A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets

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Leukocyte and platelet integrins rapidly alter their affinity and adhesiveness in response to various activation (inside-out) signals. A rare leukocyte adhesion deficiency (LAD), LAD-III, is associated with severe defects in leukocyte and platelet integrin activation. We report two new LAD cases in which lymphocytes, neutrophils, and platelets share severe defects in β_1, β_2, and β_3 integrin activation. Patients were both homozygous for a splice junction mutation in their CalDAG-GEFI gene, which is a key Rap-1/2 guanine exchange factor (GEF). Both mRNA and protein levels of the GEF were diminished in LAD lymphocytes, neutrophils, and platelets. Consequently, LAD-III platelets failed to aggregate because of an impaired $\alpha_{IIb}\beta_3$ activation by key agonists. β_2 integrins on LAD-III neutrophils were unable to mediate leukocyte arrest on TNFα-stimulated endothelium, despite normal selectin-mediated rolling. In situ subsecond activation of neutrophil β_2 integrin adhesiveness by surface-bound chemoattractants and of primary T lymphocyte LFA-1 by the CXCL12 chemokine was abolished. Chemokine inside-out signals also failed to stimulate lymphocyte LFA-1 extension and high affinity epitopes. Chemokine-triggered VLA-4 adhesiveness in T lymphocytes was partially defective as well. These studies identify CalDAG-GEFI as a critical regulator of inside-out integrin activation in human T lymphocytes, neutrophils, and platelets.

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Abbreviations used: GEF, guanine exchange factor; GPCR, G protein–coupled receptor; HUVEC, human umbilical vein endothelial cell; LAD, leukocyte adhesion deficiency; NMD, nonsense-mediated decay; PAF, platelet-activating factor; PLC, phospholipase C; SNP, single-nucleotide polymorphism.

Leukocyte arrest at target endothelial sites is nearly exclusively mediated by integrin receptors (1). As circulating leukocytes maintain their integrins in a generally nonadhesive state, a key checkpoint in leukocyte arrest is the rapid modulation of integrin affinity and avidity to endothelial ligands (2) by various agonists, predominantly chemoattractants or chemokines, presented on the endothelium (3, 4). Likewise, platelets maintain their major fibrinogen receptor, the integrin $\alpha_{IIb}\beta_3$, in an inactive conformation, which is converted by multiple agonists, predominately ligands to G protein–coupled receptors (GPCRs), into an activated receptor with high affinity to multiple ligands (5). The small GTPase Rap-1 has been implicated in the activation of leukocyte, platelet, and megakaryocyte integrins by multiple receptors, including GPCRs, the TCR, receptors to various inflammatory cytokines, and shear stress signals (6–13).

We recently described a human genetic deficiency of leukocyte adhesion to endothelium leukocyte adhesion deficiency (LAD) III that is

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distinct from LAD-I. Whereas LAD-I is a genetic defect in β_2 integrin expression or function (14), LAD-III leukocytes express intact integrins with an impaired ability to generate high avidity to their endothelial ligands at vascular endothelial contacts in response to rapid endothelial chemoattractant signals (15, 16). Patient leukocytes, however, express functionally intact GPCRs. A role for Rap-1 malfunction in the LAD-III syndrome was inferred by our finding that LAD-III lymphoblasts express normal levels of Rap-1, which fails to undergo activation in response to the prototypic chemokine CXCL12. Although Rap-1 is also implicated in the survival and function of many nonhematopoietic tissues (17–19), no severe developmental disorders or abnormalities in nonhematopoietic tissues were reported in the LAD-III patients (14). Thus, we suggested that LAD-III and related integrin activation defects in hematopoietic systems are the result of a loss in a key Rap-1 guanine exchange factor (GEF) that is essential for the transduction of leukocyte and platelet GPCR signals into Rap-1 and integrin activation.

In this study, we report two similar LAD-III cases in which neutrophil and lymphocyte integrins cannot acquire adhesiveness upon rapid activation by GPCR agonists under shear flow conditions. Chemokine-induced activation of two LFA-1 conformations associated with integrin extension and high affinity states is also largely impaired in LAD-III T lymphocytes. In addition, platelets derived from the two patients fail to aggregate in response to prototypic inside-out signals, and their key integrin, $\alpha_{IIb}\beta_3$, does not acquire a high affinity state essential for ligand binding and aggregation. These LAD cases share a homozygous mutation in the acceptor splice junction at the beginning of exon 16 of the Rap-1 GEF, CalDAG-GEFI (RasGRP2). Furthermore, LAD-derived total blood levels of CalDAG-GEFI mRNA and the protein expression in LAD platelets, neutrophils, and lymphocytes are diminished. These results are the first example of a human inherited disease caused by a Rap GEF deficiency that is linked to profound defects in both leukocyte and platelet integrin activation and adhesive functions in the vasculature.

RESULTS

Patients

The two patients, a 1-yr-old male (patient K) and a 2-yr-old female (patient A) of Turkish origin were each born to consanguineous parents. The two families, although not related, originally lived in the close villages in the eastern region of Turkey, suggesting a common ancestor. Both sets of parents are asymptomatic. Both patients shared a similar clinical presentation consisting of petechia from birth caused by a severe bleeding tendency and requiring repeated blood transfusions. They suffered recurrent and severe bacterial infections associated with marked leukocytosis (25–70,000/mm^3, 60% neutrophils and 30% lymphocytes), but normal platelet counts. More than 95% of patient neutrophils expressed CD18, CD11a, and CD11b integrins, ruling out a LAD-I syndrome. Lymphocyte subsets and immunoglobulin levels were within normal range. Patient A suffered from recurrent severe pneumonias, necessitating constant antibiotic treatment. Patient K suffered from recurrent severe pneumonias and sepsis. He had surgery to correct a bowel intususception when he was 14 mo old, resulting in a large, nonhealing wound after the operation. Blood culture yielded B-hemolytic *Pseudomonas aeruginosa*. Patient K died at 2 yr of age from sepsis and pulmonary bleeding. Laboratory data showed hemoglobin around 8 gr%. Failure to thrive was consistent in both patients from early life, and both children were below the fifth percentile for both height and weight. Patient A's sister died at age 15 mo from pneumonia and sepsis and had suffered from anemia and bleeding tendency from early life. Patient A has only one healthy brother aged 6 yr. Patient K does not have siblings. Neither of the patients displayed overt neurological disorders.

Platelets derived from LAD patients fail to aggregate because of the inability of agonists to trigger high affinity $\alpha_{IIb}\beta_3$

As both patients displayed major bleeding disorders in addition to their severe leukocytosis, we first analyzed their agonist-stimulated platelet aggregation. Platelet aggregation triggered by all GPCR agonists tested, i.e., ADP, epinephrine, arachidonic acid, and thrombin, was completely absent in patient-derived platelets (Fig. 1 A and not depicted), despite only moderate reduction in surface $\alpha_{IIb}\beta_3$ (GpIIbβ3) expression (Fig. 1 B, inset). As this integrin is the main fibrinogen receptor on platelets (5), these results suggested a major defect in GPCR-mediated inside-out activation of patient $\alpha_{IIb}\beta_3$ (Fig. 1 A). An alternative GPCR-independent inside-out activation pathway that stimulates $\alpha_{IIb}\beta_3$-mediated platelet aggregation involves the binding of collagen to platelet receptors such as GPVI and the $\alpha_2\beta_1$ integrin (20). Patient-derived platelets were also unable to aggregate in response to collagen signals (Fig. 1 A). However, these multiple defects were not caused by a global platelet aggregation defect because when GPCR signaling to $\alpha_{IIb}\beta_3$ was bypassed with the vWF agonist ristocetin, patient platelets underwent robust aggregation, although at a twofold lower magnitude (Fig. 1 A). Defective platelet aggregation could result from a failure of $\alpha_{IIb}\beta_3$ to undergo activation or from cytoskeletal defects that impair postligand occupancy of this integrin (5). Direct activation by agonists of the $\alpha_{IIb}\beta_3$ integrin, from an inactive state to a fully active integrin with high affinity to ligands, can be probed by binding of the ligand mimetic activation reporter antibody, PAC-1 (21). PAC-1–specific staining of activated platelets derived from the LAD patients was slightly reduced in response to platelet GPCR agonists, whereas PAC-1 staining on control platelets was dramatically increased (Fig. 1 B). In contrast, P- selectin was normally activated by the same agonists in LAD-derived platelets (unpublished data). These results collectively indicate that affinity up-regulation of $\alpha_{IIb}\beta_3$ by inside-out signals is severely impaired in patient platelets.

