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Review

Glucocorticoid induced TNF receptor family-related protein (GITR) – A novel driver of atherosclerosis

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ABSTRACT

Atherosclerosis is a lipid-driven, chronic inflammatory disease. In spite of efficient lipid lowering treatments, such as statins and PCSK9 inhibitors, patients, especially those with elevated inflammatory biomarkers, still have a significant residual cardiovascular disease risk. Novel drugs targeting inflammatory mediators are needed to further reduce this residual risk.

Agonistic immune checkpoint proteins, including CD86, CD40L and CD40, have been shown to be drivers of atherosclerosis. Recently, glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR), a co-stimulatory immune checkpoint protein, was identified to be pivotal in cardiovascular disease. Cardiovascular patients have elevated soluble GITR plasma levels compared to healthy controls. Furthermore, in human carotid endarterectomy plaques, GITR expression was higher in plaques from symptomatic compared to asymptomatic patients and correlated with features of plaque vulnerability. Moreover, depleting GITR reduced atherosclerotic plaque development in mice. GITR-deficient monocytes and macrophages exhibited less inflammatory potential and reduced migratory capacity. In this review, we discuss GITR's effects on various immune cells, mechanisms, signalling pathways and finally GITR's potential as a novel drug target in atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are responsible for the largest amount of annual deaths worldwide [1]. A major underlying cause of CVD is atherosclerosis, which is characterized by chronic, low-grade inflammation that goes hand in hand with the build-up of lipid rich lesions in medium- and large sized arteries [2]. Standard treatment for cardiovascular disease patients is lowering circulating lipids with statins [3] or anti-proprotein convertase subtilisin/kexin type 9 (α-PCSK9) monoclonal antibodies [4]. However, a residual risk of ~30% remains for these patients, especially for those with increased levels of C-reactive protein (CRP) and/or other pro-inflammatory mediators [5,6]. Therefore, new therapeutic strategies are needed.

Recently, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial showed that inhibiting the interleukin (IL)-1β significantly decreased recurrence of cardiovascular events [7]. Unfortunately, canakinumab has a negative cost-effectiveness ratio and is therefore an unlikely candidate to be used as standard of care to reduce

Abbreviations: PCSK9, proprotein convertase subtilisin/kexin type 9; GITR, Glucocorticoid induced TNF receptor family-related protein; CVD, Cardiovascular disease; TNFRSF, Tumour necrosis factor receptor superfamily; TRAF, Tumour necrosis factor receptor associated factors; BMDM, bone marrow derived macrophages; ILC, innate lymphoid cells; VCAM, vascular cell adhesion molecule; ICAM, intracellular cell adhesion molecule.

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vascular inflammation [8]. Moreover, anti-IL-1β treatment caused an increase in the number of infections. The Cardiovascular Inflammation Reduction Trial (CIRT) investigated the effect of low-dose methotrexate, a wide-spectrum anti-inflammatory drug, on the reduction of the residual inflammatory risk factor in cardiovascular patients. Methotrexate did neither reduce myocardial infarction, stroke or cardiovascular death, nor altered levels of IL-1β, IL-6 and CRP. [9]. These data taught us that targeting general inflammation in CVD is not effective and that specific inflammatory pathways driving atherosclerosis need to be investigated. Colchicine is a drug with anti-inflammatory properties as it targets tubulin and thereby inhibits polymerization into microtubules, which are widely present in leukocytes. Furthermore, colchicine has an inhibitory effect on chemotaxis of leukocytes and on adhesion molecules, leading to decreased leukocyte migration [10]. In accordance, the Colchicine Cardiovascular Outcomes Trial (COLCOT) showed a reduction in cardiovascular events, however no differences in high sensitive CRP levels were identified in a subgroup containing 207 patients [11]. The low-dose colchicine trials (LoDoCo and LoDoCo2), which included patients with acute or chronic coronary symptoms and revealed that colchicine reduced the risk of cardiovascular death, myocardial infarction and ischemic stroke [12,13]. However, the LoDoCo2 trial does have its limitations as CRP or plasma lipid levels were not routinely measured. Interestingly, the effect of colchicine on the primary endpoints in the LoDoCo2 trial was larger in Australian than Dutch patients. Overall, these clinical trials prove that the immune system plays an important role in human CVD.

Atherosclerosis, an inflammatory disease, could benefit greatly from targets specifically aimed at the immune system. Inflammation is initiated when low density lipoprotein (LDL) surpasses the dysregulated endothelium barrier where it is modified LDL in the sub-endothelial space, e.g. by oxidation or glycosylated [14]. Such modifications attract monocytes, and once they have migrated towards the intima, they differentiate into macrophages. Macrophages phagocytose modified LDL, thereby forming foam cells and initial atherosclerotic lesions. Macrophages secrete pro- and anti-inflammatory cytokines, attracting other innate and adaptive immune cells, including T-and B-cells [15,16]. Excessive uptake of modified LDL induces apoptosis of foam cells, which triggers efferocytosis. However, this process is mostly ineffective, resulting in necrosis, inflammation and, thus, progression of atherosclerosis [17].

