Platelet Fragmentation Requires a Specific Structural Conformation of Human Monoclonal Antibody against β3 Integrin*

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We have described an autoantibody against β3 (GPIIIa49–66), a region of platelet integrin αIIbβ3 that is unique. It induces platelet fragmentation in the absence of complement via antibody activation of platelet NADPH oxidase and 12-lipoxygenase to release reactive oxygen species, which destroy platelets. To study the mechanism of anti-GPIIIa antibody-induced platelet fragmentation, we screened a human single chain Fv antibody library with the GPIIIa49–66 peptide. Nine monoclonal antibodies were identified that were capable of binding to GPIIIa49–66. Surprisingly, binding avidity for GPIIIa49–66 did not correlate with activity of induction of platelet fragmentation. We therefore investigated the requirements for platelet fragmentation. Mutations were introduced into the heavy chain complementary-determining region-3 of clones 11, 43, and 54 by site-directed mutagenesis. The capability of these clones to induce platelet fragmentation or bind to GPIIIa49–66 subsequently changed. Molecular modeling of these clones with their mutants revealed that the ability to induce platelet fragmentation is affected by the side chain orientation of positively charged amino acids in the heavy chain of residues 99–102. Thus, a structural change in the conformation of anti-GPIIIa49–66 antibody contributes to its binding to the β3 integrin and subsequent antibody-induced platelet fragmentation and aggregate dissolution.

Patients with early HIV-1 infection commonly develop an immunologic thrombocytopenia (HIV-1-ITP) with shortened platelet survival because of an autoantibody specific against an epitope of integrin subunit β3 (GPIIIa) on the surface of platelets, GPIIIa49–66 (CAPESIEFPVSEARVLED) (1, 2). Anti-GPIIIa49–66 autoantibody induces thrombocytopenia in mice and correlates inversely with platelet count in HIV-1-ITP patients. Rabbit polyclonal antibody raised against the GPIIIa49–66 peptide induces platelet fragmentation in the same manner as the antibody from HIV-1-ITP patients (3). The development of this antibody in HIV-1-ITP patients is due to molecular mimicry of epitopes on polymorphic regions of HIV protein: Nef, Env, and Gag (4). The antibody is unique in that it induces complement-independent platelet fragmentation in vitro by the generation of reactive oxygen species released through activation of 12-lipoxygenase and NADPH oxidase (3, 5). Because anti-GPIIIa antibodies against other regions of β3 do not induce platelet fragmentation (3), it is likely that a unique mechanism is employed in anti-GPIIIa49–66 antibody-induced platelet fragmentation.

The extracellular domain of the β3 integrin subunit consists of a head and stalk region (6). The head includes a βA domain with a metal ion-dependent adhesion site motif and one hybrid domain. The stalk consists of a PSI (plexin-semaphorin/integrin) domain, four tandem epidermal growth factor repeats, and a β tail domain (7). Although the function of the PSI domain is incompletely understood, a PI12 polymorphism of the β3 integrin (Leu-33 to Pro-33) has been linked to this region, which leads to an increased risk of thrombotic events (8, 9). It has been suggested that the PI12 allele is associated with greater affinity of the αIIbβ3 integrin for fibrinogen (10, 11). In addition, the PI12 polymorphism can result in a severe form of alloimmune thrombocytopenia (12, 13). Thus, the PSI domain can be considered as a domain with strong immunogenicity as well as the potential to regulate the binding of αIIbβ3 integrin to fibrinogen. We have reported previously that the anti-platelet autoantibody developed in HIV-1-ITP patients is immunodominant against GPIIIa49–66 (a region that crosses the PSI and hybrid domains) (3–5). However, it is unclear how antibody interacts with this region between the PSI and hybrid domains to induce the downstream signaling and oxidative platelet fragmentation.

To investigate the detailed mechanism of antibody-induced binding and fragmentation, monoclonal antibody is required because some polyclonal antibodies often have cross-reactivity with other antigens. However, attempts at raising monoclonal antibody against human GPIIIa49–66 in mice were unsuccessful. This is likely due to homology sequences shared with human GPIIIa49–66 leading to mouse immune tolerance. We therefore switched to the phage surface display antibody technology system.

