マウス骨髄移植モデルおよび発現クローニング法を利用した解析. 第 66 回日本癌学会学術総会、シンポジウム

7) 渡辺（大河内）直子、沖俊彦、小埜良一、原田浩徳、湯地晃一郎、東條有伸、中島秀明, 野阪哲哉、稲葉俊哉、北浦次郎、北村俊雄 (2007 年 10 月) RasGRP4 と変異型 AML1 はマウス BMT モデルにおいて協調的に働き、T 細胞性白血病を誘発する. 第 69 回日本血液学会総会、口演

8) 小埜良一、熊谷英敏、中島秀明、殿塚行雄、菱谷愛、滝智彦、林泰秀、北村俊雄、野阪哲哉. (2007 年 10 月) MLL 融合蛋白は Ras-MAP キナーゼ系の活性化と相乗的に協調して急性白血病を発症する. 第 69 回日本血液学会総会、口演

9) 川島敏行、北村俊雄. (2007 年 10 月) STAT3/5 の活性化メカニズムと分子標的療法
第 69 回日本血液学会総会、シンポジウム

G. 知的財産権の出願・登録状況(予定を含む)

1.特許取得

シグナルシークエンストラップ法
特許第３４９９５２８号
中外製薬株式会社、北村俊雄
発明者：北村俊雄、小嶋哲郎

パッケイジング細胞
特許第３９０４４５１号
北村俊雄、中外製薬株式会社
発明者：北村俊雄、森田純代

2. 実用新案登録

なし

3.その他

なし

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分担研究報告書

ユーイング肉腫における新規膜抗原・分泌蛋白の探索

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研究要旨

ユーイング肉腫（Ewing sarcoma）は若年者に好発する骨腫瘍である。化学療法の発達により予後は改善されつつあるが、依然不良であり、早期診断が望まれる。当研究ではシグナルシークエンストラップ法を用いて、ユーイング肉腫に特異的な新規膜抗原や分泌蛋白のスクリーニングを行い、3種の興味深い遺伝子を得た。

A．研究目的

ユーイング肉腫は1921年、ユーイングによって報告された高悪性度小円形細胞肉腫で、5—15 歳で発症し、骨肉腫よりも若年者に好発する傾向がある。大腿骨、骨盤、肋骨に好発し、画像的には玉ねぎの皮様と表現される骨膜反応を伴った骨吸収破壊像が特徴的である。ユーイング肉腫の細胞起源は不明であり、骨の腫瘍であるにもかかわらず軟部への進展が速い。放射線や化学療法に対する反応はよいが、再発しやすく、骨や肺への転移のため、5年生存率は50%程度と予後の悪い腫瘍である。ユーイング肉腫は、骨を覆っている軟部組織が厚いために早期発見が困難な場合も多く、ユーイング肉腫に比較的特異的とされる膜蛋白 MIC2（CD99）は診断の補助にはなるが、完全に特異的というわけではなく、ある種のリンパ腫や横紋筋肉腫、肺小細胞癌の一部でも発現が見られる。したがって、早期診断と鑑別診断の目的で、ユーイング肉腫特異的な分泌蛋白、膜蛋白を同定することが望まれている。

本研究はユーイング肉腫の腫瘍マーカーとなる分子の探索を目的としており、将来的には分子標的療法への応用も視野に入れている。

B．研究方法

レトロウイルスを用いたシグナルシークエンストラップ法によるユーイング肉腫特異的分泌蛋白、膜蛋白遺伝子の同定

当研究代表者らが開発したシグナルシークエンストラップ法 SST-REX（Kojima T and Kitamura T, Nat Biotechnol 1999）は、IL-3 依存性のマウス血液細胞株 Ba/F3 が活性型 c-mpl によってトランスフォームし、IL-3 非依存性に増殖するようになる性質を応用したものである。すなわち、細胞膜に局在できないように細工した活性型 c-mpl 遺伝子との融合遺伝子ライブラリーを作成し、融合遺伝子がシグナルシークエンスを有する場合にのみ、活性型 c-mpl 遺伝子が細胞膜に移行し、Ba/F3 細胞をトランスフォームすることを利用して、シグナルシークエンスをもつ遺伝子を単離するのが SST-REX 法の原理である。ライブラリーの作成に用いる mRNA が由来する細胞を選択することにより、組織特異的な分泌蛋白、膜蛋白遺伝子を単離することが可能となる。研究分担者らはユーイング肉腫由来のヒト細胞株（SJES-2, SJES-3, SJES-5, SJES-6, SJES-7, SJES-8）を用いて、各々から mRNA を抽出し、それらを等量ずつ混合し、そこからシグナルシークエンストラップ用のライブラリーを作成し、Ba/F3 細胞でスクリーニングを行った。得られたクローンからゲノム DNA を抽出し、ライブラリーの作成に用いたベクター特異的配列を primer にした PCR を行い、増幅された DNA の塩基配列を決定した。ユーイング肉腫の細胞起源に関する定説はないが、間葉系幹細胞(mesenchymal stem cells; MSCs)由来説が優勢であり、末梢原始神経外胚葉性腫瘍（PNET）は非常に近縁であるとされる。したがって、まず、ヒト間葉系幹細胞株から mRNA を抽出し、候補遺伝子の発現を RT-PCR で調べる計画である。並行して、候補遺伝子の正常組織での発現パターンも解析する。

（倫理面への配慮）

ここまでの研究ではすでに他所で樹立されたヒトの細胞株を使用しているが、直接の患者検体は使用してい

ない。ヒトの直接の検体を利用する場合は、三重大学の倫理審査委員会の指針に従って該当者に説明を行い、同意書に署名をいただく。

C．研究結果

レトロウイルス発現系を用いたシグナルシークエンストラップ法にて81種類257クローンが単離した。内訳は膜蛋白が58種、分泌蛋白が14種、その他（機能不明も含む）が9種であった。例えば、ユーイング肉腫とPNETに特異的とされるt(11;22)(q24;q12)由来のEWS-FLI-1キメラ遺伝子を間葉系前駆細胞に発現させた時に発現が誘導される分泌蛋白であるIGFBP3 (Insulin-like Growth Factor Binding Protein 3)およびIGFBP5 (Riggi N. et al. Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res 65: 11459-11468, 2005)は、予想通り単離された。また、神経系に発現する種々の膜蛋白、軟骨、骨格組織の形成に関与する分泌蛋白、腫瘍・炎症細胞に高レベルで発現する膜蛋白、分泌蛋白などが単離された。これらのうち、明らかに組織特異性のない遺伝子や、他の組織の癌細胞で強く発現されているものは除外し、興味深い組織特異的mRNA発現パターンを示す3遺伝子に焦点を絞ってさらに解析した。遺伝子Aは分泌蛋白であり、正常マウス組織では脳において特異的に強く発現し、ヒトMSCsでの発現は検出されなかった。腫瘍細胞株では神経芽腫、横紋筋肉腫、Ewing肉腫由来のものでmRNAの強い発現が検出された。遺伝子Bは膜蛋白であり、正常マウス組織では脳において強く発現しており、他に脾、胸腺、骨髄、精巣、筋肉で発現がみられ、ヒトMSCsでの発現は検出されなかった。腫瘍細胞株では横紋筋肉腫、Ewing肉腫由来のものでmRNAの強い発現が検出された。遺伝子Cは分泌蛋白であり、正常マウスの様々な組織で発現していたが、ヒトMSCsでの発現は検出されなかった。腫瘍細胞株ではEwing肉腫由来のものでmRNAの強い発現が検出された。

D．考察

現在、上記3遺伝子産物に対するモノクローナル抗体を作製中であるが、今後、疾患特異性、予後との関連など、腫瘍マーカーになりうるか、さらには生物学的、病理学的機能を検討していく予定である。

E．結論

レトロウイルス発現クローニング法を用いたシグナルシークエンストラップ法にて 6 種のユーイング肉腫細胞株mRNA の混合物から、81 種、257 クローンの遺伝子を単離し、特に3種の遺伝子に関して、現在解析中である。

F．研究発表

1．論文発表

1) Lu Y, Kitaura J, Oki T, Komeno Y, Ozaki K, Kiyono M, Kumagai H, Nakajima H, Nosaka T, Aburatani H, Kitamura T. Identification of TSC-22 as a potential tumor suppressor that is upregulated by Flt3-D835V but not Flt3-ITD. Leukemia 21: 2246-2257, 2007.

2) Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y, Nakajima H, Nosaka T, Inaba T, Kitamura T. AML1 mutations induced MDS and MDS/AML in a mouse BMT model. Blood in press.

3) 小埜良一、野阪哲哉. MLL 関連白血病の分子病態. 血液・腫瘍科 52(6): 615-624, 2006.

4) 野阪哲哉. 造血器腫瘍マウスモデル. がん分子標的治療. 印刷中.

2．学会発表

1) 小埜良一、北村俊雄、野阪哲哉（演者）.（2007 年 3 月）MLL 融合蛋白による多段階発癌における Raf-MAP キナーゼ系活性化の重要性. 日本プロテインホスファターゼ研究会 第 3 回国内集会.

2) 渡辺-大河内直子、北浦次郎、小埜良一、原田浩徳、原田結花、中島秀明、野阪哲哉、稲葉俊哉、北村俊雄.（2007 年 5 月） AML1 点変異はマウス BMT モデルにおいて MDS/ AML を発症させる. 第 5 回幹細胞シンポジウム.

3) 小埜良一、熊谷英敏、中島秀明、殿塚行雄、菱谷愛、滝智彦、林泰秀、北村俊雄、野阪哲哉.（2007 年 10 月）Hoxa9 と Ras-MAP キナーゼ系の協調作用が MLL 融合蛋白による急性白血病の発症に重要である. 第 66 回日本癌学会学術総会.

4) 北村俊雄、野阪哲哉 （2007 年 10 月）白血病および MDS の分子病態：マウス骨髄移植モデルおよび発現ク

ローニング法を利用した解析. 第 66 回日本癌学会学術総会、シンポジウム

5) 渡辺（大河内）直子、沖俊彦、小埜良一、原田浩徳、湯地晃一郎、東條有伸、中島秀明,野阪哲哉、稲葉俊哉、北浦次郎、北村俊雄. (2007 年 10 月) RasGRP4 と変異型 AML1 はマウス BMT モデルにおいて協調的に働き、T 細胞性白血病を誘発する. 第 69 回日本血液学会総会.

6) 松下弘道、中島秀明、中村嘉彦、塚本秀雄、田中由美子、浅井さとみ、小埜良一、野阪哲哉、安藤潔、宮地勇人. (2007 年 10 月)活性誘導型 C/EBP α および C/EBP ε による MLL キメラ遺伝子を有する骨髄単球性白血病細胞の単球系分化. 第 69 回日本血液学会総会.

7) 小埜良一、熊谷英敏、中島秀明、殿塚行雄、菱谷愛、滝智彦、林泰秀、北村俊雄、野阪哲哉. (2007 年 10 月) MLL 融合蛋白は Ras-MAP キナーゼ系の活性化と相乗的に協調して急性白血病を発症する. 第 69 回日本血液学会総会.

