

Leucine-rich α-2 glycoprotein 1 (LRG1) during inflammatory complications after allogeneic stem cell transplantation and CAR-T cell therapy

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ABSTRACT

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Dr Olaf Penack; olaf.penack@charite.de **Background** Previous data indicated that the leucine-rich α -2 glycoprotein 1 (LRG1) pathway contributes to vascular dysfunction during cancer growth. Therapeutic targeting of LRG1 normalized tumor vessel dysfunction and enhanced the efficacy of anti-cancer adoptive T cell therapy. A major clinical problem after allogeneic hematopoietic stem cell transplantation (alloHSCT) and after chimeric antigen receptor (CAR) T-cell therapy is the induction of hyperinflammatory side effects, which are typically associated with severe endothelial dysfunction. **Methods** We investigated LRG1 in preclinical models and in patient samples.

Results In prospective studies, we found elevated LRG1 serum levels in patients with cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome after CAR-T-cell therapy as well as in patients with acute graft-versus-host disease (aGVHD) after alloHSCT.

In preclinical models of aGVHD, we found vasculatureassociated LRG1 upregulation as well as LRG1 pathway gene upregulation. The genetic deletion of LRG1 in alloHSCT donors and in alloHSCT recipients led to reduced clinical and histological aGVHD. In line with this, LRG1 deletion led to clinically and histologically reduced disease severity in experimental inflammatory models of colitis (dextran sulfate sodium colitis) and paw edema. LRG1 deletion reduced inflammation-related vascular leakiness, endothelial cell proliferation, and migration.

Conclusions The current data support the hypothesis that LRG1 is an attractive therapeutic target after alloHSCT and after CAR-T cell therapy for cancer because of its role in dysfunctional tumor vessels as well as in inflammatory complications.

INTRODUCTION

The vasculature in malignant tumors is dysfunctional, and normalization of vascular function is already an established therapeutic concept.¹ The field of immunotherapy for malignant tumors is rapidly expanding. Due

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Leucine-rich α -2 glycoprotein 1 (LRG1) is associated with immature and dysfunctional blood vessels that are formed during pathological angiogenesis such as in tumor growth and was found to be elevated in patients during inflammatory conditions.

WHAT THIS STUDY ADDS

⇒ This study addresses the relevance of LRG1 for severe side effects after the tumor immunotherapies allogeneic hematopoietic stem cell transplantation and CAR T cell treatment. The influence of LRG1 on vascular integrity under pathological conditions is examined in preclinical mouse models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study contributes to the search for suitable targets for the treatment of inflammatory diseases associated with dysfunctional vasculature, in particular the serious side effects graft-versus-host disease, cytokine release syndrome, and immune effector cell-associated neurotoxicity syndrome, which currently severely compromise the success of cancer immunotherapies. Furthermore, these results will trigger preclinical and clinical studies on the inhibition of LRG1 as a new therapeutic option.

to the severe endothelial dysfunction associated with immunotherapy-related inflammatory complications, the area of cancer immunotherapy has developed an increasing interest in interventions aiming at normalization of vascular dysfunction.

Allogeneic hematopoietic stem cell transplantation (alloHSCT) and chimeric antigen receptor (CAR) T-cell therapy are cellular immunotherapies that are in standard use to treat patients with hematological malignancies. Graft-versus-host disease (GVHD) after alloHSCT and cytokine release syndrome (CRS) after CAR T-cell therapy are severe systemic inflammatory complications that have been associated with vascular dysfunction. In patients undergoing alloHSCT at risk of GVHD, biomarkers of endothelial dysfunction are already in clinical use to predict survival.²⁻⁴ As an endothelial-associated initiator of aGVHD, angiogenesis has been identified.⁵ In patients after CAR T-cell therapy, recent data demonstrate that severe CRS is associated with elevated endothelial stress and activation factors in blood serum.⁶⁻⁸ CRS is also a risk factor for the second serious side effect of CAR T cell therapy, immune effector cell-associated neurotoxicity syndrome (ICANS). The increased amount of cytokines leads to endothelial activation and consequently to a permeable blood-brain barrier and cytokine-induced brain inflammation and neurotoxicity.910

The main hurdle for the development of therapeutic strategies targeting the vasculature in cancer immunotherapy is the discovery of ideal targets. There is concern over the use of anti-angiogenic strategies because the considered therapeutic targets are in parallel crucial for physiological angiogenesis and regeneration. As an example, blocking vascular endothelial growth factor signaling using antivascular endothelial growth factor receptor 2 antibodies led to the early death of alloHSCT recipients in experimental models.¹¹ Currently, suitable targets that are differentially regulated during pathological angiogenesis (eg, tumors and inflammatory diseases) and physiological angiogenesis are lacking.

The secreted glycoprotein leucine-rich α-2 glycoprotein 1 (LRG1) is a promising new target because it is induced in tumor vessels and disrupts normal vessel growth.¹² LRG1 acts by modulating the TGFB signaling pathway. In a preclinical tumor model using CD8⁺ T cells to target previously injected melanoma cells in an antigen-specific manner, the additional inhibition of LRG1 showed remarkable effects. The blocking of LRG1 with the specific 15C4 antibody resulted in a 30% improvement of tumor reduction and normalization of vascular dysfunction.¹² LRG1 is constitutively expressed by hepatocytes and neutrophils and secreted into the serum, but is upregulated in various cell types and expressed locally at sites of inflammation.¹³ LRG1 is specifically upregulated under inflammatory conditions in the eye and displays no major effects on physiological vascularization.¹⁴

Based on these data, we were interested in investigating LRG1 as a potential therapeutic target in the context of cancer immunotherapy-associated inflammation. We hypothesized that: (a) LRG1 serum levels are elevated in patients with inflammatory complications after cancer immunotherapies and (b) the lack of LRG1 leads to amelioration of inflammation in preclinical models.