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§The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; CDR, complementary-determining region; HCDR, heavy chain CDR; LCDR, light chain CDR; scFv, single chain Fv; ITP, immunologic thrombocytopenia; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
Phage surface display antibody technology has been successfully employed to generate antibody that specifically blocks the activated form of \( \alpha_{IIb}\beta_3 \) integrin (14) and to investigate the anti-\( \alpha_{IIb}\beta_3 \) autoantibody profile from patients with autoimmune thrombocytopenic purpura or Glanzmann thrombasthenia after receiving blood transfusions (15). In the present study, we have employed human scFv (single chain fragment of the variable region) Tomlinson I\(^1\)\( ^{11001} \) and J\(^2\)\( ^{11002} \) libraries to generate human anti-GPIIIa\( ^{49–66} \) monoclonal antibody. Each library contains over 100 million different scFv fragments cloned in the phagemid vector pIT2. The library sizes of I and J are \( 1.47 \times 10^8 \) and \( 1.37 \times 10^8 \) constructs, respectively (16–18). Both libraries are based on a single human framework for VH (V3–23/DP-47 and JH4b) andVk (O12/O2/DPK9 and Jk1) with side chain diversity incorporated at positions in the antigen-binding site that make contacts to antigen in known structures and are highly diverse in the mature repertoire. Thus, these libraries can be used to screen antibodies against virtually any given antigen.

Therefore, we used these libraries to better understand the mechanism of antibody-induced oxidative platelet fragmentation with respect to interaction with the PSI and hybrid domains of the \( \beta_3 \) integrin subunit. In the present study, we show that

1) eight scFv monoclonal antibodies induce platelet fragmentation;
2) there is no correlation between binding activity and ability to induce platelet fragmentation;
3) the presence and position of positively charged amino acids in the complementary-determining region-3 (CDR3) of the heavy chain can affect platelet fragmentation as well as binding;
4) these scFv monoclonal antibodies are capable of destroying platelet aggregates; and
5) one of the scFv antibodies tested decreases the platelet count and prolongs the bleeding time in mice. These results strongly suggest that a specific structural conformation is required for antibody-induced platelet fragmentation and that appropriate scFv antibodies might be useful clinically in the dissolution of platelet thrombi.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were obtained from Sigma unless otherwise designated. Human scFv Tomlinson I + J libraries were kindly provided by MRC Geneservice (Cambridge, UK).

**Screening of the Phage Display Peptide Library**—GPIIIa\( ^{49–66} \)-biotin peptide (130 nM) was incubated with \( 10^12–10^13 \) phage from the Tomlinson I or J library in 2% nonfat milk in PBS at room temperature for 3 h. Avidin-agarose beads were then added overnight at 4 °C, centrifuged, and washed with 0.1% Tween 20 and PBS 20 times, and positive phages were eluted with trypsin/PBS by shaking for 10 min at room temperature.

**FIGURE 1. Generation of anti-GPIIIa\( ^{49–66} \) human scFv monoclonal antibodies. A, ELISA with scFv library I and J clones from each round of panning against the GPIIIa\( ^{49–66} \) peptide coated on a microtiter 96-well microplate (\( n = 3 \)). B, ELISA after three rounds of panning. Individual clones from library J were measured for their ability to bind to the GPIIIa\( ^{49–66} \) peptide coated on the microplate. The concentration of phage antibodies was normalized, and the dilutions (black and gray columns) were 1:3 and 1:9 in PBS, respectively (\( n = 3 \)). C, binding ability of clones 43 and 54 with respect to clone 11, measured by standard curve. x axis numbers 1–8 refer to dilutions of 1:2–1:9, respectively (\( n = 3 \)). 13CG2 is a control scFv antibody.
Eluted phages were then titered and amplified following the protocol provided by MRC Geneservice for the next round of panning. After three rounds of panning, positive phage clones were randomly picked up from the plate.