1.1. Immune checkpoints

Immune checkpoint proteins, including co-stimulatory and co-inhibitory proteins, are key regulators of the immune response, also in atherosclerosis. In a classic view, immune checkpoints are known as ‘signal 2’: after the activation of the T-cell receptor, the immune checkpoint proteins provide the second signal to either activate or inhibit the reaction. Cytokines are produced to fine-tune the immune response [18]. Nowadays, we know that these membrane proteins not only facilitate the interactions between T-cells and antigen presenting cells (APC), including dendritic cells (DCs), macrophages and B-cells, but play also a pivotal role in interactions between immune cells and endothelial cells (ECs), vascular smooth muscle cells, platelets, epithelial and cancer cells [19–21].

An important superfamily belonging to the class of immune checkpoints is the tumour necrosis factor (receptor) superfamily (TNF(*R*)SF) [20]. Several of its members, including CD40-CD40L, CD70-CD27, OX40L-OX40 and CD30L-CD30 [21–23] have been shown to play a role in atherogenesis. Remarkably, each of these co-stimulatory immune checkpoints play a different role in the progression of atherosclerosis. For instance, inhibition of CD70-CD27 aggravates atherogenesis by disrupting regulatory immune responses, whereas inhibition of CD40-CD40L or CD30L-CD30 ameliorate atherogenesis [24–26].

Recently, we investigated another immune checkpoint of this family: glucocorticoid-induced TNF receptor family-related protein (GITR; TNFRSF18). We found that GITR plays a pivotal role in atherosclerosis, both in humans and in mice. GITR was associated with a vulnerable atherosclerotic plaque phenotype and the occurrence of cerebrovascular events in humans, and its inhibition reduced atherosclerosis in a murine model [27]. As we consider GITR as a therapeutic target of high potential to combat CVD, we here review GITR's effect in different cell types and in atherosclerosis.

GITR is ubiquitously expressed on T-cells, B-cells, monocytes, DCs, macrophages, ECs as well as on epithelial cells [27–34]. The ligand for GITR, GITR-L, is expressed mainly on ECs and APCs (including immature and mature DCs, Langerhans cells and B-cells), macrophages and epithelial cells [29,35–37]. In 1997, GITR was identified in activated T-cells and was found to protect T-cells from undergoing apoptosis [22]. Soon after, a GITR knock-out (*Gitr*$^{-/-}$) mouse was generated [38]. *Gitr*$^{-/-}$ mice developed normally and were viable and fertile. Remarkably, the homeostasis of the T-cells in the spleen and lymph nodes in *Gitr*$^{-/-}$ mice were similar to their wild-type littermates. However, when challenged with an inflammatory stimulus, *Gitr*$^{-/-}$ mice had a reduced inflammatory response, which, depending on the stimulus, either resulted in a decreased innate immune response, characterized by a reduction in neutrophils and decreased levels of tumour necrosis factor alpha (TNF-α), IL-1β and inducible nitric oxide (iNOS) concentrations, as well as reduced adhesion molecules [39–41] or an altered adaptive immune response, with a downregulation in regulatory T-cells (Tregs) and memory T cells numbers, as well as a lack of mature B-cells with decreased IgG antibody and IL-10 levels.

The different responses GITR induces reflects GITR's expression on different immune cells and, thus, the capability of GITR to modulate the immune response. However, it is still unknown how GITR modulates the individual immune cell types.

2. Signalling pathway of GITR

GITR was classified as a TNFRSF member as its cytoplasmic domain contains a characteristic amino acid sequence, characterized by cysteine pseudo-repeats consisting of six disulfide-bridged cysteines (C): C1-C2, C3-C5 and C4-C6, which is preserved in all other TNFRSF members [19,22]. When GITR-L binds to GITR, adaptor proteins adhere to GITR's cytoplasmic domain and extracellular signals can be transmitted, activating the downstream signalling pathways [42–44]. Similar to other members of the TNFRSF, GITR signalling mainly occurs through tumour necrosis factor receptor associated factors (TRAFs). GITR was found to bind TRAF1 [45], TRAF2 [35,46], TRAF3 [47], TRAF4 [44] and TRAF5 [48]. Although so far, 5 different TRAFs can bind to GITR, their exact binding sites, as well as their cell-type specificity and function still need to be detailed.

Specific GITR-TRAF interactions either activate mitogen-activated protein kinase (MAPKs) – stimulating cell proliferation, differentiation, or apoptosis – or NF-κB – inducing inflammation by activating the immune response pathways in addition to cell growth, survival, or development (Fig. 1). Splenocytes stimulated with agonistic α-GITR antibodies showed activation of downstream MAPKs proteins, e.g. extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 [49]. The effect of the NF-κB mechanism was studied in a *Gitr*$^{-/-}$ pancreatitis model, where *Gitr*$^{-/-}$ mice were found to have a reduced protein concentration of IκBα in pancreatic tissue [49]. Moreover, *Gitr*$^{-/-}$ pancreatic tissue had lower NF-κB p65 protein expression compared to wild-type tissue. Furthermore, pro-apoptotic markers, e.g., Bax, and increased anti-apoptotic markers, such as Bcl-2, were reduced when GITR was deficient. Taken together, these results clearly show that GITR increases inflammation by altering the downstream signals from the canonical NF-κB pathway and, therefore, can aggravate immune responses.