MATERIAL & METHODS

Given is a summary description. Please see online supplemental file 1 for a full description of the material and methods.
 Table 1
 Patient and treatment characteristics for the aGVHD versus matched-control patients without GVHD

	aGVHD≥grade II (N=9)	No aGVHD (N=9)
Patient (gender)		()
Male	5	5
Female	4	4
Age at transplant (years)	•	•
Median	61	59
(Min. Max)	33–69	30–69
Karnofsky Performance Score		
<90	4	3
≥90	5	6
HCT Comorbidity Index		
0	3	3
1–2	4	3
>=3	2	3
Hematological malignancies		
Acute myeloid leukemia	4	5
Myelodysplastic syndrome	2	2
Acute lymphoblastic leukemia	2	1
Myeloproliferative neoplasm	1	1
Stem cell source		
Peripheral blood	9	9
Bone marrow	0	0
Donor type		
Matched related	2	2
Matched unrelated	5	5
Mismatched unrelated 9/10	2	2
Myeloablative conditioning		
No	8	8
Yes	1	1
Total body irradiation		
No	8	8
Yes	1	1
In vivo T cell depletion (ATG)		
No	0	0
Yes	9	9
GVHD prevention regimen		
CSA+MTX based	1	1
CSA+MMF based	8	8
Maximum aGVHD grade		
0	0	9
I	0	0
II	6	0
III	3	0
IV	0	0

 Table 2
 Patient and treatment characteristics for patients

 with CRS after CAR T-cell therapy

	Patients with CRS (N=27)	Patients without CRS (N=7)	
Patient (gender)			
Male	18	5	
Female	9	2	
Age at CAR-T infusion (years)			
median	59	60	
(Min, Max)	18–74	51–72	
Karnofsky Performance Score			
<90	17	7	
≥90	10	0	
Diagnosis			
Large cell B NHL	20	4	
Other high-grade NHL	7	3	
Previous autologous stem cell transplantation			
Yes	5	3	
No	22	4	
Lymphodepletion chemotherapy			
Fludarabine/cyclophosphamide	27	7	
Other	0	0	
Disease status at CAR T-cell infusion			
Chemorefractory/progressive disease	20	2	
Stable disease (no change, no response)	5	0	
Partial remission	2	4	
Complete response	0	1	
CAR T-cell product			
Yescarta (AxiCel)	15	1	
Kymriah (TisaCel)	11	6	
Breyanzi (LisoCel)	1	0	
Number of prior lines of treatment, including bridging therapy			
1	1	0	
2	6	1	
3	5	0	
≥4	15	6	
Cytokine release syndrome grade			
0	0	7	
1	3	0	
2	23	0	
3	1	0	
4	0	0	
Immune effector cell-associated neurotoxicity syndrome grade			
0	15	7	
1	7	0	
2	1	0	
3	4	0	
4	0	0	

Patient samples

We prospectively collected serum samples between March and November 2020 from all patients undergoing alloHSCT at Charité who gave their informed consent (n=70). All patients with grades II and III aGVHD who had no proven infection during the relevant time period were included in the current analysis. After selection under these criteria, the total number of patients with clinically relevant (≥grade II) aGVHD was n=9. Each patient was assigned a control patient without aGVHD. Control patients were selected based on age, gender, conditioning regimen, source of stem cells, and time point of blood collection matching the respective aGVHD patient. Further explanation can be found in online supplemental methods and table S1, S3-S5. Furthermore, blood samples from CAR T patients were collected in a second cohort. These blood samples were prospectively collected three times a week during hospitalization between August 2020 and August 2024. All patients suffered from non-Hodgkin's lymphoma and were treated with CD19-targeted CAR T cells. For the analysis, blood samples taken on days -5, +3, +6, +10, and +14 before or after CAR T infusion were used. Patient characteristics for both cohorts are described in table 1 (alloHSCT/aGVHD) and table 2 (CAR T/CRS/ICANS).

Mice and GVHD experiments

LRG1 knockout (LRG1 KO=LRG1-/-, H-2Kb) mice were a gift from Prof. John Greenwood (UCL, London, England) and were generated by the knockout mouse project (KOMP) repository (University of California, Davis, USA; http://www.komp.org/). Mice were bred as heterozygotes and wild-type (WT) littermates served as control mice for LRG1 KO mice. GVHD models have been described previously in more detail.^{15 16} For aGVHD experiments, different minor and major mismatch mouse models were used to demonstrate reproducibility and generalizability. For LP \rightarrow B6, 129 \rightarrow B6, and 129 \rightarrow LRG1 models, chemotherapy started 7 days before bone marrow transplantation (BMT) and consisted of daily intraperitoneal injections of 20 mg/kg busulfan (day -7 to day -3) and 100 mg/kg cyclophosphamide (day -7 and day -6). Recipient mice were injected intravenously with 1.5×10^7 BM cells and 2×10^6 splenic T-cells from allogeneic donor mice on day 0. For $B6 \rightarrow BALB/c$ and $LRG1 \rightarrow$ BALB/c models, 850cGy total body irradiation from a 137Cs source was performed in two split doses on the day of BMT (day 0). In the B6 \rightarrow BALB/c model, recipient mice were injected with 5×10^{6} BM cells and 10^{6} splenic T-cells from allogeneic donor mice. Timelines of GVHD development and assessment differ slightly between the models.