**ELISAs**—Plastic microtiter plates (Corning) were coated with 1/102 g/well GPIIIa 49–66 at 4 °C overnight. The plate was then blocked and washed with Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl) and 2% nonfat milk. Our stock concentration of scFv was standardized to 5 × 10^12 phage/ml or 12.4 μg/ml, which is 8.3 nM. 100 μl of serially diluted (1:10 initial dilution, 1:2/step) human scFv antibody was then added to the microtiter plate and incubated at 37 °C for 1 h. Plates were washed in the same buffer. The dilution of the second antibody, horseradish peroxidase-conjugated anti-M13 monoclonal antibody (GE Healthcare, 27-9421-01), was 1:500.

**Induction of Platelet Particle Formation**—Gel-filtered human platelets were prepared from platelet-rich plasma obtained from blood collected in 0.38% sodium citrate utilizing a Sepharose 2B column preincubated with Tyrode’s buffer (pH 7.4). 1 × 10^7 gel-filtered platelets/ml were labeled with either anti-GPIIb-fluorescein isothiocyanate-labeled monoclonal antibody (3B2) or anti-GPIIIa-fluorescein isothiocyanate-labeled monoclonal antibody (Ancell, Bayport, MN) to 10 μg/ml for 30 min at 4 °C, centrifuged at 1000 × g for 6 min at room temperature, and resuspended in Tyrode’s buffer. 10 μl of fluorescein isothiocyanate-labeled platelets (10^7/ml) were then incubated with human scFv anti-GPIIIa 49–66 antibody (5–40 μg/ml) and 75 μl of Tyrode’s buffer for 0–4 h at 37 °C and stored in an ice bucket prior to measurement of the percentage of platelet particles by flow cytometry. Further particle formation was arrested at 0 °C.

Fluorescently labeled platelet particles were measured by flow cytometry, employing a FACScan (BD Biosciences) as reported previously (3). Debris and dead cells were excluded using scatter gates. Only cells with low orthogonal light scattering were included in the sorting gates. Gates were adjusted for control platelets by exclusion of other blood cells. Fluorescently labeled intact platelets were monitored in the right upper quadrant with the y axis measuring forward scatter and the x axis measuring fluorescence. A shift in fluorescent particles from the right upper quadrant to the left upper and lower quadrants reflected the percentage of platelet particle induction of 10,000 counted platelets/particles.

**Destruction of Platelet-Fibrinogen Aggregates in Vitro**—1 × 10^7 platelets were incubated with 100 mg/ml fibrinogen and 10 μM ADP for 30 min with shaking at 37° to create platelet aggregates. Excess reagents were removed by centrifugation at
1000 × g for 2 min. The platelet aggregates were then resuspended in PBS on a counting chamber, and the number of platelets/aggregate was counted. Anti-GPⅢⅠa49–66 antibody was then added for various time intervals, and the remaining number of platelets/aggregate was enumerated.

**Bleeding Time**—The mouse tail vein was severed 2 mm from its tip and blotted every 30 s on a circular sheet of filter paper to obtain an objective measurement. The variation of the bleeding time was recorded after absence of blood on the filter paper. Bleeding time differences were recorded by an unbiased observer and confirmed by two other observers.

**Site-directed Mutagenesis**—Two mutation primers (forward and backward) targeted for amino acids at positions 99 and 101 were synthesized by Sigma Genosys. The 5′ and 3′ fragments of mutant heavy chain cDNA were amplified using the mutant primers. The full-length mutant heavy chain cDNA fragment was assembled by splicing overlap extension PCR. The mutant heavy chain cDNA was then cloned into the pTi2 expression vector between the restriction enzyme cutting sites of SfiI and XhoI.

**Molecular Modeling**—The heavy and light chain amino acid sequences of clones 11, 43, and 54 and their mutants were used to generate the molecular model through WAM-Web antibody modeling. The side chains of amino acids at positions 99 and 101 in the heavy chain have been labeled with different colors using Rasmol software.