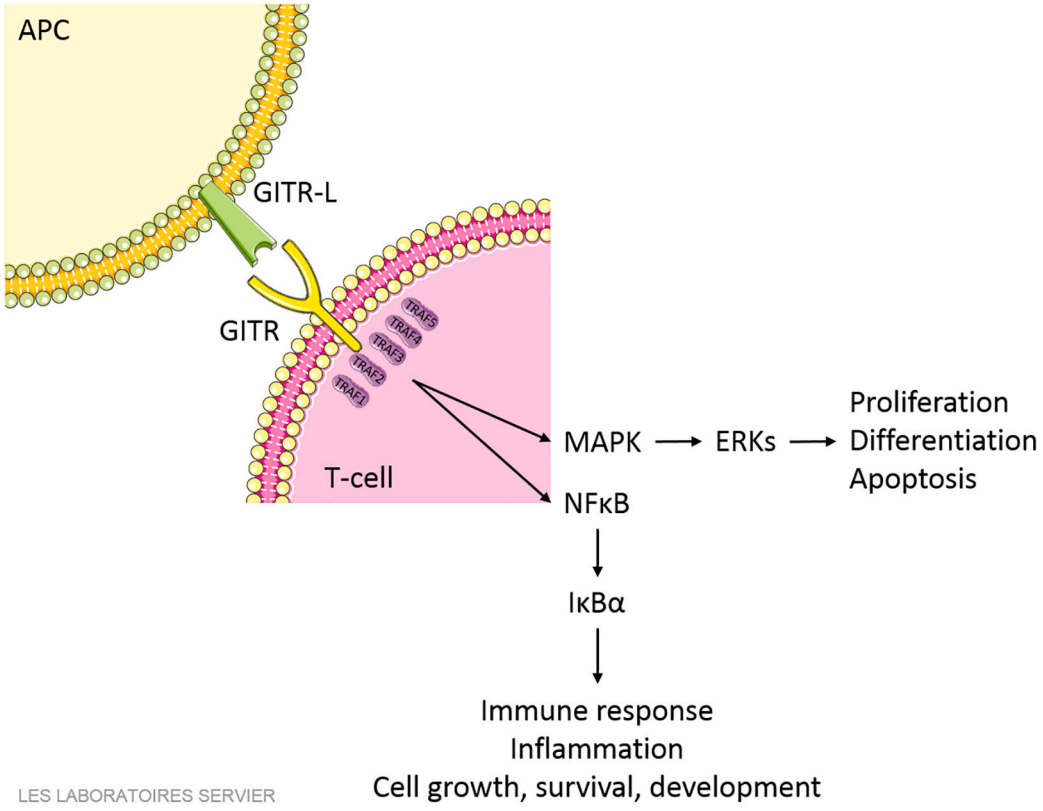

Fig. 1. The GITR-GITR-L signalling pathway. Once GITR becomes activated by its ligand, GITR-L, TRAF1, 2, 3, 4 or 5 can bind to GITR leading to the initiation of different signalling pathways: the MAPK pathway or the NF-κB pathway. The MAPK pathway induces proliferation, differentiation, and apoptosis of several cells. The NF-κB pathway stimulates immune response, inflammation and cell growth, survival, and development.

3. The effect of the GITR-GITR-L dyad in individual cell types

As mentioned previously, GITR and GITR-L are present on a large variety of cell-types, each responding differently to the activation of the GITR-GITR-L dyad. In the immune system, every immune cell has a specific role to play in inflammation and due to the wide availability of GITR or GITR-L, GITR-dependent (inter)actions of innate and the adaptive immune responses are closely linked. We here provide an overview of the effects of GITR and GITR-L on different cell types.

3.1. T-cells

T-cells are part of the adaptive immune response and help maintain the inflammatory response, as well as forming immune memory. The T-cell becomes activated when the antigen presented on MHCI or MHCII binds to the T-cell receptor (TCR). Then, the T-cell receives co-stimulatory or co-inhibitory signals from co-stimulatory or co-inhibitory immune checkpoint proteins. This signal steers the T-cell into a stimulating or inhibiting function. Lastly, T-cell activation is fine-tuned by cytokines, directing the T-cell to polarize into a T helper 1, 2, 17 cell (Th1; Th2; Th17, respectively), or regulatory T cell (Tregs) [50].

GITR is expressed on several T cell subsets but is mostly known for its expression and function in Tregs. Both human [51] and mouse T-regs express GITR, which was shown to be crucial for Treg homeostasis [52,53]. GITR could already be detected on $CD4^+CD8^-CD25^+Foxp3^-$ regulatory progenitors, indicating GITR's importance in Treg development [54]. In mature Tregs, the activation of GITR enhances proliferation [55], reflected by a decreased amount of regulatory T-cell progenitors when GITR is deficient [54]. However, $CD4^+CD25^+$ Tregs can proliferate independent of GITR when co-cultured with DCs [56]. On mature Tregs, GITR expression can be mediated through other regulatory genes, including inducible protein tyrosine phosphatase non-receptor type22 (*Ptpn22*). Its deficiency upregulates GITR expression on Tregs and promotes their expansion [53].

Recently, another type of Tregs have been found that can be identified by high GITR levels: B-cell-induced $CD4^+$ $CD25^+$ $Foxp3^-$ regulatory T-cells (Treg-of-B-cells) [57]. These cells are induced by several B-cell subsets, such as naïve splenic B2 cells, peritoneal B1 cells and Peyer's patch B-cells, and secrete IL-10, in mice protective against graft-vs-host disease, asthma and collagen-induced arthritis. GITR is, so far, one of the few markers to identify Treg-of-B-cell cells.

