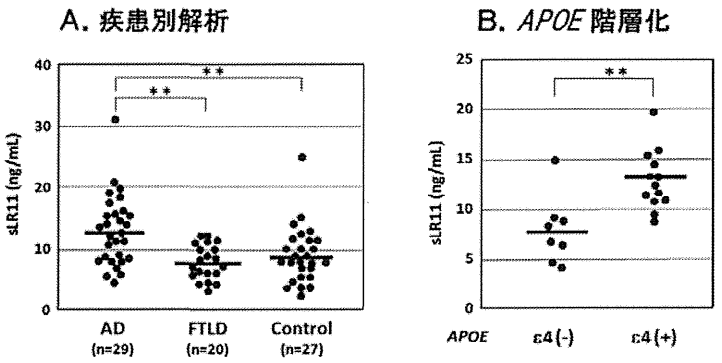
厚生労働科学研究費補助金医療技術実用化総合研究事業
「バイオマーカー可溶性 LR11 による病的未分化細胞疾患の新規診断と標的治療の開発」
総合研究報告書：病的未（脱）分化細胞疾患の診断ならびに治療指針案のための基礎資料

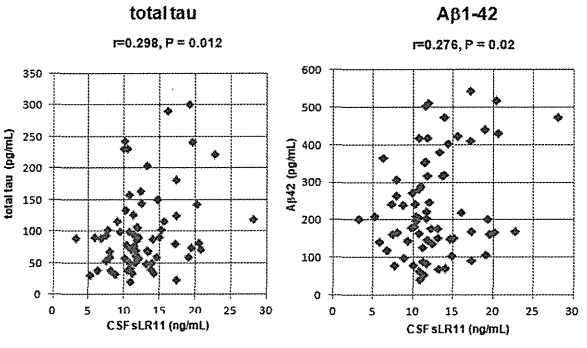
作成年月日：２０１２年　１２月　３０日
分担研究者名： 池内 健，三井田孝
研究協力者名： 徳武孝允，矢島隆二，平山哲

１． 病的未（脱）分化細胞疾患対象疾患名： 認知症
２． 評価項目：診断・病期・合併症・治療反応性・予後・機序・その他（　　）
３． 測定検体：血清・血しょう・尿・髄液・組織・細胞・その他（　　）
４． 試験方法：後ろ向き試験・横断試験・前向き試験・介入試験・基礎解析・モデル解析・その他（　　）
５． 試験内容（対象・人数・期間・単一または多施設・モデル内容・モデル条件など）：
認知症（アルツハイマー病 29 例，前頭側頭型認知症 20 例，健常対照者 27 例），CDR=0（認知機能正常群）34 例
６． 試験結果（抄録程度で４－５行に）：
アルツハイマー病患者髄液中の可溶型 LR11 は 13.1±5.6（平均±標準偏差）ng/mL であり，前頭側頭型認知症患者（8.3±2.4）および健常対照者（9.3±4.5）よりも有意に高値を示した．アルツハイマー病の重症度を３群（I 群：MMSE 21-26，II 群:MMSE 11-20，III 群: MMSE 10 以下）で階層化した解析では，各群間で可溶型 LR11 の値に有意な変化なかった．一方，*APOE4* 陽性アルツハイマー病患者の髄液中可溶型 LR11（13.1±2.9）は，*APOE4* 陰性患者（8.1±3.3）よりも有意に高値を示した．CDR=0 群の認知機能正常群では，髄液中可溶型 LR11 と総タウ，アミロイドβ42（Aβ42）との間に有意な相関を示した．
７． 代表的図表と簡易説明：
図A）アルツハイマー病，前頭側頭型認知症患者および健常対照者の髄液中可溶型 LR11 定量．図B）*APOE4* 陽性および陰性アルツハイマー病患者髄液中の可溶型 LR11 定量．図C）CDR=0（認知機能正常者）髄液中の可溶型 LR11 と総タウおよび Aβ42 の相関



８． まとめ（1-2文）：
アルツハイマー病患者，特に APOE4 陽性患者の髄液中の可溶型 LR11 は有意に高値をとる．CDR=0（認知機能正常者）では，髄液中において可溶型 LR11 とアルツハイマー病の既知のバイオマーカーである総タウおよび Aβ42 と強く相関する．
９． 臨床的有用性：
髄液中の可溶型LR11はアルツハイマー病の診断マーカーとなる可能性がある．また，認知機能正常者の髄液において可溶型 LR11 と総タウおよび Aβ42 が強く相関したことから，可溶型 LR11 は未発症期における脳内のアルツハイマー病の病態変化を反映する代理マーカーとして有望である．

C. CDR=0 における可溶型LR11とタウ，Aβ42相関



１０．現時点の診断または治療・予後における基準案もしくは発症や病態への機序案：
髄液中可溶型 LR11 のカットオフ値を 11.3ng/mL に設定すると，アルツハイマー病の診断感度 66%，特異度 78%，AUC 71%で診断が可能である．可溶型 LR11/Aβ42 の比率のカットオフ値を 1.4 で設定すると，診断感度 71%，特異度 88%，AUC 79%と上昇する．
１１．臨床的問題点（今後解析すべき調査等も含めて）：
多施設共同研究で集積したアルツハイマー病髄液サンプルでの検証試験が必要である．また前向き試験による経

時的な変化，病気に伴う髄液中可溶型 LR11 の変化の検討が必要である．CDR=0（認知機能正常者）における髄液中可溶型 LR11 と総タウ，Aβ42 の相関については，これらの分子の挙動を剖検脳を用いて検証し，その病態機序を明らかにする必要がある．

１２．学会発表・雑誌（投稿中含めて）

西野香織，矢島隆二，徳武孝允，手塚敏之，西澤正豊，池内 健．リポタンパク受容体 LR11 によるアポリポ蛋白 E（ApoE）アイソフォーム依存性の調節機構．分子生物学会．2012.12.14. 福岡

矢島隆二，西野香織，徳武孝允，手塚敏之，西澤正豊，池内　健．リポ蛋白受容体 LR11 による ApoE 産生代謝調節機構の解析．第 31 回日本認知症学会　ポスター発表 2012.10.26. つくば市

矢島隆二，池内 健，徳武孝允，西澤正豊．リポタンパク受容体 LR11 によるアミロイドβ代謝産物調整機構の解析．第５３回日本神経学会 2012.5. 東京

T. Ikeuchi, S. Hirayama, T. Miida, T. Tokutake, R. Yajima, M. Jiang, H. Bujo, M. Nishizawa Quantification of soluble LR11/SorLA in CSF and plasma of patients with Alzheimer disease. Keystone Symposia. ApoE, Alzheimer's, and lipoprotein biology, 2012.2.27. Keystone, USA

矢島隆二，池内　健．リポ蛋白受容体 LR11/SorLA によるアミロイド産生調節機構 The Lipid 50:390-395, 2012

矢島隆二, 池内　健, 徳武孝允, 石原智彦, 西澤正豊. アルツハイマー病の病態に関与する脳由来神経栄養因子（BDNF）による LR11 発現調節の解析．第５２回日本神経学会 ポスター発表 2011.5.19　名古屋

池内　健，平山哲，三井田孝，深町勇，矢島隆二，徳武孝允，春日健作，金子博之，田久保耕平，海老沼宏幸，武城英明，西澤正豊．アルツハイマー病患者・髄液中では可溶性 LR11 が増加する．第 29 回日本認知症学会学術集会 ポスター発表 2010.11.5 名古屋