G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
該当なし							

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
伊沢　久未	Functional analysis of an activating receptor LMIR4 as a counterpart of an inhibitory receptor LMIR3.	The Journal of Biological Chemistry	25	17997-18008.	2007
呂　洋	Identification of TSC-22 as a potential tumor suppressor that is up-regulated by Flt3-D835V but not Flt3-ITD.	Leukemia	21	2246-2257.	2007
山西　吉典	Analysis of mouse LMIR5/CLM7 as an activating receptor : differential regulation of LMIR5/CLM7 between mouse and human.	Blood	111	688-698.	2008

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Functional Analysis of Activating Receptor LMIR4 as a Counterpart of Inhibitory Receptor LMIR3*

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The leukocyte mono-Ig-like receptor (LMIR) belongs to a new family of paired immunoreceptors. In this study, we analyzed activating receptor LMIR4/CLM-5 as a counterpart of inhibitory receptor LMIR3/CLM-1. LMIR4 is expressed in myeloid cells, including granulocytes, macrophages, and mast cells, whereas LMIR3 is more broadly expressed. The association of LMIR4 with Fc receptor-γ among immunoreceptor tyrosine-based activation motif-bearing molecules was indispensable for LMIR4-mediated functions of bone marrow-derived mast cells, but dispensable for its surface expression. Cross-linking of LMIR4 led to Lyn- and Syk-dependent activation of bone marrow-derived mast cells, resulting in cytokine production and degranulation, whereas that of LMIR3 did not. The triggering of LMIR4 and TLR4 synergistically caused robust cytokine production in accordance with enhanced activation of ERK, whereas the co-ligation of LMIR4 and LMIR3 dramatically abrogated cytokine production. Notably, intraperitoneal administration of lipopolysaccharide strikingly up-regulated LMIR3 and down-regulated LMIR4, whereas that of granulocyte colony-stimulating factor up-regulated both LMIR3 and LMIR4 in granulocytes. Cross-linking of LMIR4 in bone marrow granulocytes also resulted in their activation, which was enhanced by lipopolysaccharide. Collectively, these results suggest that the innate immune system is at least in part regulated by the qualitative and quantitative balance of the paired receptors LMIR3 and LMIR4.

The Ig-like receptors provide positive and negative regulation of immune cells upon recognition of various ligands (1–5). We identified previously leukocyte mono-Ig-like receptors (LMIRs)[2] from a cDNA library of bone marrow-derived mast cells (BMMCs). We (6) and others (7–9) demonstrated that LMIR1/MAIRI (myeloid-associated Ig-like receptor I)/CLM-8 (CMRF-35-like molecules-8) and LMIR2/MAIRII/CLM-4/DIgR1 (dendritic cell-derived Ig-like receptor 1) are expressed mainly in myeloid cells. The human homolog of LMIR1 is CMRF-35H/IRp60 (inhibitory receptor protein of 60 kDa)/CD300a (10–14). The inhibitory effects of LMIR1 on mast cells and eosinophils and the activatory roles of LMIR2 in macrophages have been described recently (6, 7, 11). In addition to LMIRs, a variety of Ig-like paired receptors are expressed by myeloid cells (2, 15–17), but the biological significance of a paired receptor remains incompletely understood. Despite the similarity in the extracellular Ig-like domains, a striking structural difference between activating and inhibitory receptors exists in the transmembrane and cytoplasmic regions. In general, the former associate with an immunoreceptor tyrosine-based activation motif (ITAM)- or the related activating motif-bearing adaptor transmembrane protein, including DAP10, DAP12, or Fc receptor-γ (FcRγ), via a positively charged residue in the transmembrane domain, whereas the latter include an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain (1, 5, 18, 19).

Cells of the myeloid lineage such as granulocytes and mast cells are the major component of the innate immune response. Mast cells are implicated in a wide variety of inflammatory processes through a high affinity IgE receptor (FcεRI) or other immune receptors (20, 21). Aggregation of FcεRI with IgE plus antigen or highly cytokinergic IgE alone induces the activation of mast cells, leading to the secretion of preformed and newly synthesized pro-inflammatory mediators (22, 23). Alternatively, inhibitory receptors such as FcRγIIb, paired Ig-like receptor B, gp49B1, and LMIR1 are also expressed on the same cell surface, probably preventing excessive activation or decreasing the background activation levels before stimulation (3, 6, 7, 24–26).

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB292061 and AB292062.

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[2] The abbreviations used are: LMIRs, leukocyte mono-Ig-like receptors; BMMCs, bone marrow-derived mast cells; ITAM, immunoreceptor tyrosine-based activation motif; FcRγ, Fc receptor-γ; ITIM, immunoreceptor tyrosine-based inhibitory motif; FcεRI, high affinity IgE receptor I; TLR, Toll-like receptor; LPS, lipopolysaccharide; G-CSF, granulocyte colony-stimulating factor; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; Ab, antibody; TNP, trinitrophenyl; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FLMCs, fetal liver-derived mast cells; IL, interleukin; RT, reverse transcription; MAPKs, mitogen-activated protein kinases.

In this study, we identified new members of the LMIR family (LMIR3 and LMIR4) from a BMMC cDNA library. Sequence analysis showed that LMIR3 and LMIR4 are basically identical to CLM-1 and CLM-5, respectively (8). The human homolog of LMIR3 is also called IREM-1 (immune receptor expressed by myeloid cell-1) (27). Inhibitory receptor LMIR3/CLM-1 controls osteoclast differentiation (8). On the other hand, the function of LMIR4 was not fully understood; Fujimoto *et al.* (28) recently reported the expression of the LMIR4/CLM-5 transcript in myeloid cells and its association with FcRγ in transfected cells. In view of the high homology of LMIR3 and LMIR4 in the Ig-like domain, we have characterized LMIR4 as a counterpart of LMIR3. On the basis of the finding that LMIR3 and LMIR4 are expressed mainly in myeloid cells, we have utilized BMMCs or granulocytes to analyze the functions. The cross-talk between LMIR4 and LMIR3 or other receptors such as TLR4 or FcεRI leads us to postulate that LMIR4 is involved in a wide array of immune responses, including innate immunity and allergy. Moreover, the change in the relative expression levels of LMIR3 and LMIR4 in granulocytes in response to lipopolysaccharide (LPS) or granulocyte colony-stimulating factor (G-CSF) might suggest the relationship between innate immunity and differentiation of granulocytes upon bacterial infection.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rat anti-LMIR3 and anti-LMIR4 (derived from CBA/J mice) IgG2a monoclonal antibodies (mAbs), designated anti-LMIR3 and anti-LMIR4 (CBA) mAbs, respectively, was obtained from R&D Systems (Minneapolis, MN). Anti-FLAG mAb M2, fluorescein isothiocyanate (FITC)-conjugated anti-FLAG mAb M2, and mouse anti-dinitrophenyl IgE mAb (clone SPE-7; designated SPE-7 IgE) were all purchased from Sigma. Anti-Myc mAb and R-phycoerythrin (PE)-conjugated goat anti-rat IgG2a antibody (Ab) were from Roche Diagnostics (Mannheim, Germany) and Southern Biotech (Birmingham, AL), respectively. Mouse anti-trinitrophenyl (TNP) IgE mAb (C-38) and FITC-conjugated anti-mouse IgE mAb were from BD Biosciences. PE- or FITC-conjugated anti-CD3, anti-c-Kit, anti-CD45R/B220, anti-CD11b, and anti-Gr-1 Abs were from eBioscience (San Diego, CA). Anti-ERK, anti-p38, anti-JNK, and anti-Akt Abs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All phospho-specific Abs were purchased from Cell Signaling Biotechnology (Beverly, MA). $F(ab')_2$ fragments were prepared by digesting anti-FLAG mAb M2 or mouse IgG1 mAb with immobilized pepsin, followed by removing intact mAb by protein A affinity chromatography (Pierce). Cytokines were obtained from R&D Systems. PP2, PP3, and piceatannol were from EMD Biosciences, Inc. (San Diego, CA). All other reagents were from Sigma unless stated otherwise.

Cell Culture and Isolation—All hematopoietic cell lines were cultured as described (6). CBA/J or C57BL/6 (B6) mice (Charles River Laboratories, Inc.) were used at 8–10 weeks of age for isolation of tissues and cells. To generate BMMCs or fetal liver-derived mast cells (FLMCs) with 90% purity (c-Kit$^+$/FcεRI$^+$ by flow cytometry), BMMCs or FLMCs were cultured for 4–6 weeks in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng/ml IL (interleukin)-3 alone or with 20 ng/ml stem cell factor, respectively, as described previously (22, 29). The following mutant mice were used: *FcRγ*$^{-/-}$, *Dap12*$^{-/-}$, *FcRγ*$^{-/-}$/*Dap12*$^{-/-}$, *lyn*$^{-/-}$, and *syk*$^{+/-}$. Bone marrow-derived macrophages, plasmacytoid dendritic cells, and myeloid dendritic cells were generated as described (30, 31). Peritoneal cells were isolated by peritoneal lavage with 8 ml of phosphate-buffered saline. Granulocytes were obtained as reported previously (32, 33). Briefly, the whole bone marrow prepared from mice was centrifuged and washed with phosphate-buffered saline. After the red blood cells were hypotonically lysed with 0.2% NaCl, this solution was returned to isotonicity with 1.2% NaCl. After washing, the solution was delicately applied over a 62% Percoll gradient before it was centrifuged for 30 min at 1500 × *g*. The neutrophil pellet was then isolated. >90% neutrophil purity was confirmed with a Cytospin preparation. For *in vivo* experiments, 10 ng of G-CSF or 20 ng of LPS was intraperitoneally injected into mice 12 h before analysis of granulocytes.

Cell Stimulation—BMMCs were sensitized with 0.5 μg/ml anti-TNP IgE for 12 h, washed twice, and stimulated with various concentrations of TNP-conjugated bovine serum albumin. Alternatively, BMMCs were directly stimulated with SPE-7 IgE, LPS, $F(ab')_2$ anti-FLAG mAb, or their combinations. For granulocytes, these cells preincubated with 20 μg/ml anti-LMIR4 (CBA) Ab or rat IgG2a for 1 h on ice were washed before stimulation with 10 μg/ml $F(ab')_2$ anti-rat IgG2a Ab. Alternatively, granulocytes were incubated with RPMI 1640 medium including 10% fetal calf serum in the presence of 1000 ng/ml LPS or 100 ng/ml G-CSF for 24 h before analysis.

Cloning of LMIR3 and LMIR4—The DDBJ/EBI/GenBank™/Data Bank was searched using the amino acid sequence of the Ig-like domain of LMIR1. Accession numbers AY457049 (LMIR3 (B6)) and AY457051 (LMIR4 (B6)), which were derived from B6 mice, with close similarity in the extracellular domain were selected for cloning. On the basis of these sequence data, the cDNAs of LMIR3 and LMIR4 were isolated from a BMMC cDNA library (derived from CBA/J or C57BL/6 mice) by PCR and confirmed by sequencing (accession numbers AB292061 (LMIR3 (CBA)) and AB292062 (LMIR4 (CBA))) as described (6).

Plasmid Constructs—An expression plasmid encoding FLAG epitope-tagged DAP10, DAP12, or FcRγ was generated as described (6). LMIR3 (CBA), LMIR3 (B6), LMIR4 (CBA), or LMIR4 (B6) was ligated into pMXs-IRES-neo^r or pMXs-IRES-puro^r (34). The entire sequence excluding the leader sequence of LMIR3 (B6) or LMIR4 (B6) was amplified, and the resulting fragment was ligated into a pME vector including the signal sequence of SLAM (CD150; provided by Dr. H. Arase, Osaka University) (35). The resulting SLAM signal sequence-FLAG- or -Myc-LMIR3 (B6) or -LMIR4 (B6) fragment was subcloned into pMXs-IRES-puro^r or pMXs-IRES-neo^r, generating pMXs-FLAG- or -Myc- LMIR3- or -LMIR4-IRES-puro^r or -IRES-neo^r. All constructs were verified by DNA sequencing.

