Glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR) drives atherosclerosis in mice and is associated with an unstable plaque phenotype and cerebrovascular events in humans

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Aims	GITR—a co-stimulatory immune checkpoint protein—is known for both its activating and regulating effects on T-cells. As atherosclerosis bears features of chronic inflammation and autoimmunity, we investigated the relevance of GITR in cardiovascular disease (CVD).
Methods and results	GITR expression was elevated in carotid endarterectomy specimens obtained from patients with cerebrovascular events ($n = 100$) compared to asymptomatic patients ($n = 93$) and correlated with parameters of plaque vulnerability, including plaque macrophage, lipid and glycophorin A content, and levels of interleukin (IL)-6, IL-12, and C-C-chemokine ligand 2. Soluble GITR levels were elevated in plasma from subjects with CVD compared to healthy controls. Plaque area in 28-week-old $Gitr^{-/-}Apoe^{-/-}$ mice was reduced, and plaques had a favourable phenotype with less macrophages, a smaller necrotic core and a thicker fibrous cap. GITR deficiency did not affect the

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lymphoid population. RNA sequencing of $Gitr^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ monocytes and macrophages revealed altered pathways of cell migration, activation, and mitochondrial function. Indeed, $Gitr^{-/-}Apoe^{-/-}$ monocytes displayed decreased integrin levels, reduced recruitment to endothelium, and produced less reactive oxygen species. Likewise, GITR-deficient macrophages produced less cytokines and had a reduced migratory capacity.

Conclusion   Our data reveal a novel role for the immune checkpoint GITR in driving myeloid cell recruitment and activation in atherosclerosis, thereby inducing plaque growth and vulnerability. In humans, elevated GITR expression in carotid plaques is associated with a vulnerable plaque phenotype and adverse cerebrovascular events. GITR has the potential to become a novel therapeutic target in atherosclerosis as it reduces myeloid cell recruitment to the arterial wall and impedes atherosclerosis progression.

Graphical Abstract
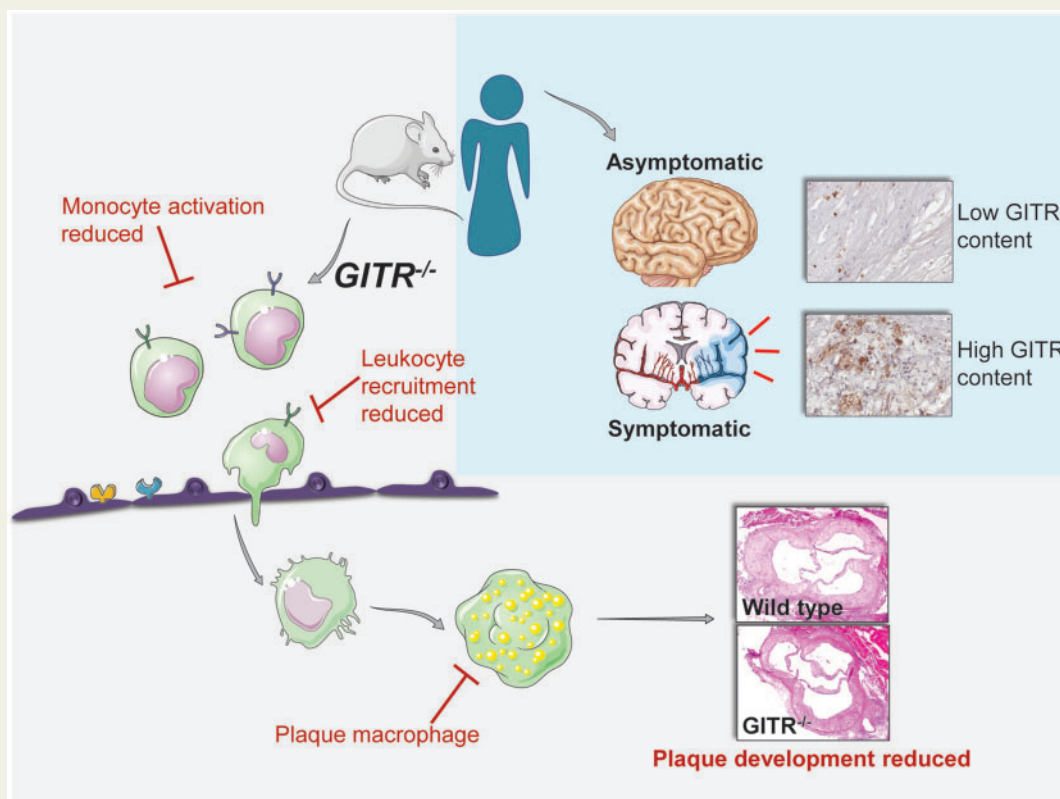
Translational perspective

With this study, we demonstrate that high levels of the co-stimulatory immune checkpoint GITR (GITR) in carotid artery atherosclerotic plaques are associated with occurrence of cerebrovascular symptoms in humans. Our experimental data establish GITR as a driving force in atherosclerosis during both plaque development and progression. Deficiency of GITR reduces monocyte activation and attenuates leucocyte recruitment, thereby slowing down plaque progression. Notably, no effects on the lymphoid population were observed. Thus, GITR may pose a promising novel therapeutic target in atherosclerosis to slow plaque progression and prevent plaque rupture, while leaving the adaptive immune system intact.

Introduction

Atherosclerosis, the underlying cause of the majority of cardiovascular disease (CVD) is a chronic, dyslipidaemia-driven inflammatory disease resulting from complex local and systemic immune reactions.[1,2] Key players modulating these immune interactions are co-stimulatory and co-inhibitory immune checkpoint proteins.[3–6]

Glucocorticoid-induced tumour necrosis factor (TNF) receptor family-related protein (GITR) or TNF-receptor superfamily-18 (TNFRSF18), a 70 kDa homodimeric glycoprotein, is a powerful co-stimulatory immune checkpoint protein that is expressed on T-cells [both regulatory (Treg) and effector T-cells],[7,8] dendritic cells (DCs), macrophages,[9] and endothelial cells.[10] Its ligand, GITR ligand (GITRL), is found on antigen-presenting cells[11] and endothelial cells.[12]

GITR/GITRL signalling regulates the extravasation and activation of innate immune cells but is better known for its role in regulating T-cell activation.[13] Increased numbers of GITR[high] Tregs and effector T-cells, as well as increased soluble plasma GITR levels, have been reported in inflammatory conditions, including rheumatoid arthritis,[14] systemic lupus erythematosus (SLE),[15–17] and Sjögren's syndrome.[18] Enhanced numbers of GITR-expressing Tregs were also reported in the endomyocardium from patients with dilated cardiomyopathy.[19]

Although the presence of GITR in human atherosclerotic plaques has been reported,[7] its functional role in atherosclerosis remains elusive. Immunohistochemical analyses of small collections of carotid endarterectomy plaques (6–11 specimens) showed that GITR is present in plaque macrophages, smooth muscle cells (SMCs), and endothelial cells,[20] solely in macrophages,[21] or solely in T-cells.[22] Kim *et al.* hypothesized GITR to be pro-atherogenic based on the observation that GITR-activation in macrophages *in vitro* appears to promote expression of TNFα and matrix metalloproteinase (MMP)-9, yet statistical tests to confirm this conclusion were not included.[21] Remarkably, our previous study using an *Apoe*^{−/−} mouse model revealed an atheroprotective function of constitutive GITRL expression by B-cells: the resulting plaques were smaller and had a more stable phenotype, a response that appeared to be driven by promotion of a Treg over an effector T-cell response.[23]

Though GITR clearly appears to be present during—and likely plays a role in—atherogenesis, the diverse data reported by previous studies demonstrate a great need to elucidate the effects of GITR in atherosclerosis. With this study, we present an extensive report on the important role of GITR in human atherosclerotic disease, as explored in endarterectomy plaques from a large patient cohort, and reveal the main underlying mechanisms using a GITR-deficient mouse model.