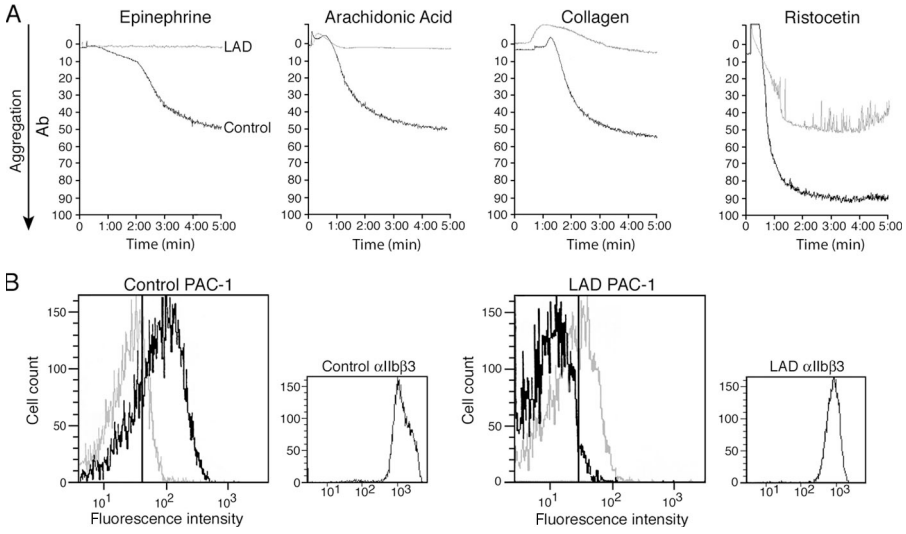

Figure 1. GPCR and collagen agonists fail to trigger LAD-III–derived platelet aggregation caused by their inability to trigger high affinity $\alpha_{IIb}\beta_3$ integrin. (A) Platelet aggregation induced by GPCR agonists, collagen, and the vWF agonist ristocetin. Figures depict treatments at time 0 of either healthy control (black line) or LAD patient K (gray line) platelets with epinephrine (first panel), arachidonic acid (second panel), collagen (third panel), or ristocetin (fourth panel), as indicated in the Materials and methods. (B) FACS staining of resting (gray line) and agonist-stimulated (black line) platelets derived from a healthy (age-matched) donor and LAD patient K with the $\alpha_{IIb}\beta_3$ ligand mimetic mAb PAC-1. Vertical lines depict the mean intensity of background staining with an isotype-matched control mAb. Small graphs depict the total surface $\alpha_{IIb}\beta_3$ levels on these stimulated platelets. Similar results were obtained with LAD patient A.

Absence of CalDAG-GEFI transcripts and protein in LAD patient–derived platelets and neutrophils

The Rap-1 GEF CalDAG-GEFI has been implicated as a key regulator of agonist-induced $\alpha_{IIb}\beta_3$ activation and platelet aggregation (22). Therefore, we analyzed the level of CalDAG-GEFI by RT-PCR in total blood. Strikingly, several CalDAG-GEFI transcripts tested were nearly completely absent from blood obtained from both patients (Fig. 2 A and not depicted), whereas normal levels of this transcript were found in all four parents (Fig. 2 A). In contrast, levels of a second Rap-1 GEF, CalDAG-GEFIII, were completely normal in both patients compared with parents and age-matched control (Fig. 2 A). Quantitative PCR analysis revealed that CalDAG-GEFI was expressed at >30-fold lower levels in LAD patient A compared with healthy age-matched control, whereas the expression level of CalDAG-GEFIII was comparable (Fig. 2 B). In agreement with the loss of CalDAG-GEFI in LAD patients, Western blot analysis with two mAbs against CalDAG-GEFI revealed a complete loss of the protein in lysates derived from platelets and neutrophils purified from patient A (Fig. 2 C and not depicted). Consistent with the RT-PCR results, both of patient A's parents expressed normal levels of the CalDAG-GEFI protein in both neutrophil and platelet lysates (unpublished data).

Both LAD patients share an identical homozygous mutation in an intronic acceptor splice junction in CalDAG-GEFI

We next assessed whether the two patients were homozygous at the CalDAG-GEFI chromosomal locus 11q13.1. We identified a single microsatellite marker, located at chr11:64,428,757-64,429,006 (National Center for Biotechnology Information build 36), which is 159,253 bp away from the gene (chr11:64,250,960-64,269,504), and thus the closest published polymorphic marker to CalDAG-GEFI. We initially used this marker to analyze DNA of the two patients, their parents, and the healthy sibling of patient A (Fig. 3 A). In family A, two alleles, 252 and 260 bp long, were detected in the samples derived from the parents. Patient A was homozygous for the 260-bp allele, whereas his healthy sibling was homozygous for the 252-bp allele. In family K, DNA from both parents contained two alleles that were 248 and 260 bp in lengths, whereas patient K was homozygous for the 260 bp allele. These results indicated that the two patients are each homozygous for the same affected allele, which suggested that this allele may carry a mutation for CalDAG-GEFI.

We next sequenced the genomic region of CalDAG-GEFI from 2,000 bp upstream to 500 bp downstream of the cDNA sequence (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070058/DC1). 20 deviations from the reference genome were found, comprising 17 known single-nucleotide polymorphisms (SNPs) and 3 novel SNPs (Fig. S2). The PCR products containing novel SNPs were generated and sequenced in LAD patient A's healthy brother. Only one novel SNP, IVS15nt718c>a, was unique in LAD patient A (Fig. 3 B and Fig. S3). The same mutation was found in LAD patient K (Fig. 3 B and Fig. S3). Importantly, this mutation was absent in all 116 control chromosomes tested from Turkish donors (unpublished data). The mutation occurs 3 bp upstream of the beginning of exon 16, disrupting the splice junction present at that location. An automated splice site

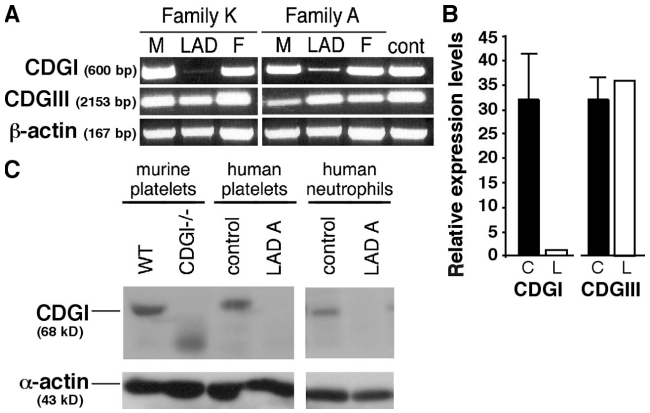

Figure 2. Loss of CalDAG-GEFI mRNA and protein in blood samples of two LAD patients. (A) CalDAG-GEFI amplified by RT-PCR from peripheral blood DNA derived from patients A and K and their parents using the primers listed in Fig. S1. Levels of CalDAG-GEFIII and actin transcripts are shown as controls. The data shown are representative of five independent experiments. (B) Relative CalDAG-GEFI and CalDAG-GEFIII mRNA expression levels were determined by Q-RT-PCR in LAD patient A (L) and an age-matched control (C). GusB was used for normalization for each gene, and normalization to HRPT1 gave similar results. The data are representative of three independent experiments. (C) Western blot analysis of platelet and neutrophil lysates derived from an age-matched control donor and LAD patient. CalDAG-GEFI (CDGI) levels were probed using the mAb 18B11. Left columns depict CalDAG-GEFI levels in wt and CalDAG-GEFI knockout murine platelets probed with the same mAb. Actin levels are shown as control.