Transfection and Infection—Retroviral transfection was as described previously (6, 34). Briefly, retroviruses were generated by transient transfection of PLAT-E packaging cells (36) with FuGENE 6 (Roche Diagnostics). Cells were infected with

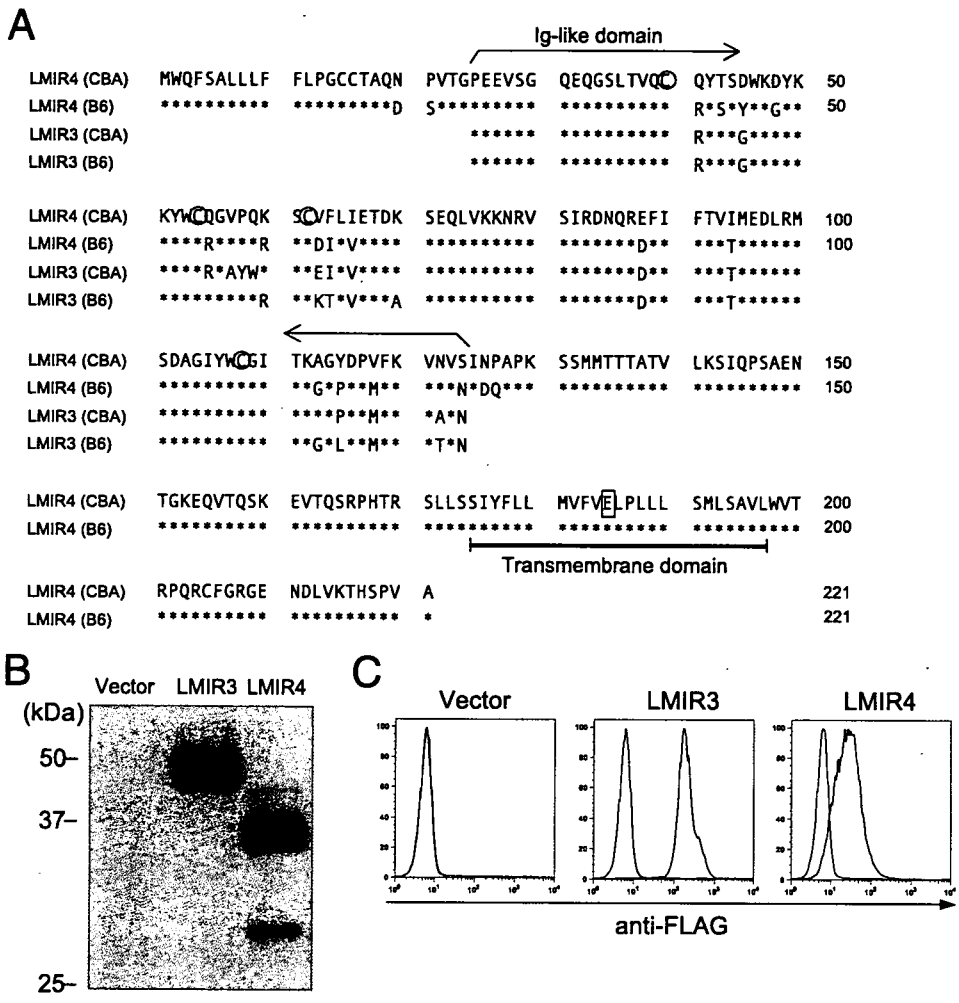


FIGURE 1. **Molecular and biochemical characteristics of LMIR4.** *A*, shown are the amino acid sequences of LMIR4 derived from different strains (CBA/J and C57BL/6). Ig-like domains are indicated. The cysteines potentially involved in generating the interchain disulfide bridge of the Ig-like domains are *circled*. The putative leader sequence is shaded. The predicted transmembrane domain is *underlined*; the negative charged glutamate residue is *boxed*. Alignment of the Ig-like domains of LMIR3 derived from CBA/J and C57BL/6 mice is also indicated. Identical amino acids are denoted by *asterisks*. *B*, Ba/F3 cells expressing FLAG-tagged LMIR3 or LMIR4 were lysed, and proteins were immunoprecipitated with anti-FLAG polyclonal Ab. Immunoprecipitates were resolved by SDS-PAGE under reducing conditions and blotted with anti-FLAG mAb. Molecular mass markers are indicated. *C*, flow cytometric analysis demonstrated that LMIR3 and LMIR4 on Ba/F3 cells expressing FLAG-tagged LMIR3 and LMIR4, respectively, were stained by FITC-conjugated anti-FLAG mAb.

retroviruses in the presence of 10 μg/ml Polybrene. Selection with G418 or puromycin was started 48 h after infection.

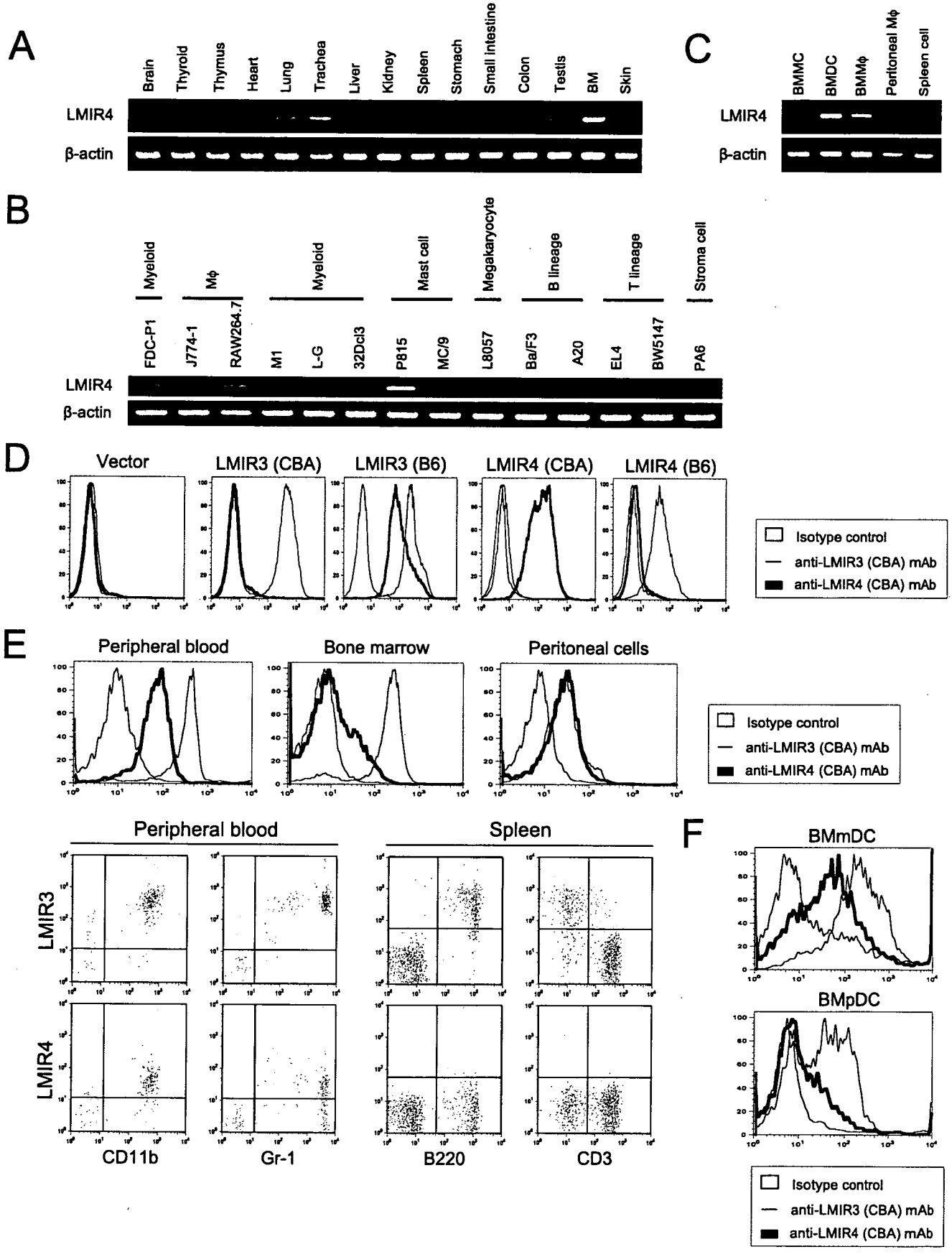
Reverse Transcription (RT)-PCR—The expression of LMIR4 was analyzed by RT-PCR amplification as described (6). Total RNAs were extracted from each cell line and CBA/J mouse-derived tissues and cells. A fragment of LMIR4 was amplified with primers 5′-ctgagattgcaagcatacacg-3′ and 5′-gattcctgcagttgacctcc-3′. This set of primers does not cross-react with LMIR3 (data not shown). For normalization, a fragment of β-actin was amplified with primers 5′-catcactattggcaacgagc-3′ and 5′-acgcagctcagtaacagtcc-3′.

Biochemistry—To detect the association of LMIR4 and ITAM- or the related activating motif-bearing molecules, COS-7 or 293T cells were cotransfected with two constructs of interest (pME-Myc-LMIR4 and pMKIT-FLAG-DAP10, -DAP12, or -FcRγ). Cells were harvested at 48 h after transfection and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, and 1% Nonidet P-40 in the presence of protease and phosphatase inhibitor mixtures (Sigma). Cleared supernatants of cell lysates were used for immunoprecipitation with appropriate Abs and protein A-Sepharose CL-4B (GE Healthcare). Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membranes were blocked and incubated with anti-FLAG mAb, followed by horseradish peroxidase-conjugated anti-mouse Ig (Sigma). To detect phosphorylation of several proteins, stimulated cells were lysed in lysis buffer, and lysates were subject to protein assay using a Bio-Rad protein assay kit. An equal amount of total lysate was separated by SDS-PAGE. The membrane was incubated with anti-phosphotyrosine mAb or phospho-specific Abs and then incubated with the appropriate horseradish peroxidase-conjugated secondary Abs (Sigma). Proteins were detected by enhanced chemiluminescence (Amersham Biosciences) as described (6, 37).

Flow Cytometry—Briefly, cells were incubated with the appropriate antibodies for 60 min on ice after blocking the Fc receptor. When necessary, the samples were incubated with secondary antibodies for an additional 60 min on ice. To monitor apoptosis, cells were incubated with 1 μg/ml PE-labeled annexin V (BD Biosciences) at room temperature for 20 min in the dark. Flow cytometric analysis of the stained cells was performed with a FACSCalibur (BD Biosciences) equipped with CellQuest software and Flowjo software (TreeStar Inc.) as described (6, 22, 37).

Measurement of Histamine and Cytokines and Adhesion Assays—Histamine released from stimulated cells during a 50-min incubation period was measured as described (22). After 12 h of stimulation, supernatants were measured using enzyme-linked immunosorbent assay kits from R&D Systems. For adhesion assays, BMMCs or granulocytes were incubated on 96-well plate with or without the indicated stimulation 1 h before evaluation of adherent cells using CellTiter-Glo and a MicroLumat Plus luminometer as described (37).

Statistical Analysis—Data are shown as the mean ± S.D., and statistical significance was determined by Student's *t* test, with $p < 0.05$ taken as statistically significant.