Methods

Methods are provided in detail in the Supplementary material online. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request, with the exception of biobank data of 'the Carotid Plaque Imaging Project' (CPIP) and the 'SUrrogate markers for Micro- and Macrovascular hard end points for Innovative diabetes Tools' (SUMMIT) cohorts due to limitations specific to each respective ethical permit.

Human studies

Endarterectomy plaques were obtained from 193 patients from the Carotid Plaque Imaging Project (CPIP, Lund University, Sweden) cohort and were snap-frozen in liquid nitrogen immediately after surgical removal. One-mm-thick portions from the most stenotic plaque region were embedded in optimal cutting medium and used for histological analyses. The remainder of the plaque was homogenized and used for protein quantification, cytokine analysis, and real-time quantitative PCR (RT-qPCR). The study fully conformed to the principles of the Declaration of Helsinki and was approved by the local ethics committee. Patient characteristics are summarized in Supplementary material online, *Table S1*.

Mouse studies

Male and female *Gitr*^{−/−}*Apoe*^{−/−}, *Apoe*^{−/−}, and *ldlr*^{−/−} mice were bred and housed at the animal facilities of the Ludwig-Maximilian's Universität München (LMU Munich, Germany) and of the Amsterdam Universitair Medische Centers (location AMC) according to institutional guidelines. All experiments were approved by the local ethical committees [TV55.2-1-54-2532-156-2015/AVD1180020171666 (17-1666-1-23)]. Mice were fed a chow diet or a Western-type diet containing 21% fat and 0.21% cholesterol (EF TD88137, ssniff-Spezialdiäten GmbH, Soest, Germany).

Results

GITR expression in human carotid plaques is associated with plaque vulnerability and cerebrovascular events

All 193 carotid endarterectomy plaques contained GITR^{+} cells that were primarily located at the base of the plaque and in the shoulder regions, with only a few GITR^{+} cells in the fibrous cap area. Double immunohistochemistry showed that GITR was predominantly located in CD68^{+} and CD11b^{+} cells (macrophages), CD31^{+} cells (endothelial cells), a subset of CD3^{+} cells (T-cells) and, to a lesser degree, in smooth muscle α-actin^{+} cells (α-SMA/differentiated vascular SMCs; *Figure 1A–D*/Supplementary material online, *Figures S1 and S2*). Flow cytometric analysis of cells from human femoral plaques confirmed this pattern and revealed the key GITR-expressing cell types to be T-cells, B-cells, and myeloid cells (Supplementary material online, *Figures S3–S5*).

Higher levels of the soluble form of GITR (sGITR) were measured in plasma from subjects with CVD compared with healthy controls (*P* < 0.0001; *Figure 1E*/Supplementary material online, *Table S2*). Furthermore, GITR immunoreactivity was significantly higher in carotid plaques obtained from patients that had cerebrovascular symptoms (transient ischaemia attack, *amaurosis fugax*, or stroke) than in those from asymptomatic patients (1.63%, IQR 0.83–3.01 vs. 0.90%, IQR 0.46–2.1; *Figure 1F–H*, Supplementary material online, *Figure S2D, E*). There was no correlation between plaque GITR content and overall stenosis degree (Spearman's rho, *r* = 0.011, *P* = 0.881). However, GITR content does correlate with the presence of CD68^{+} macrophages, lipids (Oil Red O^{+} area), cleaved collagen (type I/II neoepitope), and with necrotic core size. There was also a correlation between GITR immunoreactivity and Glycophorin A, an intraplaque haemorrhage marker. GITR content in the plaque was negatively correlated with differentiated smooth muscle cells (α-SMA[+]) as well as with collagen type III (*Figure 2*, Supplementary material online, *Table S3, Figure S6*).
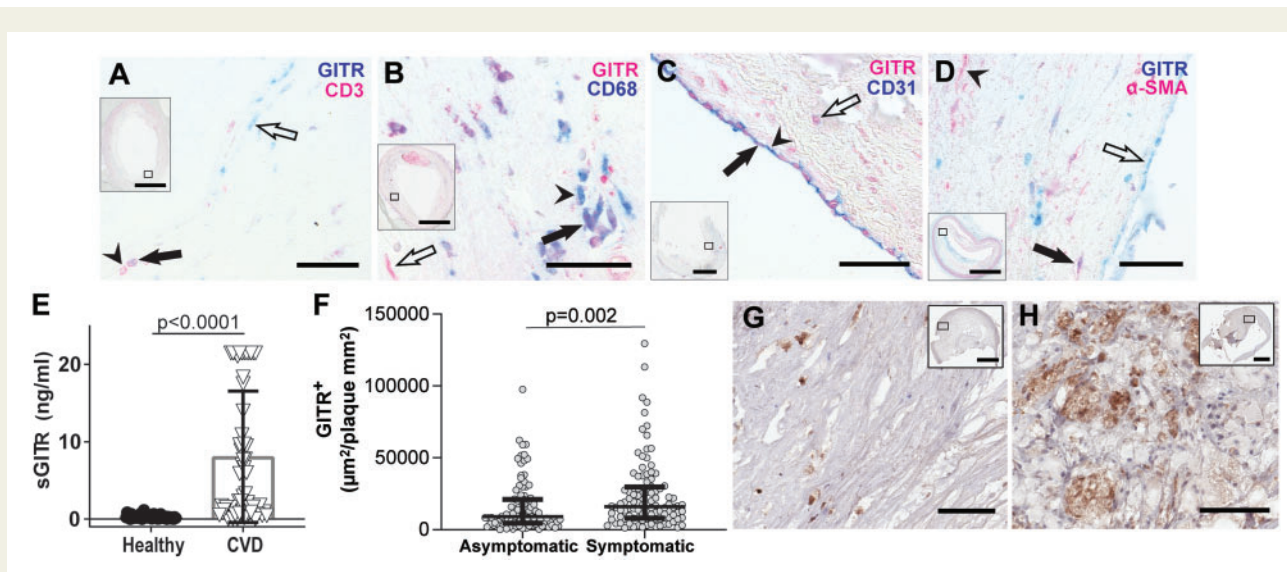
Figure 1 GITR is expressed in human carotid artery plaques and associated with cerebrovascular events. Expression of GITR (blue chromogen in *A* and *D*, pink in *B* and *C*) co-localized with expression of CD3 (*A*; pink), CD68 (*B*; blue), CD31 (*C*, blue), and with α-smooth muscle actin (*D*; pink). Co-localization is marked by black arrows, GITR expression by white arrows, and CD68/CD31/α-smooth muscle actin expression by arrowheads. Higher levels of soluble GITR (sGITR) were measured in plasma from subjects with cardiovascular disease compared with healthy controls (*E*). Expression of GITR was higher in endarterectomy plaques from symptomatic than asymptomatic patients (*F*). Representative immunohistochemical detection of GITR is shown in plaques from asymptomatic (*G*, n = 100) and symptomatic (*G*, n = 93) patients. Scale bars represent 50 µm in (*A–D*) and 100 µm in (*F, G*)—all insets are 2 mm. Statistical comparisons were performed using the unpaired *t*-test in (*E*) and the Mann–Whitney *U* test in (*F*).

Moreover, elevated GITR expression in atherosclerotic plaques correlated with the levels of the pro-inflammatory cytokines and chemokines interleukin (IL)-6, chemokine (C-C motif) ligand (CCL)-2, CCL4, and CCL5, as well as MMP1, MMP9, and tissue inhibitor of metalloproteinases-1 measured in plaque homogenates (*Table 1*, Supplementary material online, *Table S3*). Correlation was also found between GITR expression and several plaque components involved in extracellular matrix remodelling, namely fibromodulin, lumican, and urokinase receptor (uPAR; Supplementary material online, *Table S3*). Finally, GITR content also correlated with mRNA expression of (helper) T-cells (CD3$^+$ and CD4$^+$) and regulatory T-cells (FoxP3$^+$) (Supplementary material online, *Table S3*).