池内　健．アルツハイマー型認知症の脂質関連バイオマーカー．**The Lipid**　21;52-57, 2010

Ikeuchi T, Hirayama S, Miida T, Fukamachi I, Tokutake T, Ebinuma H, Takubo K, Kaneko H, Kasuga K, Kakita A, Takahashi H, Bujo H, Saito Y, Nishizawa M. Increased levels of soluble LR11 in cerebrospinal fluid of patients with Alzheimer disease. ***Dementia and Geriatric Cognitive Disorders*** 30:28-32, 2010

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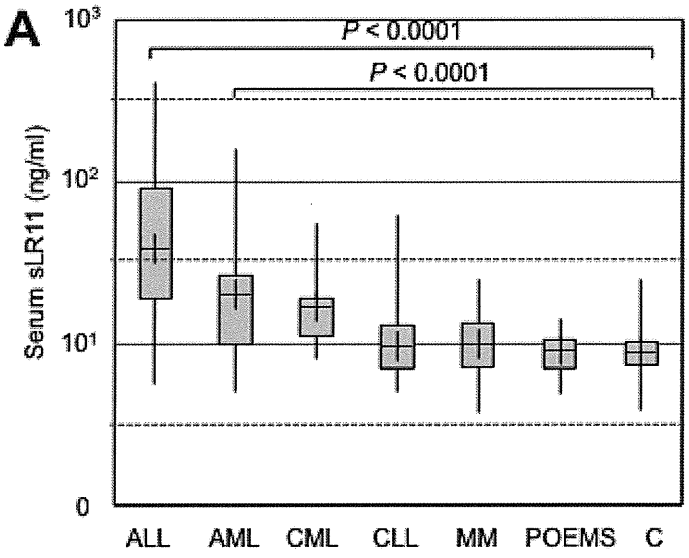
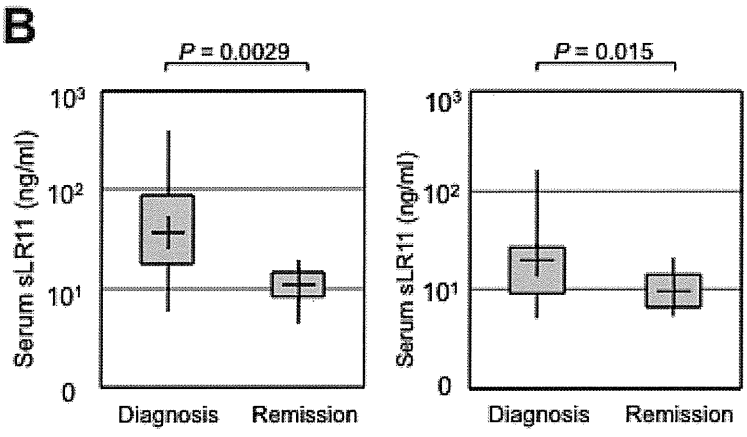
作成年月日：２０１２年　　月　　日
分担研究者名：中世古知昭
研究協力者名：清水直美、武内正博、大和田千佳子、他

１．　病的未（脱）分化細胞疾患対象疾患名：急性白血病
２．　評価項目：診断・病期・合併症・治療反応性・予後・機序・その他：治療反応性
３．　測定検体：血清・血しょう・尿・髄液・組織・細胞・その他：血清
４．　試験方法：後ろ向き試験・横断試験・前向き試験・介入試験・基礎解析・モデル解析・その他：後ろ向き試験
５．　試験内容（対象・人数・期間・単一または多施設・モデル内容・モデル条件など）：
血液疾患　AML43 症例、ALL23 症例を含む２３９症例、千葉大学附属病院および関連病院による多施設共同
６．　試験結果（抄録程度で４－５行に）：
AML および ALL 白血病症例の細胞表面で LR11 の発現が著しく亢進する。血清可溶性 LR11 値は正常対照に比べて著しく上昇する（P < 0.001）（ALL, 73.5 ± 93.5 ng/ml; AML, 26.8 ± 29.1 ng/ml、正常対照, 9.2 ± 3.3 ng/ml）。寛解時の AML、ALL 患者の sLR11 値は優位に低下し全例 20 ng/ml になる。





７．　代表的図表と簡易説明：
図 A 急性白血病や他の血液疾患の血清 LR11 値
図 B 診断時と観海時の血清 LR11 値（左　ALL 右 AML）
８．　まとめ（1-2文）：
LR11 とその可溶性たんぱくは急性白血病で上昇する。とえいわけ、血清 LR11 値は寛解とともに改善する。
９．　臨床的有用性：
細胞発現 LR11 レベルは急性白血病で上昇し、診断時の病態を表す可能性がある。
血清 LR11 レベルは急性白血病細胞の状態を反映し、治療反応性や予後をあらわす可能性がある。
１０．　現時点の診断または治療・予後における基準案もしくは発症や病態への機序案：
血清 LR11 20ng/ml 以下となることが LR11 高値をともなう急性白血病の寛解とともに随伴する血中指標となる。
１１．　臨床的問題点（今後解析すべき調査等も含めて）：
白血病各分類、治療内容を考慮した対象患者に対して行う横断試験と前向き試験による検証。
１２．　学会発表・雑誌（投稿中含めて）
Ohwada C, Nakaseko C, Sakai S, Takeda Y, Abe D, Shimizu N, Takeuchi M, Sakaida E, Kawaguchi T, Takubo K, Rbinuma H, Fukamachi I, Yokote K, Saito Y, Iwama A, ○Bujo H: Prognostic Impact of a Novel Biomarker, Serum Soluble LR11 on Acute Leukemias.

The 52th American Society of Hematology, Orlando, USA, 2010,11
Sakai S, Nakaseko C, Takeuchi M, Ohwada C, Shimizu N, Sakaida E, Abe D, Kawaguchi T, Takeda Y, Takubo K, Rbinuma H, Fukamachi I, Yokote K, Saito Y, Iwama A, ○Bujo H: Soluble LR11 is a novel biomarker for acute leukemias. 第72回日本血液学会, 横浜, 2010.9
Takeuchi M, Nakaseko C, Ohwada C, Sakai S, Takeda Y, Abe D, Shimizu N, Sakaida E, Kawaguchi T, Takubo K, Rbinuma H, Fukamachi I, Yokote K, Saito Y, Iwama A, ○Bujo H: Soluble LR11 is a novel surface marker for normsl leukocytes and leukemia cells. 第72回日本血液学会, 横浜, 2010.9
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Sakai S, Nakaseko C, Takeuchi T, Ohwada C, Shimizu N, Tsukamoto S, Kawaguchi T, Jiang M, Sato Y, Ebinuma H, Yokote K, Iwama A, Fukamachi I, Schneider WJ, Saito Y, Bujo H: Circulating soluble LR11/SorLA levels are highly increased and ameliorated by chemotherapy in acute leukemias. Clin Chim Acta 2012 in press

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分担研究者名：　中世古知昭
研究協力者名：　大和田千桂子，川口岳晴，他