Experimental dextran sulfate sodium-colitis and scoring

Experimental colitis was induced as described previously.¹⁷ Disease Activity Index (DAI) scoring parameters for LRG1 KO mice and WT littermates are shown in online supplemental table S2.



Figure 1 Serum level of LRG1 in aGVHD and CRS patients. (A) LRG1 serum levels of patients with aGVHD grades II and III versus patients without aGVHD before and after alloHSCT. (B) LRG1 serum levels of patients with CRS scores I–III before and after CAR T-cell therapy. (C) LRG1 serum level of patients without CRS versus patients with CRS grades I–III on day 3 after CAR T cell infusion. (D) LRG1 serum levels of patients with ICANS scores I–III before and after CAR T-cell therapy. (E) LRG1 serum level of patients with ICANS scores I–III before and after CAR T-cell therapy. (E) LRG1 serum level of patients with ORS grades I–III on day 3 after CAR T cell infusion. Number of patients: 9 patients without ICANS versus patients with CRS grades I–III on day 3 after CAR T cell infusion. Number of patients: 9 patients with aGVHD grades II–III, 9 matched control patients without aGVHD; 27 patients with CRS (all grades) 7 patients without CRS (grade 0); 12 patients with ICANS (all grades), 21 patients without ICANS (grade 0). Error bars indicate mean±SD. Significance was tested with paired ((A, B, D) measurements of serum from the same patients at different time points) or unpaired Student's t-test (A, C, E). aGVHD, acute graft-versus-host disease; alloHSCT, allogeneic hematopoietic stem cell transplantation; CRS, cytokine release syndrome; ICANS, immune effector cell-associated neurotoxicity syndrome.

Induction of paw edema

For paw edema experiments, mice received preemptive 0.03 mg/kg buprenorphine (Temgesic; Invidia, North Chesterfield, Virginia, USA) subcutaneously 1 hour prior to the footpad injection. For the induction of paw edema, mice were transferred to an induction chamber and anesthetized with 1%-2% isoflurane in oxygen. Anesthetized mice were injected with $30\,\mu\text{L}$ of $1\%\,\lambda$ -Carrageenan (Sigma-Aldrich) in 0.9% saline into the right footpad. The left footpad served as control and was injected with 30 µL of 0.9% saline only. Footpad thickness of both paws was measured at different time points after the injection. After 3 hours or 6 hours, mice were sacrificed, and biopsies were punched out of the footpads using a 6 mm biopsy punch (Stiefel, Research Triangle Park, North Carolina, USA). Footpad biopsies and harvested spleens were weighed and embedded in Tissue-Tek O.C.T. (Sakura Finetek) for histological examinations.

In vitro assays

For the MTT, scratch, and tube formation in vitro assays, three endothelial cell lines were used. Immortalized murine cardiac endothelial cells (MCECs, generated from H-2Kb-tsA58 transgenic mice),^{18 19} murine microvascular endothelial cells from skin tissue (MuMecs, InSCREENeX, Braunschweig, Germany) and human liver endothelial cells (TMNK-1, tebu-bio, Offenbach am Main,

Germany). Murine serum for the allogeneic stimulation was obtained from LRG1 KO mice and WT littermates.

Statistics

For statistical analysis, the unpaired Student's t-test was used. When analyzing patient sera, the paired Student's t test was used to compare values from the same patients at different time points. In the case of the woundhealing assay, exploratory data analysis was performed by comparing different time points. Values are presented as mean±SD and values of p≤0.05 were considered statistically significant.

All evaluations are exploratory, and p values are, therefore, not of a confirmatory character. No adjustment was made for multiple testing. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, California, USA).

RESULTS

Serum levels of LRG1 in patients with aGVHD and CRS

As a first step to determine the clinical relevance of LRG1 in the field of cancer immunotherapy, we started by prospectively measuring LRG1 serum levels in patients after alloHSCT. Patient- and treatment characteristics are shown in table 1.



Figure 2 Expression data of whole liver tissue and isolated liver sinusoidal endothelial cells at different time points after alloHSCT. (A) Experimental schema of the LP/J \rightarrow C57BL/6 model, detailed description in the methods section. mRNA (B) and protein (C) expression in the liver and mRNA expression in isolated sinusoidal endothelial cells (D). mRNA expression of members of the TGF β pathway (E, F), the proangiogenic co-receptor ALK1 (G) and the angiostatic co-receptor ALK5 (H). For qPCR and proteomics data, we used the chemotherapy-based minor mismatch model LP \rightarrow C57BL/6 and harvested liver tissue from syngeneic and allogeneic transplanted animals at the indicated time points after HSCT. Further, we isolated liver sinusoidal endothelial cells on day +2 and day +15 after HSCT for qPCR analysis. Fold change refers to the relative expression compared with wild-type controls. Error bars indicate mean±SD. Significance was tested with unpaired Student's t-test. N=2–12 samples per group. alloHSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; BMT, bone marrow transplantation; LSECs, liver sinusoidal endothelial cells.

We collected blood samples before transplantation and at the time of acute GVHD (aGVHD) onset. We compared LRG1 serum levels of patients who developed clinically significant aGVHD (≥grade II) with matched control patients who did not develop aGVHD after alloHSCT. The LRG1 serum levels of patients with aGVHD showed a significant increase after alloHSCT, whereas LRG1 serum levels of patients without aGVHD stayed at a similar level as compared with before the transplantation (figure 1A).