Taken together, these data suggest that in human atherosclerosis, increased expression of GITR is associated with a vulnerable atherosclerotic plaque phenotype that is prone to cause cerebrovascular events.

GITR-deficiency in atherosclerotic mice: general characteristics

To further investigate how GITR affects atherogenesis, *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice, and *Apoe*$^{-/-}$ littermates were generated, deficiency of GITR was confirmed (Supplementary material online, *Figure S7A–C*) and mice were aged until 28 weeks. Deficiency of GITR did not affect body weight, cholesterol, or triglyceride levels (Supplementary material online, *Figure S7D–F*), nor did it cause abnormalities in any of the organs investigated (see Supplementary material online). Haematologic parameters were similar in both genotypes (Supplementary material online, *Figure S7G–M*).

Flow cytometric analysis showed no abnormalities in the lymphoid (including B-cells, T-cells, and its subsets) and myeloid (including DCs, neutrophils, and monocytes) populations of blood and lymph nodes of *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice (Supplementary material online, *Figure S8*). In spleen, *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice displayed decreased dendritic cell and altered CD8$^+$ T-cell fractions (Supplementary material online, *Figure S9A–D*). There were no changes in T-cell content or subsets in the atherosclerotic aorta (Supplementary material online, *Figure S9E–G*). Furthermore, bone marrow haematopoietic stem and progenitor cell populations were also unaffected by GITR deficiency (Supplementary material online, *Figure S9H–J*). GITR deficiency thus does not markedly affect systemic inflammation.

GITR-deficiency in mice reduces atherosclerosis and promotes a favourable plaque phenotype

The pattern of GITR immunoreactivity in mouse atherosclerotic plaques mirrored that of human plaques, with GITR expression found in CD3$^+$ T-cells, CD31$^+$ endothelium, some α-SMA$^+$ SMCs and predominantly in CD68$^+$ and Mac2$^+$ macrophages (*Figure 3A–D*, Supplementary material online, *Figures S10–S11A–H*).

Female *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice developed smaller atherosclerotic lesions in the aortic root (*Figure 3E*) with reduced CD68$^+$ macrophage content (*Figure 3F*) and smaller necrotic core sizes (*Figure 3G*) and thicker fibrous caps compared (Supplementary material online, *Figure 11I*) to *Apoe*$^{-/-}$ littermates. This phenotype was confirmed in male mice (Supplementary material online, *Figure S11J,K*). Furthermore, aortic *cd68* expression was reduced in *Gitr*$^{-/-}$*Apoe*$^{-/-}$
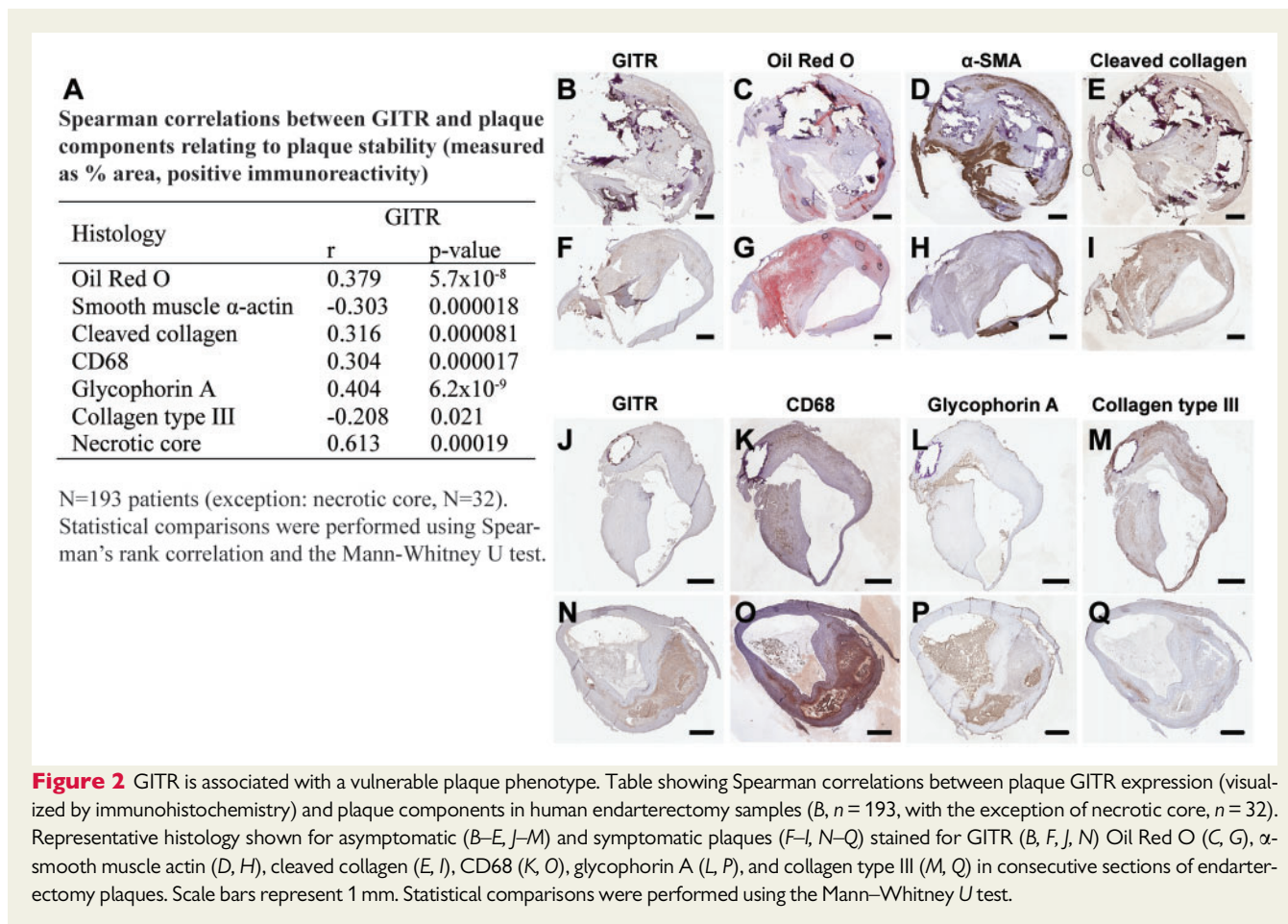
Figure 2 GITR is associated with a vulnerable plaque phenotype. Table showing Spearman correlations between plaque GITR expression (visualized by immunohistochemistry) and plaque components in human endarterectomy samples (*B, n* = 193, with the exception of necrotic core, *n* = 32). Representative histology shown for asymptomatic (*B–E, J–M*) and symptomatic plaques (*F–I, N–Q*) stained for GITR (*B, F, J, N*) Oil Red O (*C, G*), α-smooth muscle actin (*D, H*), cleaved collagen (*E, I*), CD68 (*K, O*), glycophorin A (*L, P*), and collagen type III (*M, Q*) in consecutive sections of endarterectomy plaques. Scale bars represent 1 mm. Statistical comparisons were performed using the Mann–Whitney *U* test.

compared to *Apoe*$^{-/-}$ mice (Supplementary material online, *Figure S12A*). Collagen, αSMA$^+$ SMC content, the amount of CD3$^+$ T-cells, Ki67$^+$ proliferating cells, TUNEL$^+$ apoptotic cells, intraplaque haemorrhage, and lipid content were not different (Supplementary material online, *Figure 12B–H*), although the amount of intraplaque haemorrhage tended to decrease in plaques of *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice. In order to better compare the degrees of vulnerability in *Apoe*$^{-/-}$ and *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice, a vulnerability-index was calculated.[24,25] The vulnerability indices calculated were 0.66 (SD 0.21) for *Apoe*$^{-/-}$ mice and 0.30 (SD 0.23) for *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice (*Figure 3H*). Furthermore, applying the Virmani lesion classification scheme[26] revealed a higher ratio of fibrous cap atheroma (FCA) compared to thin FCA among plaques from *Gitr*$^{-/-}$*Apoe*$^{-/-}$ compared to *Apoe*$^{-/-}$ mice (Supplementary material online, *Figure S12I*). Total gelatinase activity (mainly MMP-2 and -9) analysed in plaques by *in situ* zymography did not differ in *Gitr*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ plaques and there was no difference in aortic *mmp-2* or *-9* expression measured by qPCR (Supplementary material online, *Figure S12J–L*).

