
|                      |                       |                                  | Joint and skin inflammation |            |
| hTNFtg Doxycycline-inducible | Systemic | Progressive arthritis at a later age | Reduced body weight | (87) |
| CD2-TNF              | T cell                | Increased metabolic rate | Reduced body weight | (88) |
|                      |                       | Restricted motor activity | Vascular thrombosis |            |
|                      |                       | Psoriatic arthritis | Tissue necrosis |            |
|                      |                       | Keratinocyte activation | Lymphoid tissue abnormalities | (Continued) |
The next step in the generation of hTNF transgenic mice was a low transgene copy number model characterized by low circulating levels of hTNF (86). In this case mice developed progressive arthritis at an older age, however, increased TNF production was also associated with reduced body weight, increased metabolic rate and restricted motor activity. This is similar to symptoms of rheumatoid cachexia in humans; therefore, these mice also represent a useful model to study conditions associated with elevated TNF production (100).

To overcome limitations of constitutive TNF overexpression, mice with reversible, doxycycline-inducible hTNF overexpression were generated. Two weeks after doxycycline administration, hTNF Tg mice developed psoriatic arthritis characterized by keratinocyte activation, joint and skin inflammation (87). Interestingly, signs of inflammation in this model were observed exclusively in the digits and, to a lesser extent, in the skin and ankles, unlike in mice with systemic TNF overexpression.

Another approach to generate mice with TNF overexpression was used by Liepinsh et al. (90). In this study mice with a large human genomic segment comprising hTNF and its two closest homologues, lymphotoxin α and β, were generated. Natural genomic context allowed hTNF/LT genes to be expressed in response to physiological stimuli under the control of intrinsic
regulatory elements. These mice demonstrated thymic atrophy and affected thymic T cell development with impaired thymocyte differentiation. Taken together, TNF humanized mouse models that partially mimic inflammatory conditions in patients with autoimmune disorders, such as rheumatoid arthritis and psoriasis, were generated and evaluated. Furthermore, to address the efficacy and possible side effects of TNF/TNFRs inhibitors in other disease models, humanized mice with regulated and cell type-specific hTNF expression were also established.

**Mice With Humanization of TNF and TNFR2**

Humanized TNF knock-in (hTNFKI) mice, in which case the mouse *Tnf* gene was substituted by its human ortholog, were generated using embryonic stem cell technology. They were used as a platform to study the effects of hTNF blockade in various disease models, including infectious, autoimmune, toxicity and transplantable tumor models (92, 93, 101, 102). Also, the efficacy of a novel myeloid-specific TNF inhibitor MYSTI in blocking hTNF and its effects in mouse models of LPS/D-galactosamine-induced hepatotoxicity and collagen antibody-induced arthritis were demonstrated using these hTNFKI mice (93, 102). Additionally, these engineered mice allowed investigators to compare clinically available hTNF inhibitors such as Infliximab, Etanercept and Adalimumab (82). Furthermore, TNF ablation by pharmacological neutralization in hTNFKI mice led to the loss of the resistance to mycobacterial infection and to increased bacterial burden in the lungs (92). hTNF inhibition decreased tumor growth and MDSC accumulation in transplantable MCA 205 fibrosarcoma model, indicating a pro-tumorigenic function of TNF (94). Overall, hTNFKI mice are a useful tool to assess multiple effects of human TNF inhibition in various disease models, including adverse effects of TNF neutralization on hematopoietic compartment. To delineate the role of TNF inhibition with clinically approved blockers in myeloid cell differentiation, we isolated BM cells from hTNFKI mice, cultured them in the medium supplemented with GM-CSF and IL-4 with the addition of Infliximab and analyzed immature myeloid cell differentiation after 5 days of culturing (Figure 2A). We observed that TNF inhibition with Infliximab shifted differentiation of immature myeloid cells in vitro. Thus, TNF neutralization led to an increase in the frequency of Ly6G<sup>-</sup>Ly6C<sup>low</sup> granulocytes and to a decrease in the frequency of Ly6G<sup>+</sup>Ly6C<sup>high</sup> monocytes (Figure 2B). Since TNF is important for survival of monocytes (20), we hypothesized that decreased frequency of monocytes upon anti-TNF treatment was due to induction of apoptosis. To verify that, we analyzed expression of genes encoding anti-apoptotic proteins in purified Ly6G<sup>+</sup>Ly6C<sup>high</sup> monocytes and found down-regulation of Bcl2, Bcl2a1a and Bcl2li upon treatment with Infliximab (Figure 2C). Altogether, TNF blockade with Infliximab in BM cultures from hTNFKI mice inhibited the differentiation of immature myeloid cells into monocytes probably due to the induction of apoptosis.