RESULTS

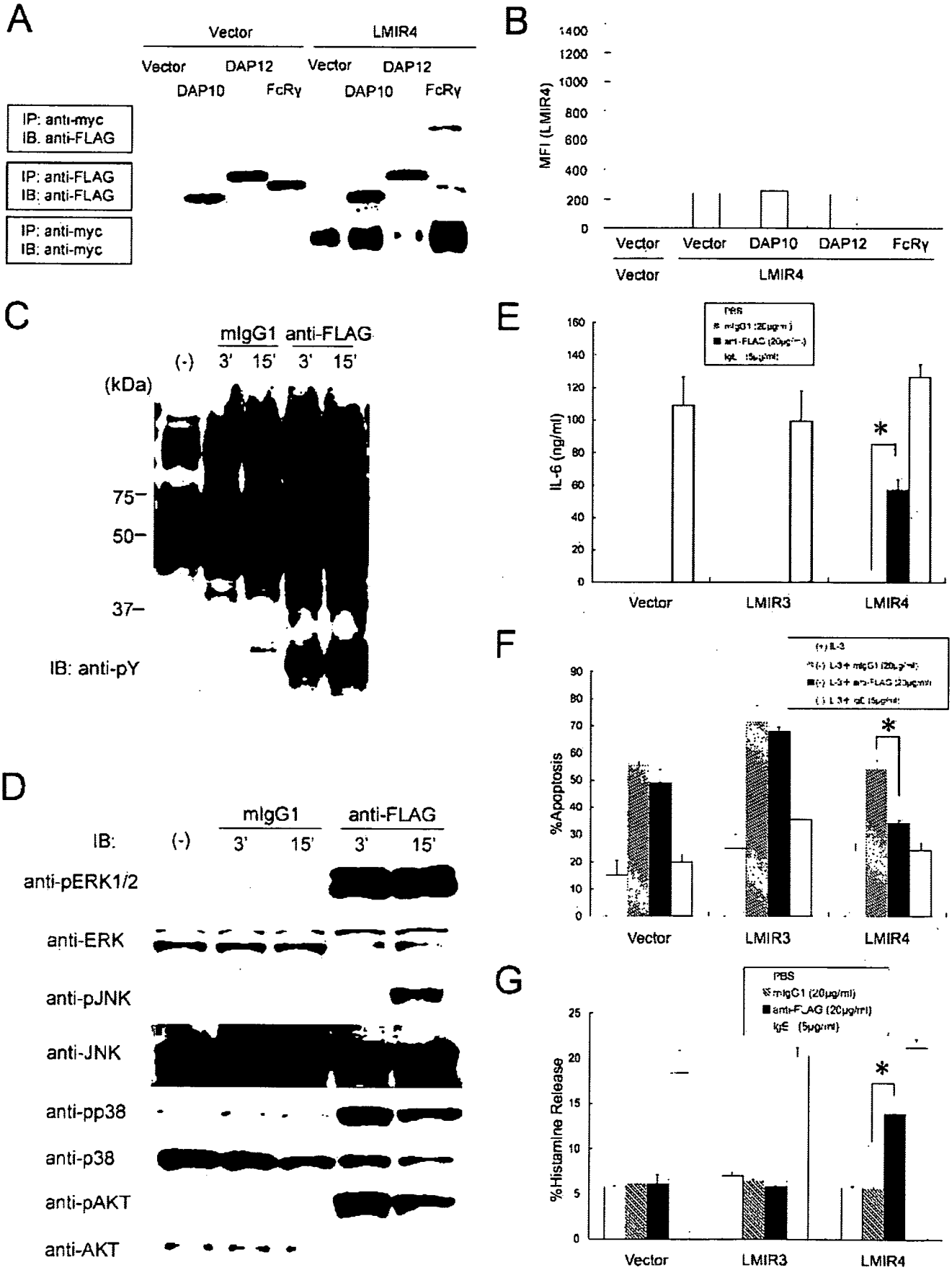
Cloning of LMIR4—We previously cloned paired immunoreceptors, LMIR1 and LMIR2, using a signal sequence trap based on retrovirus-mediated expression screening (38) and the DDBJ/EBI/GenBank™ Data Bank (6). To find new members of the LMIR family, we searched the same data base using the sequence of the Ig-like domain of LMIR1 and identified four homologous cDNA sequences. Among them, we cloned two cDNAs (1014 and 666 nucleotides in an open reading frame, termed LMIR3 and LMIR4, respectively) from a BMMC cDNA library derived from CBA/J mice. The LMIR3 and LMIR4 proteins are 337 and 221 amino acids in length, respectively, and 91% identical in the Ig-like domain (Fig. 1*A*). Both LMIR3 and LMIR4 cDNAs encode for a type I transmembrane protein containing a signal peptide, a single variable-type Ig-like domain, and a transmembrane domain, with the former harboring a cytoplasmic region containing ITIM. The latter contains only a short cytoplasmic tail. Thus, LMIR3 displays a structure typical of an inhibitory receptor. In contrast, LMIR4 has a unique property as an activating receptor, *i.e.* LMIR4 does not possess a positively charged residue such as an arginine or lysine that is supposed to associate with an ITAM-bearing adaptor protein, but instead contains a negatively charged residue, glutamic acid, in its transmembrane domain (1, 2, 5, 39). Sequence analysis showed that LMIR3 and LMIR4 are basically identical to CLM-1 and CLM-5, respectively (8). The difference of 13 amino acids between LMIR3 and CLM-1 or 19 amino acids between LMIR4 and CLM-5 is due to the difference between mouse strains. LMIR and CLM are derived from CBA/J and C57BL/6, respectively (Fig. 1*A*). Amino acid alignment showed that the LMIR4 protein does not have apparent *N*-linked glycosylation sites within its extracellular domain, but has several potential *O*-linked glycosylation sites within a serine/threonine-rich region. Immunoprecipitation of FLAG-tagged LMIR4 transduced into Ba/F3 cells revealed ~36- and ~28-kDa proteins when analyzed under both reducing and nonreducing conditions. However, *N*-glycosidase F treatment did not decrease the mobility of LMIR4 (Fig. 1*B*) (data not shown). The surface expression levels of transduced LMIR4 on Ba/F3 cells were confirmed by flow cytometry (Fig. 1*C*). Collectively, LMIR4 is a monomeric, *O*-linked glycoprotein.

LMIR4 Is Expressed Mainly in Myeloid Cells—To investigate the expression patterns of LMIR4, RT-PCR was performed on various tissues. A high expression level of LMIR4 was observed in bone marrow cells. Interestingly, LMIR4 was also highly expressed in the trachea and lung (Fig. 2*A*). To further delineate the expression pattern in hematopoietic cells, RT-PCR analysis was applied to a variety of cell lines and primary cells. We observed the expression of LMIR4 in myeloid cell lines, including mast cell lines and macrophage lines (Fig. 2*B*), and in primary myeloid cells such as bone marrow-derived macrophages, bone marrow-derived dendritic cells, and BMMCs (Fig. 2*C*). To confirm the surface expression level of LMIR4 on hematopoietic cells, we generated anti-LMIR3 (CBA) and anti-LMIR4 (CBA) mAbs. The staining of Ba/F3 cells overexpressing LMIR3 (CBA), LMIR4 (CBA), LMIR3 (B6), or LMIR4 (B6) demonstrated the sensitivity and specificity of these Abs. Anti-LMIR3 (CBA) mAb detected LMIR3 and LMIR4 (B6), but not LMIR4 (CBA). Anti-LMIR4 (CBA) mAb detected LMIR4 (CBA), but not LMIR4 (B6) and LMIR3 (Fig. 2*D*). All Abs did not cross-react with LMIR1 or LMIR2 (data not shown). Only in the analysis of the cells derived from CBA/J mice was the surface expression of LMIR3 and LMIR4 specifically detected by these mAbs. Flow cytometric analysis revealed that LMIR4 was expressed in granulocytes (Gr-1^{high}Mac-1^{high}) and monocytes/macrophages (Gr-1^{low}Mac-1^{low}). Granulocytes in peripheral blood highly expressed LMIR4 (Fig. 2*E*) (data not shown), whereas a weak but detectable level of LMIR4 was also observed in bone marrow-derived cells (Fig. 2*F*) (data not shown). LMIR4 expression was not detected in B- or T-cells (Fig. 2*E*), in accordance with the result of LMIR4 mRNA expression patterns. In contrast, LMIR3 was more broadly expressed in hematopoietic cells other than T-cells (Fig. 2, *E* and *F*). In summary, LMIR4 was expressed rather exclusively in myeloid cells compared with LMIR3.

LMIR4 Selectively Associates with FcRγ, but Not DAP10 or DAP12—LMIR4 is atypical as an activating receptor in that it has a negatively charged residue, glutamic acid, in the transmembrane domain. To clarify whether LMIR4 can associate with ITAM- and the related activating motif-bearing adaptor proteins, co-immunoprecipitation experiments were performed using COS-7 cells cotransfected with FLAG-tagged DAP10, DAP12, or FcRγ or a control construct together with Myc-tagged LMIR4. As depicted in Fig. 3*A*, only FcRγ was co-immunoprecipitated with LMIR4. The expression level of LMIR4 was significantly elevated in the presence of FcRγ. At the same time, the surface expression level of LMIR4 on COS-7 cells dramatically increased by overexpression of FcRγ, but not DAP10 and DAP12 (Fig. 3*B*). These results suggest that FcRγ associates with and stabilizes LMIR4, resulting in its increased expression.

Cross-linking of LMIR4, but Not LMIR3, Induces the Activation of BMMCs—To obtain strong activation of primary cells stimulated by LMIR4 cross-linking, we generated FLAG-tagged LMIR4-transduced BMMCs. When stimulated with $F(ab')_2$ anti-FLAG mAb, but not control mAb, LMIR4 transfectants showed increased tyrosine phosphorylation in total cellular proteins as revealed by anti-phosphotyrosine blotting (Fig. 3*C*). Moreover, the phosphorylation of Akt and MAPKs such as ERK, JNK, and p38 was recognized using phospho-specific Abs

FIGURE 2. **Expression of LMIR4.** *A–C*, various mouse tissues, hematopoietic cell lines, and bone marrow (*BM*)-derived cells were analyzed by RT-PCR using specific primers for LMIR4. β-Actin was amplified as a control. *BMMΦ*, bone marrow-derived macrophages. *D*, LMIR4 (CBA), LMIR4 (B6), LMIR3 (CBA), and LMIR3 (B6) were transduced into Ba/F3 cells. The sensitivity and specificity of anti-LMIR3 (CBA) mAb or anti-LMIR4 (CBA) mAb were confirmed by flow cytometry. *E* and *F*, single cell suspensions were prepared from peripheral blood, bone marrow, splenocytes, and peritoneal cells from CBA/J mice. The cells gated in the population forward scatter $(FSC)^{high}$ side scatter $(SSC)^{high}$ were stained with anti-LMIR3 (CBA) mAb, anti-LMIR4 (CBA) mAb, or control rat IgG2a, followed by staining with PE-conjugated anti-rat IgG2a mAb. For double staining, the FITC-conjugated mAbs indicated were used. The peripheral blood cells gated in the population ($FSC^{high}SSC^{high}$) and the splenocytes gated in the population ($FSC^{low}SSC^{low}$) were analyzed. Bone marrow-derived myeloid (*BMmDC*) and plasmacytoid (*BMpDC*) dendritic cells were generated from CBA/J mice. The cells were stained as described above.



(Fig. 3*D*). These data indicate that aggregation of LMIR4 induced activation of mast cells. As activated mast cells have the potential to produce various chemical mediators, we next examined whether BMMCs stimulated by LMIR4 cross-linking produce cytokines. In response to $F(ab')_2$ anti-FLAG mAb, the LMIR4 transfectants, but not LMIR3 or control transfectants, produced a large amount of IL-6 and tumor necrosis factor-α almost comparable with the levels obtained upon IgE stimulation (Fig. 3*E*) (data not shown). As reported recently, mast cells treated with highly cytokinergic IgE survive by an autocrine mechanism under IL-3-depleted conditions (22, 40, 41). We next explored whether the triggering of LMIR4 exerts the same survival effect. As shown in Fig. 3*F*, LMIR4 stimulation displayed an anti-apoptotic effect on mast cells, although highly cytokinergic IgE was more effective. We also measured the released histamine, indicative of degranulation in mast cells. Cross-linking of LMIR4 induced a 15% release of histamine, a relatively low but significant level compared with the stimulation by SPE-7 IgE (Fig. 3*G*). In summary, the aggregation of LMIR4 activates mast cells, resulting in secretion of newly synthesized and preformed chemical mediators.

Cross-linking of LMIR4 Induces Cytokine Production of BMMCs through Lyn and Syk Kinases—To investigate the role of two major tyrosine kinases, Lyn and Syk, in mast cell functions caused by LMIR4 aggregation, pharmacological experiments were conducted (29, 42–44). Pretreatment with piceatannol (a Syk kinase inhibitor) or PP2 (a Src family kinase inhibitor), but not PP3 (a control analog of PP2), dramatically reduced cytokine production of LMIR4-stimulated BMMCs (Fig. 4*A*). Lyn is a Src family kinase found in abundance in mast cells and can be a positive or negative regulator of mast cell functions, depending on the intensity of FcεRI aggregation (45–49). To clarify the role of Lyn in LMIR4 signaling pathways, LMIR4 was transduced into wild-type or Lyn-deficient mast cells (48). Comparable expression levels of c-Kit and FcεRI and transduced LMIR4 were confirmed by flow cytometry (Fig. 4*B*). Stimulation by IgE plus a high dose of antigen caused higher levels of IL-6 production in Lyn-deficient mast cells than in wild-type mast cells, as reported previously (22, 47). In contrast, LMIR4-induced IL-6 production was observed only in wild-type mast cells, but not in Lyn-deficient mast cells, at least in the range of the stimulation mode we used (Fig. 4*C*). Next, we performed similar experiments using Syk-deficient FLMCs with expression levels of FcεRI and c-Kit comparable with those of wild-type FLMCs (Fig. 4*D*). As shown in Fig. 4*E*, IL-6 production by LMIR4 cross-linking was completely abolished in Syk-deficient FLMCs in accordance with negligible activation of ERK (Fig. 5*D*), although that by phorbol 12-myristate 13-acetate stimulation as a control was comparable between wild-type and Syk-deficient FLMCs (29). Collectively, these data indicate that both Lyn and Syk kinases are required for positive signals downstream of LMIR4.