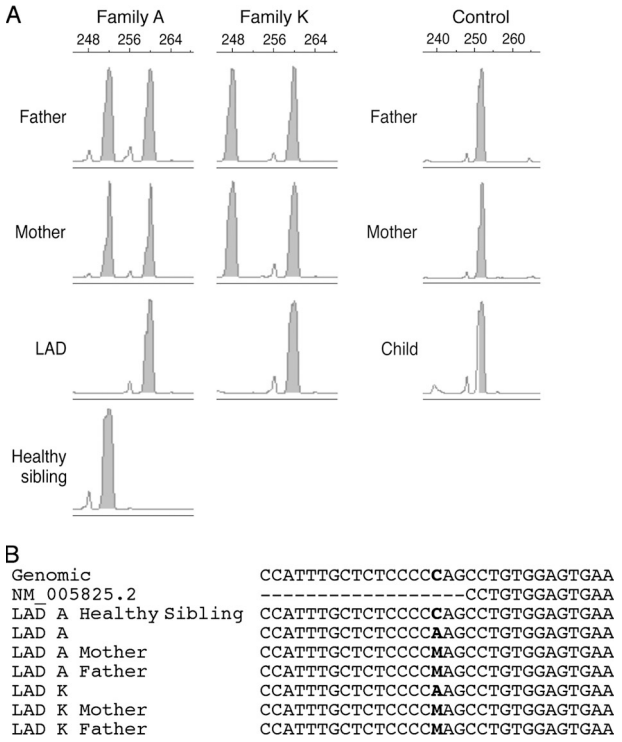

Figure 3. Both LAD patients are homozygous for a c>a mutation in a splice junction of the CalDAG-GEFI gene. (A) A microsatellite marker containing 11.75 TATC perfect repeats located on chromosome 11q13.1, which is located 159,253 bp away from the CalDAG-GEFI gene, was amplified in single plex reaction by touchdown PCR using genomic DNA prepared from the patients (A and K), their parents, and one healthy sibling from family A, as well as from an unrelated control family. The separated fluorescently labeled PCR products were analyzed using GeneScan analysis software. The allele length distribution in each detected individual is shown. (B) Multiple alignment of genomic DNA surrounding the putative disease-causing mutation. The reference genome (National Center for Biotechnology Information 36; chr11:64253078-64253108), the mRNA (NM_005825), and genomic sequence from the patients, their parents, and one healthy sibling were aligned surrounding the mutation (in bold). Note that M is the IUPAC-IUB ambiguity code for A or C. The full trace alignments are depicted, in color, in Fig. S3.

analysis program based on information theory (23) was used to predict the possible effects of this mutation on splicing. The program predicts that this mutation creates a putatively active cryptic splice site located 1 bp before the actual site, which is concomitantly weakened (Fig. S4). If this cryptic site is preferred by the transcription machinery, the products of such a splice would have a shifted reading frame and a premature stop codon, resulting in nonsense-mediated decay (NMD) (24). Collectively, the LAD patients share a homozygous mutation in a critical acceptor splice region of CalDAG-GEFI that predicts the loss of message, which was indeed observed (Fig. 2, A and B).

CalDAG-GEFI–null neutrophils fail to arrest on inflamed endothelial cells and on ICAM-1 in spite of normal selectin-mediated capture and rolling

Neutrophils from the initial LAD-III patient characterized by us (15) expressed normal levels of the two major β_2 integrins implicated in neutrophil arrest on vascular endothelium, LFA-1 and Mac-1. Neutrophils derived from the two new LAD cases also expressed normal levels of LFA-1 and Mac-1 (Fig. 4 A and not depicted). The LAD-derived CalDAG-GEFI–null neutrophils were next perfused over a monolayer of TNF-stimulated human umbilical vein endothelial cells (HUVECs) under physiological shear flow; these cells express high levels of E-selectin and multiple β_2 integrin ligands (25, 26). Patient and control neutrophils were captured by the cytokine-activated HUVEC at comparable rates and initiated normal rolling on the endothelial monolayer (Fig. 4 B). Shortly after capture on the endothelial surface, normal neutrophils spontaneously arrest on the activated endothelium, in a β_2 integrin–dependent manner (26) (unpublished data), whereas CalDAG-GEFI–null neutrophils failed to arrest and continued to roll over the cytokine-activated EC monolayer (Fig. 4 B), as was previously observed for LAD-III neutrophils (15). Inhibition of phospholipase C (PLC) in control neutrophils completely eliminated their ability to arrest in a β_2 integrin–dependent manner on the activated HUVECs (unpublished data), which is consistent with a role for PLC, an upstream regulator of CalDAG-GEFI (22) in CalDAG-GEFI–mediated β_2 integrin activation in normal neutrophils. Furthermore, whereas both normal and LAD neutrophils were efficiently captured by

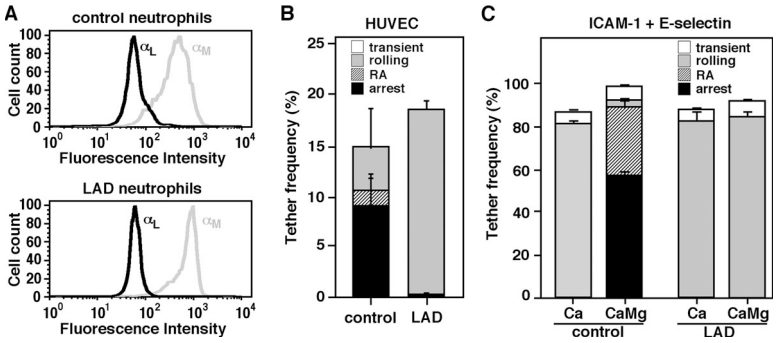

Figure 4. Firm β_2-mediated adhesion, but not capture or rolling, are defective in LAD neutrophils. (A) FACS staining of LFA-1 and Mac-1 integrins on control and LAD patient A neutrophils using the mAbs TS2.4 (anti-α_L integrin subunit) and CBRM1/2 (anti-αM integrin subunit), respectively. Background antibody stainings had fluorescence intensity values of <5. (B) Neutrophil accumulation and development of firm arrest on TNFα-activated HUVECs under physiological shear flow. Healthy age-matched donor (control) or patient A (LAD) neutrophils were perfused for 1 min at 0.75 dyn/cm^2 over the HUVEC monolayers, and accumulated leukocytes were subjected to a shear stress of 5 dyn/cm^2 for a 10-s period. The number of adherent leukocytes that either continued to roll on the monolayer or came to full arrest immediately or after a short period of rolling (RA, rolling then arrest) was determined during the 10-s period. The values shown correspond to fractions of the original leukocyte flux in immediate contact with the endothelial layer. (C) Frequency and type of tethers generated by control and LAD (patient A) neutrophils interacting with 0.2 μg/ml E-selectin-Fc coimmobilized with 5 μg/ml ICAM-1-Fc on a protein A–coated surface. Neutrophils were perfused at 1.5 dyn/cm^2 over the substrate either in the presence of Ca^{2+} alone (Ca) or in regular binding medium containing both Ca^{2+} and Mg^{2+} (CaMg), and the frequencies of each of the indicated tethers were determined. Results in B and C are each representative of three independent experiments. Data represent the mean ± the range of two fields of view. Similar results were obtained in LAD patient K.

