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Attenuation of Myeloid Specific TGFβ Signaling Induces Inflammatory Cerebrovascular Disease and Stroke

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Abstract

Rationale—Cryptogenic strokes, those of unknown cause, have been estimated as high as 30–40% of strokes. Inflammation has been suggested as a critical etiological factor. However, there is lack of experimental evidence.

Objective—In this study, we investigated inflammation associated stroke etiology using a mouse model that developed spontaneous stroke due to myeloid deficiency of TGFβ signaling.

Methods and Results—We report that mice with deletion of Tgfbr2 in myeloid cells ($Tgfbr2^{Myeko}$) developed cerebrovascular inflammation in the absence of significant pathology in other tissues, culminating in stroke and severe neurological deficits with 100% penetrance. The stroke phenotype can be transferred to syngeneic wild type mice via $Tgfbr2^{Myeko}$ bone marrow transplant, and can be rescued in $Tgfbr2^{Myeko}$ mice with wild-type bone marrow. The underlying mechanisms involved an increased type 1 inflammation, and cerebral endotheliopathy, characterized by elevated NFκB activation and TNF production by myeloid cells. A high fat diet

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DISCLOSURES

None

accelerated stroke incidence. Anti-TNF treatment, as well as metformin and methotrexate, which are associated with decreased stroke risk in population studies, delayed stroke occurrence.

Conclusions—Our studies show that TGFβ signaling in myeloid cells is required for maintenance of vascular health, and provide insight into inflammation-mediated cerebrovascular disease and stroke.

Keywords

Inflammation; cerebrovascular disease/stroke; mouse; model; prevention

Subject Terms

Animal Models of Human Disease; Inflammation; Cerebrovascular Disease/Stroke

INTRODUCTION

Stroke is the second leading cause of death worldwide, behind heart disease, and is a leading cause of disability[1]. Estimates of cryptogenic stroke, of unknown cause, have been estimated as high as 30–40%[2]. Cerebrovascular or peripheral inflammation is a contributory factor when the cause of the stroke is known, as in atherosclerosis, but is often inferred even when no “smoking gun” can be found[3]. However, there is little experimental evidence. Current animal models utilize germline mutations or artery stenosis[5], and do not provide etiological insight.

Chronic inflammation increases with aging and contributes to cerebrovascular disease, which is a risk factor for stroke[3]. Transforming growth factor beta (TGFβ) is a critical mediator in immune/inflammation homeostasis[4], and alterations of this pathway are implicated in vascular diseases[5,6]. We previously reported that deletion of the gene encoding TGFβ receptor II (TβRII) in myeloid cells ($Tgfbr2^{Myeko}$) decreased production of type 2 cytokines and cancer metastasis[7,8]. However, we show here that this type 1 skewed immune environment resulted in spontaneous stroke with 100% penetrance in C57BL/6 mice at 9–15 months of age, corresponding to middle age in humans. Characterization of these mice revealed defects in motor function, an increase in inflammatory cytokines, cerebral endotheliopathy, and ischemic brain lesions. Importantly, a high fat diet accelerated stroke incidence in mice analogous to poor diet as a risk factor for human stroke. Both metformin and methotrexate, which are associated with decreased stroke risk in population studies, as well as anti-TNF treatment, delayed stroke occurrence. Our data suggest $Tgfbr2^{Myeko}$ mice could be used to model risk factors and prevention of stroke.

METHODS

A number of experimental approaches were used including assessment of neurological impairment, MRI, bone marrow transplant, *ex vivo* and *in vitro* culture, molecular and cellular biology, immune/inflammation assays, statistical analysis etc. An expanded Materials and Methods section is available in the Online Data Supplement.

Animal experiments

All animal protocols were approved by the Animal Care and Use Committee at the NIH. Myeloid specific deletion of *Tgfbr2* was accomplished through breeding of *Tgfbr2* floxed mice with *Lysozyme 2* promoter-driven *Cre* recombinase (LysM-Cre) mice as previously reported[7]. All experiments used homozygous floxed Tgfbr2 mice and control littermate in a C57BL/6 background. Euthanasia criteria were head tilt, circling behavior, trembling, paresis and/or paralysis, inability to obtain food or water, or greater than 20% body weight loss. For cytokine array, 6 month-old mice were used. For stroke risk assessment studies (Bio-plex), 8–12 month-old pre-stroke mice were used. For stroke prevention, treatments began at 6 months of age. High fat diet was initiated at 6 weeks of age. For bone marrow transplants, bone marrow was isolated from mice and injected into the tail vein of irradiated recipient mice.

In vitro experiments

Cytokine arrays and Bio-plex assays were done using plasma from mice at indicated ages. Myeloid cells were isolated from peripheral blood of 3–6 month-old mice using fluorescence activated cell sorting for endothelial co-culture experiments. RNA was purified using Qiagen columns. Mouse macrophage cell line RAW264.7, with shRNA-mediated knockdown of Tgfbr2, was used as a surrogate for $Tgfbr2^{Myeko}$ myeloid cells in western blot and chromatin immunoprecipitation experiments. Immunofluorescence was performed on frozen sections or cytospun cells from blood.

RESULTS

Neurological impairment and spontaneous stroke in $Tgfbr2^{Myeko}$ mice

In the course of breeding mice with myeloid deletion of Tgfbr2 ($Tgfbr2^{Myeko}$) in a C57BL/6 background, it became apparent that $Tgfbr2^{Myeko}$ older than 6 months occasionally died of unknown causes. The median lifespan was 12 months, with 0% survival by 20 months of age for both males and females (Figure 1A). No mortality was observed in wt (either $Tgfbr2^{fl/fl}$ without LysM-cre, or LysM-cre alone) or LysM-cre, $Tgfbr2^{fl/+}$ (Tgfbr2 myeloid heterozygous, $Tgfbr2^{Myehet}$) over the course of the study. $Tgfbr2^{Myeko}$ mice showed signs of neurological impairment that presented as abnormal limb reflexes when lifted by the tail, head tilt, trembling, hiccups, abnormal gait, paresis and/or paralysis and weight loss (Online Figure IA and Movies 1–3). No neurological impairment or weight loss was observed in littermate wt or $Tgfbr2^{Myehet}$ mice. Most $Tgfbr2^{Myeko}$ mice in the lifespan cohort were euthanized for humane reasons due to above signs. All mice showed signs of neurological impairment at sacrifice. $Tgfbr2^{Myeko}$ mice showed motor function deficits on the ladder traversal test, where $Tgfbr2^{Myeko}$ mice had a high number of foot slips, and had a significantly higher ataxia coefficient with Digigait analysis (Figure 1B). Monthly rotarod testing revealed a precipitous decline in motor function over a short period, suggesting an event-related alteration in motor function rather than progressive neurodegeneration (Figure 1B).