Role of FcRγ in the Function of Mast Cells Stimulated by LMIR4 Cross-linking—To further explore the effect of FcRγ on LMIR4 expression on the cell surface, FcRγ- or DAP12-deficient BMMCs were used. As the genetic background strain of these mice is B57BL/6 and as anti-LMIR4 (CBA) mAb does not detect LMIR4 (B6), FLAG-tagged LMIR4 was retrovirally transduced into BMMCs derived from $FcR\gamma^{-/-}$, $Dap12^{-/-}$, or $FcR\gamma^{-/-}/Dap12^{-/-}$ mice (50–52). We first confirmed that the surface expression levels of c-Kit were comparable among these transfectants and that those of FcεRI in FcRγ-deficient mast cells were not detectable (Fig. 5*A*, *upper panels*), as reported (48, 49). Flow cytometric analysis using anti-FLAG mAb revealed that the surface expression of LMIR4 in FcRγ-deficient BMMCs was still detectable but significantly lower than that in wild-type or DAP12-deficient BMMCs (Fig. 5*A*, *lower panels*). This result confirmed the finding in Fig. 3, whereas it suggested that FcRγ was not essential for the surface expression of LMIR4. Next, we attempted to clarify the functional role of FcRγ in LMIR4 signaling. To this end, cytokine production of BMMCs derived from wild-type or FcRγ-deficient mice was measured in response to LMIR4 triggering. Strikingly, FcRγ-deficient mast cells induced no detectable production of IL-6 in accordance with no activation of ERK (Fig. 5, *B* and *C*). The same tendency was observed upon highly cytokinergic IgE stimulation; FcεRI also shared FcRγ essential to the signaling downstream of FcεRI, whereas control stimulation by phorbol 12-myristate 13-acetate induced comparable amounts of cytokine production in the wild-type and FcRγ-deficient mast cells (Fig. 5*B*). We conclude that FcRγ selectively associates with and stabilizes LMIR4 and is essential for downstream functions, although it is dispensable for surface expression.

Cooperation of LMIR4 Signaling with Others—Activating receptors adjust the functions of immune cells by cooperating with other receptors. For example, cytokine production of macrophages stimulated by LPS is enhanced by cross-linking of TREM1 (4, 17). Therefore, we examined the effects of LMIR4 aggregation on the functions downstream of other receptors. As shown in Fig. 6*A*, cross-linking of LMIR4 synergistically enhanced the cytokine production of BMMCs stimulated by LPS through TLR4 (*upper panel*) and by IgE plus antigen

FIGURE 3. **LMIR4 functions as an activating receptor.** *A*, COS-7 cells were transiently cotransfected with FLAG-tagged DAP10, DAP12, or FcRγ or a mock-transfected construct and a Myc-tagged LMIR4 construct. Lysates of these transfectants were immunoprecipitated (*IP*) with anti-Myc mAb. The precipitates were probed with anti-FLAG or anti-Myc mAb. The same series of lysates was also immunoprecipitated with anti-FLAG polyclonal Ab, and the precipitates were probed with anti-FLAG mAb. *B*, the surface expression of LMIR4 on these transfectants was analyzed using anti-Myc mAb by flow cytometry. The mean fluorescent intensity (*MFI*) of LMIR4 expression is presented. One experiment representative of three independent experiments is shown. *C*, BMMCs were infected with LMIR4-expressing retroviruses. Shown are the results of phosphotyrosine (*pY*) blot analysis of lysates from the transduced BMMCs stimulated with $F(ab')_2$ mouse (*m*) IgG1 or anti-FLAG mAb for the indicated times. *D*, the amount of phosphorylated (*p*) ERK1/2, JNK, p38, or Akt was examined by Western blot analysis using anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab, anti-phospho-JNK (Thr^{183}/Tyr^{185}) Ab, anti-phospho-p38 (Thr^{180}/Tyr^{182}) Ab, or anti-phospho-Akt (Ser^{473}) Ab, respectively. Equal loading was evaluated by reprobing immunoblots (*IB*) with Ab specific for ERK1/2, JNK, p38, or Akt. *E* and *G*, mock-, LMIR3-, or LMIR4-transduced BMMCs were generated. Cross-linking of LMIR4, but not LMIR3, in BMMCs using $F(ab')_2$ anti-FLAG mAb induced IL-6 production or histamine release. IL-6 (*E*) or histamine (*G*) secreted into the culture medium was measured. All data points correspond to the mean ± S.D. of four independent experiments. *PBS*, phosphate-buffered saline. *F*, BMMCs were deprived of IL-3 in the presence of $F(ab')_2$ anti-FLAG mAb, $F(ab')_2$ mouse IgG1, or SPE-7 IgE for the indicated periods before staining with PE-conjugated annexin V. The percent of apoptotic cells was analyzed by flow cytometry. All data points correspond to the mean ± S.D. of four independent experiments. *Asterisks* ($p < 0.05$) indicate statistical differences.

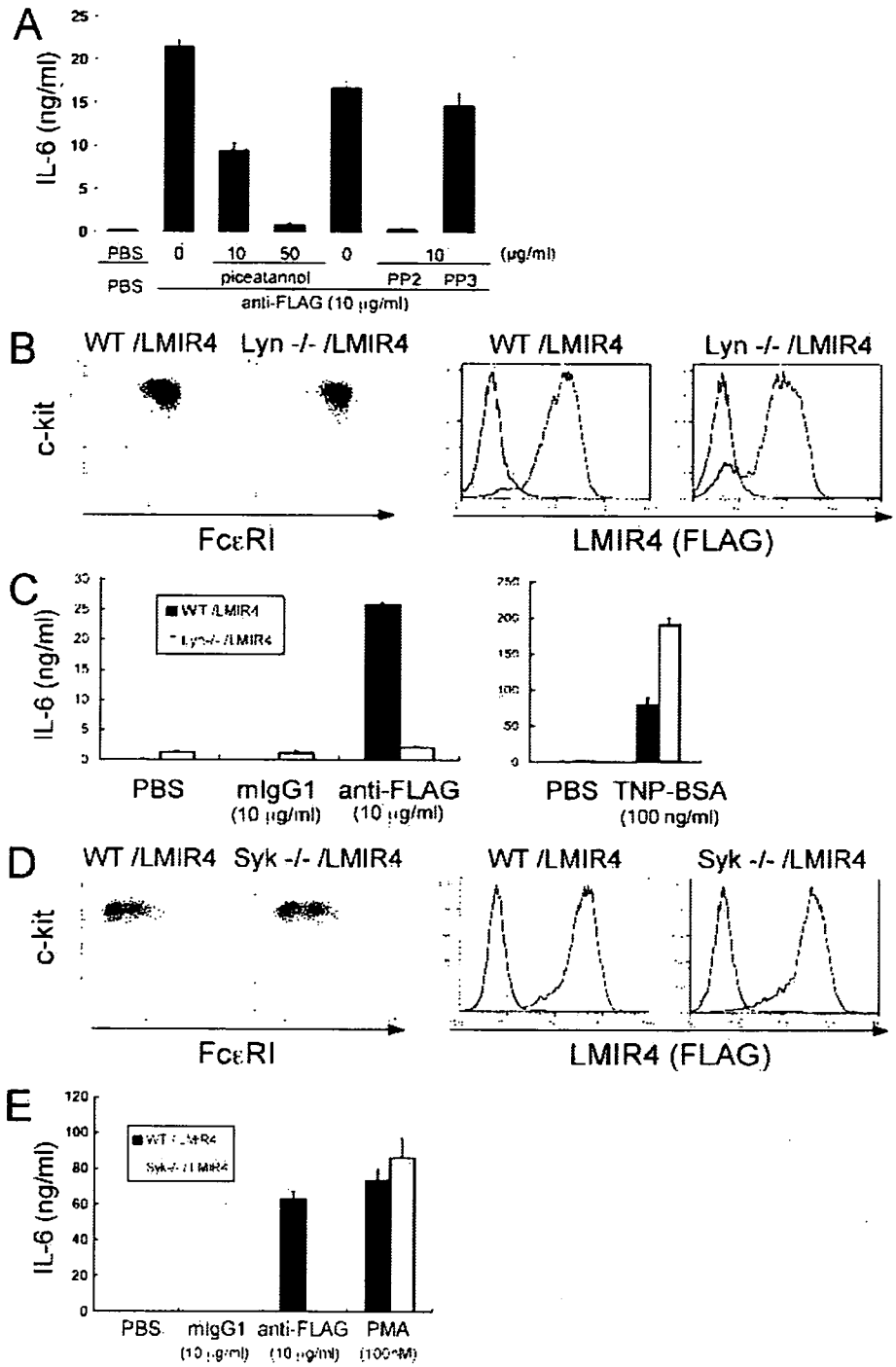


FIGURE 4. **Cytokine production of BMMCs by aggregation of LMIR4 is dependent on Lyn and Syk.** *A*, the IL-6 production of FLAG-tagged LMIR4-transduced BMMCs stimulated by $F(ab')_2$ anti-FLAG mAb was measured. Cells were preincubated for 30 min with the indicated concentrations of PP2, PP3, or piceatannol. *PBS*, phosphate-buffered saline. *B*, BMMCs derived from wild-type (*WT*) or *lyn*$^{-/-}$ mice were infected with LMIR4-expressing retroviruses. The surface expression levels of c-Kit and IgE-bound FcεRI as well as LMIR4 were analyzed by flow cytometry. *C*, the IL-6 production of these transfectants stimulated by $F(ab')_2$ mouse (*m*) IgG1 or anti-FLAG mAb (*left panel*) or by anti-TNP IgE plus TNP-conjugated bovine serum albumin (*BSA*) (*right panel*) was measured. *D*, FLMCs derived from wild-type or *syk*$^{-/-}$ mice were infected with LMIR4-expressing retroviruses. The surface expression levels of c-Kit and IgE-bound FcεRI as well as LMIR4 were analyzed by flow cytometry. *E*, the IL-6 production of these transfectants stimulated by $F(ab')_2$ mouse IgG1, anti-FLAG mAb, or phorbol 12-myristate 13-acetate was measured. All data points correspond to the mean ± S.D. of four independent experiments.

through FcεRI (*lower panel*). In particular, the strong synergy between LMIR4 and TLR4 signaling was noteworthy and was in accordance with the strong enhancement of ERK and Akt activation in BMMCs triggered by both LMIR4 and TLR4 (Fig. 6*B*).

To evaluate the effect of the co-ligation of LMIR3 and LMIR4 on mast cell functions, we generated FLAG-tagged LMIR3- and/or LMIR4-transduced BMMCs displaying comparable expression levels of FcεRI and c-Kit (Fig. 6*C*). The expression levels of LMIR3 and/or LMIR4 of these transfectants were confirmed by Western blot analysis (Fig. 6*D*). As expected, cross-linking of LMIR4 alone resulted in the production of a large amount of IL-6, whereas co-ligation of LMIR3 and LMIR4 by $F(ab')_2$ anti-FLAG mAb dramatically abrogated it (Fig. 6*E*). This result supports the notion that LMIR4 and LMIR3 function as activating and inhibitory receptors, respectively.

Modulation of the Expression Levels of LMIR3 and LMIR4 in Granulocytes by in Vivo and in Vitro Administration of LPS or G-CSF—Some activating and inhibitory receptors have been reported to increase their surface expression levels on immune cells under inflammatory conditions. For example, TLT2 expression on monocytes is up-regulated by *in vivo* administration of LPS (53). As both LMIR3 and LMIR4 are expressed predominantly in myeloid cells, the possibility arose that the expression levels of these receptors are regulated by inflammation. To test this, LPS was administrated into the peritoneal cavities of CBA/J mice. As a result, the granulocytes ($Gr\text{-}1^{high}$) included in bone marrow, peripheral blood, or the peritoneal cavity showed an increased mean fluorescence intensity for LMIR3 and a decreased mean fluorescence intensity for LMIR4 (Fig. 7*A*). On the other hand, intraperitoneal injection of G-CSF increased the expression of both LMIR3 and LMIR4 in particular in bone marrow granulocytes, but not in peripheral blood granulocytes, indicating higher expression levels of both LMIR3 and LMIR4 in mature granulocytes (Fig. 7*B*). To confirm the *in vivo* results, granulocytes purified from bone marrow cells were incubated for 24 h with either LPS or G-CSF.