**RESULTS**

**Generation of Human scFv Anti-GPⅢⅠa49–66 Monoclonal Antibodies**—Tomlinson I + J libraries were used to screen against a biotin-conjugated GPⅢⅠa49–66 peptide. After each round of screening, binding activities of phage clones were examined by ELISA (Fig. 1A). After a second round of panning, positive phage clones from library J were enriched. Because the binding activities of phage clones from library I were not increased after three rounds of panning, we focused on phage clones from library J. After three rounds of panning, scFv clones were randomly chosen from the bacterial plate, and individual scFv antibodies were made from each clone. The binding activity for GPⅢⅠa49–66 was examined by ELISA (Fig. 1B). All nine clones were found to bind to GPⅢⅠa49–66 with varying activity, with the absorbance reading varying from 3 to 40 times background (Fig. 1B). Two clones tested did not bind to β3−/− intact mouse platelets, indicating specificity of binding to intact β3 platelets (data not shown).

**Effect of scFv Antibodies on Platelet Fragmentation and Destruction of Platelet Aggregates**—We then tested the ability of each clone to induce platelet fragmentation. We found that not all clones induced platelet fragmentation (Fig. 2A) and that the binding activity (Fig. 1B) did not correlate with platelet fragmentation. This suggested that a specific conformation induced by the antibody is more important than the avidity of binding. For example, clone 43 bound to GPⅢⅠa49–66 (Fig. 1B) but did not induce platelet fragmentation. In contrast, clone 54 had weak binding to GPⅢⅠa49–66 but induced significant platelet fragmentation (Fig. 2A). We confirmed the findings with clones 43 and 54 by measuring the binding and ability to induce platelet fragmentation against a standard curve created with clone 11, a potent binding and functional antibody. Fig. 1C demonstrates similar binding for clones 43 and 54, which is about 6-fold less potent compared with clone 11. Fig. 2B demonstrates similar platelet fragmentation function for clones 11 and 54, which is about 24-fold compared with clone 43. We also tested the functional ability of clones 37, 41, 49, and 54 to dis-aggregate/destroy ADP-induced in vitro platelet aggregates. All clones tested destroyed platelet aggregates in the same manner as antibody from an HIV-1 patient (Fig. 2C). We also tested the ability of the absent functional fragmentation clone 43 to inhibit strong functional fragmentation clones 54 and 11. As noted in Fig. 2D, clone 43 inhibited fragmentation induced by clones 54 and 11, whereas no such inhibition was noted with irrelevant clone CG2. One functional clone tested, clone 11, increased the in vivo bleeding time 3-fold (n = 11; p = 0.001 compared with 43) (Fig. 2E) and decreased the platelet count by ~30% from 1.096 ± 0.039 to 0.740 ± 0.020 million/μl; p < 0.003.

**Sequence Analysis of the Heavy and Light Chains of Antibody Clones**—Nine clones had sequences for both heavy and light chains. The alignment of sequences is shown in Fig. 3. All clones shared the same framework. The CDR2 and CDR3 of both heavy and light chains are shown. Differences in the sequences were found in the HCDR2 (positions 50, 52–54, 56–57, and 59), HCDR3 (positions 99–102), LCDR2 (positions 50 and 53), and LCDR3 (positions 91–94 and 96) regions. Many positively charged amino acids (Arg, Lys, and His) were found in both the HCDRs and LCDRs. Specific conserved amino acids were not found in all functional clones. Of interest was the observation that only clone 43 had Arg at both positions 101 and 102 in HCDR3.

**Function of Mutants of Clones 11, 43, and 54**—A series of mutant clones was generated by site-directed mutagenesis of
positively charged amino acids. The amino acids in HCDR3 of clones 11 (Lys-100, Ser-101), 43 (Tyr-99, Arg-101), and 54 (Arg-99, Ser-101) were exchanged to generate mutants 11M1, 43M, and 54M (Fig. 4A). In clone 11, Lys-100 and Ser-101 were mutated to Ser-100 and Lys-101 to generate mutant 11M1. Lys-100 and His-102 were mutated to Gly to generate mutant 11M2 (Fig. 4A). In clone 43, Tyr-99 and Arg-101 were mutated to Arg-99 and Tyr-101 to generate mutant 43M. In clone 54, Arg-99 and Ser-101 were mutated to Arg-99 and Ser-101 to generate mutant 54M. The binding activities of mutants 11M1, 11M2, 43M, and 54M were then examined by ELISA (Fig. 4B). The binding activity of clones 11M2 and 43M decreased compared with their wild-type sequences, whereas 54M increased and 11M1 remained the same compared with their wild-type sequences. In addition, 43M gained the ability to induce platelet fragmentation, whereas 11M1, 11M2, and 54M decreased their platelet fragmentation function (Fig. 4C). Of particular note was 11M2, which still had some binding activity as well as function without Lys-100 and His-102 of HCDR3. These data suggest that a specific conformation is required and that the positively charged amino acids in HCDR3 may affect antibody function.