Large vessel occlusion, or decreased blood flow, such as that typically seen in the vascular territory distal to a critical stenosis, was not detected on magnetic resonance angiography

(Online Figure II). Atrial fibrillation, the most common cause of cardioembolic stroke, was not detected on electrocardiograms (data not shown). Rather histological analysis of brains from affected Tgfbr2^{Myeko} mice revealed neuronal damage, small areas of focal ischemia, similar to human lacunar stroke[9], and micro-hemorrhage (Figure 1C), as well as decreased cellularity in the olfactory lobes, brainstem and/or cortex in all affected animals (Online Figure IB). No lesions were observed in brains from 8 month-old wt and unaffected Tgfbr2^{Myeko} mice (Online Figure IB). T2-weighted MRI showed larger brain lesions (Figure 1D upper left) that were consistent with neuronal tissue loss observed on histology (Figure 1D). Positive staining for glial fibrillary acidic protein (GFAP), indicated astrogliosis, which is typical in ischemia (Online Figure IC). Neither amyloid plaques nor demyelination outside of stroke lesions were observed, which excluded neurological diseases comparable with Alzheimer's disease and multiple sclerosis (Online Figure ID). From behavioral and histological findings, the cause of neurological impairment and premature death in Tgfbr2^{Myeko} mice was attributed to spontaneous stroke.

Bone marrow from Tgfbr2^{Myeko} mice could recapitulate the phenotype in wt mice, while wt bone marrow could rescue the phenotype of Tgfbr2^{Myeko} mice (Figure 1E,F). Wt mice that received Tgfbr2^{Myeko} bone marrow exhibited mortality (Figure 1E) and brain microhemorrhages (Online Figure IF) similar to Tgfbr2^{Myeko} mice. These data exclude the contribution of brain residing microglia[10,11] and other cell types reported to be targets of LysM-cre[12]. Together, these data suggest that deficiency of TGFβ signaling in myeloid cells leads to decreased lifespan due to spontaneous stroke.

Inflammatory cerebrovascular lesions in brains of Tgfbr2^{Myeko} mice

Risk factors for human stroke were not found in Tgfbr2^{Myeko} mice, including high blood pressure, high blood glucose (Online Figure IE,F, Online Table I), high cholesterol or triglycerides (Online Table I), or elevated body weight (data not shown). Atherosclerosis in the heart and aorta was not observed by H&E or Oil Red O staining on a cereal diet, consistent with no elevation in plasma cholesterol. There also is no evidence for cerebral atherosclerosis (data not shown). Brains of affected mice showed infiltration of lymphocytes and macrophages around the cerebral arteries in areas of the brain otherwise devoid of immune cells (Figure 2A,B, Online Figure IIA,B). Tgfbr2^{Myeko} mice, as well as wt mice that received Tgfbr2^{Myeko} bone marrow, showed narrowed cerebral vessel lumens (Online Figure IIB,C). Some arterial lesions showed fibrotic scars that were consistent with thrombosis, fibrin deposition, vascular occlusion, and recanalization (Online Figure IIB). Inflamed vascular lesions were not observed in the kidneys, liver or spleen, consistent with blood chemistry that did not indicate damage to major organs (Online Table I). This might be analogous to the specific relationship between circulating immune cells and cerebral blood vessels in neurodegenerative diseases such Alzheimer's disease[13]. There was no difference in brain immune cell profile by flow cytometry prior to stroke (Online Figure IID). No Evans blue leakage was found in the brain parenchyma of pre-stroke Tgfbr2^{Myeko} mice, or in the majority of mice with signs of stroke (Online Figure IIE). These studies indicate an unlikely widespread breach of the blood brain barrier in Tgfbr2^{Myeko} mice.

Recruitment of inflammatory cells to blood vessels in normal brain adjacent to stroke lesions (Figure 2B) indicated a possible causal role of vascular inflammation in stroke occurrence. In brains from 6 month-old pre-stroke Tgfbr2^{Myeko} mice, there was significant co-localization of immune cells with the vasculature (Figure 2C). Leakage of fluorescent Dextrans into the brain parenchyma revealed focal breakdown of the blood brain barrier in regions of the brain co-staining for immune cells, but not in areas of the brain without immune infiltrate (Figure 2D). Leakage of fluorescent dextrans was more severe and widespread in mice with signs of stroke. Elevated brain expression of endothelial activation markers, intercellular adhesion molecule 1 (ICAM1) and Von Willebrand factor (VWF) was observed in pre-stroke Tgfbr2^{Myeko} mice (Figure 2E–F). To further examine whether Tgfbr2^{Myeko} mice might have enhanced susceptibility for vascular inflammation, femoral arteries of Tgfbr2^{Myeko} and wt mice were subjected to wire injury *in vivo*. This model is widely used in cardiovascular studies in mice, and produces a significant inflammatory response that is not observed with carotid wire injury[14]. At 13 days post injury, there was an increase in infiltrating CD45+ inflammatory cells surrounding the arteries in Tgfbr2^{Myeko} mice compared with wt littermates (Figure 2G). These data suggest the involvement of inflammatory cells in the cerebral vasculature in the etiology of cerebrovascular disease in Tgfbr2^{Myeko} mice.

Mechanisms of vascular inflammation

Proinflammatory cytokines cause vascular damage under pathological conditions[15]. To investigate whether an inflammatory cytokine environment plays a role in stroke etiology, cytokines were investigated in plasma from 6 month-old pre-stroke Tgfbr2^{Myeko} and wt mice. There was a significant elevation of pro-inflammatory cytokines such as TNF, CXCL10 and CCL2 (Figure 3A, Online Figure IIIA). Representative type 2 cytokines such as IL4 and IL10 were not detected in plasma from either wt or Tgfbr2^{Myeko} mice (Figure 3A). This enhanced type 1, and deficient type 2 polarization is also observed in vascular inflammation and atherosclerotic plaque formation[20,21].

Increased blood TNF is particularly interesting as it occurs with aging, vascular inflammation, and cardiovascular disease[16]. Wt mice had TNF levels that fell into a narrow range, while many Tgfbr2^{Myeko} mice had elevated levels (Figure 3B). Two mouse macrophage cell lines and one human myeloid cell line expressed lower levels of TNF RNA after treatment with TGFβ (Online Figure IIIB), suggesting that TGFβ signaling in myeloid cells is required for repression of TNF.