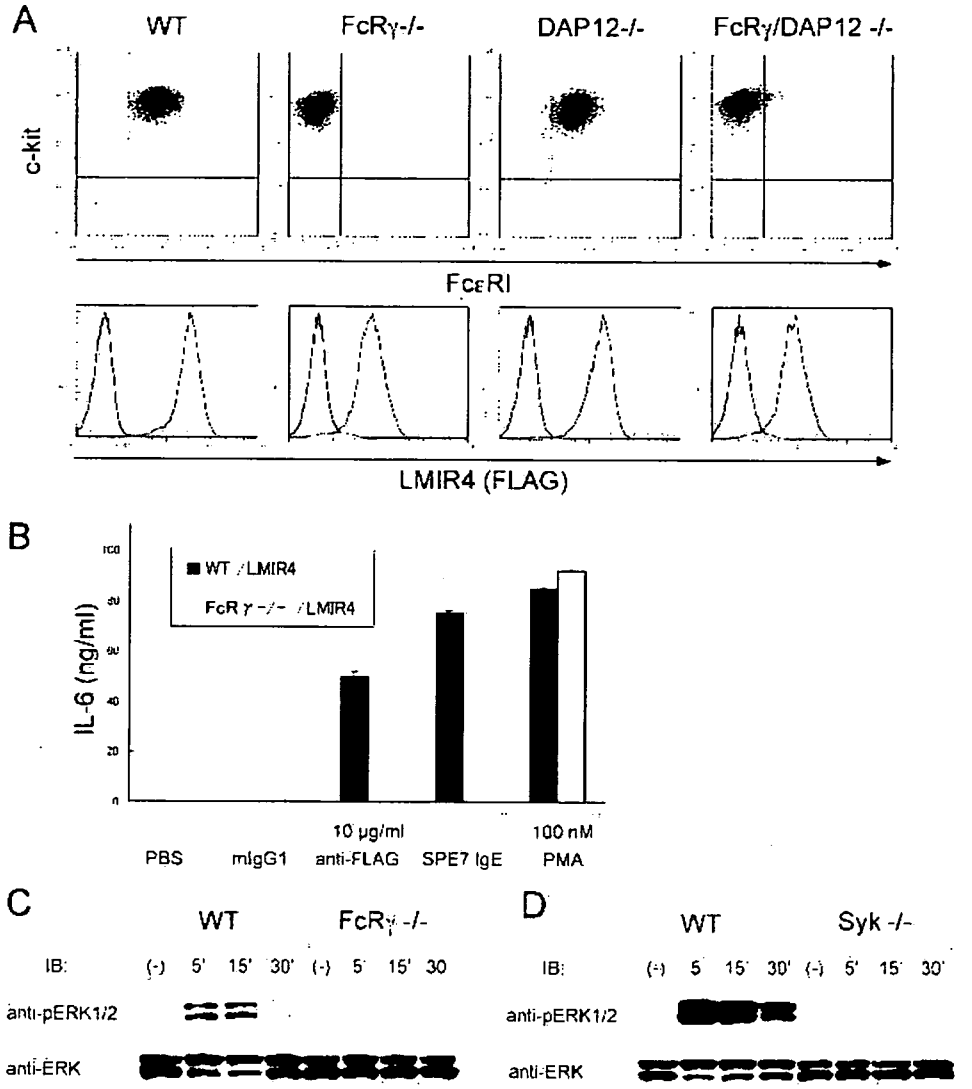


FIGURE 5. **FcRγ is essential to the activation of mast cells stimulated by cross-linking of LMIR4, but is dispensable for surface expression on mast cells.** *A*, BMMCs generated from wild-type (*WT*), *FcRγ*$^{-/-}$, *Dap12*$^{-/-}$, or *FcRγ*$^{-/-}$/*Dap12*$^{-/-}$ mice were transfected with LMIR4-expressing retroviruses. The surface expression levels of c-Kit and IgE-bound FcεRI as well as LMIR4 were analyzed by flow cytometry. *B*, IL-6 production of these transfectants stimulated by F(ab')$_2$ mouse (*m*) IgG1 or anti-FLAG mAb was measured. All data points correspond to the mean ± S.D. of four independent experiments. *PBS*, phosphate-buffered saline. *C* and *D*, the amount of phosphorylated ERK1/2 (*pERK1/2*) in lysates from wild-type or FcRγ-deficient BMMCs (*C*) or from wild-type or Syk-deficient FLMCs (*D*) was examined by Western blot analysis using anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab. Equal loading was evaluated by reprobing immunoblots (*IB*) with Abs specific for ERK1/2.

but not tumor necrosis factor-α, was observed. In addition, LPS-induced production of IL-6 and tumor necrosis factor-α in granulocytes was significantly enhanced by LMIR4 engagement (Fig. 7*E*). Collectively, the activation of granulocytes is induced by engagement of endogenous LMIR4, which is enhanced by LPS. Thus, synergistic activation by LMIR4 and TLR4 engagement is recognized in granulocytes as well as in mast cells.

DISCUSSION

In this study, we identified LMIR3 and LMIR4 from a CBA/J mouse-derived BMMC cDNA library as new members of the LMIR family. We demonstrated a diversity of LMIR/CLM molecules among mouse strains as well as a similarity in the Ig-like domain among the members of the LMIR family, possibly giving a clue to the identification of ligands for LMIRs. Staining hematopoietic cells derived from CBA/J mice with specific mAbs revealed that LMIR4 is expressed mainly in myeloid cells, whereas LMIR3 is more broadly expressed, suggesting the existence of other activating receptors of the same family in other cells and tissues. In fact, there are a couple of activating receptors related to LMIR4. Interestingly, the expression levels of LMIR4 were higher in granulocytes of peripheral blood than in those of bone marrow and were elevated after administration of G-CSF, suggesting that mature or activated granulocytes express more LMIR4. The relationship of LMIR4 expression to the differentiation of myeloid cells remains to be determined. Our results implicate LMIR4 in innate immunity because of the following reasons. First, the expression of LMIR4 is observed in myeloid cells participating in innate immunity as well as in trachea and lung exposed to incoming invaders. Second, LMIR4 ligation strongly enhances LPS-induced cytokine production of mast cells and granulocytes. In addition, LPS stimulation leads to up-regulation of LMIR3, but to down-regulation of LMIR4.

Similar to the *in vivo* findings, G-CSF increased the expression of both LMIR3 and LMIR4, whereas LPS increased the expression of LMIR3 and decreased that of LMIR4 (Fig. 7*C*). Finally, the effect of LMIR4 engagement on granulocytes was examined. To obtain mature granulocytes (CD11b^{high}Gr-1^{high}) with high expression levels of LMIR4, bone marrow granulocytes were purified from CBA/J mice given an intraperitoneal injection of G-CSF. When these granulocytes were stimulated by LMIR4 engagement with or without LPS, the adhesion of these cells to plastic plates was examined. Although LMIR4 cross-linking alone induced only a weak adhesion, additional LPS stimulation accelerated the adhesive property of granulocytes triggered by LMIR4 (Fig. 7*D*). When these granulocytes were stimulated by LMIR4 engagement, cytokine production of IL-6,

Although alignment of the amino acid sequence of LMIR4 shows that its transmembrane domain contains a negatively charged residue instead of a positively charged amino acid, our results strongly suggest that FcRγ is indispensable for the function of LMIR4 by selective binding to and stabilization of

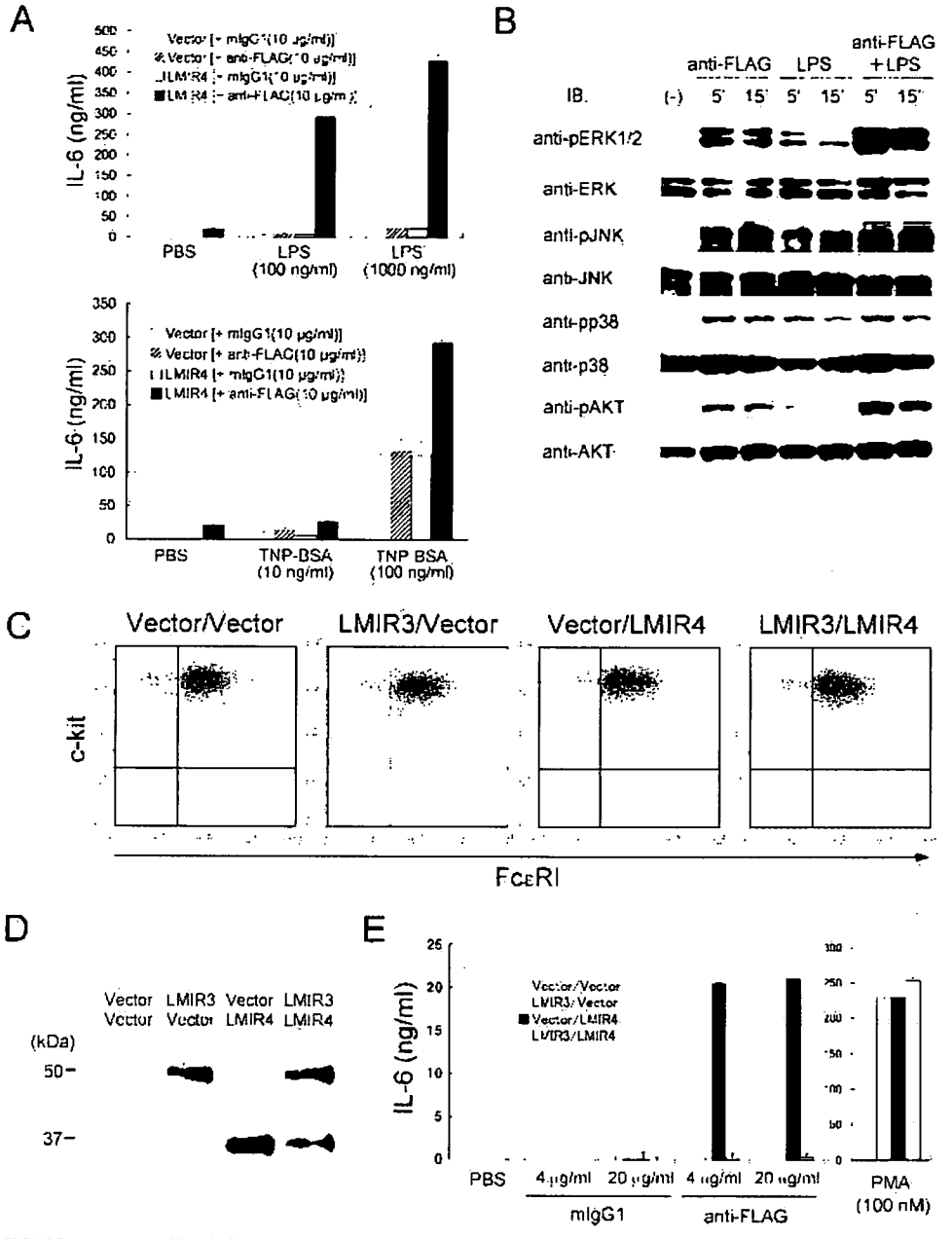


FIGURE 6. **Cross-talk of the signaling downstream of LMIR4 and other receptors.** *A*, mock- or LMIR4-transduced BMMCs were stimulated by F(ab')$_2$ anti-FLAG mAb and either LPS (*upper panel*) or IgE plus antigen (*lower panel*) as described under "Experimental Procedures." IL-6 production was measured. *PBS*, phosphate-buffered saline; *BSA*, bovine serum albumin. *B*, LMIR4-transduced BMMCs were stimulated with F(ab')$_2$ anti-FLAG mAb, LPS, or F(ab')$_2$ anti-FLAG mAb plus LPS for the indicated times. The amount of phosphorylated (*p*) ERK1/2, JNK, p38, or Akt was examined by Western blot analysis as described in the legend to Fig. 3*D*. *IB*, immunoblot. *C*, mock-transduced, FLAG-tagged LMIR3-transduced, FLAG-tagged LMIR4-transduced, or FLAG-tagged LMIR3- and LMIR4-transduced BMMCs were generated. The surface expression levels of c-Kit and IgE-bound FcεRI were examined by flow cytometry. *D*, the expression levels of LMIR3 and LMIR4 in these transfectants were confirmed by immunoprecipitation followed by immunoblotting as described in the legend to Fig. 1*B*. *E*, the IL-6 production of these transfectants stimulated by F(ab')$_2$ mouse (*m*) IgG1 or anti-FLAG mAb (*left panel*) or by phorbol 12-myristate 13-acetate (*PMA*) (*right panel*) was measured. All data points correspond to the mean ± S.D. of three independent experiments.