１．　病的未（脱）分化細胞疾患対象疾患名：悪性リンパ腫
２．　評価項目：診断・病期・合併症・治療反応性・予後・機序・その他（　　）
３．　測定検体：血清・血しょう・尿・髄液・組織・細胞・その他（　　）
４．　試験方法：後ろ向き試験・横断試験・前向き試験・介入試験・基礎解析・モデル解析・その他（　　）
５．　試験内容（対象・人数・期間・単一または多施設・モデル内容・モデル条件など）：
2002 年～2012 年に千葉大学医学部附属病院及び関連施設で診断･治療されたびまん性大細胞型 B 細胞性リンパ腫(DLBCL)55 例，ろ胞性リンパ腫(FL)61 例を含む悪性リンパ腫症例

６．　試験結果（抄録程度で４－５行に）：
DLBCL，FL ともに免疫染色によりリンパ腫組織に LR11 が高発現していた。診断時の血清可溶型 LR11(sLR11)値は，DLBCL，FL ともに健常人コントロールと比較して有意に高値であり，寛解時に正常化した。血清 sLR11 高値と関連する因子として，DLBCL では LDH，病期，節性病変数，β2-MG 等が，FL では LDH，Hb，骨髄浸潤等が抽出され，国際的予後因子である IPI（DLBCL）、FLIPI-2(FL)と有意に相関した。初診時の sLR11 により層別化すると，sLR11 高値群では DLBCL，FL ともに有意に無進行生存(PFS)が不良であった。

７．　代表的図表と簡易説明：
図 1：FL における初診時血清 sLR11 値。健常人コントロールと比較して有意に高値である。
図 2：FL における初診時血清 sLR11 で 2 群に層別化した場合の PFS。sLR11>16.7 ng/mL では有意に生存率が低下する。

図 1

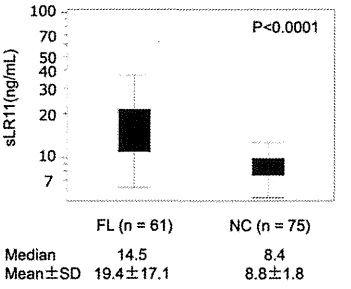
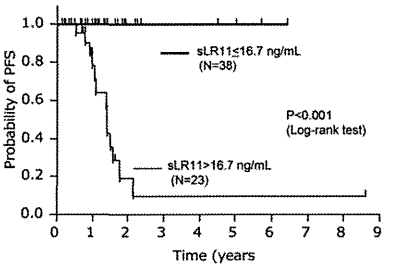


図 2



８．　まとめ（1－2 文）：
DLBCL，FL 等の B 細胞性リンパ腫において LR11 は高発現し，初診時の患者血清における sLR11 値は有意に高値となり，治療反応性と相関するとともに，予後を予測し得る有効なバイオマーカーとなることが示された。

９．　臨床的有用性：
リンパ腫組織における LR11 発現は診断に有効であり，血清 sLR11 は予後を予測しうる可能性がある。

１０．　現時点の診断または治療・予後における基準案もしくは発症や病態への機序案：
DLBCL，FL では，初診時血清 sLR11>18ng/mL、もしくは 16.7ng/mL では予後不良であり，これに基づいた治療方針の策定が必要である。

１１．　臨床的問題点（今後解析すべき調査等も含めて）：
多数例の前向き試験による検証が必要であり，既に多施設共同前向き試験を開始している。

１２．　学会発表・雑誌（投稿中含めて）

1. Kawaguchi T, Ohwada C, Sakai S, Takeda Y, Shimizu N, Takeuchi M, Sakaida E, Takubo K, Ebinuma H, Fukamachi I, Higashi M, Tamaru J, Yokote K, Bujo H, Nakaseko C. Serum soluble LR11 is a promising novel biomarker for malignant lymphoma. The 73rd Annual Meeting of the Japanese Society of Hematology, Nagoya, Oct14-16, 2011

2. Kawaguchi T, Ohwada C, Higashi M, Takeuchi M, Sakai S, Takeda Y, Shimizu N, Sakaida E, Takubo K, Ebinuma H, Fukamachi I, Tamaru J, Yokote K, Bujo H, Nakaseko C. Serum soluble LR11 is a promising

novel biomarker for B cell lymphoma. The 53rd Annual Meeting of the American Society of Hematology, San Diego, Dec 10-13, 2011

3. Kawaguchi T, Ohwada C, Shimizu N, Takeuchi M, Sakaida E, Takeda Y, Sakai S, Yamazaki A, Higashi M, Tamaru J, Yokote K, Bujo H, Nakaseko C. Serum Soluble LR11 Can Predict High FLIPI-2 Score in Follicular Lymphoma. The 53rd Annual Meeting of the American Society of Hematology, Atlanta, Dec 8-11, 2012

4. Ohwada C, Kawaguchi T, Shimizu N, Takeuchi M, Sakaida E, Takeda Y, Fujikawa K, Matsue K, Higashi M, Tamaru J, Yokote K, Bujo H, Nakaseko C. Serum Soluble LR11, a Novel Biomarker for Intravascular Large B-cell Lymphoma The 53rd Annual Meeting of the American Society of Hematology, Atlanta, Dec 8-11, 2012

5.川口岳晴, 大和田千桂子、山崎敦子、塚本祥吉、酒井紫緒、竹田勇輔、武内正博、堺田恵美子、清水直美、横手幸太郎、武城英明、中世古知昭. 濾胞性リンパ腫における可溶型 LR11 のバイオマーカーとしての有用性　第 50 回日本臨床分子医学会学術集会 2013/4/12-14

6. Kawaguchi T, Ohwada C, Takeuchi M, Shimizu N, Sakaida E, Takeda Y, Sakai S, Tsukamoto S, Yamazaki A, Sugita Y, Jiang M, Higashi M, Yokote K, Tamaru J, Bujo H, Nakaseko C. LR11: a novel biomarker identified in follicular lymphoma. Haematologica 投稿中

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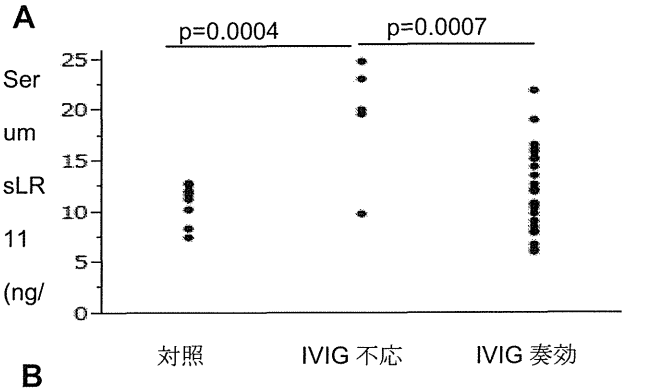
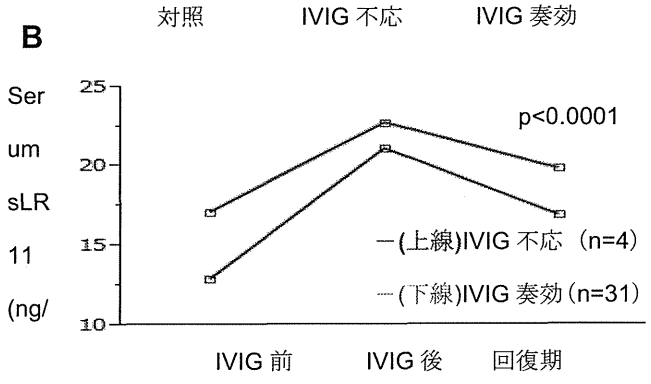
分担研究者名： 鈴木博
研究協力者名： 渡辺健一