TNF is known to cause endothelial cell damage in vitro and in vivo[16]. Unlike wt myeloid cells, Tgfbr2^{Myeko} myeloid cells caused disruption of adherens and tight junctions of C57BL/6 mouse brain microvascular endothelial cells (MBMEC) in co-culture, as visualized by fragmented border staining of VE-Cadherin, ZO-1 and Occludin (Figure 3C). A TNF neutralizing antibody or deletion of TNF also in myeloid cells prevented endothelial damage mediated by Tgfbr2^{Myeko} myeloid cells (Figure 3C). This result was further validated using human dermal microvascular endothelial cells (Online Figure IIIC). Increased TNF, and increased markers for endothelial damage, ICAM-1 and VCAM-1 RNA, were observed in MBMEC co-cultured with Tgfbr2^{Myeko} myeloid cells, which was prevented by deletion of

TNF in Tgfbr2^{Myeko} myeloid cells (Figure 3D). These results suggest a cross talk of myeloid cells with endothelial cells through TNF. Taken together, these data suggest that myeloid-produced TNF due to deficiency of TGFβ signaling is critical for endothelial damage, consistent with roles of TNF in stroke initiation and progression[17].

Transcription factor NFκB is a master regulator of inflammatory programs, and is activated in aged hematopoietic stem cells[18]. Active, phosphorylated-p65 (encoded by Rela) nuclear translocation was observed in myeloid cells sorted from Tgfbr2^{Myeko} mice (Figure 3E). Blockade of TGFβ signaling in RAW264.7 cells using a TβRI inhibitor increased phosphorylation of p65, and nuclear translocation of p65 and p50 (Figure 3F). Of note, the TNF promoter region contains 5 putative NFκB binding sites (Online Figure IIID). Chromatin Immunoprecipitation (ChIP) for p65 or RNA polymerase 2 (POLR2a) showed enrichment of TNF promoter binding by TβRII knockdown in RAW264.7 cells (Figure 3G, Online Figure IIIE). Together, our data suggest that TβRII knockdown activated a NFκB-TNF inflammatory program that contributes to the endotheliopathy in Tgfbr2^{Myeko} mice.

Stroke risk assessment and prevention

The events prior to stroke are particularly interesting and important in understanding stroke etiology and stroke risk assessment, which remain one of the toughest challenges[19]. To compile a stroke risk profile, 32 plasma cytokines were measured from a young and old wt, young and old (prestroke), and old post-stroke Tgfbr2^{Myeko} mice. Tgfbr2^{Myeko} post- stroke mice showed a unique cytokine profile that overlapped with that from pre-stroke mice (Figure 4A). A human RNA dataset of peripheral blood mononuclear cells (PBMC) from patients 6 months post ischemic stroke[20], who are at high risk for subsequent stroke[21], revealed increased expression of inflammatory cytokines, including TNF (Figure 4B). Ingenuity pathway analysis (IPA) of significantly altered pro-inflammatory genes from the human dataset, revealed striking similarity to our mouse pre-stroke cytokine signature (Online Figure IV). This suggests that Tgfbr2^{Myeko} mice model inflammatory processes important for stroke in human patients.

Spontaneous stroke has been achieved in several mouse models through germline mutation[22–24], but is not representative of the clinical conditions when specific genetic alterations are not a factor. Tgfbr2^{Myeko} mice were used to model human stroke risk factors and prevention. High fat diet (HFD), a major stroke risk factor[25], accelerated stroke occurrence in Tgfbr2^{Myeko} mice (Figure 4C). High fat diet increased blood cholesterol in both wt and Tgfbr2^{Myeko} mice to a similar extent, but did not significantly increase blood glucose or blood pressure in either group (Online Figure VA). Prevention with anti-inflammatory drugs was started at 6 months of age, just prior to earliest stroke occurrence, but when Tgfbr2^{Myeko} mice show increased inflammatory cytokines (Figure 3A). Metformin, a drug widely used for diabetes, and which decreased stroke risk in diabetics in epidemiological studies[26], delayed stroke occurrence (Figure 4D). Treatment with low dose Methotrexate, a drug used to treat auto-inflammatory diseases such as rheumatoid arthritis and severe psoriasis[27], also delayed stroke occurrence (Figure 4E). Both methotrexate and metformin treatments led to a significant decrease in plasma TNF (Figure 4F). Importantly, anti-TNF therapy with Infliximab delayed stroke occurrence in Tgfbr2^{Myeko} mice, and led to

improved neuromuscular function in pre-stroke Tgfbr2^{Myeko} mice (Figure 4G–I). Tgfbr2^{Myeko} mice with whole body deletion of TNF also showed a trend toward delayed stroke occurrence (Online Figure VB). Of interest, PTGS2 (the gene encoding cyclooxygenase 2, COX2) was elevated and suggested to be a signaling node in the human stroke risk dataset (Online Figure IV). However, Tgfbr2^{Myeko} mice given the COX2 inhibitor, Celecoxib, in the diet showed significantly accelerated stroke (Online Figure VC), consistent with the increased risk for stroke with Celecoxib use in humans[28]. These data provide evidence that Tgfbr2^{Myeko} mice can be used to model stroke risk factors and prevention.

DISCUSSION

We provide evidence using a novel mouse model, of a cause and effect relationship between inflammation, and cerebrovascular disease and stroke. In this model, myeloid cells that lack *Tgfbr2*, create a type 1 polarized inflammatory immune environment characterized by elevated inflammatory cytokines. In Tgfbr2^{Myeko} mice, stroke leads to decreased motor control, and, in particular, we show gait disturbances, which are a common cause of disability in stroke patients.

Our data suggest that inflammation is required for myeloid-mediated cerebrovascular disease that leads to stroke, and as such may be an early event in stroke etiology. Our findings are consistent with the recent identification of DADA2 (deficiency of ADA2) an autoinflammatory disease caused by loss of function mutations in ADA2, an adenosine deaminase almost exclusively produced and secreted by myeloid cells. Affected children suffer from recurrent fever early onset stroke and small vessel vasculitis[29–31]. Human monocytes and macrophages with mutant ADA2 are skewed towards a proinflammatory state, which causes endothelial cell damage *in vitro* and *in vivo*[29]. Anti-TNF treatment of these patients leads to dramatic clinical improvement of the inflammatory features, and reduction in recurrent strokes[32]. Although there is no mouse homolog of CECR1 (encoding ADA2), DADA2 patients provide valuable insight into myeloid cell function, inflammation and stroke risk. In fact there is increasing evidence for roles of inflammation in stroke etiology[33,34]. In particular, a recent clinical trial using anti-inflammatory therapy targeting interleukin-1β led to a significant decrease in strokes in cardiovascular patients with high C-reactive protein[35]. Our studies, together with others offer insight for cryptogenic strokes, approximately 30% of all strokes. Identification of high stroke risk patients without currently known risk factors, such as with a plasma cytokine profile, would allow intervention.