LMIR4 functions, further studies using knock-out mice are now under way.

In response to LMIR4 engagement, high level cytokine production was observed in wild-type mast cells, but it was dampened in FcRγ-, Lyn-, and Syk-deficient mast cells (Figs. 4 and 5). On the basis of this and previous findings on the signaling pathways downstream of ITAM-bearing molecules (5, 21, 23), we assume that the triggering of LMIR4 induces the phosphorylation of ITAM in FcRγ by Lyn, the recruitment of Syk to phosphorylated ITAM, and the activation of Syk by Lyn. In summary, all the events induced by cross-linking of LMIR4 are positively regulated by FcRγ and two tyrosine kinases, Lyn and Syk.

The cross-talk between ITAM-bearing receptors and TLRs is noteworthy in terms of innate immunity (54, 55). As clearly demonstrated in Fig. 6 (*A* and *B*) and Fig. 7 (*D* and *E*), LMIR4 signaling synergistically enhanced TLR4 signaling in mast cells and granulocytes. These data suggest that the aggregation of LMIR4 by its unknown ligands would positively regulate various signaling pathways, affecting the inflammatory responses of myeloid cells. Taking into consideration that LMIR3 and LMIR4 share high homology in the Ig-like domain, these receptors may share the ligands. Based on this hypothesis, the effects of the co-ligation of LMIR3 and LMIR4 on mast cells may mimic the physiological situations. As clearly demonstrated in Fig. 6*E*, co-ligation abrogated the cytokine production of mast cells induced by the cross-linking of LMIR4 alone. This could be because the inhibitory effect of the ITIM-bearing receptor LMIR3 was exerted through the phosphatases or because the number and size of aggregated LMIR4 were decreased by the co-ligation of both receptors. In any case, the increase or decrease of LMIR3 expression levels in mast cells should influence the activation events induced by the aggregation of LMIR4 with the same ligand. Notably, the expression levels of both LMIR3 and LMIR4 increased in granulocytes under the pre-inflammatory conditions, where G-CSF was mobilized upon

LMIR4 (Figs. 3–5). Indeed, we generated LMIR4 mutants in which the glutamic acid in the transmembrane domain was mutated to lysine with a negative charge or to glutamine with a neutral charge, but the expression levels or functions of LMIR4 in mast cells did not significantly alter whatever the residue was (data not shown). To fully understand the mechanism of

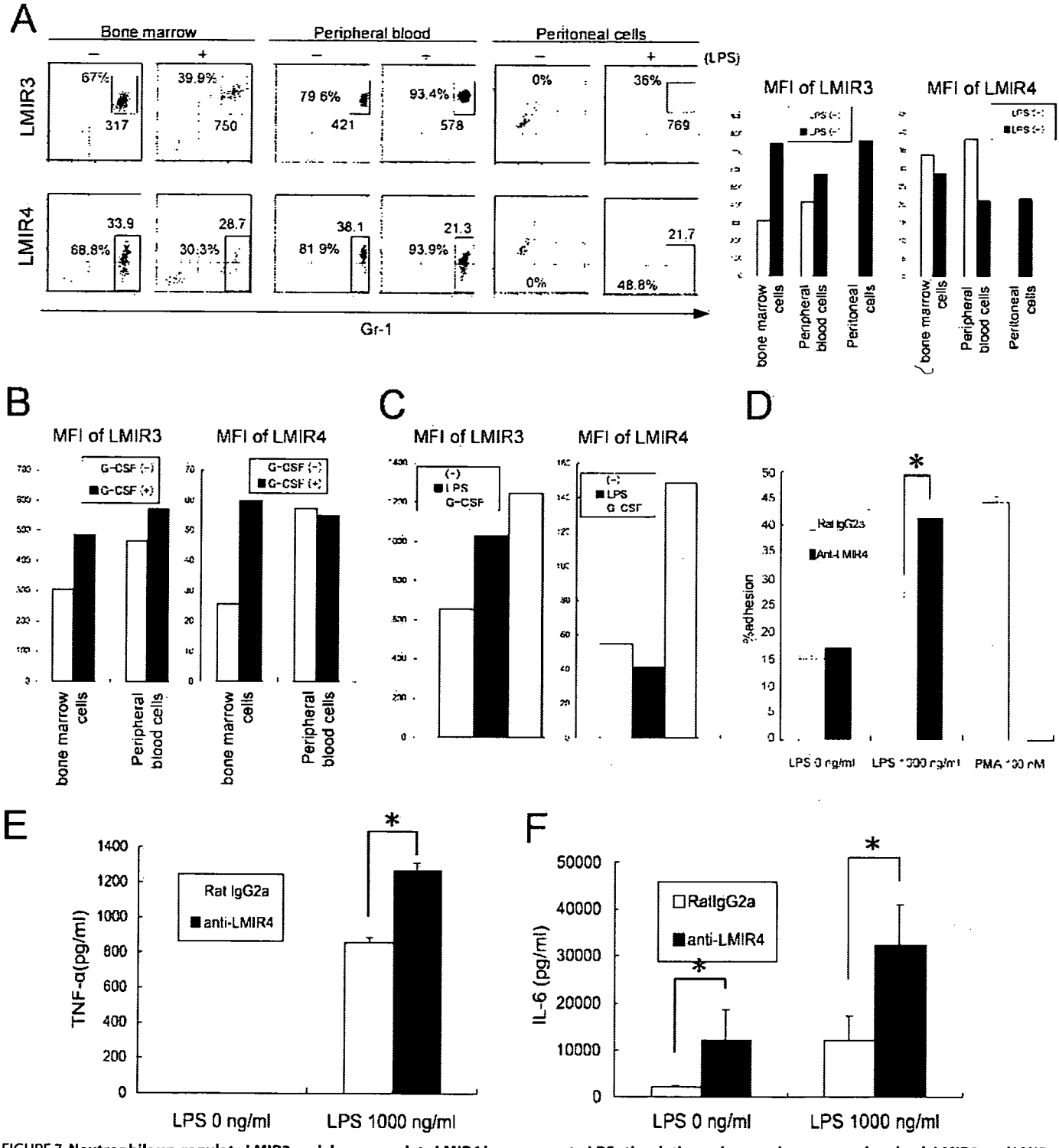


FIGURE 7. **Neutrophils up-regulate LMIR3 and down-regulate LMIR4 in response to LPS stimulation, whereas they up-regulate both LMIR3 and LMIR4 in response to G-CSF stimulation.** *A*, CBA/J mice were intraperitoneally injected with LPS (20 ng). After 12 h, cells were isolated from the peritoneal cavity, peripheral blood, or bone marrow and then stained for Gr-1, LMIR3, and LMIR4. The percent of neutrophils (Gr-1^{high}) in the population ($FSC^{high}SSC^{high}$) and the mean fluorescence intensity (*MFI*) of LMIR3 or LMIR4 on neutrophils (Gr-1^{high}) were measured as determined by flow cytometry. Data are representative of experiments done three times independently. *B*, similar experiments were done using G-CSF (10 ng) instead of LPS in an intraperitoneal injection. Data are representative of experiments done three times independently. *C*, granulocytes were isolated from CBA/J mice. The expression levels of LMIR3 and LMIR4 were measured by flow cytometry after incubation with 1000 ng/ml LPS or 100 ng/ml G-CSF for 24 h. Data are representative of experiments done four times independently. *D* and *E*, mature granulocytes were stimulated with anti-LMIR4 (CBA) Ab or rat IgG2a plus $F(ab')_2$ anti-rat IgG2a Ab for 1 h before adhesion assay (*E*) or measurement of cytokine production (*F*) of tumor necrosis factor-α (*left panel*) and IL-6 (*right panel*).

bacterial infection, whereas those of LMIR3 and LMIR4 dramatically increased and decreased, respectively, under the inflammatory conditions induced by exposure to LPS. These findings indicate that the ratio of LMIR3 and LMIR4 expression on the cell surface changes upon bacterial infection. Accordingly, it is tempting to assume that up-regulation of LMIR3 in myeloid cells under

the inflammatory situations suppresses the excessive activation induced by the aggregation of LMIR4 with the same ligand. Thus, both the qualitative and quantitative balance of the paired receptors LMIR3 and LMIR4 might regulate the inflammatory response of immune cells, suggesting a significant role for these paired receptors under pathophysiological situations (1, 4, 5, 56). Although the identification of the ligands for LMIR3 and LMIR4 is indispensable for complete understanding of the functions, it has been unsuccessful despite an extensive trial using expression cloning or biochemical approaches. Fine-tuning of LMIR3, LMIR4, and their ligands might provide a new therapeutic strategy in the regulation of allergy and innate immunity.