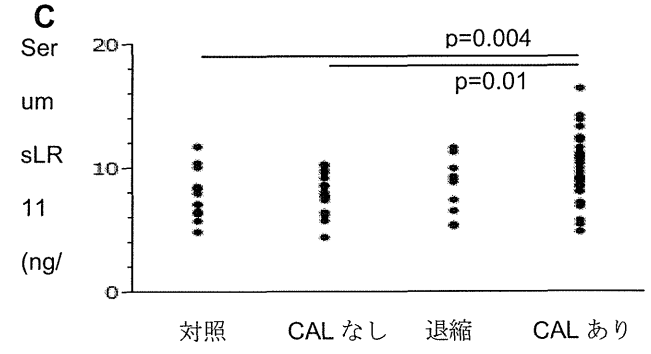
１． 病的未（脱）分化細胞疾患対象疾患名： 川崎病
２． 評価項目：[診断]・病期・合併症・[治療反応性]・予後・機序・その他（　　）
３． 測定検体：[血清]・血しょう・尿・髄液・組織・細胞・その他（　　）
４． 試験方法：後ろ向き試験・横断試験・[前向き試験]・介入試験・基礎解析・モデル解析・その他（　　）
５． 試験内容（対象・人数・期間・単一または多施設・モデル内容・モデル条件など）：
川崎病急性期患者　35 例　うちγグロブリン治療（IVIG)奏効 30 例、IVIG 不応 6 例
川崎病遠隔期患者　88 例　うち冠動脈病変(CAL)なし 18 例、退縮 12 例 、CAL あり 58 例
新潟大学医歯学総合病院および関連病院による多施設共同
６． 試験結果（抄録程度で４−５行に）：
川崎病急性期において、IVIG 不応例の治療前血清可溶性 LR11(sLR11)は、対照、奏効例に比べて有意に高値をとる（不応,19.5 ± 5.8 ng/nl; 奏効, 11.8 ± 3.1ng/ml, p<0.001)。不応例では回復期にも高値が持続する(p<0.0001)。川崎病遠隔期において、CAL あり例の sLR11 は、対照、CAL なし例、退縮例と比べ有意に高値をとる(対照, 8.1±2.1ng/ml; CAL なし, 8.3 ± 1.7ng/ml; 退縮, 8.8 ± 2.1ng/ml; CAL あり, 10.3±2.3ng.ml)。



７． 代表的図表と簡易説明：
図 A　川崎病急性期IVIG 前 sLR11 値
図 B　川崎病急性期 sLR11 値の変化（IVIG 不応と奏効の比較）
図 C　川崎病遠隔期各群における sLR11 値



８． まとめ（1-2文）：
sLR11 は川崎病急性期の冠動脈瘤形成の危険性の高い重症例で治療前から上昇し、治療後にも高値が持続する。川崎病遠隔期の冠動脈後遺症例で上昇する。
９． 臨床的有用性：
sLR11 は川崎病急性期の重症度を反映し、γグロブリン不応や冠動脈瘤形成の予測マーカーとなる可能性がある。また川崎病遠隔期の血管障害を反映するバイオマーカーとなる可能性がある。
１０． 現時点の診断または治療・予後における基準案もしくは発症や病態への機序案：
川崎病急性期γグロブリン治療前血清 sLR11 が 17.5ng/ml 以上では、γグロブリン治療不応の指標となる(感度83%, 特異度 100%)。



１１． 臨床的問題点（今後解析すべき調査等も含めて）：
急性期γグロブリン不応例を更に増やし予測マーカーとしての妥当性を検証。川崎病以外の急性期発熱疾患との

比較による診断マーカーとしての検討。

１２．　学会発表・雑誌（投稿中含めて）

Watanabe K, Suzuki H, Hasegawa S, Numano F, Uchiyama M, Bujo H: Soluble LR11 is a new biomarker for vascular lesions late after Kawasaki disease. 第46回日本小児循環器学会総会・学術集会, 東京, 2010.7

Watanabe K, Suzuki H, Ozawa J, Haniu H, Hasegawa S, Uchiyama M, Bujo H: A novel biomarker in acute phase of Kawasaki disease: Soluble LR11. 第47回日本小児循環器学会総会・学術集会, 福岡, 2011.7

Watanabe K, Suzuki H, Haniu H, Numano F, Saitoh A, Bujo H: A Novel Biomarker for Late Vascular Lesions in Patients with Kawasaki Disease: Soluble LR11. 10th International Kawasaki Disease Symposium, Kyoto, 2012.2

Watanabe K, Suzuki H, Haniu H, Numano F, Saitoh A, Bujo H: Relation between soluble LR11 and other biomarkers for atherosclerosis late after Kawasaki disease. 第48回日本小児循環器学会総会・学術集会, 京都, 2012.7

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表レイアウト

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nishii K, Nakaseko C, Jiang M, Shimizu N, Takeuchi M, Schneider WJ, Bujo H	The soluble form of LR11 is a regulator of hypoxia-induced, uPAR-mediated adhesion of immature hematological cells.	J Biol Chem.	in press		2013
Nakata Z, Nagae M, Yasui N, Bujo H, Nogi T, Takagi J	Crystallization and preliminary crystallographic analysis of human LR11 Vps10p domain.	Acta Crystallogr Sect F Struct Biol Cryst Commun	67	129-132	2011
Takahashi M, Bujo H, Jiang M, Noike H, Saito Y, Shirai K	Enhanced circulating soluble LR11 in patients with coronary organic stenosis.	Atherosclerosis	210	581-584	2010
Takahashi M, Bujo H, Shiba T, Jiang M, Maeno T, Shirai K:	Enhanced circulating soluble LR11 in patients with diabetic retinopathy.	Am J Ophthalmol	154	187-192	2012
Ogita M, Miyauchi K, Dohi T, Tsuboi S, Miyazaki T, Yokoyama T, Yokoyama K, Shimada K, Kurata T, Jiang M, Bujo H, Daida H.	Increased circulating soluble LR11 in patients with acute coronary syndrome.	*Clin Chim Acta.*	415	191-4	2013
Ikeuchi T, Hirayama S, Miida T, Fukamachi I, Tokutake T, Ebinuma H, Takubo K, Kaneko H, Kasuga K, Kakita A, Takahashi H, Bujo H, Saito Y, Nishizawa M:	Increased Levels of Soluble LR11 in Cerebrospinal Fluid of Patients with Alzheimer Disease.	Dement Geriatr Cogn Disord	30	28-32	2010
Alexopoulos P, Luo L-H, Tsolakidou A, Kratzer M, Grimmer T, Westerteicher C, Jiang M, Bujo H, Diehl-Schmid J, Kurz A, Perneczky R:	Interrelations between CSF soluble AβPPβ, amyloid-β1-42, SORL1, and tau levels in Alzheimer's disease.	J Alzheimers Dis	28	543-552	2012