In addition to Tregs, GITR expression can also be detected on other T-cell subsets, such as naïve T-cells, $CD4^+$ and $CD8^+$ effector cells and (regulatory) follicular T helper cells where its expression can be attributed to specific immunological functions [58,59]. In $CD4^+$ effector T-cells, activation of GITR results in T-cell activation, reflected by an enhanced Th1/Th2 response [60], proliferation and survival [23]. GITR has similar function in $CD8^+$ T cells [35]. In addition, GITR co-stimulation was shown to affect the formation of tissue resident memory T-cells; especially the $CD8^+Ly6C^{low}CX3CR1^{low}$ tissue resident memory T-cell subset [61].

In conclusion, GITR expression, in combination with other markers can be used to distinguish between several different T-cell subsets – ranging from Tregs to Treg-of-B-cells, and effector T cells. GITR co-stimulation has a substantial impact on the development and function of Tregs, and on the activation, proliferation and polarization of various T-cell subsets (Fig. 2).

3.2. B-cells

B-cells can interact with activated T-cells through their membrane-bound Ig receptor (B-cell antigen receptor) [62]. GITR-L is expressed

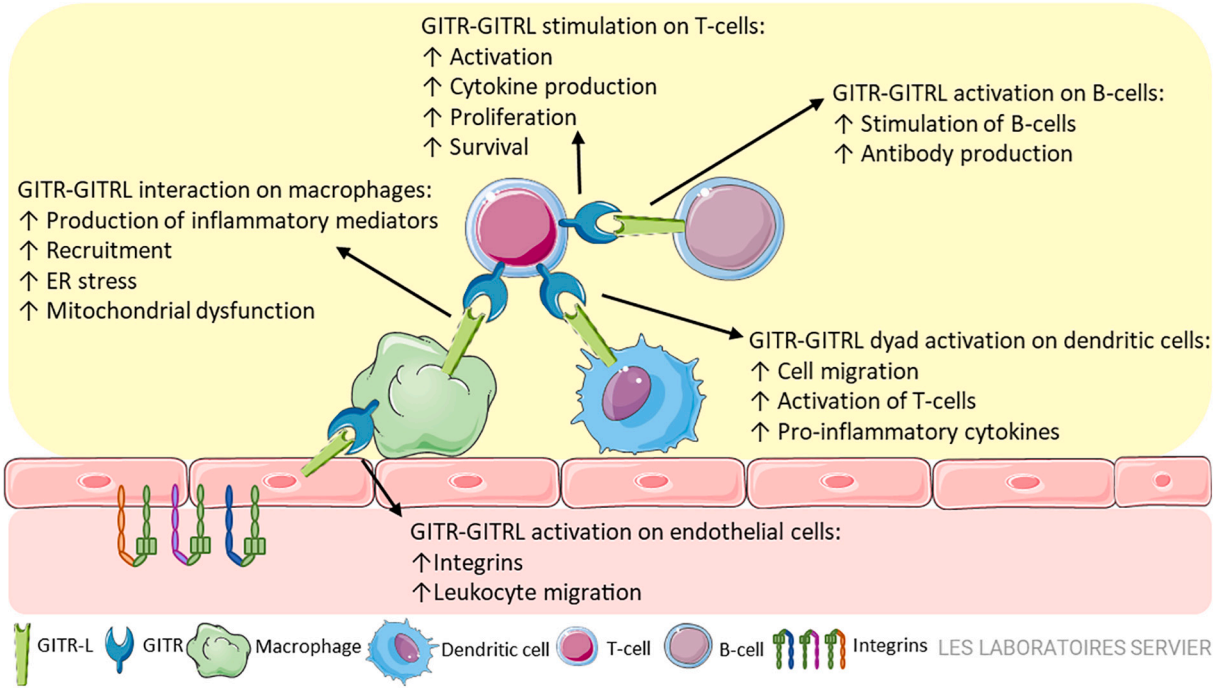

Fig. 2. Cell specific effects of GITR-GITRL interactions. Endothelial GITRL upregulates integrins, allowing for increase leukocyte migration. Interaction of the macrophage GITR-GITRL dyad induces the production of inflammatory mediators, increased recruitment and mitochondrial dysfunction and ER stress. Dendritic cell GITR-GITRL dyad activation enhances DC migration and T-cell activation, which stimulates pro-inflammatory cytokine production. GITR-GITRL interactions on T-cells activate the cell, increasing cytokine production and stimulates proliferation and cell survival. B-cell GITR-GITRL interactions activate the B-cell, which induces production of antibodies.

on B-cells [29] and GITR-L-GITR interactions between B-cells and naturally occurring Tregs stimulate the production of IgG4, most likely via an IL10 dependent mechanism [63]. Interestingly, in a different study, GITR-L expression on B-cells is needed to stimulate Treg proliferation independent of IL10 [64].

GITR can activate mature B-cells and induce antibody production (Fig. 2), as B-cells from tumour draining lymph nodes from mice treated with an agonistic GITR antibody, DTA-1, secreted more IgG1 and IgG2a into the serum [65]. However, GITR alone cannot activate resting B-cells, as its expression is low on $B220^+$ B-cells. On the other hand, when resting B-cells are stimulated with lipopolysaccharide (LPS), GITR expression increases together with CD40 expression, leading to the activation of B-cells.