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REFERENCES

1. Ravetch, J. V., and Lanier, L. L. (2000) *Science* **290,** 84–89
2. Dietrich, J., Nakajima, H., and Colonna, M. (2000) *Microbes Infect.* **2,** 323–329
3. Takai, T., and Ono, M. (2001) *Immunol. Rev.* **181,** 215–222
4. Colonna, M. (2003) *Nat. Rev. Immunol.* **3,** 445–453
5. Humphrey, M. B., Lanier, L. L., and Nakamura, M. C. (2005) *Immunol. Rev.* **208,** 50–65
6. Kumagai, H., Oki, T., Tamitsu, K., Feng, S. Z., Ono, M., Nakajima, H., Bao, Y. C., Kawakami, Y., Nagayoshi, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Kawakami, T., and Kitamura, T. (2003) *Biochem. Biophys. Res. Commun.* **307,** 719–729
7. Yotsumoto, K., Okoshi, Y., Shibuya, K., Yamazaki, S., Tahara-Hanaoka, S., Honda, S., Osawa, M., Kuroiwa, A., Matsuda, Y., Tenen, D. G., Iwama, A., Nakauchi, H., and Shibuya, A. (2003) *J. Exp. Med.* **198,** 223–233
8. Chung, D. H., Humphrey, M. B., Nakamura, M. C., Ginzinger, D. G., Seaman, W. E., and Daws, M. R. (2003) *J. Immunol.* **171,** 6541–6548
9. Luo, K., Zhang, W., Sui, L., Li, N., Zhang, M., Ma, X., Zhang, L., and Cao, X. (2001) *Biochem. Biophys. Res. Commun.* **287,** 35–41
10. Cantoni, C., Bottino, C., Augugliaro, R., Morelli, L., Marcenaro, E., Castriconi, R., Vitale, M., Pende, D., Sivori, S., Millo, R., Biassoni, R., Moretta, L., and Moretta, A. (1999) *Eur. J. Immunol.* **29,** 3148–3159
11. Munitz, A., Bachelet, I., Eliashar, R., Moretta, A., Moretta, L., and Levi-Schaffer, F. (2006) *Blood* **107,** 1996–2003
12. Clark, G. J., Cooper, B., Fitzpatrick, S., Green, B. J., and Hart, D. N. (2001) *Tissue Antigens* **57,** 415–423
13. Daish, A., Starling, G. C., McKenzie, J. L., Nimmo, J. C., Jackson, D. G., and Hart, D. N. (1993) *Immunology* **79,** 55–63
14. Green, B. J., Clark, G. J., and Hart, D. N. (1998) *Int. Immunol.* **10,** 891–899
15. Samaridis, J., and Colonna, M. (1997) *Eur. J. Immunol.* **27,** 660–665
16. Kubagawa, H., Burrows, P. D., and Cooper, M. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5261–5266
17. Bouchon, A., Dietrich, J., and Colonna, M. (2000) *J. Immunol.* **164,** 4991–4995
18. Colonna, M., Nakajima, H., and Cella, M. (1999) *J. Leukocyte Biol.* **66,** 718–722
19. Lanier, L. L., and Bakker, A. B. (2000) *Immunol. Today* **21,** 611–614
20. Galli, S. J., Maurer, M., and Lantz, C. S. (1999) *Curr. Opin. Immunol.* **11,** 53–59
21. Kinet, J. P. (1999) *Annu. Rev. Immunol.* **17,** 931–972
22. Kitaura, J., Song, J., Tsai, M., Asai, K., Maeda-Yamamoto, M., Mócsai, A., Kawakami, Y., Liu, F. T., Lowell, C. A., Barisas, B. G., Galli, S. J., and Kawakami, T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12911–12916
23. Kawakami, T., and Galli, S. J. (2002) *Nat. Rev. Immunol.* **2,** 773–786
24. Katz, H. R. (2002) *Curr. Opin. Immunol.* **14,** 698–704
25. Lu-Kuo, J. M., Joyal, D. M., Austen, K. F., and Katz, H. R. (1999) *J. Biol. Chem.* **274,** 5791–5796
26. Uehara, T., Blery, M., Kang, D. W., Chen, C. C., Ho, L. H., Gartland, G. L., Liu, F. T., Vivier, E., Cooper, M. D., and Kubagawa, H. (2001) *J. Clin. Investig.* **108,** 1041–1050
27. Alvarez-Errico, D., Aguilar, H., Kitzig, F., Brckalo, T., Sayos, J., and Lopez-Botet, M. (2004) *Eur. J. Immunol.* **34,** 3690–3701
28. Fujimoto, M., Takatsu, H., and Ohno, H. (2006) *Int. Immunol.* **18,** 1499–1508
29. Costello, P. S., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P. H., Downward, J., and Tybulewicz, V. L. (1996) *Oncogene* **13,** 2595–2605
30. Toyama-Sorimachi, N., Omatsu, Y., Onoda, A., Tsujimura, Y., Iyoda, T., Kikuchi-Maki, A., Sorimachi, H., Dohi, T., Taki, S., Inaba, K., and Karasuyama, H. (2005) *J. Immunol.* **174,** 4621–4629
31. Underhill, D. M., Rossnagle, E., Lowell, C. A., and Simmons, R. M. (2005) *Blood* **106,** 2543–2550
32. Looney, M. R., Su, X., Van Ziffle, J. A., Lowell, C. A., and Matthay, M. A. (2006) *J. Clin. Investig.* **116,** 1615–1623
33. Pereira, S., Zhou, M., Mócsai, A., and Lowell, C. A. (2001) *J. Immunol.* **166,** 4115–4123
34. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) *Exp. Hematol.* **11,** 1007–1014
35. Shiratori, I., Ogasawara, K., Saito, T., Lanier, L. L., and Arase, H. (2004) *J. Exp. Med.* **199,** 525–533
36. Morita, S., Kojima, T., and Kitamura, T. (2000) *Gene Ther.* **7,** 1063–1066
37. Oki, T., Kitaura, J., Eto, K., Lu, Y., Maeda-Yamamoto, M., Inagaki, N., Nagai, H., Yamanishi, Y., Nakajima, H., Kumagai, H., and Kitamura, T. (2006) *J. Immunol.* **176,** 52–60
38. Kojima, T., and Kitamura, T. (1999) *Nat. Biotechnol.* **17,** 487–490
39. Ono, M., Yuasa, T., Ra, C., and Takai, T. (1999) *J. Biol. Chem.* **274,** 30288–30296
40. Asai, K., Kitaura, J., Kawakami, Y., Yamagata, N., Tsai, M., Carbone, D. P., Liu, F. T., Galli, S. J., and Kawakami, T. (2001) *Immunity* **14,** 791–800
41. Kalesnikoff, J., Huber, M., Lam, V., Damen, J. E., Zhang, J., Siraganian, R. P., and Krystal, G. (2001) *Immunity* **14,** 801–811
42. Eiseman, E., and Bolen, J. B. (1992) *Nature* **355,** 78–80
43. Yamashita, T., Mao, S. Y., and Metzger, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11251–11255
44. Kihara, H., and Siraganian, R. P. (1994) *J. Biol. Chem.* **269,** 22427–22432
45. Xu, Y., Harder, K. W., Huntington, N. D., Hibbs, M. L., and Tarlinton, D. M. (2005) *Immunity* **22,** 9–18
46. Odom, S., Gomez, G., Kovarova, M., Furumoto, Y., Ryan, J. J., Wright, H. V., Gonzalez-Espinosa, C., Hibbs, M. L., Harder, K. W., and Rivera, J. (2004) *J. Exp. Med.* **199,** 1491–1502
47. Kawakami, Y., Kitaura, J., Satterthwaite, A. B., Kato, R. M., Asai, K., Hartman, S. E., Maeda-Yamamoto, M., Lowell, C. A., Rawlings, D. J., Witte, O. N., and Kawakami, T. (2000) *J. Immunol.* **165,** 1210–1219
48. Nishizumi, H., and Yamamoto, T. (1997) *J. Immunol.* **158,** 2350–2355
49. Kawakami, T., and Kitaura, J. (2005) *J. Immunol.* **175,** 4167–4173
50. Ravetch, J. V., and Bolland, S. (2001) *Annu. Rev. Immunol.* **19,** 275–290
51. Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. V. (1994) *Cell* **76,** 519–529
52. Kaifu, T., Nakahara, J., Inui, M., Mishima, K., Momiyama, T., Kaji, M., Sugahara, A., Koito, H., Ujike-Asai, A., Nakamura, A., Kanazawa, K., Tan-Takeuchi, K., Iwasaki, K., Yokoyama, W. M., Kudo, A., Fujiwara, M., Asou, H., and Takai, T. (2003) *J. Clin. Investig.* **111,** 323–332
53. King, R. G., Herrin, B. R., and Justement, L. B. (2006) *J. Immunol.* **176,** 6012–6021
54. Hamerman, J. A., Tchao, N. K., Lowell, C. A., and Lanier, L. L. (2005) *Nat. Immunol.* **6,** 579–586
55. Turnbull, I. R., McDunn, J. E., Takai, T., Townsend, R. R., Cobb, J. P., and Colonna, M. (2005) *J. Exp. Med.* **202,** 363–369
56. Speckman, R. A., Wright Daw, J. A., Helms, C., Duan, S., Cao, L., Taillon-Miller, P., Kwok, P. Y., Menter, A., and Bowcock, A. M. (2003) *Hum. Genet.* **112,** 34–41

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ORIGINAL ARTICLE

Identification of TSC-22 as a potential tumor suppressor that is upregulated by Flt3-D835V but not Flt3-ITD

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Transforming growth factor-β (TGF-β)-stimulated clone-22 (TSC-22) was originally isolated as a TGF-β-inducible gene. In this study, we identified TSC-22 as a potential leukemia suppressor. Two types of FMS-like tyrosine kinase-3 (Flt3) mutations are frequently found in acute myeloid leukemia: Flt3-ITD harboring an internal tandem duplication in the juxtamembrane domain associated with poor prognosis and Flt3-TKD harboring a point mutation in the kinase domain. Comparison of gene expression profiles between Flt3-ITD- and Flt3-TKD-transduced Ba/F3 cells revealed that constitutive activation of Flt3 by Flt3-TKD, but not Flt3-ITD, upregulated the expression of TSC-22. Importantly, treatment with an Flt3 inhibitor PKC412 or an Flt3 small interfering RNA decreased the expression level of TSC-22 in Flt3-TKD-transduced cells. Forced expression of TSC-22 suppressed the growth and accelerated the differentiation of several leukemia cell lines into monocytes, in particular, in combination with differentiation-inducing reagents. On the other hand, a dominant-negative form of TSC-22 accelerated the growth of Flt3-TKD-transduced 32Dcl.3 cells. Collectively, these results suggest that TSC-22 is a possible target of leukemia therapy.
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Keywords: TSC-22; Flt3; AML

Introduction

Fms-like tyrosine kinase 3 (Flt3), originally isolated as a hematopoietic progenitor cell-specific kinase, belongs to the class III receptor tyrosine kinase family.[1,2] Flt3 is mainly expressed by early myeloid and lymphoid cells and has a crucial role in normal hematopoiesis,[1–5] while Flt3 mutations are frequently observed in acute myeloid leukemia (AML).[4,6,7] An internal tandem duplication within the juxtamembrane domain of the Flt3 gene (Flt3-ITD) or an activating mutation at aspartic acid 835 (D835) in the kinase domain of Flt3 (Flt3-D835), including Flt3-D835V, was found in 20–30 or 7%, respectively, of patients with AML.[8–16]

Whereas both types of Flt3 mutations cause constitutive activation of Flt3, resulting in autonomous proliferation of factor-dependent hematopoietic cell lines,[17–19] only Flt3-ITD was associated with poor clinical outcomes in both pediatric and adult patients with AML.[8,12,13,16,20–23] Recent accumulated data have shown that Flt3-ITD causes the constitutive activation of both Ras/extracellular regulated kinase (ERK) and signal transducer and activator of transcription 5 (STAT5), and that the activation of STAT5 is more prominent in the signaling pathway downstream of Flt3-ITD as compared with Flt3-D835.[18,24,25] However, the reason why Flt3-ITD causes poor clinical outcomes is not fully understood.

Transforming growth factor-β (TGF-β)-stimulated clone-22 (TSC-22) was initially isolated from mouse osteoblastic cells as an immediate early response gene of TGF-β.[26] It encodes a putative transcriptional regulator containing a leucine zipper structure that is highly conserved between many species.[26–30] TSC-22 was upregulated by many different stimuli such as anticancer drugs[29] and a variety of growth factors and cytokines, including follicle-stimulating hormone,[30] fibroblast growth factor-2,[31] progesterone,[32] epidermal growth factor,[33] TGF-β,[26,27,29] tumor necrosis factor-α, interferon-γ and interleukin-1β.[27] TSC-22 was also expressed in a dynamic pattern at sites of epithelial–mesenchymal interactions during mouse development.[34,35] Accumulated data suggest that TSC-22 is a potential tumor suppressor gene, because TSC-22 was a progesterone target gene upregulated in breast cancer cells, where growth is inhibited by progestins.[32] In addition, overexpression of the TSC-22 gene caused apoptotic cell death involved in the activation of caspase-3 in a human gastric carcinoma cell line[36] and TSC-22 suppressed growth in a salivary gland carcinoma cell line and reduced tumor formation in nude mice.[29,37,38]

In this study, to understand the molecular basis for the fact that Flt3-ITD, but not Flt3-TKD, is associated with poor prognosis, we transduced Flt3-ITD and Flt3-D835V into a mouse IL-3-dependent pro-B cell line Ba/F3 and compared the gene expression profiles downstream of Flt3-ITD and Flt3-D835V. We identified TSC-22 as a gene upregulated by Flt3-D835V, but not by Flt3-ITD. We report the effect of TSC-22 on the proliferation and differentiation of leukemia cells in the context of leukemic therapy.

Materials and methods

Reagents

Recombinant murine interleukin-3 (rmIL-3), recombinant human Flt3 ligand (rhFL), and recombinant murine granulocyte colony-stimulating factor (G-CSF) were obtained from R&D Systems (Minneapolis, MN, USA). Mouse anti-human CD14 and anti-human leukocyte antigen-DR (HLA-DR) monoclonal antibodies (mAbs) were purchased from Becton Dickinson (San Jose, CA, USA). Mouse anti-human CD80 mAb were from ImmunoTech Co., Ltd (Osaka, Japan). Phycoerythrin (PE)-labeled mouse anti-human Flt3 (CD135) and CD11b mAbs were from BD Pharmingen (San Diego, CA, USA). Rabbit

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