Guo L-H, Guo, Westereicher C, Wang X-H, Kratzer M, Tsolakidou A, Jiang M, Grimmer T, Laws SM, Alexopoulos P, Bujo H, Kurz A, Perneczky R:	SORL1 genetic variants and cerebrospinal fluid biomarkers of Alzheimer's disease.	*Eur Arch Psychiatry Clin Neurosci*	262	529-34	2012
Tsolakidou, A., Alexopoulos, P., Guo, L. H., Grimmer, T., Westerteicher, C., Kratzer, M., Jiang, M., Bujo, H., Roselli, F., Leante, M.R., Livrea, P., Kurz, A. and Perneczky R.	β-Site amyloid precursor protein-cleaving enzyme 1 activity is related to cerebrospinal fluid concentrations of sortilin-related receptor with A-type repeats, soluble amyloid precursor protein, and tau.	Alzheimers Dement.	Alzheimers Dement.		2013
Sakai S, Nakaseko C, Takeuchi M, Ohwada C, Shimizu N, Tsukamoto S, Kawaguchi T, Jiang M, Sato Y, Ebinuma H, Yokote K, Iwama A, Fukamachi I, Schneider WJ, Saito Y, Bujo H.	Circulating soluble LR11/SorLA levels are highly increased and ameliorated by chemotherapy in acute leukemias.	*Clin Chim Acta.*	413	1542-8	2012

Ⅳ. 研究成果の刊行物・別冊

The soluble form of LR11 is a regulator of hypoxia-induced, uPAR-mediated adhesion of immature hematological cells*

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[4]Department of Medical Biochemistry, Max F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria.

*Running title: *sLR11 regulates hematological cell adhesion in hypoxia*

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Keywords: LR11; uPAR; HIF-1α; hypoxia; cell adhesion; HSPC; bone marrow

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Background: Serum levels of the soluble LR11 fragment (sLR11) increase in patients with acute leukemia.
Results: Hypoxia-induced HIF-1α activation increases LR11 levels, and sLR11 enhances adhesion of HSPCs to BM stromal cells via a uPAR-mediated pathway.
Conclusion: sLR11 regulates hypoxia-induced attachment of HSPCs.
Significance: sLR11 may stabilize the hematological pool size by controlling HSPC attachment to the BM niche.

SUMMARY

A key property of hematopoietic stem and progenitor cells (HSPCs) regarding differentiation from the self-renewing quiescent to the proliferating stage is their adhesion to the bone marrow (BM) niche. An important molecule involved in proliferation and pool size of HSPCs in the BM is the hypoxia-induced uPAR. Here we show that the soluble form (sLR11) of LR11 (also called SorLA or SORL1) modulates the uPAR-mediated attachment of HSPCs under hypoxic conditions. Immunohistochemical and mRNA expression analyses revealed that hypoxia increased LR11 expression in hematological c-Kit(+) Lin(-) cells. In U937 cells, hypoxia induced a transient rise in LR11 transcription, production of cellular protein, and release of sLR11. Attachment to stromal cells of c-Kit(+) Lin(-) cells of *lr11*$^{-/-}$ mice was reduced by hypoxia much more than of *lr11*$^{+/+}$ animals. sLR11 induced the adhesion of U937 and c-Kit(+) Lin(-) cells to stromal cells. Cell attachment was increased by sLR11 and reduced in the presence of anti-uPAR antibodies. Furthermore, the fraction of uPAR co-immunoprecipitated with LR11 in membrane extracts of U937 cells was increased by hypoxia. $CoCl_2$, a chemical inducer of HIF-1α, enhanced the levels of LR11 and sLR11 in U937 cells. The decrease

in hypoxia-induced attachment of HIF-1α knockdown cells was largely prevented by exogenously added sLR11. Finally, hypoxia induced HIF-1α binding to a consensus binding site in the LR11 promoter. Thus, we conclude that sLR11 regulates the hypoxia-enhanced adhesion of HSPCs via an uPAR-mediated pathway that stabilizes the hematological pool size by controlling cell attachment to the BM niche.

Hypoxic conditions play a key role in the regulation of the pool size of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) through the regulation of many molecules expressed in HSPCs (1,2) . The partial pressure of oxygen (pO_2) of the endosteal sites is known to be much lower than that of the nearest capillaries (3). Long-term hematopoietic stem cells (LT-HSCs) reside mainly in the endosteum (4-6), and are stained with pimonidazole, a chemical probe for hypoxia (7-9). Accordingly, human cord blood HSCs transplanted into immuno-deficient mice require a hypoxic status to maintain cell cycle quiescence in the BM (10).

One of the key regulatory functions of the molecules in the HSPCs under hypoxia is the modulation of cell adhesion to the osteoblastic niche in order to facilitate the differentiation from the immature quiescent self-renewal stage to the down-stream proliferating mature hematological cell stage (1,2,11). Many proteins including Sca-1, cKit, CD34, and urokinase-type plasminogen activator receptor (uPAR; CD87) have been identified as regulators of HSPCs adhesion to osteoblastic niches (12-17). In fact, recent studies using overexpressing or knockout mice and cells for Angiopoietin-1 (Ang-1), Thrombopoietin (TPO), BMP-4, Secreted frizzled related protein-1 (Sfrp-1), or uPAR have shown that changes in expression of the respective gene cause disturbed maintenance of normal HSPC pool size and lead to pathological conditions typical of hematological proliferative disease or severe anemia (2). In fact, uPAR has been shown to be a major regulator of proliferation, marrow pool size, engraftment, and mobilization of murine HSPCs (17). Together with previous results of analyses of uPAR as a prognosis marker in leukemic patients (18), disturbed regulation of uPAR may be important in the pathogenesis of leukemias involving uPAR-expressing malignant cells. Although uPAR expression is known to be induced by hypoxia in cultured hematological cells (19), the mechanism underlying the upregulation of uPAR under hypoxic conditions has not yet been elucidated.

We have identified and characterized a regulator of uPAR function, LR11 (also called SorLA or SORL1), in vascular smooth muscle cells (SMCs) (20,21). LR11 is a type I membrane protein, from which a large soluble extracellular part, sLR11, is released by proteolytic shedding (20,22-24). sLR11 accelerates intimal thickening and macrophage-foam cell formation in the process of atherosclerosis (25). Recent studies in humans and animals have shown that sLR11 is produced by myeloid cells after G-CSF treatment, and that the released sLR11 plays an important role in the G-CSF-induced leukocyte mobilization from BM to the circulation (Shimizu et al., unpublished observation). Zhang et al. reported high levels of LR11 mRNA in human CD34(+) CD38(-) immature hematopoietic precursors (26). Both LR11 mRNA and cell surface protein levels are elevated in immature leukemic cells, in turn leading to increased levels of sLR11 in acute leukemias (27). Thus, it is conceivable that in hypoxic environments, modulation of uPAR expression by sLR11 may be important for maintenance of the HSPC pool size.

Here, we have studied the regulation of LR11 expression in hematological cells under hypoxic conditions such as those found in the BM niche. Immature and mature hematological cells in the BM express LR11 in hypoxia-sensitive fashion. HIF-1α activation by hypoxia or chemical means leads to increased LR11 expression, which in turn enhances the adhesion of leukemia cells to stromal cells through direct interaction of sLR11 with uPAR. Regulation of uPAR by LR11 may provide the basis for a novel strategy towards maintenance of the hematological cell pool size via modification of uPAR functions in hypoxic niches of the BM.

EXPERIMENTAL PROCEDURES

Mice—All animal studies were reviewed and approved by the Special Committee on Animal Welfare, School of Medicine, at the Inohana Campus of Chiba University. *Lr11*$^{-/-}$ mice (21) were maintained under standard animal house conditions with a 12 h light/dark cycle, and were fed ad libitum with regular chow diet.