Earlier biochemical studies indicated that hTNF can efficiently bind to TNFR1, but not to TNFR2 (103, 104). Indeed, inefficiency of hTNF interaction with murine TNFR2 led to disease exacerbation and decrease in Treg numbers in the periphery and CNS in a mouse model of multiple sclerosis (EAE), in which case TNFR2 signaling is protective (95). Therefore, it was desirable to generate a mouse with humanization of TNFR2 to provide efficient TNF-TNFR2 signaling. Mice were genetically designed to include two LoxP sites into human TNFR2 locus, which allowed conditional Cre-mediated deletion of TNFR2 extracellular part in the desired cell type. These doubly humanized hTNF x hTNFR2KI mice showed EAE disease severity and Treg numbers comparable to wild-type mice, confirming the restoration of protective TNF/TNFR2 signaling. Cre-mediated genetic deletion of TNFR2 gene in Treg cells resulted in EAE exacerbation and malfunction of T cells. Intrinsic TNFR2 signaling was important for the maintenance of suppressive functions of Tregs by sustaining expression of Treg signature molecules, such as FoxP3, CD25, CTLA-4 and GITR (95). Further, hTNFR2 agonists applied to Treg cells from doubly humanized mice induced increased Treg cells proliferation (95).

Dong et al. also generated useful TNF receptor humanized mouse models, namely hTNFR1 knock-in and hTNFR2 knock-in mice, and demonstrated decreased neuroinflammation in response to TNFR1 antagonist ATROSAB or TNFR2 agonist EHD2-scTNFR2 (TNF hexamer oligomerized using the CH2 domain of IgE) in a model of NMDA-induced neurodegeneration (96). Additionally, ATROSAB administration inhibited development of EAE, decreased CNS infiltration and demyelination in hTNFR1KI mice (97). Yet another transgenic hTNFR1 mouse strain was used for studying the efficacy of VHH (antigen binding fragment of heavy chain only camelid antibody)-based nanobody against human TNFR1 (TNFR one silencer, TROS) in EAE, in which case a prophylactic administration of TROS resulted in disease amelioration (91).

In summary, there is a growing panel of useful humanized mouse models for evaluation of biologics that affect TNF/TNFR1/ TNFR2 systems, including their effects on hematopoiesis. Furthermore, restoration of affected signaling by humanization of both TNF and its receptor, TNFR2, makes it possible to comparatively evaluate not only anti-TNF drugs, but also hTNFR2 agonists and antagonists. Taken together, humanized mouse models will allow investigators to study efficacy of various TNF/TNFRs-targeting biologics and assess possible side effects on other systems for further clinical translation.

**CONCLUDING REMARKS**

For many years TNF was mainly considered a proinflammatory cytokine with its role in host defense, but also with detrimental effects on autoimmunity. However, basic studies on TNF biology, as well as reported side effects in patients receiving anti-TNF therapy, highlighted its homeostatic functions in many physiological processes, including hematopoiesis. Regulation of
TNF/TNFRs expression in various tissues modulates the cross-talk between immune and non-immune cells, which subsequently determines the outcome of TNF action.

Future investigation of pathological versus regulatory functions of TNF and deciphering its systemic and local effects in tissues may help to improve current therapeutic approaches. Therefore, mouse models with humanized TNF/TNFRs system represent a powerful tool to study side effects of anti-TNF therapy on hematopoiesis.

AUTHOR CONTRIBUTIONS

VG, KA, MD, and SAN designed research. VG and KA performed research and analyzed data. VG, KA, AD, TY, MSD, and SN discussed the concept and wrote the manuscript.

All authors contributed to the article and approved the submitted version.

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