Decreased expression of Tgfbr2 in PBMC with age has been reported recently[36], and reduced TGFß signaling was observed in aged hematopoietic stem cells[37]. Mutation or polymorphism in TGFß pathway genes, TGFB1, TGFBR1 and TGFBR2 are associated with vascular disorders that have high stroke risk and inflammation such as Loeys Dietz syndrome[6,38], Kawasaki disease[39,40], and Moyamoya disease[41,42]. Tgfbr2 polymorphism is also associated with intracerebral hemorrhage[43]. Genetic attenuation of TGFβ signaling in myeloid cells thus constitutes human relevance, and is an experimental approach to investigate inflammation associated stroke etiology. We propose the consequential

upregulation of NFκB and enhanced production of TNF is one significant mechanism, consistent with the association of TNF with vascular diseases, atherosclerosis and stroke[15–17]. Anti-TNF therapy appears to be efficacious in prevention of cardiovascular events in rheumatoid arthritis[44,45]. Anti-TNF therapy has also been shown to decrease motor impairment when given to patients greater than one year after stroke, presumably by decreasing persistent neuroinflammation[46]. Comparably, we found that anti-TNF therapy of Tgfbr2Myeko mice with Infliximab delayed stroke occurrence, and decreased neuromuscular deficits in Tgfbr2^{Myeko} pre-stroke mice. Deletion of TNF in Tgfbr2Myeko mice also trended toward delayed stroke occurrence, supporting our assertion that TNF is an important factor leading to stroke in Tgfbr2^{Myeko} mice. However, the immune system is a very complex web of cell types and mechanisms that are intricately entwined. Given the scope of inflammatory cytokine elevation, there may be more than one mechanism or cytokine critical for the stroke phenotype in Tgfbr2^{Myeko} mice.

We have demonstrated the utility of Tgfbr2^{Myeko} mice in modeling inflammation-mediated vascular risk factors for the prevention of stroke. Tgfbr2^{Myeko} mice showed delayed stroke occurrence with anti-inflammation treatments, and accelerated stroke occurrence when adding a risk factor, HFD. Although there is limited data regarding methotrexate as a prevention for stroke, the cardiovascular inflammation reduction trial (CIRT) is addressing this question in hopes of shedding light on the “inflammatory hypothesis of atherothrombosis”[47]. If high stroke risk patients could be identified, such as with a plasma cytokine profile, treatment with drugs identified using Tgfbr2^{Myeko} mice might prevent or delay strokes, prolong life and decrease disability.

In summary, we propose that deficiency in myeloid TGFβ signaling activates an NFκB-TNF inflammatory program leading to cerebral inflammatory endotheliopathy and cerebrovascular atherosclerosis, culminating in spontaneous stroke (Figure 4J). Tgfbr2^{Myeko} mice developed smaller lesions more closely resembling lacunar strokes, which are thought to result from cerebral vascular lesions, as in Tgfbr2^{Myeko} mice[9]. We anticipate that our studies provide insight into inflammation and vascular disease-associated stroke etiology. Myeloid TGFβ signaling could be exploited for intervention of these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ChIP	chromatin immunoprecipitation
Cox2	Cyclooxygenase (protein encoded by PTGS2)
GFAP	glial fibrillary acidic protein
ICAM1	Intercellular Adhesion Molecule 1
LysM-cre	Lysozyme 2 promoter driven Cre recombinase
MRI	magnetic resonance imaging
NFκB	nuclear factor-κB
PBMC	peripheral blood mononuclear cells
Polr2a	polymerase (RNA) II (DNA directed) polypeptide A
Pre-stroke	mice prior to discernable signs of stroke
Post-stroke	mice that showed obvious signs of stroke as detailed in Methods
PTGS2	prostaglandin-endoperoxide synthase 2
TβRI	transforming growth factor beta receptor 1 (protein)
TβRII	Transforming growth factor beta receptor 2 (protein)
Tgfbr2^{Myeko}	Transforming growth factor receptor 2, myeloid knockout (Tgfbr2−/−)
TGFβ	Transforming growth factor beta
TNF	tumor necrosis factor
VWF	Von Willebrand factor
wt	wild type

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Stroke is a leading cause of disability and death worldwide.
- Approximately 30% of ischemic strokes are cryptogenic (of unknown cause).
- Common stroke risk factors such as hypertension, poor diet, smoking, physical inactivity, and aging are associated with systemic inflammation, although no direct cause and effect relationship has been demonstrated.

What New Information Does This Article Contribute?

- Inflammation, mediated by myeloid cells deficient in TGFß receptor 2 (Tgfbr2), leads to cerebrovascular disease, neurological deficits, and stroke in 100% of mice, at approximately middle age, in the absence of any other stroke risk factors.
- Inflammatory plasma cytokines, most notably Tumor Necrosis Factor (TNF), are elevated prior to stroke in these mice, and are largely conserved after stroke, similar to what has been observed in post-stroke patients.
- Metformin and low dose methotrexate lower plasma TNF levels, and similar to anti-TNF therapy, can delay stroke occurrence in these mice.

In this study, we report that spontaneous stroke in mice leads to disability and death, in the absence of exogenous treatment. This is the only animal model of stroke caused by inflammation that provides evidence for a cause and effect relationship between inflammation and stroke. Mice lacking Tgfbr2 in myeloid cells ($Tgfbr2^{Myeko}$) developed stroke in approximately middle age, in the absence of any known stroke risk factors. $Tgfbr2^{Myeko}$ mice had high plasma levels of inflammatory cytokines prior to stroke, and after stroke, similar to what has been observed in post-stroke patients. Cerebrovascular inflammation, and associated focal breach of the blood brain barrier was observed prior to stroke in these mice. Modeling stroke prevention, using metformin (anti-diabetic), low dose methotrexate (anti-inflammatory) and Infliximab (anti-TNF antibody), reduced inflammation and delayed stroke occurrence in mice. These data suggest that patients with specific cytokine profiles might benefit from anti-inflammatory treatment for stroke prevention. $Tgfbr2^{Myeko}$ mice thus provide a valuable tool to investigate the etiology and progression of vascular inflammation to cerebral vascular disease to stroke.