For patients who received CAR T-cell therapy, we collected blood samples once before CAR T-cell treatment and regularly after CAR T-cell treatment until discharge from hospital or at the latest on day 14 after CAR T-cell infusion. Patient and treatment characteristics are shown in table 2. In line with the results, we saw in aGVHD patients, we found a significant increase in LRG1 serum levels during the onset of the inflammatory CRS reaction, usually around day +3 after CAR T-cell infusion (figure 1B). On day +3 after CAR T cell infusion, we further observed a trend but no significant increase in LRG1 levels in patients with CRS I-III compared with patients without CRS (figure 1C). On day +3 after CAR T cell infusion, the amount of LRG1 in patients with ICANS grades I-III was significantly higher than before CAR T cell infusion (figure 1D). However, there was no significant difference in LRG1 level of patients without ICANS versus patients with ICANS grades I-III on day +3 after CAR T cell infusion because patients without ICANS had elevated LRG1 levels as well (figure 1E). Apart from day +3, the other time points also showed no significant differences between CRS/ICANS grade 0 and grades I-III (online supplemental figure S1). Despite this, we can observe that LRG1 tends to increase in CRS patients with CRS grades I-III until



Figure 3 LRG1 in blood vessels and knockout of LRG1 in aGVHD. (A) Experimental schema of the 129/SV \rightarrow C57BL/6 model, detailed description in the methods section. (B) Quantification of LRG1 positive area (left) and LRG1/CD31 ratio (right) in the liver on day 15 after HSCT. (C) Representative images of increased LRG1 expression in the liver during aGVHD. (D) Quantification of LRG1 positive area (left) and LRG1/CD31 ratio (right) in the colon on day 15 after HSCT. (E) Representative images of increased LRG1 expression in the lover during aGVHD. (D) Quantification of LRG1 positive area (left) and LRG1/CD31 ratio (right) in the colon on day 15 after HSCT. (E) Representative images of increased LRG1 expression in the colon during aGVHD. For immunohistological staining (B–E) we used the chemotherapy-based minor mismatch model 129 \rightarrow C57BL/6 and harvested tissue on day 15 after HSCT. Colon and liver sections were stained against LRG1 and CD31 and counterstained with 4'6-diamino-2-phenylindole (DAPI). (B, D) n=4–5 per group. (F) Clinical aGVHD scores of B6 WT and LRG1–/– mice used as alloHSCT recipients on day 8 after transplantation. (G) Experimental schema of the 129/SV \rightarrow C57BL/6 model, detailed description in the methods section (H) Clinical GVHD scores of mice receiving either B6 WT or LRG1–/– donor cells on day 12 after transplantation. (I) Experimental schema of the B6 WT/LRG1–/– \rightarrow Balb/C model, detailed description in the methods section (H) Clinical GVHD scores of mice receiving either B6 WT or LRG1–/– \rightarrow Balb/C. (J) Quantification of vessel density in the liver. (K) n=4–5 mice per group, mouse model: LRG1–/– \rightarrow Balb/C. (J) Quantification of vessel density in the liver. (K) n=4–5 mice per group, mouse model: B6 WT/LRG1–/– \rightarrow Balb/C. (J) Quantification of vessel density in the liver. (K) n=4–5 mice per group, mouse model: B6 WT/LRG1–/– \rightarrow BDF. Error bars indicate mean±SD, significance tested by unpaired Student's t-test. aGVHD, acute graft-versus-host disease; BMT, bone marrow transplantation; HSCT,

day +6 after CAR T cell infusion, whereas LRG1 starts to decrease again from day 0 in patients without CRS (online supplemental figure S1C), indicating that lymphodepletion no longer triggers LRG1 expression after day 0. Online supplemental figure S2 shows individual curves of LRG1 levels over time of each patient sorted by CRS score.

In addition, we evaluated CRS-relevant serum markers as well as clinical parameters of patients as far as possible. These include the Endothelial Activation and Stress Index (EASIX), which predicts the occurrence of CRS and ICANS in patients after CAR T cell therapy, as well as IL-6 values (see online supplemental table S6). While the EASIX scores show no correlation with the LRG1 values, we see similarities between IL-6 and LRG1 progression in CRS patients. Already on day+3, we observed elevated IL-6 values in patients with CRS in contrast to patients without CRS. This difference reached significance on day+6 (online supplemental figure S3), thus strengthening the link between LRG1 and the development of CRS.

The results from serum analyses of aGVHD and CRS patients show that LRG1 serum levels are increased during these inflammatory complications. These



Figure 4 Role of LRG1 in DSS Colitis. (A) Experimental schema of the DSS-induced colitis model. LRG1–/– mice and B6 WT littermates were challenged with 2.5% DSS in their drinking water for 8 days. Mice were monitored for DAI score every second day. On day 9 mice were sacrificed, and organs were taken for detailed examinations. (B) Colitis DAI of LRG1–/– mice and WT littermates from day 2 to day 8. (C) Quantitative comparison of colon length and spleen weight of LRG1–/– and B6 WT on day 9 after DSS treatment start. (D) Representative pictures of colon and spleen from LRG1–/– and B6 WT mouse. (E) Histopathological score determined on H&E-stained colon sections of B6 WT and LRG1–/– mice. (F) Representative pictures of H&E staining of colon sections from B6 WT and LRG1–/– mice. Expression of CD11b (G) and CD3 (I) of B6 WT and LRG1–/– mice without colon inflammation and during experimental colitis. (H, J) Representative images of immunological staining against CD11b (H) and CD3 (J) with and without colitis. (K) Histological examination of CD31 expression and ZO1⁺ vessels in B6 WT and LRG1–/– mice during colitis. (L) Representative images of CD31 and ZO1 staining with and without colitis. (M) mRNA expression of the typical pathway gene TGF β in B6 WT and LRG1–/– mice during colitis. N=12–15 per group (B–E), n=5–9 per group (G), n=7–9 per group (I), n=4 per group (K). Error bars indicate mean±SD, significance tested by unpaired Student's t-test. DAI, Disease Activity Index; DSS, dextran sulfate sodium; WT, wildtype.