3.3. Macrophages

Macrophages produce a wide range of pro-inflammatory mediators. Several studies showed that activation of GITR in these cells increased the release of various inflammatory mediators, such as TNFα, IL-6, IL-8, C-C motif chemokine ligand 2 (CCL2) and matrix metalloproteinases (MMP)-9 [43,66,67]. Even though the basal expression level of GITR on macrophages is low, its expression increases in activated monocytes and macrophages [28,67]. GITR activation in macrophages increased MMP-9 protein expression along with TNF-α and similar results were obtained in murine peritoneal and bone marrow derived macrophages (BMDMs) [28]. In human rheumatoid arthritis, GITR co-localized with $CD68^+$ macrophages in the synovial tissue, as did GITR-L [67]. Recently, we found that GITR expression in human carotid endarterectomy plaques was co-localized with $CD11b^+CD68^+$ macrophages, as well as with $Mac3^+$ macrophages in murine atherosclerotic plaques [27]. Moreover, the same study found that $Gitr^{-/-}$ monocytes and BMDMs displayed impaired cell recruitment, and that $Gitr^{-/-}$ monocytes and macrophages were protected from endoplasmic reticulum (ER) stress and mitochondrial dysfunction. This demonstrates how GITR can modulate macrophages not only by altering production of cytokines and migratory capacity, but also by affecting its mitochondrial function (Fig. 2).

Both RAW264.7 cells and peritoneal macrophages express GITR and GITR-L constitutively [66]. Macrophages stimulated with GITR agonists express a high level of iNOS, which leads to a high synthesis of nitric oxide (NO). GITR stimulation of RAW264.7 cells and thioglycollate-induced peritoneal macrophages also induced expression of cyclo-oxygenase (COX)-2 [43], an enzyme involved in production of prostaglandins, which are needed for the regulation of blood pressure and inflammation [68]. The induction of COX-2 was abrogated when macrophages were treated with a GITR blocking antibody [43]. An interesting thought is that activation of GITR signalling in the macrophage can increase COX-2 production and stimulate a pro-inflammatory reaction by producing more prostaglandin E2 (PGE_2). PGE_2 in turn induces cytokine synthesis (e.g., IL-6) to, by extension, stimulate T-cell differentiation towards a pro-inflammatory phenotype, which can propagate an immune response.

3.4. Dendritic cells

DCs are the main APCs that interact with and modulate T-cells. Multiple studies showed that GITR and GITR-L are present on both immature and mature DCs [29]. Studies reported that GITR-L is also present on plasmacytoid DCs found in the lymph nodes [37]. However, no effects of GITR-L deficiency were found on these cells. Interestingly, the migratory capacity of cutaneous DCs, including Langerhans cells, were decreased when GITR-L was blocked. This demonstrates that GITR-L can have varying effects on DCs in different tissues. Co-culturing $Gitr^{-/-}$ bone marrow derived DCs with $Gitr^{+/+}$ T-cells resulted in reduced T-cell proliferation [56]. Furthermore, when $Gitr^{-/-}$ T-cells were co-cultured with $Gitr^{-/-}$ bone marrow derived DCs, there was a lower amount of IL-2 and IL-6, while IL-10 was increased. The increase in IL-10 is unexpected, as mice that received an α-GITR agonistic antibody, as well as tumour lysate-pulsed DCs, had lower serum IL-10 concentration

than mice receiving either tumour lysate or α-GITR agonistic antibody alone [69]. This indicates that deficiency of GITR on DCs can affect proliferation and in this model decrease IL-10 which, therefore, decreased the differentiation of Tregs (Fig. 2).

3.5. Type 2 innate lymphoid cells

GITR was also detected on innate lymphoid cells (ILC)2 s, a GATA3 expressing cell type known to regulate type 2 inflammation by producing IL-4, −5, −9 and − 13 as well as affecting macrophages, granulocytes and smooth muscle cell activation [70]. Interestingly, ILC2 was shown to limit adiposity and GITR activation reduces visceral adipose tissue. Furthermore, GITR expression was found on ILC2s and when mice on a high fat diet were treated with DTA-1, glucose homeostasis was improved [71]. This study suggests that GITR could have an additional protective role in diabetes, delaying its onset and progression of obesity cooperating with alternatively activated macrophages steered this phenotype due to GITR activation. Another study showed a role for GITR on ILC2s in lung inflammation [72]. *Gitr*$^{-/-}$ mice had fewer inflammatory cells in the bronchial tissues compared to wild-type mice, which could be as deficiency of GITR attenuates migration of leukocytes [27], in addition to reduced cytokine production. The ILC2s that were present in the lung were more apoptotic in the *Gitr*$^{-/-}$ mice, suggesting a role for GITR in survival of ILC2s. Moreover, the cytokines produced, IL-5 and IL-13, were decreased in *Gitr*$^{-/-}$ ILC2s. This illustrates that GITR has similar effects on ILC2 as in T-cells i.e., reducing cytokine production and proliferation.