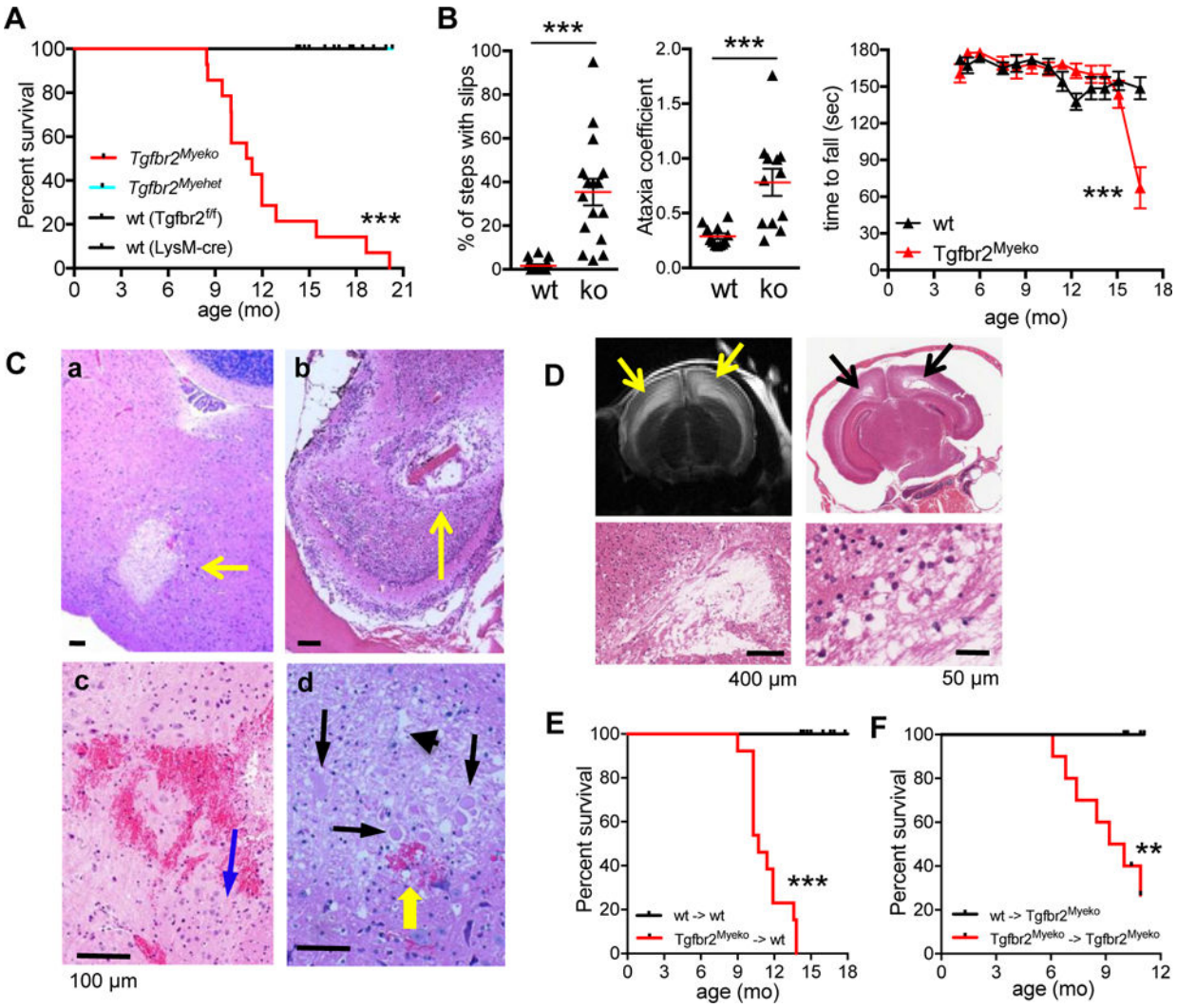

Figure 1. Tgfbr2^{myeko} mice develop severe neurodeficits and spontaneous stroke

(**A**) Decreased overall survival of Tgfbr2^{Myeko} (n=19) and compared with wt (Tgfbr2$^{fl/fl}$, n=25 and LysM-cre, n=3) and heterozygous (Tgfbr2^{Myehet}, n=9) littermates. Mice were sacrificed for humane endpoints, which included head tilt, abnormal gait, severe trembling, paresis or paralysis, or weight loss of >20% due to severe neurological impairments. p, Tgfbr2^{Myeko} vs wt. (**B**) Decreased motor function and gait disturbance in Tgfbr2^{Myeko} (ko) mice. Foot slips on ladder traversal test (n=4, left panel) and ataxia coefficient from Digigait analysis (n=3, center panel) in 9 mo-old pre-stroke Tgfbr2^{Myeko} mice and wt littermates. Each data point is from one foot from one mouse, 4 points per mouse. Serial rotarod testing of young mice until decline in performance (n=5, right panel). (**C**) Post-ischemic brain lesions in Tgfbr2^{Myeko} mice. H&E staining of brain sections from Tgfbr2^{Myeko} stroke mice with neurological impairment: a, focal sub-acute infarction in brainstem; b, focal infarction in olfactory bulb; c, micro-hemorrhage in cortex; d, dead and degenerating neurons (black arrows); eosinophilic neurons with neuronal shrinkage (blue arrow); and neuropil vacuolation (arrow head); micro-hemorrhage (yellow arrow). Scale bars for all panels are 100 µm. (**D**) T2-weighted MRI of brain from Tgfbr2^{Myeko} mouse with signs of stroke (upper

left panel), with hyperintensities (yellow arrows); H&E from same mouse in corresponding regions on MRI, (black arrows) showing loss of brain tissue. Lower subpanels, zoom. (**E**) $Tgfbr2^{Myeko}$ bone marrow causes stroke in wt mice, (n=13 for $Tgfbr2^{Myeko}$ donors, n=4 for wt donors). wt->wt denotes donor->recipient mice. (**F**) wt bone marrow rescues stroke in $Tgfbr2^{Myeko}$ mice (n=10 per group). For E-F, tick marks are censored subjects (alive at termination of experiment). **p<0.01, ***p<0.001