To substantiate the role of haematopoietic GITR in atherosclerosis, we generated bone marrow chimeras. In line with our results in *Gitr*$^{-/-}$*Apoe*$^{-/-}$ mice, plaque macrophage content (mac3$^+$) and necrotic core sizes were reduced, while minimal fibrous cap thickness was increased in atherosclerotic plaques from *ldlr*$^{-/-}$ mice transplanted with *Gitr*$^{-/-}$ bone marrow (*Gitr*$^{-/-}$ → *ldlr*$^{-/-}$) compared to *ldlr*$^{-/-}$ mice transplanted with *Gitr*$^{+/+}$ bone marrow (*Gitr*$^{+/+}$→

Table I   Spearman correlations between GITR (% area) and cytokines (pg/g wet weight plaque)

Cytokine	GITR	
	R	*P*-value
Interleukin (IL)-6	0.182	0.011
IL-10	-0.010	0.894
Interferon γ	0.050	0.494
Chemokine (C-C motif) ligand (CCL)-4	0.240	0.001
CCL2	0.143	0.047
Tumour necrosis factor α	0.113	0.119
Regulated on activation, normal T-cell expressed and secreted (RANTES)	0.164	0.023
sCD40L	0.076	0.294
Vascular endothelial growth factor	-0.001	0.979
Eotaxin	-0.140	0.072

n = 193 patients. Statistical comparisons were performed using Spearman's rank correlation and the Mann–Whitney *U* test.

ldlr$^{-/-}$, *P* = 0.0029, Supplementary material online, *Figure S13A–D*). In addition, *Gitr*$^{-/-}$ → *ldlr*$^{-/-}$ mice contained more fibrous FCA and less thin FCA than *Gitr*$^{+/+}$→ *ldlr*$^{-/-}$ mice. Plaque T cell content did not differ. Although plaque composition phenocopied the results of
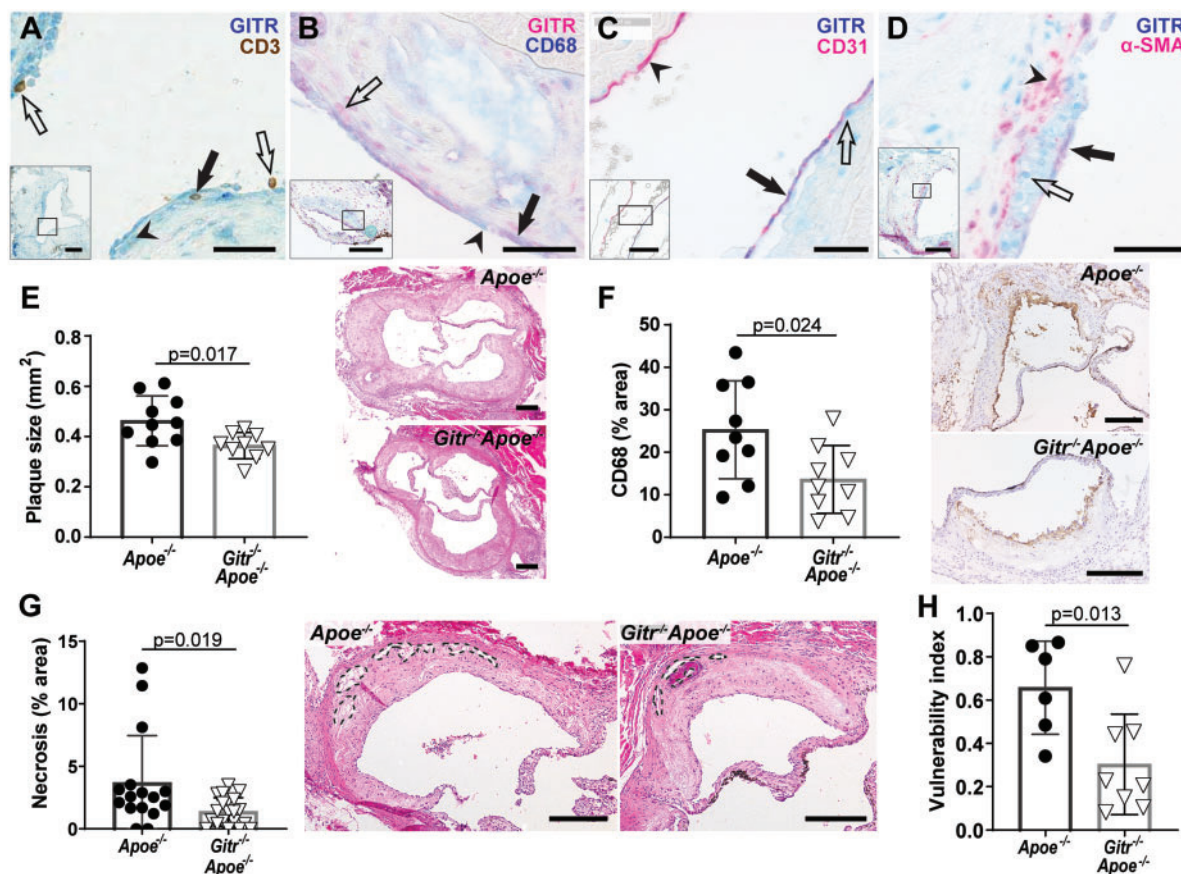
Figure 3 GITR expression and plaque phenotype in murine aortic root plaques. Expression of GITR in aortic root plaques (brown chromogen in *A*, pink in *B*, blue in *C, D*) co-localized with expression of CD3 (*A*; blue), CD68 (*B*; blue), CD31 (*C*, pink), and with α-smooth muscle actin (*D*; pink). Co-localization (dark brown in *A*, purple in *B–D*) is marked by black arrows, GITR expression by white arrows, and CD68/CD31/α-smooth muscle actin expression by arrowheads. In $Gitr^{-/-}Apoe^{-/-}$ mice aortic root plaque size (*E*; representative plaques stained by haematoxylin and eosin, $n = 10$ mice) and CD68^{+} macrophages (*F*; $n = 9$ mice) was reduced. Necrotic regions of aortic root plaques were smaller in $Gitr^{-/-}Apoe^{-/-}$ mice compared to $Apoe^{-/-}$ mice (*G*; $Apoe^{-/-}$, $n = 16$ plaques from six mice, $Gitr^{-/-}Apoe^{-/-}$, $n = 20$ plaques from eight mice). The vulnerability-index was lower in aortic root plaques of $Gitr^{-/-}Apoe^{-/-}$ than in $Apoe^{-/-}$ mice (*H*; $Apoe^{-/-}$, $n = 6$, $Gitr^{-/-}Apoe^{-/-}$, $n = 8$ mice). Scale bars represent 50 μm in (*A–D*) (with 200 μm in insets), 200 μm in (*E, F*) and 1 mm in (*G*). Analyses were performed on female mice and statistical comparisons were performed using the unpaired *t*-test.

the $Gitr^{-/-}Apoe^{-/-}$ mice, plaque area did not differ, most likely due to an increase in serum cholesterol levels, induced by the bone marrow transplant procedure (BMT) (Supplementary material online, Figure S13E–G).