Antibodies, Recombinant Proteins—Monoclonal antibodies (A2-2-3, M3, R14) against LR11 have been described previously (28). M3 was used for immunoprecipitation and ELISA, A2-2-3 for immunoblotting, and R14 for immunohistochemistry and ELISA. Polyclonal antibodies against uPAR and HIF-1α were from R&D systems and Cell Signaling Technology, respectively. Recombinant LR11 protein lacking the 104 C-terminal amino acids containing the transmembrane region (sLR11) was prepared as described (22).

Cells—The human promonocytic cell line U937 and the human myeloid cell line K562 were purchased from ATCC. Human mesenchymal stem cells (MSCs) were purchased from Lonza. The mouse stromal cells, OP-9, were provided by Dr. Osawa (Chiba University). For murine cell sorting, BM cells were first stained with biotinylated-anti-Lineage (Lin) [CD5, B220, CD11b, Gr-1, 7-4, Ter-119] followed by incubating with streptavidin-microbeads (Miltenyi Biotec). After washing with staining buffer (PBS containing 0.5% BSA and 2mM EDTA), Lin(+) and Lin(-) cells, respectively, were enriched using magnetically activated cell sorting (MACS) columns. For mouse c-Kit(+) Lin(-) cell sorting, Lin(-) enriched cells were stained with anti-c-Kit microbeads (Miltenyi Biotec), then cKit(+) Lin(-) cells were enriched using MACS columns. U937 cells and K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. MSCs were cultured in MSC growth medium, MSCGM (basal medium with growth supplements, Lonza), and were used between passages 2 and 5. OP-9 cells were cultured in DMEM, supplemented with 20% FBS. Lin(-) cells and c-Kit(+) Lin(-) cells were cultured in IMDM with 20% FBS. For hypoxia treatment, the cells were cultured in a humidified multigas incubator (APM-30D; Astec) with 1% O_2 and 5% CO_2 at 37 °C.

Cell Adhesion Assay—Cell adhesion was determined in 96-well plates as described (22). For experiments using vitronectin-coated plates, wells were coated with 10 ng/well vitronectin for 2 h at 37 °C. For the preparation of OP-9- and MSCs-coated plates, OP-9 and MSCs were seeded onto 96-well plates 24 h at 37 °C, respectively, to obtain a confluent cell layer before experiments. Freshly purified mouse primary cells or U937 cells were fluorescently labeled by loading with Calcein acetoxymethylester (Calcein AM, BD Bioscience) for 1 h at 1 x 10^7 cells/ml in HBSS containing 1% BSA. Calcein-loaded cells were added to the vitronectin-, OP-9-, or MSCs-coated plates at 3 x 10^4 cells/well. After centrifugation, the culture plates were incubated for 20 min at 37 °C to allow the cells to attach to the coated plates. Non-attached cells were removed by gently washing 3 times with PBS, and the attached cells were quantitated by measuring fluorescence intensity using a fluorescence microplate reader (SPECTRAmax GEMINI XS, Molecular Devices). The numbers of attached cells were determined from standard curves generated by serial dilutions of known numbers of labeled cells.

LR11-overexpressing cells, LR11-knockdown cells, and HIF-1α knockdown cells—For the generation of LR11-overexpressing cells, transient transfection of U937 cells and transfection for stable expression in K562 cells were carried out with pBKCMVhLR11 (29) or pBK-CMV (mock) by using the Neon electroporation device (Invitrogen) according to the manufacturer's guidelines. Stable transfectants were selected in medium supplemented with 800 μg/ml G418 (Roche), and maintained in medium containing 400 μg/ml G418. For the generation of LR11-knockdown cells, Lentiviral vectors (CS-H1-shRNA-EF-1α-EGFP) expressing short hairpin RNAs (shRNAs) that target two different sequence regions in human LR11 cDNA (#1, 5',-GGATCATGATTCAGGAACA-3' and #5,

5'-GGAGAGAGCATATGGAAGA-3 '), and that target luciferase as control (Cosmo Bio) were constructed. The virus particles were produced as previously described (30). Briefly, plasmid DNA was transfected into 293T cells along with the packaging plasmid (pCAG-HIVgp) and the VSV-G and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) by calcium phosphate coprecipitation. Stable shRNA-expressing U937 cells were generated by infection with the supernatants from transfected 293T cells in the presence of 5 μg/ml protamine sulfate for 24 h with subsequent sorting of the GFP-positive cells using FACS Aria (BD Biosciences). For the generation of HIF-1α knockdown cells, HIF-1α small interfering RNA (siRNA) and control siRNA were designed and synthesized by Ambion. These siRNAs were transfected into U937 cells by using the Neon electroporation device according to the manufacturer's guidelines.

Western Blotting, Immunoprecipitation—Sorted cells or cultured cells were washed 3 times with PBS and harvested in ice-cold RIPA buffer with protease inhibitors (Complete Mini, Roche). Cell lysates were recovered in the supernatant after centrifugation at 20,000 g for 30 min. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific). Samples were mixed with an equal volume of 2x Laemmli sample buffer containing 5% β-mercaptoethanol and heated for 5 min at 90 °C. Where indicated, cells were incubated with sLR11 (1 μg/ml) at 37 °C for 15 min, harvested, and 10 ng of mouse anti-LR11 antibody (M3) or mouse IgG was added and incubated at 4°C overnight under mixing. The LR11/uPAR/antibody complex was bound to Protein G Sepharose. The proteins were released into Laemmli sample buffer containing 5% β-mercaptoethanol by heating to 90 °C for 10 min. Protein samples were subjected to SDS-PAGE, and the immunoreactive signals were detected by mouse monoclonal antibody against LR11 (A2-2-3), goat polyclonal antibody against uPAR, or rabbit polyclonal antibody against human HIF-1α, followed by horseradish peroxidase (HRP)-conjugated anti-mouse, anti-goat, or anti-rabbit IgG, respectively. Development was performed with the ECL detection reagents (GE Healthcare). The signals were quantified with ChemiDoc XRS+ system using Image-Lab software (Bio-Rad).

RNA Extraction and Real-Time Quantitative PCR Analysis—Total RNA was isolated using the RNeasy Kit (QIAGEN). RNA was eluted and quantified using the Nanodrop spectrometer (Thermo Scientific). The reverse transcription step was performed with the TaqMan Reverse Transcription Reagent Kit (Applied Biosystems) according to the manufacturer's guidelines. LR11 mRNA levels were determined by quantitative real-time PCR on the cDNA samples using the TaqMan assay-on-demand kit with the ABI-PRISM 7000 (Applied Biosystems). Analysis was carried out in triplicates in a volume of 20 μl for LR11 and the endogenous reference gene 18S rRNA, which does not change in hypoxia (31), and the comparative threshold cycle method was used. In each experiment, the RNA prepared from a sample obtained from normoxic conditions was used as calibrator to allow comparison of relative mRNA levels.

Flow Cytometric Analysis—For the analysis of uPAR expression, cells were washed with PBS and incubated at 4 °C in the dark for 30 min with phycoerythrin (PE)-conjugated anti-uPAR antibody (BioLegend). Isotype control antibody (BD pharmingen) was used as a negative control. Flow cytometric analyses were performed with a FACSCant II (BD Biosciences).