findings suggest that there is a link between LRG1 and inflammatory diseases following alloSCT and CAR-T cell therapy. We, therefore, proceeded to investigate LRG1 in preclinical disease models using LRG1 deficient versus LRG1 WT mice.

aGVHD is associated and expression of LRG1 pathway genes in the liver

To confirm the clinical relevance of LRG1 in our experimental aGVHD mouse models, we investigated the role of LRG1 expression in the development of aGVHD. We collected expression data from qPCR and proteomic analyses (ProteomeXchange; accession number PXD004606).⁵ To measure the expression in one of the main target organs of aGVHD, we used liver tissue harvested from an MHC-matched, minor histocompatibility antigen mismatched murine alloHSCT model as described previously¹⁵ ¹⁶ and shown in figure 2A. Using whole liver tissue, we found a significant increase in mRNA expression of LRG1

on day 2 and day 15 after alloHSCT (figure 2B), indicating a potential role for LRG1 during the initiation phase of aGVHD as well as during the acute phase of aGVHD. Further, proteomic analysis showed significantly higher LRG1 levels in alloHSCT recipients as compared with syngeneic transplanted controls (figure 2C). To specifically check the expression of LRG1 in endothelial cells, we FACS-sorted liver sinusoidal endothelial cells (LSECs) out of whole liver tissue and used the isolated LSECs for qPCR analyses. Again, we found significantly higher LRG1 levels in allogeneic transplanted mice compared with syngeneic controls on day 2 and day 15 after alloHSCT (figure 2D). Because LRG1 modulates transforming growth factor beta (TGF- β) signaling,¹¹ we also examined expression levels of TGF- β (figure 2E) and TGF- β receptor 2 (TGF- β R2) (figure 2F). For both genes, we found elevated expression levels in alloHSCT recipients compared with syngeneic controls. Subsequently,



Figure 5 Influence of LRG1 on local inflammation. (A) Experimental schema of the paw edema model of local inflammation. LRG1–/– mice and B6 wild-type (WT) littermates were injected with a 1% carrageenan solution into one footpad and 0.9% saline into the other footpad. Footpad swelling was determined by measuring footpad thickness every hour (indicated by the ruler), using the footpad thickness before injection as a baseline (B) Amount of increase in paw thickness of the footpad injected with carrageenan in WT and KO mice. (C) Extent of the increase in paw thickness excluding the swelling of the control foot injected with NaCl. (D) Sections from footpad biopsies were stained for CD31 and analyzed with ImageJ. (E, F) The additional in vivo Evans blue assay provided information about the vascular integrity on local inflammation. 3 hours after carrageenan injection, mice were intravenously injected with Evans blue, and punches of footpads were taken 30 min later to determine the amount of extravasated Evans blue into the vessel surrounding tissue. n=25–27 per group (B, C), n=5 per group (D), n=11–14 per group (E, F). Error bars indicate mean±SD, significance tested by unpaired Student's t-test.

we analyzed the expression of the two coreceptors, type 1 and type 5 Activin A Receptor-like kinases (ALK1 and ALK5). In endothelial cells, ALK1 is described as being essential for activation of the proangiogenic TGF-B R2-Smad 1,5.8 signaling pathway and ALK5 for the angiostatic TGF- β R2-Smad 2,3 signaling pathway. We found significantly increased levels of ALK1 in allogeneic transplanted mice during the acute phase of aGVHD (figure 2G). ALK5 showed a significantly higher expression in allogeneic transplanted mice on day 2 post-transplantation (initiation of aGVHD) but no difference during the acute phase GVHD (figure 2H), fitting with the hypothesis that ALK5 is needed for complex formation with ALK1. Using a specific reporter assay, we also determined the signaling competence of TGF- β and BMP in the serum of syngeneic and allogeneic transplanted mice but found no significant differences (online supplemental figure S4).

As the number of samples in the individual experiments was relatively small, no clear conclusion can be drawn. Nevertheless, we see trends in LRG1 and the factors of the TGF- β signaling pathway that point to increased expression during aGVHD.

LRG1 is associated with blood vessels and knockout of LRG1 ameliorates experimental aGVHD

To determine the importance of LRG1 in the vasculature during aGVHD, we used liver and colon tissue from our minor mismatch mouse model (figure 3A) for the histological examination of vessel-associated LRG1. In liver tissue, we found a significantly higher expression of LRG1 (figure 3B, left) and a significantly higher expression of LRG1 in endothelial cells (LRG1+/CD31+cells) (figure 3B, right) in alloHSCT recipients compared with syngeneic controls. As shown in figure 3C, we observed coexpression of LRG1 and CD31 in liver sections of allogeneic and syngeneic transplanted mice.