E-selectin coimmobilized with ICAM-1 (Fig. 4 C), nearly all normal neutrophils either immediately arrested on the E-selectin/ICAM-1 surface or rolled for several seconds before arrest, but none of the LAD neutrophils were able to arrest (Fig. 4 C). Exclusion of Mg^{2+} from the binding medium resulted in integrin inactivation and loss of arrest on the E-selectin/ICAM-1 surface only in control neutrophils (Fig. 4 C). As expected, this integrin inactivation did not affect the capture and rolling adhesions initiated by either control or LAD neutrophils. These results collectively indicate intact rolling capacity, but loss of adhesiveness of both LFA-1 and Mac-1 integrins in LAD neutrophils interacting with inflamed endothelial cells, as well as with purified ICAM-1 under shear flow conditions.

Impaired spontaneous β_2 integrin adhesiveness to ICAM-1 in CalDAG-GEFI–null neutrophils is associated with reduced levels of high affinity integrin conformations

The inability of both LFA-1 and Mac-1 expressed by LAD neutrophils to develop firm adhesions could result from improper inside-out activation through E-selectin (27, 28). We therefore next compared β_2-mediated attachments of normal and LAD neutrophils to isolated ICAM-1 coated at high density in the absence of E-selectin. Control blood-derived neutrophils retained moderate levels of spontaneous β_2 integrin adhesiveness to high density ICAM-1, manifested by the ability of these leukocytes to generate both transient and firm tethers on the ligand under low shear flow (Fig. 5 A). Notably, β_2 integrins on LAD-derived CalDAG-GEFI–null neutrophils failed to interact with ICAM-1 even under these permissive flow conditions (Fig. 5 A). Furthermore, prolonged association with ICAM-1 under static conditions allowed β_2 integrins on normal, but not on LAD, neutrophils to generate high shear–resistant adhesions (Fig. 5 B). Thus, a considerable fraction of normal neutrophils settled on ICAM-1 for 1 min developed resistance to detachment by high shear stress, whereas only a negligible fraction of LAD neutrophils developed shear resistance on ICAM-1 after prolonged association with the ligand under static conditions (Fig. 5 B). These results suggested that LAD β_2 integrins are inherently defective in their acquisition of the intermediate and/or high affinity states necessary for rapid ligand recognition and contact-dependent adhesion strengthening on ligands (29, 30). Comparing the levels of these β_2 conformational states probed by specific reporter mAbs, we found, however, identical levels of the β_2 integrin extension reporter KIM127 on both normal and LAD neutrophils (Fig. 5 C, top). Nevertheless, the appearance of the high affinity 327C β_2 I domain epitope on LAD β_2 integrins was reduced by fivefold (Fig. 5 C, bottom), which is consistent with defective acquisition of high affinity states by LAD β_2 integrins.

Mac-1 in CalDAG-GEFI–null leukocytes fails to undergo subsecond activation of adhesiveness by chemoattractants

In a previous study on LAD-III neutrophils, we found that the neutrophil Mac-1 could undergo normal conformational activation by chemoattractants, but failed to respond to in situ inside-out stimulation by the same surface-bound chemoattractants under shear flow (15). To directly assess in the present LAD syndrome the ability of Mac-1 to undergo inside-out activation by prototypic chemoattractants, we next assessed whether platelet-activating factor (PAF) can alter the integrin activation state in patient neutrophils. CBRM1/5 is a ligand mimetic mAb that detects high affinity Mac-1 subsets (31).

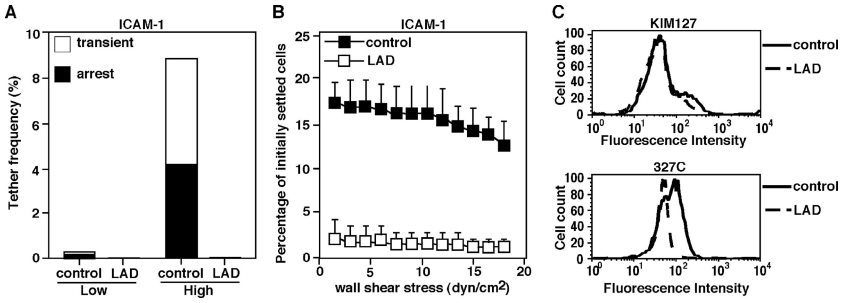

Figure 5. Reduced levels of spontaneous β_2 integrin adhesiveness and β_2 activation states in LAD neutrophils. (A) β_2 integrin–mediated capture and arrest on ICAM-1 are defective in LAD (patient A) neutrophils. The categories of the different type of adhesive tethers (transient or arrest) measured at a shear stress of 0.5 dyn/cm^2 are expressed as the percentage of the total neutrophils in direct contact with the indicated ICAM-1–coated substrates. Low and high density ICAM-1–coated fields (380 and 1,900 sites/μm^2, respectively) were prepared by overlaying 1 and 5 μg/ml of ICAM-1-Fc, respectively, on protein A precoated at 1 and 20 μg/ml. (B) Spontaneous β_2-mediated neutrophil adhesion to the high density ICAM-1 depicted in A developed upon 1-min static contact. Level and strength of adhesion were determined by the relative resistance of either healthy (age-matched) or LAD neutrophils to detachment by the indicated incremented shear stresses. Results in A and B are representative of four independent experiments. (C) Basal expression of the β_2 extension epitope KIM127 (top) and the β_2 I-like domain activation epitope 327C (bottom) on either control or LAD (patient A) neutrophils analyzed by FACS staining. Background antibody stainings had fluorescence intensity values of <5. Results are given as the mean ± the range of determinations in two fields of view, and the experiment shown is representative of three independent determinations. Similar results were obtained in LAD patient K.

In contrast to platelets, PAF, as well as IL-8, effectively triggered the CBRM1/5 Mac-1 neoepitope in patient CalDAG-GEFI–null neutrophils, which is consistent with normal PAF and IL-8 signaling in these cells (Fig. 6 A and not depicted). Nevertheless CalDAG-GEFI–null neutrophils failed to mount any PAF- or IL-8–dependent in situ activation of their Mac-1 when perfused over fibrinogen coimmobilized with these chemokines (Fig. 6 B). The ability of IL-8 to trigger LAD neutrophil attachments to ICAM-1, which is a shared ligand of Mac-1 and LFA-1 (32), was also abrogated (Fig. 6 C). Notably, neutrophils from the patient's mother, although confirmed to be a heterozygote carrier of the mutated gene (Fig. 3 and Fig. S3), expressed normal levels of CalDAG-GEFI protein (not depicted) and underwent comparable in situ activation of Mac-1 by IL-8 (Fig. 6 B). This suggests that one normal allele is sufficient for full protein expression and function. As expected, neutrophils from the healthy sibling, who was a confirmed homozygote for the two normal CalDAG-GEFI alleles (Fig. 3 and Fig. S3), were indistinguishable from healthy neutrophils in all adhesion assays tested (not depicted). Collectively, these results suggest that β_2 integrins in LAD-derived, CalDAG-GEFI–null neutrophils are completely defective in their ability to undergo rapid, chemoattractant-triggered activation under shear stress conditions, despite retained chemoattractant-triggered conformational activation of their Mac-1 β_2 integrin.