GITR-deficient monocytes display impaired recruitment and limited reactive oxygen species production

The reduction in atherosclerotic plaque size, the decrease in the number of plaque macrophages in GITR-deficient mice and the strong correlation of GITR expression to plaque macrophage and lipid content in human atherosclerotic plaques prompted us to further elucidate the role of GITR in myeloid cells.

As the spleen contributes to the pool of circulating monocytes that infiltrate atherosclerotic lesions and matures into macrophages giving rise to foam cells to the same extent as monocytes/macrophages of medullary origin,[27–29] we performed RNA sequencing on

splenic classical and non-classical monocytes (as defined by CD11b^{+}Ly6G^{-}CD115^{+}Ly6C^{+} and CD11b^{+}Ly6G^{-}CD115^{+}Ly6C^{-}, respectively) obtained from $Apoe^{-/-}$ and $Gitr^{-/-}Apoe^{-/-}$ mice.

In non-classical monocytes, GITR deficiency resulted in significant down-regulation of 460 genes and up-regulation of 292 genes (Supplementary material online, Figure S14, Table SI). Ingenuity pathways analysis (IPA) canonical pathway analysis revealed that GITR deficiency affected pathways of mitochondrial function and immunity and inflammation, while IPA downstream effects analysis also indicated effects on cell homing (Figure 4A–C). In classical monocytes, the number of differentially expressed genes was only 174, and involved pathways with few common patterns (e.g. translation, cell division, melatonin degradation and cholesterol biosynthesis) (Supplementary material online, Figure S15A,B, Table SII).

Validation experiments confirmed that GITR drives mitochondrial ROS production in non-classical, but not in classical, isolated blood monocytes (Figure 4D,E). However, mitochondrial mass or

transmembrane potential were not affected by GITR deficiency (data not shown).

IPA downstream effects analysis also showed that GITR affected biological functions related to homing and trans-endothelial leucocyte migration (*Figure 4C*). Pursuing these findings, we tested whether GITR-deficient monocytes displayed hampered recruitment to the arterial wall. Carotid arteries of $Apoe^{-/-}$ and $Gitr^{-/-}Apoe^{-/-}$ mice were perfused *ex vivo* with Green CMFDA- and Deep Red-labelled bone marrow-derived leucocytes obtained from $Gitr^{-/-}Apoe^{-/-}$ or $Apoe^{-/-}$ mice, respectively. Leucocyte adhesion to the arterial wall was studied using an arterial adhesion assay and multi-photon microscopy. Significantly less $Gitr^{-/-}$ leucocytes adhered to both GITR$^+$ and GITR$^-$ endothelium compared to $Gitr^{+/+}$-derived leucocytes (*Figure 5A*). Accordingly, expression of the integrins CD11b and L-selectin (CD62L) were reduced in circulating classical and non-classical monocytes (*Figure 5B*). Similar effects were observed in splenic classical monocytes through reduced CD11a and L-selectin expression, and in non-classical monocytes from bone marrow through reduced L-selectin expression. Classical monocytes in bone marrow exhibited increased expression of CD18 (integrin β2; *Figure 5B*). GITR did not affect integrin and CD62L expression on granulocytes (*Supplementary material online, Figure S16*).

Finally, to explore whether an altered endothelium activation state may also contribute to decreased adhesion of $Gitr^{-/-}$ leucocytes, immunohistochemical staining of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) was performed on atherosclerotic aortic root lesions from the female 28-week-old cohort. Their expression was not confined to the endothelium (as visualized by positive immunoreactivity for CD31 in an adjacent tissue section) and content of both molecules was similar in plaques from $Apoe^{-/-}$ and $Gitr^{-/-}Apoe^{-/-}$ mice, both taking the whole plaque into account and in endothelial regions only (*Supplementary material online, Figure S17*).

GITR-deficient macrophages display decreased mitochondrial activation and inflammation

In a follow-up experiment, bone marrow-derived macrophages (BMDMs) of $Gitr^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ mice were cultured, matured, stimulated with the agonistic GITR antibody DTA-1 and subjected to RNA sequencing. GITR activation resulted in significant up-regulation of 487 genes and down-regulation of 164 genes (*Supplementary material online, Figure S18A, Table SIII*). IPA revealed that GITR deficiency affected pathways of immunity and inflammation, migration, and mitochondrial function (*Figure 6A,B*).

Validation experiments indeed confirmed decreased mitochondrial membrane potential (ΔΨm), mitochondrial mass, and nitric oxide (NO) production in $Gitr^{-/-}Apoe^{-/-}$ BMDMs (*Figure 6C–E*), while ROS production was unaltered (*Supplementary material online, Figure S18B*). Moreover, BMDMs from $Gitr^{-/-}$ mice migrated to a lower degree than BMDMs from GITR-expressing mice in a transwell assay (*Supplementary material online, Figure S18C*). Macrophage foam cell formation and cytokine secretion are other significant contributors to plaque burden. Though *in vitro* uptake of acetylated low-density lipoprotein, and consequently foam cell formation, was similar between BMDMs from $Gitr^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ mice (*Supplementary material online, Figure S18D*), $Gitr^{-/-}Apoe^{-/-}$

BMDMs exhibited decreased capacity for chemokine and cytokine production. Multiplex cytokine analysis revealed decreased levels of CCL3, CCL4, CXCL2, IL-6, IL-10, and IL-17A in $Gitr^{-/-}Apoe^{-/-}$ BMDMs (*Supplementary material online, Figure S18E*), and qPCR analysis showed decreased levels of *ccr5, ccl5, ccl3, Il-6*, and *Il-10* in lipopolysaccharide (LPS) and IFNγ-treated $Gitr^{-/-}Apoe^{-/-}$ BMDMs compared to $Apoe^{-/-}$ controls, while levels of *ccl2, ccl7, ccl4,* and *cxcl2* were unaltered (*Supplementary material online, Figure S18F*). GITR-deficient macrophages thus exhibited a decreased capacity for promoting an inflammatory response.

Discussion

Immune checkpoint proteins, especially co-stimulatory proteins from the TNFRSF, including CD40L-CD40, CD27-CD70, OX40L-OX40, and CD137-CD137L, are important drivers of atherosclerosis.[3,30–36] Each member impacts atherosclerosis via distinct pathways, ranging from macrophage or T-cell activation, antibody production to Treg development. Interventions targeting these molecules have shown to be promising therapeutic targets for atherosclerosis.[33,37]

In the present paper, data on human material and experimental models revealed a prominent role for the co-stimulatory immune checkpoint protein GITR in atherosclerosis. Not only was GITR expression higher in carotid plaques from patients with previous cerebrovascular symptoms, a high plaque GITR content was also associated with an inflammatory, vulnerable plaque phenotype. Moreover, we found that sGITR plasma levels had increased in patients suffering from CVD when compared to healthy controls. Via mechanistic studies we discovered that GITR is an important mediator of monocyte recruitment and macrophage migration and activation, thereby driving atherosclerosis.

The role of GITR in human pathologies has often been related to T-cells. Increased numbers of GITR-expressing Tregs are observed in dilated cardiomyopathy,[19] SLE,[15] and rheumatoid arthritis,[14] where numbers of GITR-expressing effector T-cells were also increased.[14] Surprisingly, activation of GITR via an agonistic antibody caused a decrease in Tregs and an enhanced Th1 and Th2 responses in a model of hapten-induced colitis or experimental autoimmune thyroiditis in mice,[38,39] whereas B-cell specific overexpression of GITRL promoted a Treg response, thereby delaying the onset and severity of experimental autoimmune encephalomyelitis.[40] In oncology, treatment with the agonistic GITR antibody TRX518 also reduced the amount of Tregs in patients but failed to induce a sufficient anti-tumour response. In mice, a similar pattern was seen, and GITR agonism caused cytolytic T cell exhaustion, which could be overcome by using a combination therapy with PD-1 blockade.[41] The fate of T cells upon GITR triggering thus appears to be heavily context dependent, and seems to depend on the type of disease, the effector or regulatory T-cell response, and severity of inflammation (acute vs. chronic).