In the colon, we also found a significant increase in LRG1 (figure 3D, left) as well as in LRG1+/CD31+ratio (figure 3D, right) during aGVHD compared with syngeneic controls without aGVHD. Figure 3E shows examples of LRG1 and CD31 staining in colon tissue of syngeneic and allogeneic transplanted recipients. After confirming endothelial expression of LRG1 during aGVHD, we used LRG1 knockout mice as recipients (figure 3G) or as donors (figure 3I) for alloHSCT. The use of recipient LRG1 deficient cells allows us to investigate the effect of



Figure 6 Impact of LRG1 on endothelial cell behavior. (A) Freshly isolated liver sinusoidal endothelial cells were stained for the endothelial-specific markers CD31 and VCAM. (B–D) Migration rate of endothelial cells during wound closure with addition of LRG1–/– and B6 WT serum to the growth medium. n=3 runs per group for each assay. Error bars indicate mean±SD, significance tested by unpaired Student's t-test. MCECs, murine cardiac endothelial cells; WT, wild-type.

LRG1 in the non-hematopoietic system, whereas the use of LRG1 deficient donor cells enables the analyses of LRG1 in the hematopoietic system on GVHD. We found significantly reduced clinical aGVHD scores in LRG1 knockout recipients compared with B6 WT recipients (figure 3F). Also, in the other setting, using LRG1 mice as alloHSCT donors, we found significantly reduced clinical aGVHD scores in recipient mice of LRG1 knockout donor cells (figure 3H). Taken together, we conclude that LRG1 is expressed in blood vessels and that knockout of LRG1 either on the donor side or the recipient side ameliorates experimental aGVHD.

To analyze if the impact of LRG1 on aGVHD regulation was mainly mediated by its effect on pathological angiogenesis, we quantified endothelial cells in the liver of mice receiving LRG1 WT or knockout donor cells (figure 3K) via section immunofluorescence staining against CD31. Unexpectedly, we found that vessel density was not statistically different in recipients of LRG1-/alloHSCTs versus WT alloHSCTs (figure 3]). Therefore, we speculated that the LRG1 effect on the endothelium during inflammation may be mediated by its effect on important endothelial processes during inflammation, for example, leakiness for inflammatory cells. We subsequently performed experiments in alternative inflammatory disease models that allowed us a complete deletion of LRG1, such as DSS (dextran sulfate sodium) induced colitis and paw edema to further investigate this aspect.

Knockout of LRG1 ameliorates inflammation in experimental DSS-induced colitis

Since LRG1 -/- mice are only available on the C57B6 background, we could not completely remove LRG1 in our aGVHD models; instead, it was still present on the donor or recipient side. As a next step, we proceeded to determine the role of LRG1 in another inflammatory model with known vascular involvement that allowed us to completely delete LRG1.²⁰ We used a mouse model of experimental DSS-induced colitis as described previously⁵ and shown schematically in figure 4A. As seen in figure 4B, LRG1–/– mice developed a significantly lower DAI than B6 WT littermates during the whole period of disease progression up to day 8 after colitis induction. As further signs of decreased inflammation, we found significantly lower spleen weight and greater colon lengths in DSS-treated LRG1-/- mice compared with B6 WT mice (figure 4C,D). To confirm the impact of reduced inflammation on organ damage, H&E stains were obtained and histological scores were determined. As shown by quantification (figure 4E) and illustrated in images (figure 4F), DSS-induced LRG1-/- mice showed significantly less inflammation and organ damage than B6 WT littermates.

We then investigated immune cell distribution and endothelial alterations. Based on our hypothesis that LRG1 is only relevant under pathological conditions, we measured the expression of the immune cell marker CD11b and CD3 both in untreated animals without inflammation and in animals with DSS-induced colitis. As expected, we see no differences in the expression of CD11b and CD3 in animals without colitis (figure 4G,I, left). Representative images of staining are shown in figure 4H,J. However, we found significantly less CD3 expression in the colon of LRG1 -/- mice during colitis, indicating reduced colonic inflammation in LRG1-/mice during experimental colitis (figure 4I, right). To examine blood vessels both quantitatively and functionally, we stained colon sections for the vessel marker CD31 and the tight junction marker ZO-1. Again, we could not detect a difference in CD31 expression between WT mice and LRG1-/- mice without inflammation. On the induction of DSS-colitis, we found a non-significant tendency of reduced vascular density in LRG1-/- mice (figure 4K, upper panel). The ratio of the tight junction marker ZO1 to CD31 showed the same trend, indicating that the expression of ZO-1 does not differ between the two groups (figure 4G, lower panel). Figure 4L represents images of CD31 and ZO-1 co-staining in LRG1 WT and LRG1-/- mice with and without colon inflammation.

To confirm the impact of the TGF- β signaling pathway, we further checked the mRNA expression of TGF- β in the colon during DSS-colitis. Here, we found a nearly significant trend toward a higher expression of TGF- β in LRG1–/– mice compared with B6 WT mice (figure 4M), possibly compensating for the lack of LRG1. In conclusion, we found less colonic inflammation in LRG1–/– mice during DSS-colitis, indicated by lower clinical colitis scores and histological scores. Results from immuno-histological staining need to be strengthened to draw a clear conclusion. However, we found tendencies toward reduced CD3 expression and less vessel density in LRG1–/– mice.