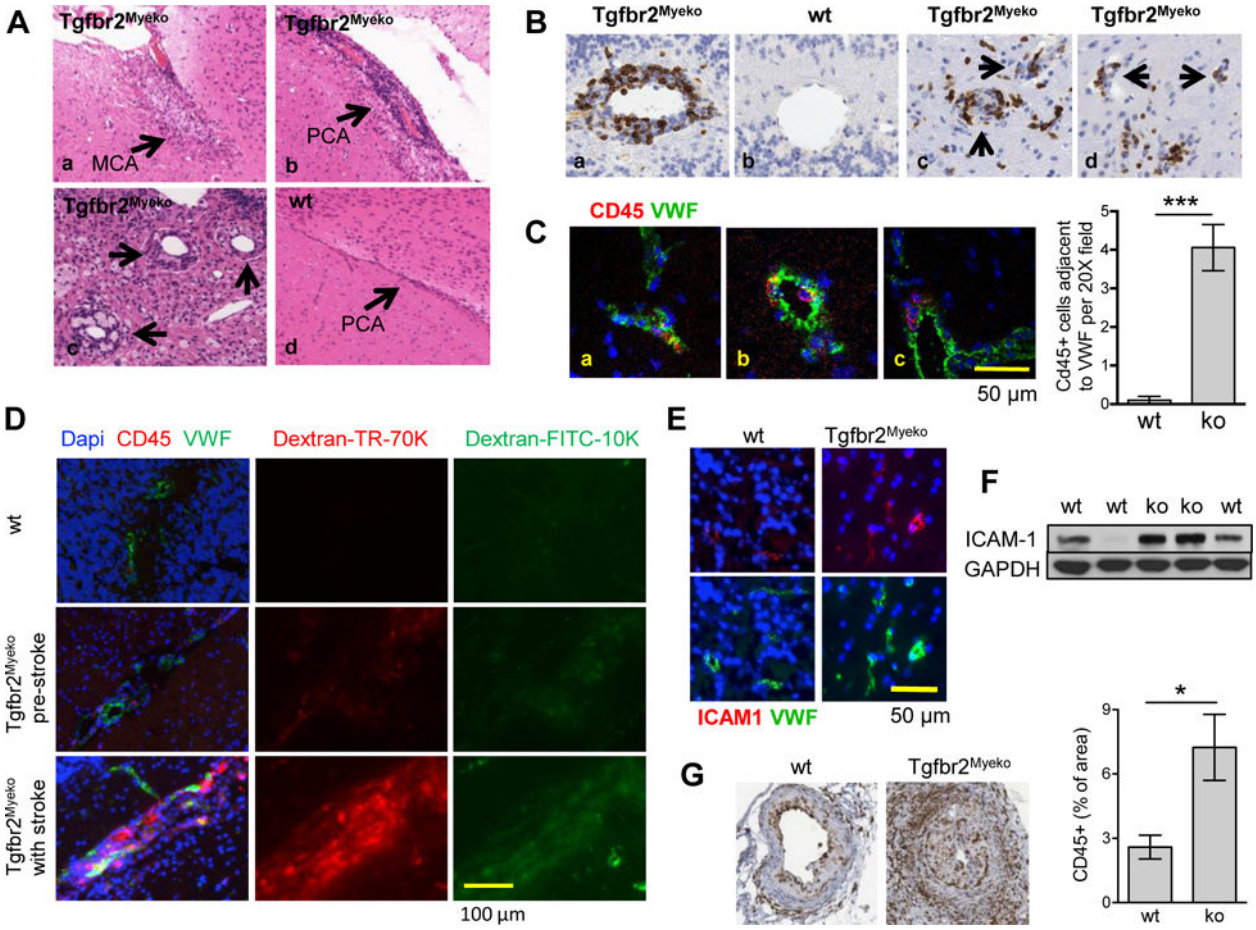

Figure 2. Vascular inflammation and endotheliopathy in Tgfbr2^{Myeko} mice

(**A**) H&E showing vascular inflammation in brain sections from Tgfbr2^{Myeko} mice with signs of stroke. (a) middle cerebral artery (MCA); (b,d) posterior cerebral artery (PCA) (c) inflammation surrounding small vessels in the brainstem of Tgfbr2^{Myeko} and wt mice. Arrows indicate inflammatory vascular lesions, or normal PCA for panel 4. (**B**) Inflammatory cerebral vascular lesions (CD45 immune cells, brown) in brain from Tgfbr2^{Myeko} mice with stroke. Medium sized vessel of the cerebellum (a) compared with wt (b); Immune infiltrate surrounding small vessels in Tgfbr2^{Myeko} brains (c,d). (**C**) Immune infiltration surrounding small and medium vessels in Tgfbr2^{Myeko} mice pre-stroke. Von Willebrand factor (VWF, green) and immune cells (CD45, red) in brain of Tgfbr2^{Myeko} mice. Nuclei in blue (DAPI). CD45+ cells within a small blood vessel, notice elongated shape (a). Localization of CD45 outside of blood vessels and adjacent to VWF (all Tgfbr2^{Myeko} shown). Quantitative data on the right. (**D**) Breach of the blood brain barrier in areas of cerebrovascular inflammation in pre-stroke and post-stroke Tgfbr2^{Myeko} mice. Mice were sacrificed 1h after IV injection of fluorescent dextrans. (**E**) Increased endothelial activation in brains of Tgfbr2^{Myeko} mice pre-stroke. Inflammatory endothelium (ICAM1, red) and blood vessels (VWF, green). (**F**) ICAM1 Western blot of brain protein extracts from Tgfbr2^{Myeko} mice and wt controls. (**G**) Increased inflammatory infiltrate surrounding femoral arteries of Tgfbr2^{Myeko} mice following experimental wire injury. Immune cell IHC

staining (CD45, brown) of tissue sections from femoral arteries 13 days after wire injury of $Tgfbr2^{Myeko}$ mice compared with wt littermates (n=3). Shown are representative images. Quantitative data at right, ***p<0.001, *p<0.05.