Interestingly, GITR-deficient mice harboured normal T-cell and B-cell numbers. Mahmud *et al.* found that Treg development was collectively driven by TNFRSF members: while deletion of any one TNFRSF family member alone, including GITR, had only modest effects on the development of Tregs, neutralization of three or four members imposed a substantial reduction in the frequency of mature Tregs.[42] These observations may in part explain why deficiency of
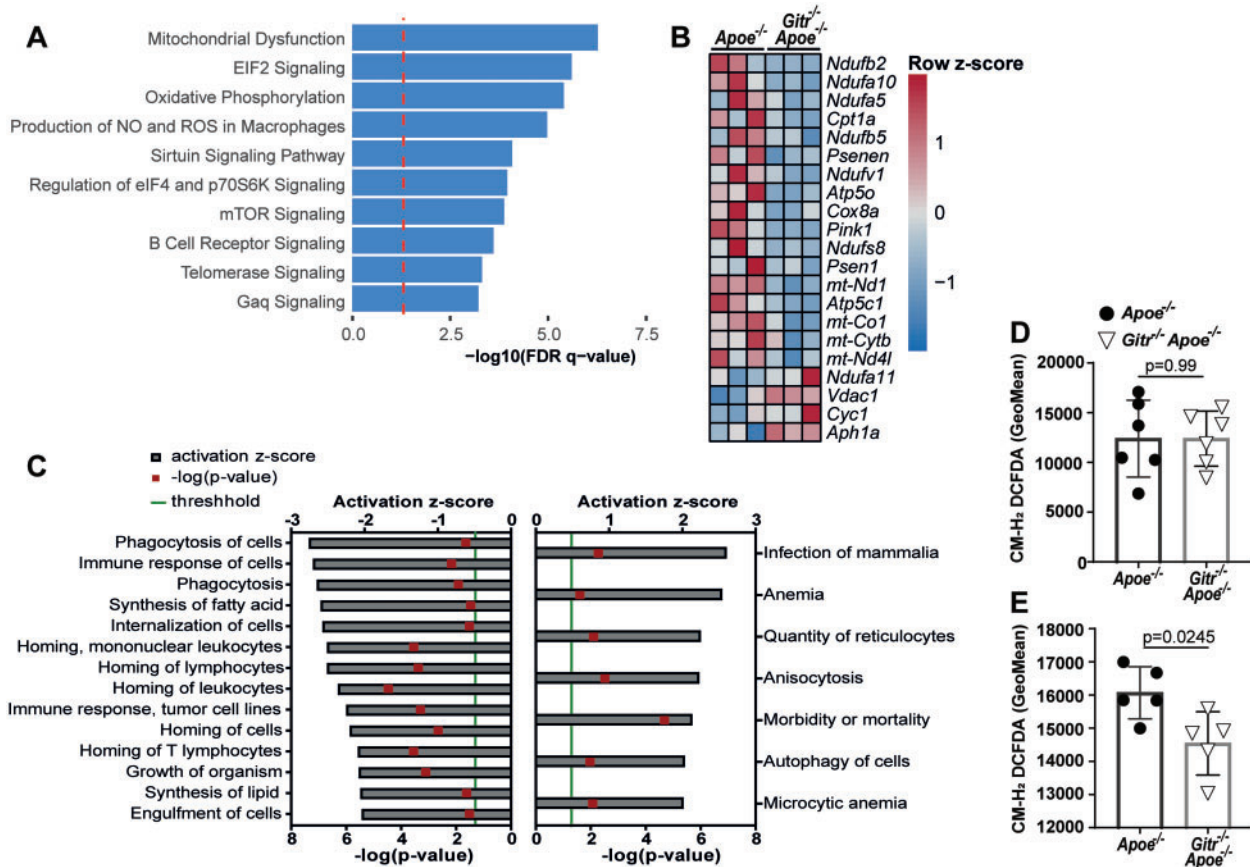
Figure 4 Mitochondrial dysfunction in non-classical monocytes $Gitr^{-/-}Apoe^{-/-}$. Dysregulated canonical pathways in $Gitr^{-/-}Apoe^{-/-}$ mice compared to $Apoe^{-/-}$ mice identified by IPA (A). Genes of the mitochondrial dysfunction pathway shown as a heatmap with each column representing one sample/mouse (B). Top affected functions in $Gitr^{-/-}Apoe^{-/-}$ mice acquired via IPA downstream effects analysis are shown in (C). Activation Z-score is calculated by the IPA software and predicts whether a specific function is increased (positive z-score) or decreased (negative z-score) based on the experimental dataset. Production of reactive oxygen species (ROS) in classical (D) and non-classical (E) blood monocytes from $Gitr^{-/-}Apoe^{-/-}$ compared to $Apoe^{-/-}$ mice as measured via flow cytometry. Statistical comparisons were performed using the unpaired t-test in (D, E). $n = 3$ mice in (A–C), and $n = 5$ replicates from a total of three mice in (D, E).

only GITR did not significantly affect T-cell subsets in our GITR-deficient $Apoe^{-/-}$ mouse model.

Although GITR was previously reported to be expressed in plaque T-cells[22] and the proportion of circulating $GITR^+Foxp3^+$ Tregs was increased by statin treatment,[43] we found that besides T-cells, GITR is also expressed in macrophages, vascular SMCs, and the endothelium of the arterial wall. We demonstrate in our study that monocyte- and macrophage-expressed GITR have a key role in driving atherosclerosis—a role that does not rule out, but seemingly outweighs parallel (and even opposite) effects that may be occurring in other cell types such as T-cells. Similar results were observed in a model of acute pleurisy and pancreatitis, where deficiency of GITR predominantly reduced the amount of macrophages and neutrophils in the lungs and pancreas.[44,45]

GITR signalling was also ascribed a central role in promoting expression of ICAM-1 and VCAM-1. GITR-deficient splenocytes adhere less to endothelial cells, and pre-treatment of the endothelium with an agonist GITR-Fc fusion protein enhanced the expression of

endothelial ICAM-1 and VCAM-1.[46] Zymosan induced shock resulted in a decrease in ICAM-1 expression in lungs and intestines of $GITR^{-/-}$ mice.[47] Macrophages obtained from rheumatoid arthritis patients that are cultured with an agonistic GITR antibody express high levels of ICAM and exhibit increased cell-cell adhesion.[48] However, we did not observe any decrease in ICAM-1 or VCAM-1 expression in plaques—in the lesion as a whole or specifically in the endothelial regions—and no decrease in leucocyte adhesion when GITR is absent in the arterial wall. Therefore, although we found expression of GITR on the endothelium, these considerations support our notion that myeloid GITR is a more important driver of atherosclerosis.