ELISA—Amounts of soluble LR11 released into the culture medium were determined by sandwich ELISA as previously reported (27). Briefly, culture media were concentrated by using Amicon Ultra-15 (100,000 NMWL membranes from Millipore), the concentrated sample was reacted with the capture monoclonal antibody M3, and then incubated with the biotinylated reporter rat monoclonal antibody R14. The LR11-Mab complex was reacted with HRP-conjugated streptavidin. A standard curve was constructed using purified LR11 protein.

Chromatin Immunoprecipitation Assay—U937 cells were cross-linked with 1% formaldehyde for 10 min at room temperature, followed by the

addition of 0.125 M glycine and incubation for 5 min at 4 °C. After washing with PBS, 1 x 10^7 cells were lysed with cell lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 0.5% SDS, and protease inhibitors on ice, and sonicated until the DNA fragments had an average size 200-500 base pairs using a Bioruptor (Cosmo Bio). After centrifugation at 15,000 rpm for 10 min, sheared chromatin was diluted 10-fold in dilution buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, and protease inhibitors. After incubation with Dynabeads Protein G (Invitrogen) for 1 h at 4°C, the precleared chromatin was immunoprecipitated overnight at 4°C with anti-HIF-1α antibodies (clone H1alpha67, Abcam)/Dynabeads Protein G mix. Beads were then sequentially washed with the following combination of wash buffers; twice each with low salt wash buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% Sodium deoxycholate), high salt wash buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% Sodium deoxycholate), and LiCl wash buffer (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% NP40, and 0.5% Sodium deoxycholate), and TE buffer. Bound chromatin together with input DNA was released from the antibodies/Protein G beads by elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS), and crosslinking was reversed by incubation in the same buffer overnight at 65 °C. Immunoprecipitated DNA or input DNA was treated with RNaseA (Sigma) and proteinase K (Roche), and then extracted with phenol:chloroform for subsequent PCR analysis. The primers used in this analysis spanned 144 bp around the putative HIF-1-binding site within the LR11 promoter (sense, 5'-GCGCTCGCTGCCTTAACTTC-3', and antisense, 5'-CCAGGTCCCGCTCGGTTC-3'), and 166 bp around that in the CD18 promoter as control (32).

Statistics—The results are shown as mean ± SD for each index. 1-way ANOVA was used to compare between 2 groups, and Dunnett's multiple-range test was used for comparison of multiple groups. A value of $P < 0.05$ was considered significant.

RESULTS

Expression of LR11 in murine HSPCs and human U937 cells is induced by hypoxic conditions—First, we investigated LR11 expression levels of HSPCs under low-oxygen conditions by immunochemistry. Protein extracts of c-Kit(+) Lin(-) cells or Lin(+) cells, representing HSPCs, were subjected to immunoblot analysis with the monoclonal antibody against LR11, R14 (28) (Fig. 1). The LR11 levels in the c-Kit(+) Lin(-) cells were significantly lower than those of the differentiated Lin(+) cells under normoxic conditions. However, the LR11 expression levels in the c-Kit(+) Lin(-) cells progressively increased with prolonged exposure to the hypoxic conditions, whereas the levels in the Lin(+) cells remained relatively constant. These results indicate that the hematological cells in the BM express LR11, and that the expression in HSPCs is induced by hypoxic conditions. In order to analyze the regulation of sLR11 expression in hypoxic environments in detail, we investigated the effects of hypoxic conditions on LR11 expression in U937 cells, an undifferentiated leukemia cell line (33). When the cells were incubated under hypoxic conditions, the LR11 mRNA levels increased 15-fold over the levels under normoxic conditions after 3 h, and then declined within 24 h (Fig. 2A). Importantly, the LR11 protein in the hypoxic cells was elevated after 6 h compared to normoxic cells, and increased to 3-fold of the unchanged control levels after incubation for 48 h (Fig. 2B). The amount of sLR11 released from the cells was also increased after 48 h incubation under hypoxia (Fig. 2C). Thus, hypoxia induced a transient increase in the transcription of the LR11 gene and subsequent production of cellular protein, accompanied by release of the shed form into the media.

LR11 mediates the hypoxia-induced adhesion of immature hematological cells and HSPCs to stromal cells—As hypoxia has been shown to induce HSPCs adhesion (34,35), the above

results suggested that the enhanced expression of LR11 may contribute to the hypoxia-induced adhesion of HSPCs to osteoblastic niches. In order to test for such a role of LR11, we analyzed the effects of LR11 knockdown on the adhesion of U937 cells to MSCs. As shown in Fig. 3A, hypoxic conditions failed to stimulate adhesion of the U937 clone with largely reduced LR11 expression (see insert in Fig. 3A), but the enhanced adhesion was readily observed in control cells. We, therefore, analyzed the effect of LR11 knockout on the adhesion of HSPCs to OP-9 murine stromal cells under hypoxia. Under normoxic conditions, the numbers of c-Kit(+) Lin(-) cells from *lr11*$^{-/-}$ and *lr11*$^{+/+}$ mice that attached to the stromal cells were not significantly different. However, while the adhesion of both *lr11*$^{-/-}$ and *lr11*$^{+/+}$ cells was significantly increased under hypoxic conditions (Fig. 3B), the enhancement of adhesion of the *lr11*$^{-/-}$ cells was significantly less than that of *lr11*$^{+/+}$ cells. Thus, LR11 functions in the hypoxia-increased adhesion to stromal cells of HSPCs and undifferentiated hematological cells. In order to identify the exact function of sLR11 in hypoxia-induced adhesion of HSPCs to stromal cells, we analyzed the effects of exogenously added sLR11 on this process. Incubation of U937 cells with sLR11 for 2 h drastically increased the numbers of cells attached to MSCs (Fig. 4A). Consistent with the effects of exogenously added sLR11 on cell adhesion, LR11-overexpressing U937 cells showed significantly increased adhesion in comparison to the control U937 cells (Fig. 4B). Furthermore, incubation with sLR11 induced the adhesion of c-Kit(+) Lin(-) cells to OP-9 cells in dose-dependent fashion (Fig. 4C). These results indicate that sLR11 is an important component of the pathway that mediates the stimulation of HSPC adhesion to stromal cells.

Hypoxia induces the adhesion of U937 cells to stromal cells via formation of an LR11/uPAR-complex—sLR11 enhances adhesion of SMCs through signaling via uPAR and integrins (21), and hypoxia leads to increased expression of uPAR in cultured hematological and other cells (19,36,37). Therefore, we examined whether uPAR also is a player in the sLR11-mediated adhesion of hematological cells. Incubation of U937 cells with sLR11 for 2h increased in dose-dependent fashion the number of cells attached to vitronectin-coated plates (Fig. 5A, left). Vitronectin is an extracellular partner of uPAR in receptor-mediated processes underlying cell attachment (38). Indeed, the adhesion of c-Kit(+) Lin(-) cells to vitronectin-coated plates was increased by incubation with sLR11 in the same dose range (Fig. 5A, right) as that of U937 cells. The increase in numbers of attached cells upon incubation with sLR11 was completely abolished in the presence of anti-uPAR antibodies, but was only non-significantly reduced in the absence of exogenously added sLR11 (Fig. 5B). Immunoprecipitation analysis of membrane extracts of U937 previously incubated with sLR11 showed that uPAR was co-precipitated with sLR11, and that the amounts of both proteins increased after exposure of the cells to hypoxia for 24 h (Fig. 5C). Finally, flow cytometric analysis (Fig. 5D) of LR11-overexpressing K562 cells (see insert; LR11 was not immunologically detectable in control K562 cells) showed increased surface uPAR levels in comparison to mock-transfected cells. These data suggest that under hypoxic conditions, the increased amounts of sLR11 stimulate uPAR-mediated adhesion of immature hematological cells by enhancing the formation of LR11/uPAR-complexes.