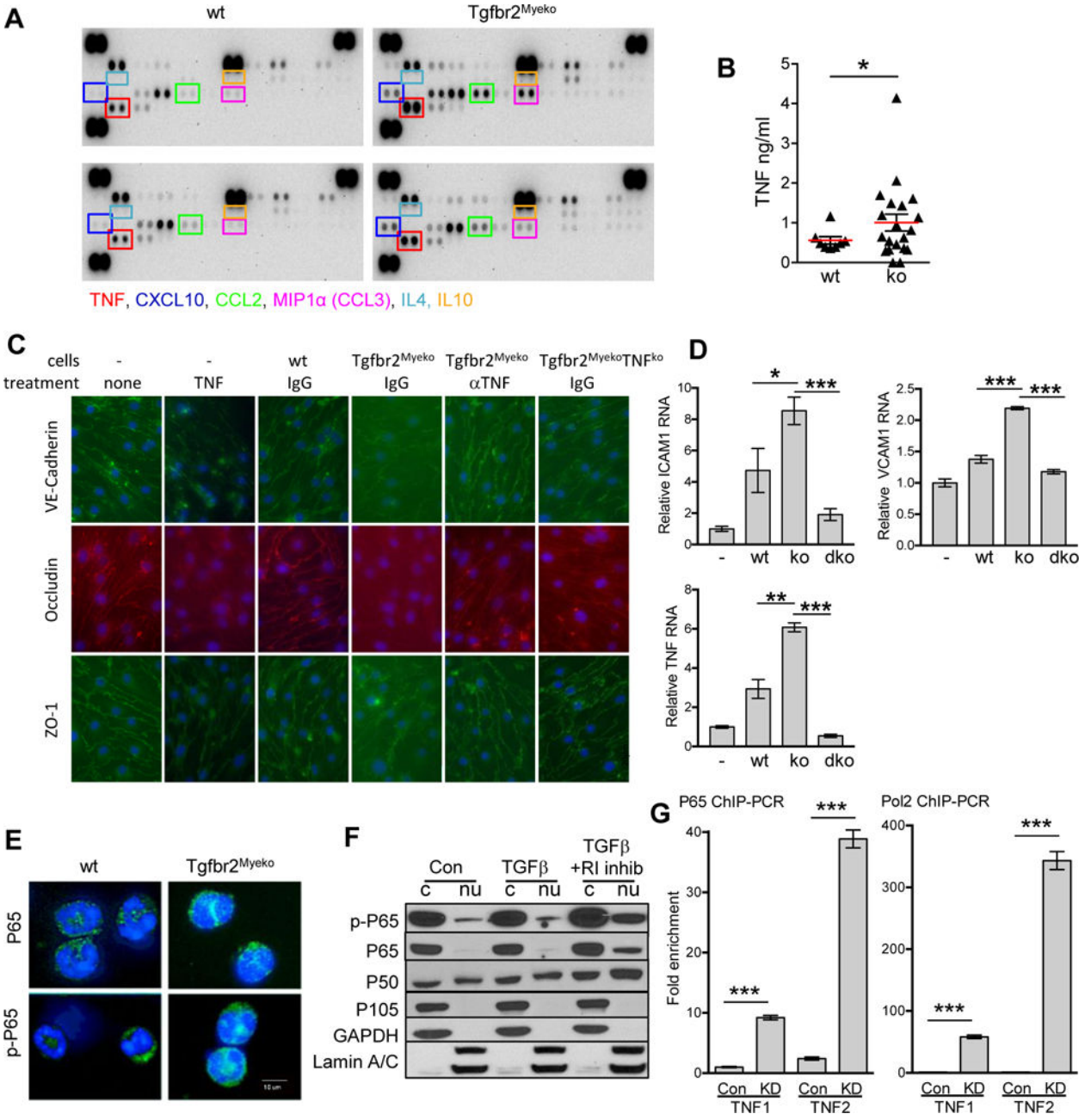

Figure 3. Increased inflammatory cytokines and mediators resulting from deletion of myeloid specific TGFβ signaling

(**A**) Cytokine antibody array of plasma from one male and one females 6 month-old pre-stroke Tgfbr2^{Myeko} mice, compared one male and one female wt (n=2 for each group). Boxed cytokines are indicated with same color text below. (**B**) TNF ELISA of plasma from 6–8 month wt and Tgfbr2^{Myeko} mixed male and female mice pre-stroke (n=10 wt and n=20 ko). (**C**) Tgfbr2^{Myeko} myeloid cells cause interruption in adherens junctions and tight junctions between co-cultured C57BL/6 mouse brain microvascular endothelial cells. Co-culture with peripheral blood myeloid cells (cells) from wt, Tgfbr2^{Myeko} or Tgfbr2^{Myeko}TNF−/− (ko) mice. Some samples were treated with TNF, aTNF neutralizing

antibody or IgG isotype antibody. Notice disruption in the endothelial junctions with TNF treatment and by Tgfbr2^{Myeko} myeloid cells. (**D**) C57BL/6 brain microvascular endothelial cells have elevated RNA for markers of endothelial damage (top panels) and TNF (lower panel) following co-culture with Tgfbr2^{Myeko} (ko) myeloid cells that can be reduced when myeloid cells lack TNF also (dko, Tgfbr2^{Myeko}TNF−/−). (**E**) Increased NFkB transcription factor subunit P65 (encoded by Rela) and active, phosphorylated P65 in myeloid cells from Tgfbr2^{Myeko} mice. P65 and p-P65 (green), nuclei (blue, Dapi). Shown are representative pictures. (**F**) Increased nuclear NFkB subunits P65 and P50 (encoded by Nfkb1) and active p-P65 upon blockade of TGFb signaling using a TbR1 (R1) inhibitor. Western blots of nuclear (nu) and cytoplasmic (c) fractions from RAW264.7 cells. (**G**) Increased binding of NFkB to the TNF promoter after knock-down (KD) of Tgfbr2. Fold enrichment of TNF from P65 Chromatin immunoprecipitation (ChIP) of RAW264.7 cells. TNF1 or TNF2 indicates two different regions in the TNF promoter (see Online Figure 3C).