Abolished chemokine activation of LFA-1 and of VLA-4–mediated arrest in CalDAG-GEFI–deficient LAD lymphocytes

Murine splenocytes express negligible levels of CalDAG-GEFI (Fig. 7 A) (22). Strikingly, high levels of the GEF were found in primary freshly isolated T lymphocytes from healthy donors (Fig. 7 A). As CalDAG-GEFI was completely lost in primary LAD T lymphocytes (Fig. 7 A), we next assessed how the GEF deficiency affects the function of the two major lymphocyte integrins, the β_2 integrin LFA-1 and the β_1 integrin VLA-4. LAD lymphocytes expressed both integrins, as well as CXCR4, which is the main receptor for the prototypic lymphocyte chemokine CXCL12 (SDF-1α) at normal levels (Fig. 7 B). Nevertheless, CXCL12-mediated activation of LFA-1, assessed by the induction of the integrin extension epitope KIM127, as well as the high affinity integrin conformation, probed by the 327C mAb (33), was greatly reduced in LAD-derived, CalDAG-GEFI–deficient T lymphocytes (Fig. 7 C). In agreement with defective chemokine-induced inside-out activation of LFA-1 in CalDAG-GEFI–deficient lymphocytes, CXCL12 failed to trigger any LFA-1 adhesions to ICAM-1 under shear flow (Fig. 7 D). Thus, CalDAG-GEFI is critical for CXCL12-mediated inside-out activation of LFA-1 in primary human T cells. We next assessed the role of CalDAG-GEFI in chemokine-mediated activation of VLA-4 in T cells interacting with VCAM-1 and coimmobilized CXCL12 under shear flow. In healthy T lymphocytes, this prototypic chemokine triggers robust VLA-4–dependent lymphocyte tethering, rolling, and arrest on VCAM-1 under shear flow (25). Notably, VLA-4 of patient CalDAG-GEFI–null T lymphocytes could still undergo in situ activation by CXCL12 (Fig. 7 E), but CXCL12-triggered VLA-4 failed to arrest patient CalDAG-GEFI–null T lymphocytes on VCAM-1. These data suggest that CalDAG-GEFI is only partially required for inside-out activation of VLA-4. As VLA-4 activation epitopes are not induced in T lymphocytes by soluble CXCL12 (25), and there are no available VLA-4 ligand mimetic mAbs, we could not confirm an inside-out defect in chemokine-triggered VLA-4 conformation or affinity state based on activation epitope analysis, as we could for LFA-1 (Fig. 7 C). We could rule out a defect in outside-in activation of VLA-4 by VCAM-1 because CalDAG-GEFI–null

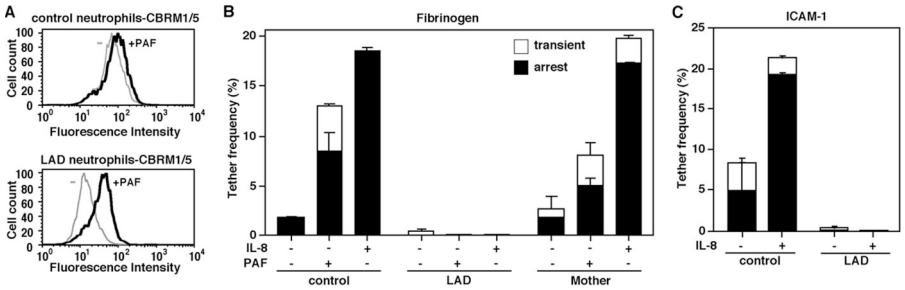

Figure 6. Chemoattractants fail to trigger Mac-1 adhesiveness in LAD neutrophils at subsecond contacts. (A) Normal PAF-triggered induction of the activation Mac-1 neoepitope CBRM1/5 on LAD (patient A) neutrophils. Control or LAD neutrophils were either left intact (−) or stimulated with 100 nM PAF (+). Background antibody stainings had fluorescence intensity values of <5. (B) Capture and arrest of neutrophils on low density fibrinogen (coated at 0.5 μg/ml) coated alone or in the presence of immobilized 100 nM PAF or 2 μg/ml IL-8. Control or LAD neutrophils were perfused over the substrates at a shear stress of 0.5 dyn/cm^2. Analysis of neutrophils derived from patient's mother and analyzed on identical substrates is included. Results are given as the mean ± the range. The data shown are representative of three experiments. (C) Capture and arrest of neutrophils on ICAM-1 coated at 5 μg/ml alone or in the presence of 2 μg/ml IL-8. Analysis was conducted as in B. The experiment shown is representative of three. In B and C, the number of leukocytes transiently or stably interacting with the substrate was determined in two fields of view and expressed in frequency units as in Figs. 4 and 5. The experiment shown is representative of three. Similar results were obtained in LAD patient K.

T lymphocytes arrested normally on high density VCAM-1 (unpublished data). Our data collectively suggest a defect in chemokine stimulation of high avidity VLA-4 bonds critical for immediate adhesion strengthening and lymphocyte arrest on VCAM-1 under shear stress conditions.

DISCUSSION

Integration of calcium and DAG signals triggered by PLC is a common theme shared by many biological targets, including immunoreceptors and integrins (34). Although these two second messengers can promote integrin adhesiveness via their cooperative activation of classical protein kinase C family members (35), chemokine activation of leukocyte integrins takes place independently of this kinase family (7, 25, 30). Alternative effectors triggered by DAG and calcium are members of the CalDAG-GEF/RasGRP family, but the importance of these GEFs in leukocyte integrin activation just begins to unfold (36). In mice, a Rap-1/2 and R-Ras–specific GEF, CalDAG-GEFI, was recently implicated in integrin activation underlying platelet aggregation, fibrinogen binding, and thrombus formation (22). Genetic ablation of CalDAG-GEFI results in severely compromised integrin-dependent platelet aggregation and bleeding disorders (22), but the role of this or other Rap-1 GEFs in leukocyte integrin activation has been vague.

We now report the first human genetic adhesion disorder that is associated with an aberrant expression of this GEF in lymphocytes, neutrophils, and platelets. In agreement with data on murine platelets derived from CalDAG-GEFI knockout mice (22), patient-derived platelets that lack CalDAG-GEFI exhibit major defects in both GPCR- and collagen-induced platelet aggregation, as well as in GPCR-triggered $\alpha_{IIb}\beta_3$ activation. We also provide the first indication for a key role of CalDAG-GEFI in neutrophil and lymphocyte integrin activation by various chemoattractant signals under shear stress conditions relevant for leukocyte interactions with blood vessels. Our genetic analysis has identified a homozygous mutation in the acceptor splice junction at the beginning of exon 16 of the CalDAG-GEFI gene in both LAD patients. Familial segregation analysis also indicates that the patients' parents are heterozygous for this mutation, yet they are healthy and their leukocytes express normal levels of the GEF. Thus, we establish for the first time an autosomal recessive mutation in a LAD-III syndrome. Furthermore, this is a first implication of a single Rap-1 GEF as a critical inside-out regulator of integrin activation in two major types of human leukocytes and platelets.