The central role of myeloid GITR in atherogenesis is also affirmed by the present study, reflected by the lack of any pronounced dysregulation of $CD4^+$ T-cells or Tregs in our GITR-deficient $Apoe^{-/-}$ mouse model. Accordingly, no difference in the plaque content of $CD3^+$ T-cells was observed. Nevertheless, in a previous study using a transgenic, dyslipidaemic mouse with B-cell-restricted
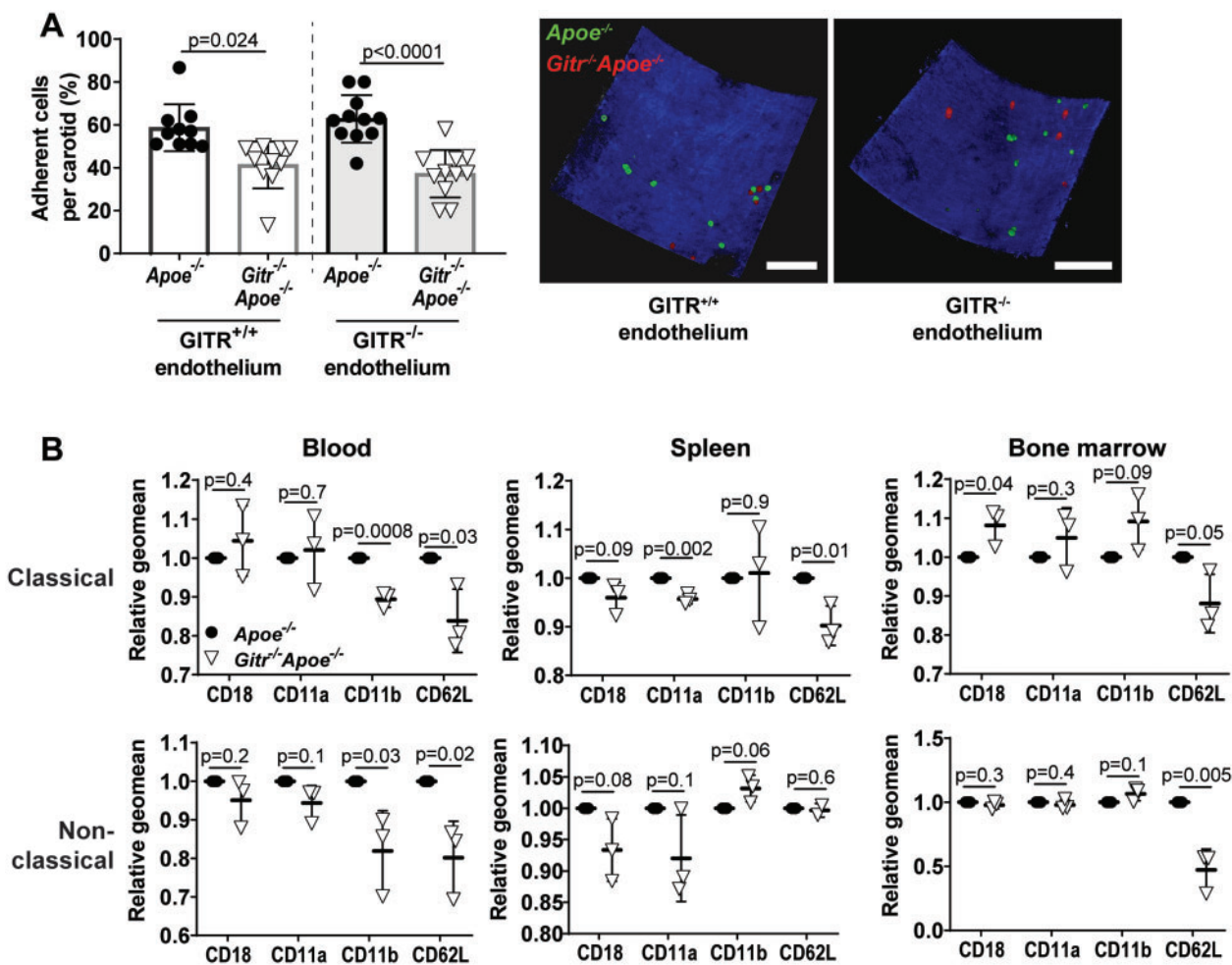
Figure 5 Altered leucocyte recruitment in GITR-deficient mice. Adherence to carotid arteries *ex vivo* was reduced in $Gitr^{-/-}Apoe^{-/-}$ leucocytes to both $GITR^{+/+}$ and $GITR^{-/-}$ endothelium (*A*, $Apoe^{-/-}$ cells are green and $Gitr^{-/-}Apoe^{-/-}$ cells are red, $n = 5$ mice/10 carotid arteries per group, experiment repeated twice). Flow cytometric analysis showing altered expression of activation markers on classical and non-classical monocytes among circulating cells in the blood, in the spleen and in the bone marrow in $Gitr^{-/-}Apoe^{-/-}$ compared to $Apoe^{-/-}$ mice (*B*, $n = 3$ mice). Scale bar represents 100 μm. Statistical comparisons were performed using Student's unpaired *t*-test (*A*) or a multiple *t*-test (*B*).

overexpression of GITRL, we found that such continuous GITRL-GITR stimulation between B- and T-cells confers atheroprotection via regulation of both effector CD4$^+$ T-cells and Tregs.[23] The present study using a model with global GITR deficiency allowed us to assess the role of GITR in atherosclerosis in a more physiological system where effects on all cell types are considered. This approach thus suggests that GITR activation by antigen-presenting cells other than B-cells, such as macrophages and DCs, could be most crucial in promoting atherosclerosis. Moreover, the opposite effects shown on plaque growth further support the idea of distinctive cell-specific mechanisms promoted/inhibited by GITR in atherogenesis. Constitutive GITR triggering on T-cells is atheroprotective, most likely through the effect of enhanced Treg presence. However, as revealed by our GITR-deficiency mouse model, GITR signalling in leucocytes is required for proper endothelial adhesion in order to initiate plaque growth, a dependency that outweighs the lack of (suppressive effects from) GITR$^+$ Tregs. Intriguingly, this facet of

GITR signalling in driving inflammation proposed by the present study may also play a role in other inflammatory conditions where GITR correlates with disease severity.