Molecular Modeling—To examine the structural differences between the wild-type and mutant clones, molecular models of the αIIbβ3 integrin transmit conformational changes bidirectionally: inside-out and outside-in signaling (19). Clustering of the αIIbβ3 integrin subunit is required for outside-in signal transmission. It has been proposed that during the conformational change of αIIbβ3 integrin, the reorganization of hydrogen bonds in the interface between the a7 helix of the βA domain allows the hybrid domain and the rigidly connected PSI domain to swing out. This results in a 70-Å separation of the αIIb and β3 stalks at their “knees,” a feature noted in electron microscopic images of active forms of αIIbβ3 integrin in the presence or absence of ligand (7). We hypothesize that our antibody may induce the PSI domain to swing out and cluster the β3 subunit. This is supported by our recent discovery that a biotin-labeled peptide capable of binding to GPIIIa49–66 requires anti-biotin antibody-induced clustering to induce oxidative platelet fragmentation.5 How such a movement leads to activation of 12-lipoxygenase and NADPH oxidase in platelets is still not clear.

In the present study, we have clearly shown that nine monoclonal antibodies against GPIIIa49–66 can be generated by panning with Tomlinson I + J scFv libraries. It is unlikely that these antibodies target other molecules on the platelet surface,

5 Z. Li, M. A. Nardi, R. Pan, W. Zhang, and S. Karpatick, unpublished data.

FIGURE 4. Site-directed mutagenesis. A, a schematic map illustrating the amino acid position changes. In 11M1, amino acids Lys-100 and Ser-101 were exchanged for Ser-100 and Lys-101. In 11M2, both Lys-100 and His-102 were mutated to glycine. In 43M, amino acids Tyr-99 and Arg-101 were exchanged for Arg-99 and Tyr-101. In 54M, amino acids Arg-99 and Ser-101 were exchanged for Ser-99 and Arg-101. B, ELISA showing individual clones with their mutants (11, 43, 54, 11M1, 11M2, 43M, and 54M) binding to the GPIIIa49–66 peptide coated on a microtiter 96-well microplate (n = 3). Ctl, control. C, in vitro platelet fragmentation induced by individual clones with their mutants (11, 43, 54, 11M1, 11M2, 43M, and 54M) (n = 3). PtG, patient IgG.
as could be the case with polyclonal antibody. Further specificity of our scFv monoclonal antibody was demonstrated by its inability to react with β3−/− mouse platelets. Of great interest was the observation that binding avidity for the GPIIIα49–66 peptide did not correlate with the ability to induce platelet fragmentation. Clone 43 does not induce platelet fragmentation despite its avid binding to the GPIIIα49–66 peptide, whereas the other eight clones induce fragmentation. It is likely that the 43 binding conformation is different compared with the antibodies that induce fragmentation. Those antibodies that induce platelet fragmentation are indeed doing this through the β3 integrin because they fail to induce platelet fragmentation with platelets from β3−/− mice. Clones 11 and 43 bind to wild-type mouse platelets by flow cytometry in a similar manner as patient antibody (data not shown). In addition, mutation analysis of clones 11, 43, and 54 shows that changing the positions of positively charged amino acids in the HCDR3 region alters the function of antibody-induced platelet fragmentation, suggesting that the positively charged amino acid side chain orientation may affect antibody-induced platelet fragmentation.