3.6. Endothelial cells

The GITR-GITR-L dyad also affects non-immune cells, such as ECs. GITR-L is expressed on ECs and once activated, the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), are increased [39,40]. A pro-inflammatory environment induces GITR-GITR-L activation, leading to the recruitment of leukocytes occurring via the upregulation of ICAM-1 and VCAM-1 [67,73]. Deficiency of GITR decreases the expression of ICAM-1 and P-selectin, another adhesion molecule on ECs [49] (Fig. 2). In addition, LPS-activated *Gitr*$^{-/-}$ splenocytes adhered less than wild-type splenocytes to an activated endothelial layer [73]. Activating GITR in vitro upregulated ICAM-1 and VCAM-1 expression on ECs, which lead to an increase in IL-6 driven leukocyte migration [40,73]. Recently, it also was shown that GITR affects leukocyte migration as the expression of adhesion molecules were decreased [27] (Fig. 2). These data portray the extensive reach GITR has on various (non)immune cells.

4. The GITR-GITR-L dyad in atherosclerosis

We recently identified a key role for GITR-GITRL interactions in atherosclerosis, the underlying cause of the majority of cardiovascular diseases and a disease characterized by lipid-driven inflammation.

In CVD patients, immunohistochemical analysis of atherosclerotic plaques revealed that GITR is present in plaque macrophages, SMCs, ECs and in T-cells [27,28,74]. Endarterectomy plaques from patients who experienced ipsilateral symptoms, including transient ischemic attacks or stroke, showed an increase in plaque GITR expression compared to asymptomatic patients [27]. Further correlation analysis of GITR expression with plaque characteristics revealed that GITR expression positively correlated with plaque lipid content, cleaved collagen, and necrotic core size and negatively correlated with differentiated smooth muscle cell content and with the intraplaque haemorrhage marker glycophorin A, all features of a vulnerable plaque. Furthermore, an increased concentration of soluble GITR (sGITR) was found in plasma of CVD patients compared to healthy controls [27]. As plaque GITR expression correlates with plaque vulnerability, and sGITR plasma levels can distinguish healthy vs CVD patients, GITR has the potential to become a potent biomarker to detect CVD.

The first in vivo evidence of GITR-GITR-L dyad's involvement in atherosclerosis was in a transgenic mouse model with constitutive activation of T-cell GITR through overexpression of B-cell GITR-L [75]. We found that constitutive activation of GITR signalling in T-cells resulted in a decrease in atherosclerosis, mostly due to an increase in regulatory T-cells within the atherosclerotic plaque itself, but also in its secondary lymphoid organs (Fig. 3).

Remarkably, when studying an atherosclerotic mouse model with a full-body deficiency of GITR, we observed a similar decrease in atherosclerosis. GITR deficiency resulted in smaller plaques with a reduced amount of macrophages and necrotic cores, and an increase in fibrous cap thickness, indicative of a stable atherosclerotic plaque phenotype [27]. Interestingly, in this mouse model, we could not detect any effects of GITR deficiency on T-cells, which is of great clinical interest by promising minimal deleterious immune suppressive side-effects. In fact, GITR deficiency had a profound effect on the myeloid lineage, as GITR deficient monocytes displayed decreased levels of integrins and adhered less to the arterial wall. Transcriptomic analysis of these monocytes showed that absence of GITR protected them from ER stress and mitochondrial dysfunction, in addition to inhibiting migratory and inflammatory pathways. Furthermore, transcriptomics analysis of DTA-1 stimulated *Gitr*$^{-/-}$ BMDMs also portrayed altered mitochondrial dysfunction and cell migration, these results further reflect the importance of GITR signalling in innate immune cells (Fig. 3). These data show that GITR is an interesting therapeutic target in atherosclerosis.

These data show that GITR is an interesting therapeutic target in atherosclerosis. Especially (targeted) inhibition of GITR in myeloid cells has a great potential to ameliorate atherosclerosis. Not only will inhibition of GITR block monocyte/macrophage recruitment and migration, but it will also reduce ER stress (e.g. *ros*), mitochondrial dysfunction (e.g. *Ndufb2*, *Ndufb5* and *Ndufb10*), and thereby mitigate excessive inflammatory responses. As the reactive oxygen species (ROS) production is reduced and the mitochondria are less dysregulated, the oxidative stress, and therefore the inflammation, decrease. Moreover, our study showed no T-cell effects, which suggests that GITR activation in myeloid cells drives atherosclerosis. Furthermore, as little differences in other cell types were seen, the immune suppressive side effects seem to be minimal. In addition, both GITR and sGITR can be considered as novel biomarkers, where GITR labelling could be used in PET-CT scans and sGITR can be detected in plasma. Larger studies need to be conducted where sGITR and hsCRP levels are measured to determine the correlation between these biomarkers.

4.1. The role of GITR in chronic inflammatory diseases and cancer

As GITR-GITR-L interactions mediate pivotal inflammatory responses in immune and non-immune cells, it is no surprise that GITR and GITR-L play an important role in many diseases with an inflammation-associated pathogenesis.

In a mouse model of asthma, GITR activation increased the number of eosinophils in the lungs, and induces T-cell proliferation and cytokine production [60]. GITR activation in a mouse model of arthritis aggravated the severity of the disease due to increased cytokines and Th17 cells in spleen and lymph nodes (Fig. 4)(53,78). Activation of GITR-L in an atopic dermatitis mouse model, increases the production of CCL17 and CCL27 by keratinocytes, attracting Tregs [77]. The activation induces Th2 cytokine production, e.g. IL-4 and IL-13, which are present in the skin with acute atopic dermatitis [78]. This indicates that inhibition of GITR-GITR-L dyad in asthma, arthritis and atopic dermatitis could reduce disease severity.