Knockout of *Lrg1* ameliorates inflammation in experimental paw edema

A common model that allows the detailed investigation of a rapid localized inflammatory reaction is experimental paw edema. In this model, an inflammatory reaction is initiated by injecting polysaccharide λ -carrageenan into one footpad to induce an inflammatory response that leads to swelling of the footpad, which peaks 3-5 hours after the injection. The other footpad is injected with a solution of sodium chloride and serves as control. The carrageenan-induced swelling of the footpad correlates with the severity of the inflammation (figure 5A). As seen in figure 5B, we found 3 hours after the injection significantly thicker footpads in the carrageenan-injected footpad of B6 WT mice than of LRG1-/- mice. The same significant result was obtained when the swelling of the control footpad was subtracted from the swelling of the carrageenan-injected footpad (figure 5C).

As with the other inflammation models described before, we next examined vessel density and functionality of B6 WT mice versus LRG1-/- mice during the local inflammatory reaction. Sections of footpads were stained for the vessel marker CD31. As seen before in models of aGVHD and DSS-induced colitis, there was no difference in CD31 expression in footpads of B6 WT mice and LRG1–/– mice 3 hours after carrageenan injection (figure 5D).

Since our results from previous experiments in other mouse models using LRG1-/- mice pointed toward impairments in vessel functionality rather than in vessel density, we decided to perform the in vivo Evans blue assay in the paw edema model in order to investigate vascular permeability. 30 min after the intravenous injection of Evans blue, footpad punches were collected, and the amount of extravasated Evans blue from vessels into the tissue was measured. The amount of extravasated Evans blue provides a measure of vessel integrity, where higher amounts of Evans blue imply increased pathological permeability. As seen in figure 5E, the amount of extravasated Evans blue was significantly higher in the carrageenan-injected footpad of B6 WT mice than the amount of Evans blue in LRG1-/- mice. Also, subtracting the diffused amount of Evans blue from the NaCl-injected control footpad revealed significantly less vascular permeability in LRG1-/- mice (figure 5F).

Overall, knockout of LRG1 resulted in a reduced local inflammatory response with reduced vascular leakage during inflammation in LRG1–/– animals being a potential mechanism.

Effect of serum from WT versus *LRG1*—/— mice on isolated cells in vitro

To gain a deeper understanding of how LRG1 affects vascular function during inflammation, we performed in vitro experiments with endothelial cells. First, we freshly isolated and short time cultivated liver sinusoidal endothelial cells from B6 WT and LRG1-/- mice. As depicted in figure 6A (representative image shown), endothelial cell proliferation appeared lower for isolated endothelial cells from LRG1-/- mice than for B6 WT mice.

To investigate in more detail proliferation behavior, but also the potential for migration and vessel formation, we performed different in vitro assays using isolated serum from B6 WT and LRG1-/- animals on endothelial cells. Due to the difficulty of long-time culture of primary isolated murine endothelial cells, we worked with three endothelial cell lines (MCEC, MuMec, and TMNK1, see material and methods). We performed scratch tests to evaluate the effect of LRG1 on migration, again with the addition of serum from either LRG1-/- mice or B6-WT littermates. We found that wound closure was significantly reduced in endothelial cells that were incubated with LRG1-/- serum versus WT serum (figure 6B-D), indicating a reduced migration potential when LRG1 is depleted. Graphs including positive control for illustration are shown in online supplemental figure S5A-C.

Proliferation and tube formation assays also showed tendencies toward reduced proliferation as well as reduced formation of branched structures and shorter tube lengths when endothelial cells were coincubated with LRG1-/- serum versus WT serum (online supplemental figure S5D-F).

These experiments give a first impression that the absence of LRG1 could lead to reduced angiogenic activity in endothelial cells. The effects on proliferation and vessel formation point in the same direction, but due to the small number of experiments, no significance could be achieved here.

DISCUSSION

One main clinical problem of alloSCT and CAR-T therapy is the induction of unwanted inflammation at non-tumor sites. The standard treatment for inflammatory side effects is the administration of immunosuppressive drugs, leading to inhibition or inactivation of the cancer immunotherapeutic and to reduced immunity against infections. To improve outcomes, novel therapeutic strategies are needed that allow control of unwanted inflammation without compromising the therapeutic efficacy of cancer immunotherapies. The vasculature and its inner lining, the endothelium, are involved in inflammation as well as in tumor growth, making this an attractive therapeutic target.

The basis for our present work was research on the significance of angiogenesis/endothelial dysfunction in $\text{GVHD}^{2\,5\,21}$ as well as CRS and ICANS after CAR T-cell therapy^{6–8} and findings that LRG1 is involved in the formation of disorganized and dysfunctional vessels.^{12 14} Greenwood *et al* proposed that LRG1 acts as a key regulator of the TGF- β signaling pathway and directly binds to TGF- β R2. This in turn causes it to form a complex with the proangiogenic coreceptor ALK1 instead of with the angiostatic coreceptor ALK5, thereby triggering angiogenesis.¹⁴ Our current data on components of this signaling cascade confirms this concept since we found similar changes in the different inflammatory disease settings.