The role of CalDAG-GEFI in lymphocyte integrin activation has been obscure because this GEF was not detected in the white pulp of the murine spleen or in the thymus (22). We confirmed these results, but found considerable expression of the protein in healthy human peripheral blood T cells. Because primary LAD T cells lacked this GEF, we were prompted to dissect the contribution of CalDAG-GEFI to rapid chemokine-induced integrin activation in T lymphocytes. Chemokine activation of lymphocyte integrins involves simultaneous bidirectional activation by both inside-out and outside-in rearrangements in integrin headpieces and tails (37, 38). Choosing CXCL12 as a prototypic chemokine for lymphocyte integrin activation (39), we assessed the ability of both LFA-1 and VLA-4 in CalDAG-GEFI–null LAD T lymphocytes to undergo in situ stimulation of adhesiveness under physiological conditions of shear flow. Because integrin tethers form within leukocyte contacts with integrin ligands in the range of 0.04–0.1 s (25, 30), any immediate adhesive tethers in situ stimulated by the surface-bound chemokine involves the contact of the integrin and the GPCR with their cognate ligands in this short time frame. Our results therefore indicate an indispensable role for CalDAG-GEFI in the earliest stages (i.e., subsecond-lived contacts)

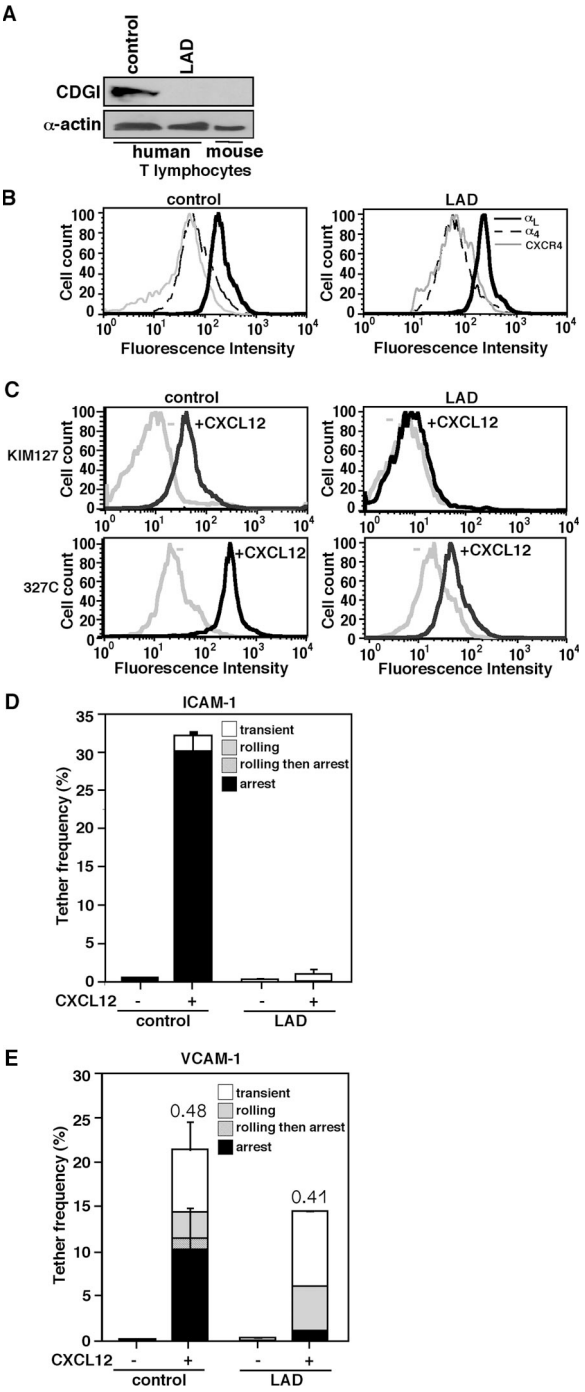

Figure 7. Defective chemokine triggering of LFA-1– and VLA-4–mediated PBL arrest on ICAM-1 and VCAM-1. (A) Western blot analysis of T lymphocyte lysates derived from a C57BL mouse, a healthy donor (control), and LAD patient A (LAD). CalDAG-GEFI (CDGI) levels were probed with an anti–CalDAG-GEFI mAb (top). Actin levels are shown as controls (bottom). (B) FACS staining of LFA-1, VLA-4, and CXCR4 on control and LAD PBLs using the α_L subunit–specific mAb TS2.4, the α_4 subunit-specific mAb HP1.2, and 12G5, respectively. (C) LFA-1 extension epitope detected by the reporter mAb KIM127 (top row) and high affinity LFA-1 epitope detected by the mAb 327C (bottom row) in intact (−) and CXCL12-treated (+) control and LAD PBLs, assessed by FACS staining. Background antibody stainings in B and C had fluorescence intensity values of <5. (D) Tethering and immediate arrest of control (left) and LAD (right) PBLs on ICAM-1-Fc (coated at 2 μg/ml) triggered by immobilized CXCL12 (2 μg/ml). Control or LAD PBLs were perfused over the substrates at a shear stress of 0.5 dyn/cm^2, and both the frequency and strength of all tethers were determined in two fields. Results are given as the mean ± the range. All adhesive tethers were blocked in the presence of the LFA-1 blocking mAb, TS1.18, or EDTA. The experiment shown is representative of three. (E) Tethering and arrest of control and LAD PBLs measured at a shear stress of 0.5 dyn/cm^2 on sVCAM-1 (coated at 2 μg/ml) triggered by immobilized CXCL12 (2 μg/ml). The lifetimes of the transient tethers are depicted above the bars. All tethers were blocked in the presence of the VLA-4 blocking mAb HP1.2, or the low mol wt ligand, Bio1211 (not depicted). The different tether categories were determined as in D.

of chemokine-induced LFA-1 firm adhesiveness. Correspondingly, we also find this GEF to be essential for the triggering in T cells of two conformational states of LFA-1 associated with integrin extension and high affinity to ligand (30). Furthermore, VLA-4 in CalDAG-GEFI–null T cells also fails to develop high avidity binding to its major endothelial ligand VCAM-1 in response to in situ activation signals from CXCL12, although a significant fraction of VLA-4 on CalDAG-GEFI–null lymphocytes could still undergo normal in situ activation by CXCL12 at subsecond contacts. Importantly, α_4 integrin conformations can be regulated by shear forces without noticeable changes in integrin affinity to ligand under shear-free conditions (40, 41). Interestingly, the subset of firm arrest-mediating VLA-4–VCAM-1 tethers developed by healthy, but not by GEF-deficient, T cells is sensitive to inhibition by low levels of soluble VLA-4 ligands known to selectively block high affinity VLA-4 (25). Although VLA-4–mediated adhesions require proper integrin anchorage to the cytoskeleton, we have ruled out a VLA-4 anchorage defect in LAD T cells because these cells normally tethered and arrested on high density VCAM-1 (unpublished data). Nevertheless, we cannot exclude the possibility that defective VLA-4–mediated T cell arrest in LAD T cells is caused by a failure of chemokine-activated VLA-4 to anchor to the cytoskeleton and develop optimal shear resistance in CalDAG-GEFI–deficient T cells. The GEF is dispensable, however, for chemokine-triggered VLA-4–mediated transient and rolling adhesions, which are generally mediated by low or intermediate affinity VLA-4 subsets (64). We thus conclude that T cells lacking CalDAG-GEFI undergo incomplete chemokine-triggered activation of VLA-4, suggesting that unlike LFA-1, VLA-4 regulation by chemokines involves additional and redundant CalDAG-GEFI–independent signaling pathways.