In cancer, GITR is considered a potent immunotherapeutic target and several agonistic compounds, such as MEDI1873, AMG-228, BMS-986156 and MK-4166, have already entered phase I/IIa clinical trials. In

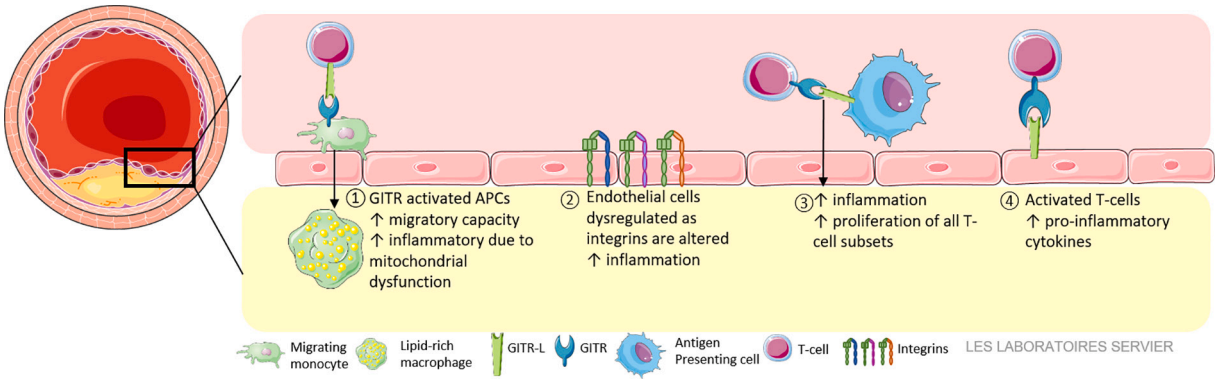

Fig. 3. In atherosclerotic plaques, GITR exerts several mechanisms of action. First, GITR can activate myeloidcells), which increases the migratory capacity and inflammatory profile of these cells. Second, endothelial cells become dysregulated as GITR affects the integrins, increasing inflammation. Third, the GITR-GITR-L dyad induces inflammation, allowing several T-cell subsets to become pro-inflammatory and produce pro-inflammatory cytokines.

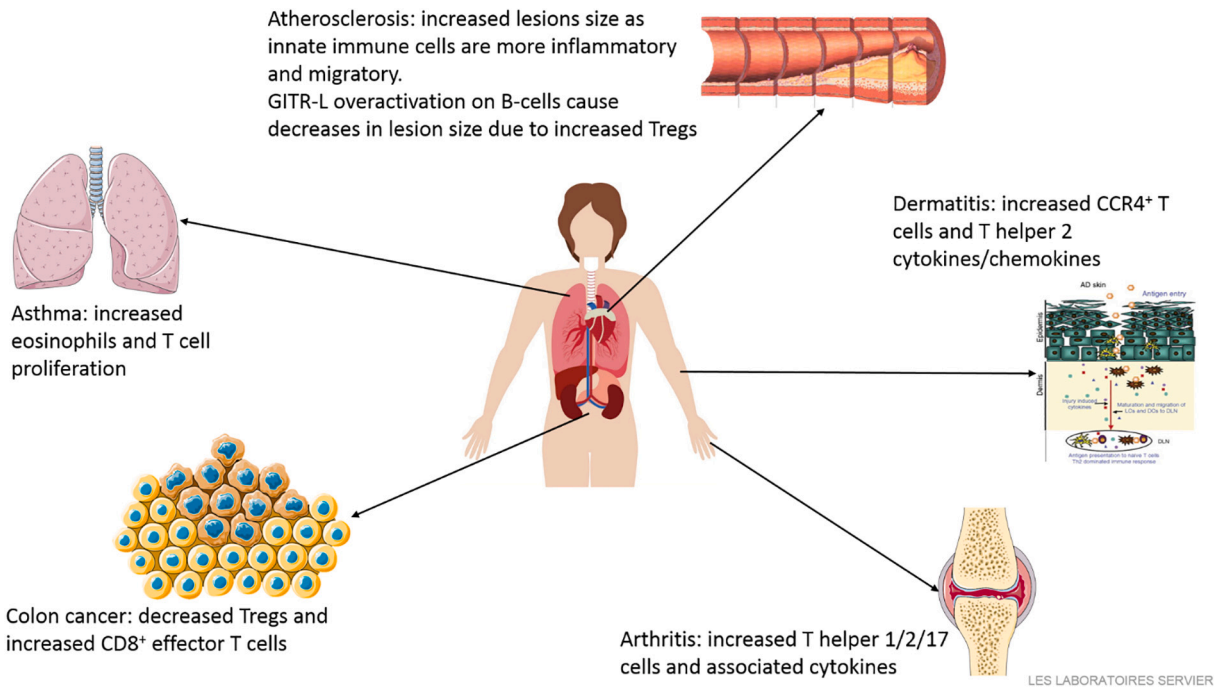

Fig. 4. The GITR-GITR-L dyad affects different diseases using different mechanisms. In atherosclerosis, GITR activation increases lesion development due to active innate immune cells, however, overexpression of GITR-L on B-cells is athero-protective due to increased numbers of regulatory T-cells (Tregs). In cancer, GITR activation decreases Treg numbers and increases effector T-cell numbers to reduce tumour growth. In asthma patients, GITR activation causes an increase in eosinophils and T-cell proliferation. Rheumatoid arthritis and atopic dermatitis are aggravated by GITR activation as it increases T helper cells.