In the prospective current study, we found elevated LRG1 levels in patients with aGVHD versus matched controls. This finding is in line with previous findings of elevated endothelium-related factors predicting survival in patients with aGVHD: the EASIX² as well as mutations in the endothelium-related Thrombomodulin gene.²² In addition, high Angiopoietin 2/Thrombomodulin levels²³ as well as ST2 levels²⁴ were found to be associated with steroid-refractory aGVHD.²⁵ In the present study, we also included EASIX from day +3 of CAR-T cell patients and found no significant association between EASIX and LRG or CRS severity. The main reason is likely because of the relatively low patient number in comparison to our previous study on CAR-T and EASIX.⁷

Our initial focus was on investigating LRG1-mediated pathological inflammatory angiogenesis as the main reason how LRG1 regulates inflammation. However, in the different experimental inflammatory disease models (GVHD, colitis, paw edema), we found no significant differences in vascular density in target organs in LRG1-/- mice versus WT mice. This pointed toward another mechanism to explain how depletion of LRG1 leads to reduced inflammation in these preclinical models. In the same direction, we would like to point out the fact that in our mouse model for aGVHD, we had two settings of LRG1 reduction: (a) we used LRG1 -/cells as donor source (hematopoietic stem cells as well as immune cells were LRG1 deficient); or (b) recipient mice were LRG1 deficient. In both settings, we found ameliorated aGVHD disease severity scores demonstrating that LRG1 produced by donor immune cells as well as by recipient non-hematopoietic cells contributes to inflammation during aGVHD. Since, as mentioned above, the vascular density did not change, it is likely that LRG1 influences other mechanisms of GVHD. Previous data provide evidence that LRG1 is also involved in other critical inflammatory and vascular functions. While LRG1 is mainly expressed by hepatocytes and neutrophils under physiological conditions, inflammatory stimuli lead to upregulation and expression in endothelial cells, epithelial cells, fibroblasts, and other myeloid cells.¹³ Released LRG1 is able to modulate the microenvironment and act in a proinflammatory manner via the binding of cytochrome C and preventing the apoptosis of immune cells.^{26 27} Moreover, LRG1 can promote neutrophilendothelial adhesion by enhancing the expression of the cell adhesion molecule L-selectin on neutrophils²⁸ and promotes the differentiation of naïve CD4⁺ T cells into proinflammatory Th17 lymphocytes.²⁹

Pathological conditions often trigger the formation of new blood vessels, which, however, are immature and unstably formed.³⁰ In response to an inflammatory stimulus, vascular permeability increases to allow the extravasation of plasma proteins and the transmigration of immune cells.³¹ Various diseases are associated with increased vascular permeability, for example, different types of edema, infectious diseases, diabetic vasculopathy, and cancer.³¹ Investigating vascular permeability in the paw edema model, we were able to demonstrate that inflammation-related vascular leakiness was massively reduced as a result of genetic LRG1 depletion. Evans blue assays in GVHD and colitis mouse models would be useful to draw a complete picture and to investigate the influence of LRG1 on vascular integrity under pathological conditions. Of note, we could previously show increased vascular permeability in target organs during aGVHD.²¹ Our current data in inflammation-associated vascular dysfunction fit well to the concept of vascular normalization by LRG1 depletion in dysfunctional tumor vessels.¹² A role for LRG1 in processes that are involved in vascular stabilization has been previously described. LRG1 causes the detachment of pericytes and thus promotes immature and permeable blood vessels.¹³ The addition of LRG1 significantly reduced the coverage of endothelial cells with mural cells in in vitro assays.³² In combination with this previous knowledge, our findings strengthen the hypothesis that LRG1 is able to initiate vascular permeability during inflammation.

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Although cellular immunotherapy settings, such as aGVHD and CRS, are our main foci and interest, we decided to expand our experiments to a colitis model and a paw edema model. Main reasons were (1) these models have a strong vascular phenotype, and it is possible to reliably investigate endothelium-related inflammatory changes; (2) the aGVHD model did not allow complete depletion of LRG1 because either the donor or the recipient were LRG1 WT; and (3) there were no reliable and easily reproducible CAR T-cell CRS murine models available. Our findings on the biological relevance of LRG1 in these inflammatory disease models fit well with clinical data on elevated LRG1 levels in different inflammatory diseases, such as inflammatory bowel disease,³³ rheumatoid arthritis³⁴ and acute appendicitis,³⁵ dermatomyositis-associated pneumonia³⁶ and asthma.³⁷ Taken together, the available data demonstrate that inflammation is often associated with increased LRG1 serum levels. The clinical perspective is now to validate results in independent cohorts. Next, there is a blocking LRG1 antibody which has been tested to therapeutically modify the tumor environment in murine models.¹² It will be interesting in the future to investigate the effect of LRG1 blockade on inflammatory disease in preclinical models as well as in the human setting. Safety is not yet established in the human setting, and it needs to be determined if LRG1 blockade is better tolerated as compared with VEGFR blockade, which is in clinical use and often causes hypertonia, proteinuria, and thrombembolism.³⁸

Another aspect that is worthy of further investigation in the future is the connection between LRG1 and the inflammatory marker interleukin-6 (IL-6).³² Dritsoula *et al* proposed the hypothesis that IL-6 can activate the secretion of LRG1 that can (1) directly affect endothelial cells via TGF- β signaling and activate the expression of vascular destabilizing genes or (2) act on nearby mural cells and reduce pericyte coverage, both leading to vascular dysfunction.³² In the case of CRS, it is very well known that IL-6 is strongly upregulated on the onset of inflammation. Also in aGVHD, IL-6 levels are elevated, and anti-IL-6 therapy has been attempted as a therapeutic approach.³⁹ However, if LRG1 is the downstream pathogenic activator, then inhibiting IL-6 may not be efficient to reduce LRG1 during inflammation, as other proinflammatory cytokines can still drive LRG1 induction.

In summary, we found elevated LRG1 serum levels in patients with aGVHD after alloHSCT and with CRS after CAR-T cell therapy. Genetic LRG1 depletion attenuated inflammatory disease severity in experimental models. LRG1 is an attractive therapeutic target because of its role in dysfunctional tumor vessels as well as during inflammation.

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