LR11 expression is dependent on HIF-1α-mediated signals—In order to gain insight into the mechanism underlying the induction of LR11 production by hypoxia, we tested whether changes in HIF-1α levels affect LR11 production. HIF-1α expression was increased by exposure of U937 cells to 1%, but not to 5%, oxygen (Fig. 6A). The response of HIF-1α expression to oxygen deprivation was the same as that of LR11, suggesting a functional link between LR11 and hypoxia-induced molecules such as HIF-1α. We therefore analyzed the effects of cobalt chloride ($CoCl_2$), a chemical inducer of HIF-1α (39), on the expression of LR11 in the cells under normoxia. $CoCl_2$ dose-dependently increased the LR11 levels in U937 cells (Fig. 6B) and also the

amounts of sLR11 released into the conditioned media (Fig. 6C). Thus, a chemical enhancer of HIF-1α induced the production of sLR11 independent of hypoxia. Finally, using HIF-1α-knockdown cells, we directly examined the role of HIF-1α in the sLR11-mediated adhesion of U937 cells under hypoxic conditions. The attachment of HIF-1α-knockdown U937 cells to MSCs was not enhanced by exposure to hypoxia, but importantly, the enhancing effect was largely recovered by addition of sLR11 (Fig. 6D). These results show that activation of HIF-1α by hypoxia increases the expression of LR11, and in turn the production of sLR11. Thus, the action of HIF-1α in increasing the sLR11 levels under hypoxic conditions is responsible for enhancing the adhesion of hematological cells to stromal cells.

Hypoxia induces HIF-1α binding to the potential binding site in LR11 promoter—We finally investigated the molecular mechanism underlying the interaction between the adhesion modulator of hematological cells, LR11, and the hypoxia-induced molecule, HIF-1α. HIF-1 has been shown to be a dominant effector of changes in transcription in response to hypoxia (39). In this context, we identified a potential HIF-1-binding site in the human LR11 gene promoter sequence spanning 5000 bp before the LR11 transcription start point. This site contains the HIF-1 core sequence 5′ -ACGTG-3′ (40) between nucleotides -65 and -61 (Fig. 7A). We therefore performed chromatin immunoprecipitation (ChIP) analysis in order to determine whether HIF-1α binds to this region of the LR11 promoter in U937 cells subjected to hypoxic conditions. As shown in Fig. 7B, ChIP analysis of nuclei derived from U937 cells grown under hypoxia revealed an increased level of amplified 144 bp product corresponding to the region encompassing the potential HIF-1 binding when compared to the nuclei from cells in normoxia. The pattern of amplified products was similar to that for the HIF-1-binding site in the promoter of CD18 (32), while no significant differences were observed in preimmunoprecipitation input samples between normoxia and hypoxia. These results strongly suggest that HIF-1α binds to the proximal 144-bp LR11 promoter in a region that bears the potential HIF-1-binding site, and that this binding is induced by hypoxia.

DISCUSSION

In this study, we have shed light on the mechanism underlying the regulation of HSPC homeostasis by LR11 under the hypoxic conditions found in the BM. Hypoxia increases the level of LR11 in, and of sLR11 produced by, undifferentiated leukemic U937 and c-Kit(+) Lin(-) cells. LR11 levels correlate with the extent of adhesion of HSPCs and U937 cells to stromal cells and exogenously added sLR11 enhances HSPC adhesion to BM stromal cells. sLR11 originates from cellular LR11, and therefore the induction of LR11 under hypoxic conditions is crucial for regulating the adhesion of HSPCs. Interestingly, previous studies have shown that uPAR expression is induced by hypoxia in cultured hematological cells (19). Furthermore, using uPAR-deficient mice, Tjwa et al. reported that membrane-anchored uPAR regulates HSPC adhesion and BM engraftment (17), and we have shown that sLR11 binds to and colocalizes with uPAR on the cell surface of SMCs (41). On the basis of these findings, we now demonstrate that the hypoxia-induced increase in LR11 was accompanied by an elevated level of uPAR, which forms a complex with LR11, and leads to enhanced HIF-1α- dependent adhesion of HSPCs. Taken together, these data suggest that the HIF-1α-mediated induction of LR11 expression by the hypoxic conditions in endosteal sites plays a key role in the adhesion of immature hematological cells to stromal cells via modulation of uPAR activity.

We suggest that the regulatory pathway operates via an increase of LR11, enhanced release of sLR11, and subsequent binding of uPAR to the cell surface of HSPCs in an autocrine and/or paracrine fashion. In this context, we previously showed that sLR11 derived from the cell surface enhances cell adhesion through the activation of uPAR and integrin-mediated signals (21,41,42). On the other hand, LR11 in intracellular vesicles (SorLA) is important for the intracellular traffic

of amyloidβ protein in neurons (43), and SNPs in the *LR11/SORL1* gene and/or sLR11 levels in the cerebrospinal fluid have been reported to be a prospective marker of Alzheimer's disease (44,45). Thus, both the released soluble form and the intracellular vesicle-enclosed form of LR11 may contribute to the regulation of adhesion properties of HSPCs under hypoxic conditions.

The present study revealed a high sensitivity of HSPCs, but not of mature hematological cells, to hypoxic conditions (see Fig. 1). Together with the previous observations that sLR11 is produced only by immature SMCs and not in mature SMCs in atherosclerotic arteries (21), and that high levels of LR11 mRNA are expressed in human CD34(+) CD38(-) immature hematopoietic progenitors (26), sLR11 released from immature cells may strengthen cell attachment to other stromal cells or extracellular matrices. In this context, preliminary results suggest that sLR11 is a potent enhancer of TNF-α-induced attachment of hematological cells to stromal cells in response to G-CSF treatment (Shimizu et al., unpublished observation).

The pO_2 in BM is approximately 55 mmHg, and the oxygen saturation is 87.5% (46). Several studies have suggested that long term HSCs reside mainly in the endosteal sites of the BM, in which the pO_2 is very low (3,6). Cell biological studies using HS(P)Cs indicated that their functions as well as their quiescence state is maintained most effectively under hypoxic conditions (10,47-52). Hypoxia stabilizes the HIF-1α protein, a master regulator of oxygen homeostasis, and activates HIF-1α-mediated signals in HSCs (48,53,54). Furthermore, leukocyte adhesion to activated endothelial cells was shown to be HIF-1α-dependent (32). However, acute severe hypoxia induces HIF-1α-independent cell adhesion of monocyte/macrophage to endothelial cells (55). Therefore, although the hypoxia-mediated LR11 expression clearly is regulated by HIF-1α, the possible roles of HIF-independent cascades in LR11 regulation need to be further investigated. In any case, the current observation of HIF-1α-dependent regulation of LR11 expression contributes novel details to our understanding of the mechanism(s) underlying hypoxia-inducible adhesion of U937 cells to endothelial cells in the stem cell niche.