cancer, immunotherapies are directed to activate the T cell response to facilitate the killing of tumour cells. In T cells, GITR has a dual effect, as it can promote regulatory T cell function, but at the same time, promote effector T cell function. In cancer, it has been shown that agonizing GITR especially boosts its T effector cell function, thereby facilitating tumour cell killing. The agonistic MEDI1873 compound was administered in patients with advanced solid tumours (AST) in a dose escalating study, where 500 mg was the best tolerated dosage [79]. For the AMG-228 compound, the phase I trial showed that patients with AST could tolerate up to 1200 mg. Interestingly, no T-cell activation was seen [80]. Similar results were seen with the BMS-986156 compound in combination with nivolumab in patients with AST [81]. The MK-4166 antibody was also tested in patients with AST as a monotherapy or in combination with pembrolizumab, which were received well, up to a dosage of 900 mg for MK-4166 [82]. In murine cancer models, activation of GITR through administration of the agonistic antibody DTA-1 reduced tumour progression due to increased influx of $CD8^+$ T-cells and decreased Tregs in the tumour [62,70,71]. Due to these changes in T-cell composition, intratumoural Tregs activation [83], number of apoptotic cells [84], anti-angiogenic transcription factor expression [84], and serum IFN-γ concentration [69] are increased when DTA-1 is administered. Furthermore, treatment with a GITR-L fusion protein also reduced tumour volume due to decreased Tregs and increased $CD8^+$ T-cells in the tumour as well as in spleen [85]. GITR also plays an interesting role in Chimeric Antigen Receptor (CAR) T-cell therapy, as CD3 CAR T-cells linked to the activating GITR domain increased the effectiveness of this treatment against the targeted ovarian, pancreatic and breast tumour cells [86]. Moreover, activating the GITR-GITR-L dyad in tumour infiltrating lymphocytes (TILs) from patients enhanced the proliferation of $CD4^+$ and $CD8^+$ TILs ex vivo [87]. These studies show how effective targeting GITR is in various murine cancer models.

Besides its potency as immunotherapeutic in oncology, this review shows that GITR may also have the potential to combat chronic inflammatory diseases, especially atherosclerosis. Envisioned strategies

are the development of antibodies against GITR or – a much more cost-effective alternative – via development of small molecules or antisense technology. The challenges of blocking GITR-GITRL interactions are potential immune suppressive side effects, as GITR and its ligand are expressed on a multitude of cell types. When GITR is inhibited in T cells, it can potentially impair T reg function, resulting in more inflammation, but it can also impair effector T cell function, thereby dampening inflammation. In atherosclerosis, macrophage GITR plays a pivotal role, and inhibiting GITR in macrophages reduces atherosclerosis. A favourable strategy to block the activity of agonistic immune checkpoint proteins of the TNF(R) specifically in myeloid cells, is to develop small molecules blocking the TRAF interaction site, which is often cell-type specific. Moreover, these the small molecules could then be incorporated into in HDL-nanoparticles, thereby directly targeting myeloid cells. Seijkens et al. followed such an approach. A small molecule inhibitor designed against the CD40 binding domain on TRAF6, and incorporation into HDL nanoparticles successfully blocked CD40 signalling in macrophages, without inducing B- or T cell associated immune suppressive side effects. [88]. Alternatively, the extracellular domain of GITR could be combined with a fusion protein that targets a specific cell type [89]. Other possible approaches include attaching compounds such as alum adjuvants to antagonistic GITR antibodies or molecules, thereby targeting GITR on myeloid cells [90] or by creating bispecific antibodies – attaching a macrophage specific antibody to an antagonistic GITR antibody [91].

5. Conclusion

As detailed in this review, inhibiting the GITR-GITR-L dyad in experimental inflammatory diseases has beneficial effects to ameliorate disease progression, especially for chronic low-inflammatory diseases such as atherosclerosis. Depleting GITR activation within an atherosclerotic mouse model has proven to diminish the inflammatory state of the innate immune cells as well as reducing their migratory capacity. Specific myeloid inhibition of the GITR-GITR-L dyad holds an interesting therapeutic potential, not only for CVD, but also for other chronic inflammatory diseases to reduce disease burden, but also to reduce the risk of developing CVD. By altering the specific signalling pathways of costimulatory molecules, such as GITR, can help reduce the cardiovascular inflammatory risk residue in patients that do not respond to lipid-lowering therapies.

Author contributions

Conceptualisation: LB, AS and EL; Data curation: LB; Formal analysis: LB; Funding acquisition: EL, AS, CW, DA and IG; Investigation: LB and EL; Methodology: LB and EL; Project administration: LB and EL; Resources: LB and EL; Software Microsoft Office; Supervision: EL and AS; Validation: LB and EL; Visualisation: LB and EL; Writing – original draft: LB, AS and EL; Writing – review & editing: LB and EL.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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