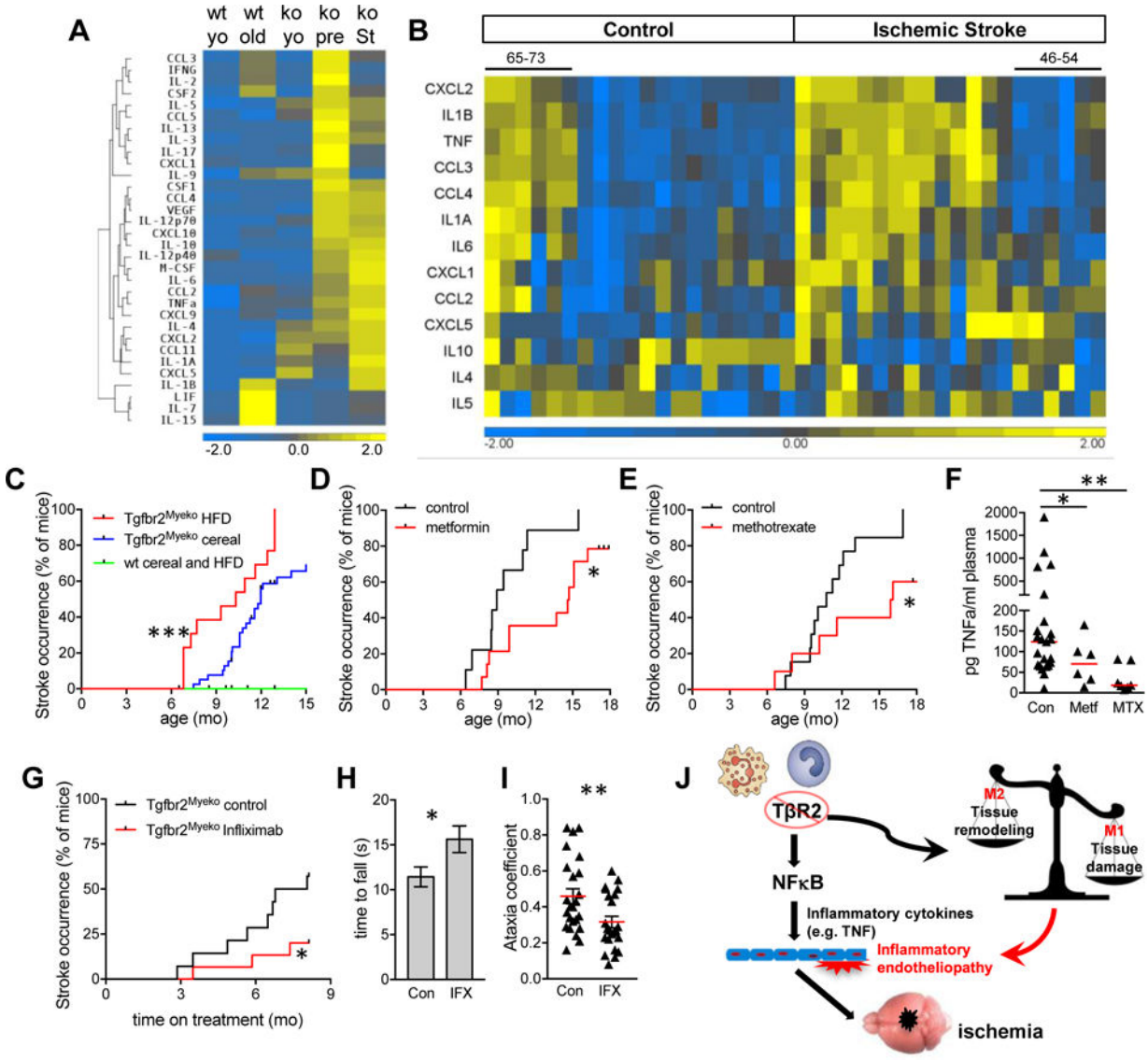

Figure 4. Increased inflammatory plasma cytokines, human correlation, and modeling risk/ prevention

(**A**) Increased inflammatory cytokines in plasma from Tgfbr2^{Myeko} mice pre-stroke, and following signs of stroke. Heatmap of plasma inflammatory cytokines from Tgfbr2^{Myeko} (ko) young (yo) and older mice pre-stroke (pre) and after signs of stroke (St), compared with young and older wt mice. Each lane is the average of all mice within each group: Young wt and ko (n=4 for each, 6 weeks old); Old wt (10 months old, n=4); Old ko pre-stroke (7–11 months old, n=17); Old ko with stroke (7–12 months old, n=10). (**B**) Increased inflammatory cytokine RNA in PBMC from patients 6 months after acute stroke, who are at high risk for subsequent stroke. Heatmap of human dataset (GSE22255) from stroke patients, compared with non-stroke controls. Each lane is one patient. 65–73 in control group are older males in this group that were more similar to the ischemic stroke group. 46–54 in the ischemic stroke group are younger females that were more similar to the control group. (**C**) High fat diet (HFD) accelerates stroke occurrence in Tgfbr2^{Myeko} mice (n=13–17). (**D**) Metformin delays

stroke occurrence in Tgfbr2^{Myeko} mice. Female Tgfbr2^{Myeko} mice were given Metformin in the chow starting at 6 months of age (n=14), compared with female Tgfbr2^{Myeko} given chow without metformin (n=14). (**E**) Methotrexate delays stroke occurrence in Tgfbr2^{Myeko} mice. Male and female Tgfbr2^{Myeko} mice that received weekly Methotrexate injections starting at 6 months of age (n=10), compared to Tgfbr2^{Myeko} mice injected with vehicle (n=13). Tick marks on survival curves are censored subjects (alive at termination of experiment). Stoke criteria are in the online methods. (**F**) Plasma TNF levels in mice treated with metformin (Metf) or methotrexate (MTX), control, n=23 males and females; metf, n=6 females; MTX, n=8 males and females. (**G**) Delayed stroke occurrence with anti-TNF therapy. Tgfbr2^{Myeko} mice were injected with Infliximab or saline (control) weekly beginning at 6–10 months of age (n=4–5 females plus 9–10 males/group). Mice in each group are age matched. **(H–I)** Infliximab improves neuromuscular function in Tgfbr2^{Myeko} mice. **(H)** Time to fall on inverted screen test for mice treated with Infliximab (IFX) or saline (Con) for 6 mo. n=4–5 females and 9–10 males per group. **(I)** Decreased Ataxia coefficient in mice treated with IFX for 8 mo (Digigait analyis, n=6 males/group). Stroke occurrence was assessed by head tilt, abnormal gait, severe trembling, paresis or paralysis, or weight loss of >20% due to severe neurological impairment. **(J)** Schematic hypothesis for inflammatory endotheliopathy and stroke in Tgfbr2^{Myeko} mice. *Tgfbr2* deletion in myeloid cells led to increased production of type 1 inflammatory cytokines including TNF, which is mediated through increased NFkB transcriptional activity. Increased circulating TNF leads to damage of cerebral blood vessels, and subsequent recruitment of inflammatory cells to the cerebral endothelium. Increased type 1 and decreased type 2 inflammatory responses lead to endothelial damage, resulting in vascular stenosis/occlusion, and ultimately ischemic stroke. ***p<0.001, **p<0.01, *p<0.05.