A final step in integrin activation is the binding of the cytoskeletal adaptor talin to integrin tails (42). A novel integrin activation pathway, which links inside-out signals to $\alpha_{IIb}\beta_3$ affinity modulation via a Rap-1–RIAM–talin signaling complex was recently identified (43). It is likely that CalDAG-GEFI acts upstream of this complex not only in platelets (20, 22) but also in neutrophils and lymphocytes. Neutrophil Rap-1 is strongly activated by cytosolic calcium and by

phorbolester DAG analogues (44), implicating these two secondary messengers and their key regulatory enzyme, PLC, in Rap-1 activation of neutrophil integrins. Indeed, β_2-mediated neutrophil arrest on inflamed endothelium is highly sensitive to inhibition of PLC (unpublished data). Rap-1 is found in multiple membranal pools in various cell types (45), but its activation by GPCRs occurs mainly in the plasma membrane (46, 47). Because these signals are transmitted within a fraction of a second at leukocyte–endothelial contacts (48), at least on circulating cells, CalDAG-GEFI and its immediate targets are expected to preexist near the plasma membrane in proximity to their target integrins. CalDAG-GEFI may also activate integrins by triggering the small GTPase R-Ras, which was previously implicated in affinity modulation of the β_1 integrin VLA-5 (49). It is noteworthy that, apart from the loss of CalDAG-GEFI, we did not find any expression defect in either Rap-1 or talin in patient platelets and leukocytes (unpublished data). The involvement of CalDAG-GEFI in platelet, neutrophil, and lymphocyte integrin activation suggests that some of the previously published clinical studies of integrin activation defects in leukocytes and platelets (50–53) may involve deficiency in this key GEF. With the exception of RAPL, none of the aforementioned Rap-1 effectors are specific to hematopoietic cells; thus, a genetic defect in any of these effectors would not be restricted to the hematopoietic lineage, in contrast to the LAD-III defect described here. Indeed, the loss of CalDAG-GEFI in LAD patient blood did not appear to impair any Rap-1–related functions in nonhematopoietic cellular environments; although the two patients exhibited dense bone in x-ray scans, this is apparently caused by impaired migration of bone remodeling precursors into their skull tissues (54). Similar to the phenotype of our patients, CalDAG-GEFI–null mice do not display any severe nonhematopoietic-associated disorder (22).

It is quite surprising that the loss of CalDAG-GEFI in platelets and leukocytes could not be compensated by any other Rap-1 activating GEF. Leukocytes and platelets express multiple Rap-1 GEFs in addition to CalDAG-GEFI, including CalDAG-GEFIII, the receptor tyrosine kinase–stimulated GEF C3G, and the cAMP-triggered GEF EPAC (17, 55). EPAC is expressed at very low levels in neutrophils (53), and thus cannot serve as a major Rap1 GEF in these cells. Importantly, we detected comparable levels of the two other Rap1 GEFs, CalDAG-GEFIII and C3G (Fig. 2 B and not depicted), in LAD neutrophils and lymphocytes, which suggest that these Rap-1 GEFs cannot functionally compensate for a loss of CalDAG-GEFI. Future studies will be required to address this point. Another open question is whether deficiency in CalDAG-GEFI in murine neutrophils results in a similarly dramatic loss of integrin inside-out activation under the experimental conditions studied in the present work. Lastly, although this Rap-1 GEF is missing in murine lymphocytes, Rap-1 activation is considered as important in murine integrin regulation as it is in human integrin regulation (56). If so, one should expect to find in murine lymphocytes an alternative Rap-1 GEF that is critical for lymphocyte integrin activation.

In addition to regulating inside-out integrin activation in platelets, neutrophils, and lymphocytes, Rap-1 and CalDAG-GEFI may also control critical outside-in activation steps, imposed by ligand-induced rearrangements (2, 57). Indeed, in the present work, as well as in a study on neutrophils from a previous LAD-III patient (15), the Mac-1 integrin could undergo normal inside-out activation in the presence of the prototypic chemoattractant PAF, but still failed to generate adhesiveness in response to rapid PAF signals under shear stress conditions, suggesting a defect in outside-in integrin activation of this integrin. Recent studies suggest that without proper anchorage to the cytoskeleton and a series of rearrangements of the β_2 integrin LFA-1 by its ligand during subsecond contacts, this integrin, and possibly other integrins operating at leukocyte–endothelial contacts, may fail to generate shear-resistant adhesiveness (30). Rearrangement of integrins by their ligands transduces specific cytoplasmic changes in integrin tails (58), which may be stabilized by in situ GPCR-mediated Rap-1 activation (16). Indeed, inhibition of Rap-1 by overexpression of its GAP, SPA-1, can interfere with integrin adhesiveness, even when the integrin ectodomain is artificially stabilized at a high affinity state by Mn^{2+} or by activating mAbs (59), which is consistent with a major role of Rap-1, and potentially of CalDAG-GEFI, in outside-in integrin activation. Rap-1 is also implicated in diverse signaling pathways that link shear stress signals to integrin activation in various cell types (12, 60). Future studies will need to address these multiple potential roles of CalDAG-GEFI as an integrator of both inside-out and outside-in integrin activation events in different neutrophil and lymphocyte subsets.

MATERIALS AND METHODS

Reagents and mAbs. Recombinant ICAM-1-IgG1 and E-selectin-IgG1 fusion protein, as well as human SDF-1α (CXCL12) and CXCL8 (IL-8), were purchased from R&D Systems. Recombinant soluble seven-domain human VCAM-1, sVCAM-1 (61), was a gift from R. Lobb (Biogen, Cambridge, MA). BSA (fraction V), Ca^{2+}, and Mg^{2+}-free HBSS, EGTA, Hepes, fibrinogen, and Ficoll-Hypaque 1077 were obtained from Sigma-Aldrich. Human serum albumin (fraction V) was purchased from Calbiochem. The anti–β_2 integrin subunit mAb TS1.18, the anti–LFA-1 TS2.4, and the anti–Mac-1 integrin mAbs CBRM1/2 and CBRM1/5 (62) were gifts from T. Springer (Harvard University, Boston, MA). The Alexa Fluor 488–conjugated anti–β_2 integrin neoepitope 327C mAb (33) was a gift from D. Staunton (ICOS Corporation, Bothell, WA). The KIM127 mAb (68) was a gift from M.K. Robinson (UCB Celltech, Slough, UK). The FITC-labeled antiactivated $\alpha_{IIb}\beta_3$ mAb PAC-1 was purchased from Becton Dickinson. Anti–P-selectin mAb and the anti-$\alpha_{IIb}\beta_3$ mAb were both purchased from Immunotech Coulter. Polyclonal goat anti-actin antibody (sc-1616) was purchased from Santa Cruz Biotechnology.

Platelet isolation and aggregation studies. Informed consent was obtained from each individual studied. This study was approved by the Institutional Review Board of the Rambam Medical Center, which is consistent with the provisions of the Declaration of Helsinki. Citrated blood was centrifuged at 150 *g* for 10 min at RT, and platelet-rich plasma was isolated (63). Platelets were adjusted to a concentration of 3×10^8/ml with 1/10 volume of 3.2% buffered sodium citrate. Aggregation was initiated by adding 5 μM ADP, 10 μM epinephrine, 10 μg/ml arachidonic acid, 2 μg/ml collagen, or 1.25 mg/ml ristocetin. Aggregation was monitored by light transmission using a two-channel aggregometer (Chrono-Log Corp.).

Isolation and culture of leukocytes. Human peripheral blood neutrophils and T lymphocytes were isolated from citrate-anticoagulated whole blood, as previously described (25, 64). Murine T lymphocytes were purified from C57BL/6 splenocytes, as previously described (65). Murine platelets were isolated and purified from wild-type and CalDAG-GEFI knockout mice, as previously described (22). Leukocytes were stored in cation-free HBSS containing 10 mM Hepes, pH 7.4, and 2 mg/ml BSA at room temperature for up to 2 h before experimentation.