It is important to note that the murine and human plaques analysed in this study feature different stages of atherosclerosis; the murine plaques have developed over months and correspond to relatively early lesions, whereas the human plaques have grown over decades and constitute complex advanced, late stages of the disease including plaque ruptures (that do not spontaneously occur in mice). We observed a prominent role for leucocyte GITR in murine atherosclerosis strongly affecting leucocyte recruitment and their infiltration into the atherosclerotic plaque. The elevated GITR content in rupture-prone plaques in human disease may indicate an additional role for GITR within the advanced plaque. The variety of correlations with traditionally inflammatory plaque features—relating to cell types, cytokines, and plaque components—suggests a role for GITR in promoting an overall inflammatory plaque profile. Such a role
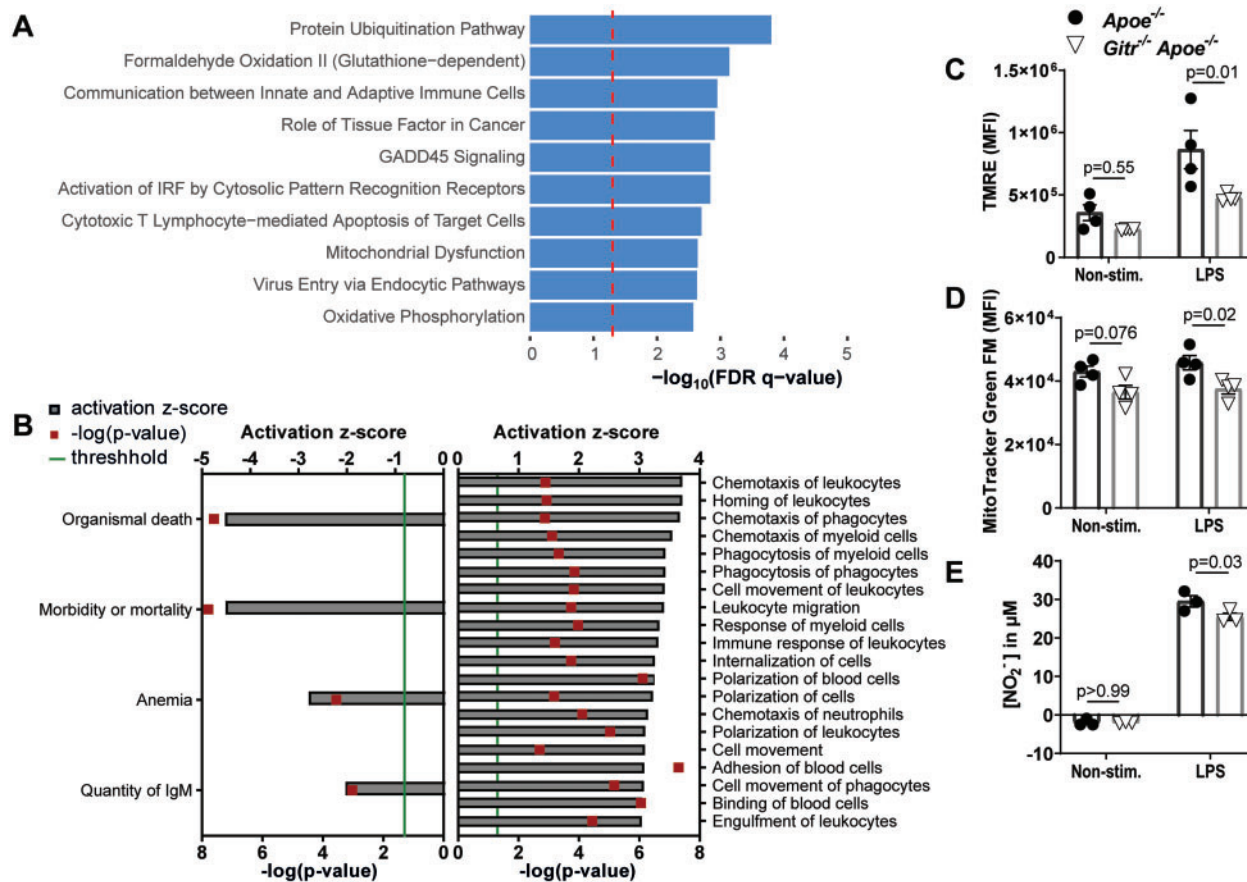
Figure 6 RNA sequencing of bone marrow-derived macrophages show mitochondrial dysfunction and altered cell migration. Dysregulated canonical pathways in $Apoe^{-/-}$ mice compared to $Gitr^{-/-}Apoe^{-/-}$ mice as identified via IPA (A). Top affected functions in DTA-stimulated GITR-deficient bone marrow-derived macrophages acquired via IPA downstream effects analysis are shown in (B). Activation z-score is calculated by the IPA software and predicts whether a specific function is increased (positive z-score) or decreased (negative z-score) based on the experimental dataset. Mitochondrial membrane potential ($\Delta\Psi$m, C), mass (D), and nitric oxide (NO) production (E) was decreased in $Gitr^{-/-}Apoe^{-/-}$ mice compared to $Apoe^{-/-}$ mice. Statistical comparisons were performed using the unpaired t-test in (C–E). $n = 3$ mice (A, B). $n = 4$ replicates in (C, D), and $n = 3$ replicates in (E) (each from three mice in total).

could be exerted via effects on activation of plaque macrophages, for example to promote lipid-uptake, cytokine production, MMP release, and to affect SMC differentiation and collagen-synthesis. It is important to note that association does not necessarily indicate causation, and we cannot exclude the possibility that GITR is upregulated in rupture-prone plaques due to stimulatory actions from another upstream destabilizing effector as part of overall increased inflammatory response or even as a defence mechanism in ongoing repair attempts. Finally, while GITRL expression patterns appear similar in humans and mice, tendencies for multimer formation differ as a result of structural differences in the human and murine orthologues which turn affect the multimerization state of GITR.[49,50] Though our results from both human and murine atherosclerosis indeed seem to imply similar expression patterns and effects of GITR triggering within the scope of the current study, minor differences of actions cannot be excluded.

In conclusion, we have identified GITR as a driving force for leucocyte recruitment in atherosclerosis and found GITR expression to be associated with plaque vulnerability and cerebrovascular symptoms in human atherosclerosis. Thus, our data identify GITR as a promising novel therapeutic target in atherosclerosis. As such, blocking GITR signalling may attenuate arterial leucocyte recruitment, in turn slowing lesion progression and promoting a plaque phenotype less prone to be rich in macrophages and destabilizing necrotic regions. As our data confirm that lack of GITR has a profound effect on monocyte activation, no effects were found on T-cell or B-cell numbers or activation status. This presents an opportunity to develop strategies for inhibiting GITR through therapeutic intervention while leaving the adaptive immune system intact. Accordingly, GITR antagonists bear an imminent potential as safe cardiovascular immunotherapies.

Supplementary material

Supplementary material is available at *European Heart Journal* online.
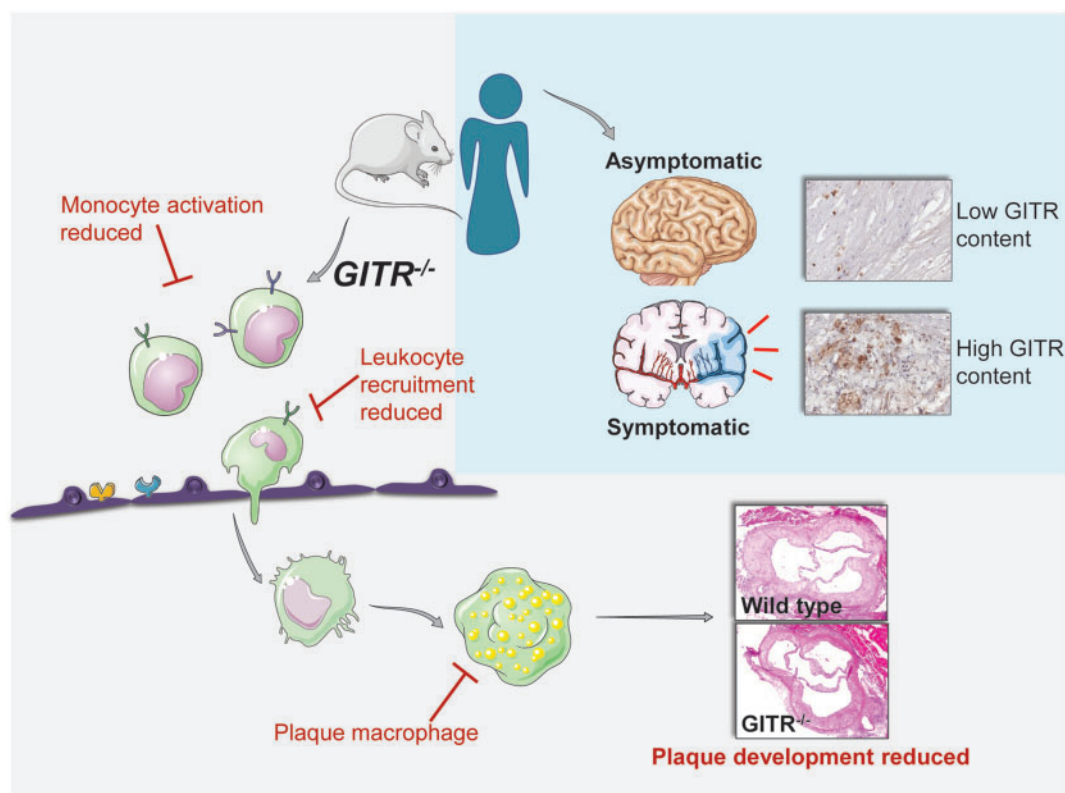
Take home figure Our data reveal a novel role for the immune checkpoint GITR in driving myeloid cell recruitment and activation in atherosclerosis, thereby inducing plaque growth and vulnerability in mice. In humans, elevated GITR expression in carotid plaques is associated with a vulnerable plaque phenotype and adverse cerebrovascular events. (This figure was created in part using templates modified from Servier Medical Art (Mountain View, CA, USA, www.servier.com, licensed under a Creative Commons Attribution 3.0 Unported Licence).

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