RT-PCR analysis. RNA prepared from EDTA-anticoagulated peripheral blood was extracted using the RNeasy mini kit (QIAGEN). cDNA was synthesized using random hexamer primers (Promega) with Superscript II RNase H-negative reverse transcriptase (Invitrogen). cDNA was amplified by PCR using specific primers, which are listed in Fig. S1. QRT-PCR was performed as previously described (66), using a LightCycler (Roche) according to the manufacturer's instructions. PCR reactions were performed in duplicate. PCR amplification consisted of 35–50 cycles of denaturation, annealing, and extension. Denaturation was performed for 15 s at 95°C, annealing was performed at 60°C, and the extension was performed at 72°C for 20 s, with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses of all samples were performed within the range of 62–99°C. Expression levels of GusB and of HPRT1 were used for sample normalization. A standard curve was obtained with serial dilutions of a reference cDNA sample amplified concomitantly with the tested samples. CalDAG-GEFI and CalDAG-GEFIII mRNA levels were determined by comparing experimental levels to the standard curves and are expressed as arbitrary units. The primers used are listed in Fig. S1.

Microsatellite analysis. Blood was drawn and DNA was isolated by standard methods. PCR amplification of one microsatellite marker with a maximal length of 260 bp, which contained 11.75 TATC perfect repeats and was located 159,253 bp upstream of CALDAG-GEFI on the minus strand on chromosome 11q13.1, was performed in single plex reaction by touchdown PCR (MJ Research). Two primers, flanking the microsatellite marker, were used in the reaction, one of which was fluorescently labeled: forward PCR primer, Fam-CGCCGAGACATATAAACACCC; reverse PCR primer, ACTTGAATCTGGGAGGCG. PCR products were separated on a 16-capillary automated genetic analyzer (AB 3100; Applied Biosystems). Results were analyzed using the GeneScan analysis software (AB 3100).

DNA sequencing. The full CalDAG-GEFI gene, including the 2,000-bp region upstream of the first exon and the 500-bp region downstream of the last exon (chr11:64,250,405-64,271,447), were PCR amplified, and 90% of these segments were successfully sequenced in LAD patient A as previously described (67). The primers used for each segment are listed in Fig. S1. Primers of fragment 32 (Fig. S1) were used to amplify the region containing the IVS15nt718c>a mutation, and thus were subsequently used to screen the genomic DNA of LAD patient K, the healthy brother of LAD patient A, the 4 parents of both patients, and 58 Turkish control donors. Sequence analysis was performed using Sequencher Version 4.7 (Gene Codes Corporation).

Flow cytometry. Leukocyte surface staining was performed and analyzed by FACScan, as previously described (64). For analysis of neutrophil expression of the Mac-1 activation neoepitope CBRM1/5, washed neutrophils were either left intact or stimulated with agonists for 10 min at 37°C, in the presence of the appropriate reporter mAb. Cells were washed at 4°C, stained with PE-conjugated secondary Ab (Jackson ImmunoResearch Laboratories), and FACScan analysis was performed as previously described (15). Spontaneous β_2 integrin activation was assayed by incubating the intact leukocytes with the reporter mAbs 327C (33) or KIM127 (68) for 10 min at 37°C, followed by secondary staining. CXCL12-induced activation of these epitopes was assayed in T lymphocytes as previously described (29). Activation of $\alpha_{IIb}\beta_3$ was measured on washed platelets incubated with 1.0 μg/ml ADP and 1.0 μg/ml epinephrine or control buffer in the presence of 10 μg/ml of FITC-labeled PAC-1 mAb (21).

Western blot analysis. For Western blot analysis, platelet, neutrophil, human lymphocyte, or murine lymphocyte lysates were first centrifuged at 13,000 rpm for 20 min at 4°C before their protein concentrations were determined. Lysates were applied to a 7.5% SDS-PAGE under reducing conditions and then transferred to a nitrocellulose membrane. Cell lysis, gel electrophoresis, and transfer to membranes were performed as previously described (41). The membrane was blocked and reacted overnight at 4°C with either monoclonal anti–CalDAG-GEFI antibody 18B11 (69), polyclonal rabbit anti–CalDAG-GEFI antibody 3753 (22), or polyclonal goat anti-actin control antibodies (Santa Cruz Biotechnology). After several washes, the membrane was probed with appropriate secondary IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories), and developed by ECL reaction.

Preparation of adhesive substrates and HUVEC monolayers. Preparation of substrates for the laminar flow adhesion assays were performed as previously described (64, 70). In brief, ICAM-1-Fc–coated substrates were prepared by plating polystyrene dishes with 1 μg/ml protein A, followed by coimmobilization of either heat-inactivated (−) or intact (+) chemokines and human serum albumin, each at 2 μg/ml. The protein A-chemokine substrates were overlaid overnight at 4°C with the indicated concentrations of ICAM-1-Fc. sVCAM-1 was coimmobilized with CXCL12 (both at 2 μg/ml), as previously described (25). E-selectin/ICAM-1 substrates were prepared by coating 0.2 μg/ml E-selectin-Fc on high density protein A, followed by ICAM-1-Fc coating (5 ng/ml). Neutrophil arrests on this substrate were totally blocked by pretreatment with the β_2 integrin–blocking mAb TS1.18. Fibrinogen was coated directly at 0.5 μg/ml and washed, and PAF (Sigma-Aldrich) was overlaid at 100 nM for an additional hour. PAF was verified to potently activate neutrophil integrins via their PAF receptors (26). For adhesion experiments on resting or TNFα-activated endothelial cells, primary HUVECs (passage 2 or 3) were left intact or stimulated for 18 h with heparin-free culture media supplemented with TNFα (2 ng/ml, 100 U/ml; R&D Systems) (70).

Analysis of leukocyte attachments and resistance to detachment developed during short, static contacts. All shear flow experiments were performed at 37°C. Neutrophils and T lymphocytes were suspended in binding medium (cation-free HBSS, containing 10 mM Hepes, pH 7.4, and 2 mg/ml BSA supplemented with Ca^{2+} and Mg^{2+} at 1 mM each) and immediately perfused through the chamber at controlled flow rates, as previously described (25). All cellular interactions with the adhesive substrates were determined by a MatLab-based computerized tracking of individual cell motion within at least two fields of view (each 0.17 mm^2 in area). "Transient" tethers were defined as cells attached briefly (<2 s) to the substrate; "rolling tethers" were attached cells that persistently rolled for at least 2 s over the substrate; "rolling then arrested" were cells that stopped for at least 3 s after a rolling period; "arrest (firm) tethers" were defined as tethered cells that immediately stopped for at least 3 s (30). For analysis of integrin-mediated adhesion strengthening at short stationary contacts, leukocytes were perfused into the flow chamber and allowed to settle onto the substrate for 1 min. Flow was then initiated and increased step-wise every 5 s through a programmed set of flow rates. At the end of each 5-s interval, the number of cells that remained bound was expressed relative to the number of cells originally settled on the substrate.

Online supplemental material. Fig. S1 shows the primers used for PCR amplification and sequencing of CalDAG-GEFI. Fig. S2 shows the polymorphisms found in the genomic sequencing. Note that all alleles refer to the minus strand. Fig. S3 depicts the sequence of the genomic DNA surrounding the putative mutation. The genomic DNA was amplified using primer set 32 (Fig. S1), and analyzed as described in Fig. 3. The base of the mutation is highlighted. Shown are the base alignment (left) and the trace alignment (right). Fig. S4 shows splice site analysis using information theory (23). The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070058/DC1.

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Note added in proof. A paper that describes multiple integrin activation in neutrophils derived from CalDAG-GEF1$^{-/-}$ mice associated with loss of firm adherence to inflamed vessels was published online on May 10, 2007 in the *Journal of Clinical Investigation* (Bergmeier, W., T. George, H.-W. Wang, J.R. Crittendon, A.C.W. Baldwin, S.M. Cifuni, D.E. Housman, A.M. Graybiel, and D.D. Wagner. *J. Clin. Invest.* 117:1699–1797).

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