GPIIIα49–66 (CAPESIEFPVSEARVLED) contains five negatively charged amino acids, four glutamic and one aspartic acid. It is therefore not surprising that there are many positively charged amino acids (Arg, Lys, and His) in the CDRs of both heavy and light chains of our antibodies. Compared with other αIIbβ3 antibodies, our antibodies do not contain RGD sequences; therefore, it is unlikely that our antibodies can cross-react with the RGD binding site of αIIbβ3 integrin (20). Although we have shown that the positively charged amino acids in the HCDR3 play a role in inducing platelet fragmentation, this result does not exclude the possible role(s) of other CDRs in heavy and light chains. In fact, after we mutated both positively charged

**FIGURE 5. Molecular modeling.** Molecular models of 11, 11M1, 11M2, 43, 43M, and 54M are shown. The heavy chain is in red and the light chain in blue. The amino acid at position 102 in all clones is shown in purple. Lys-100 (in 11), Lys-101 (in 11M1), Gly-100 (in 11M2), Arg-101 (in 43), Arg-99 (in 43M), Arg-99 (in 54), and Arg-101 (in 54M) are shown in green.
amino acids in 11 HCDR3, 11M2 still retained some platelet fragmentation capability, suggesting that other regions also contribute to induce platelet fragmentation. Nevertheless, our data establish the concept that a specific conformation with β3 is required for antibody-induced platelet fragmentation.

The αIIbβ3 integrin has been utilized as a therapeutic target for thrombosis and occlusion due to coronary artery stenting (21, 22). Side effects of these drugs are thrombocytopenia and bleeding. Development of a different approach to inhibit arterial platelet thrombi by disaggregation/lysis of platelet thrombi with the specific scFv anti-GPIIIa49–66 antibody may be of clinical value.

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REFERENCES
1. Morris, L., Distenfeld, A., Amorosi, E., and Karpatkin, S. (1982) Ann. Intern. Med. 96, 714–717
2. Najean, Y., and Rain, J. D. (1994) J. Lab. Clin. Med. 123, 415–420
3. Nardi, M., Tomlinson, S., Greco, M. A., and Karpatkin, S. (2001) Cell 106, 551–561
4. Li, Z., Nardi, M. A., and Karpatkin, S. (2005) Blood 106, 572–576
5. Nardi, M., Feinmark, S. J., Hu, L., Li, Z., and Karpatkin, S. (2004) J. Clin. Investig. 113, 973–980
6. Bennett, J. S. (2005) J. Clin. Investig. 115, 3363–3369
7. Xia, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) Nature 432, 59–67
8. Undas, A., Brummel, K., Musial, J., Mann, K. G., and Szczeklik, A. (2001) Circulation 104, 2666–2672
9. Szczeklik, A., Undas, A., Sanak, M., Frolov, M., and Wegrzyn, W. (2000) Br. J. Haematol. 110, 965–967
10. Andrioli, G., Minuz, P., Solero, P., Pincelli, S., Ortolani, R., Lussignoli, S., and Bellavite, P. (2000) Br. J. Haematol. 110, 911–918
11. Michelson, A. D., Furman, M. L., Goldschmidt-Clermont, P., Mascelli, M. A., Hendrix, C., Coleeman, L., Hamlington, J., Barnard, M. R., Kickler, T., Christie, D. J., Kundu, S., and Bray, P. F. (2000) Circulation 101, 1013–1018
12. Davoren, A., Curtis, B. R., Aster, R. H., and McFarland, J. G. (2004) Transfusion (Bethesda) 44, 1220–1225
13. Winters, J. L., Jennings, C. D., Desai, N. S., Dickson, L. G., and Ford, R. F. (1998) Vox Sang. 74, 256–259
14. Schwarz, M., Rottgen, P., Takada, Y., Le Gall, F., Knackmuss, S., Bassler, N., Burtner, C., Little, M., Bode, C., and Peter, K. (2004) FASEB J. 18, 1704–1706
15. Jacobin, M. J., Laroche-Traineau, J., Little, M., Keller, A., Peter, K., Welschof, M., Nurden, A., and Clofent-Sanchez, G. (2002) J. Immunol. 168, 2035–2045
16. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Nature 352, 624–628
17. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994) EMBO J. 13, 692–698
18. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaczok, M., Gherardi, E., and Winter, G. (1994) EMBO J. 13, 3245–3260
19. Shattil, S. J., and Newman, P. J. (2004) Blood 104, 1606–1615
20. Jacobin, M. J., Robert, R., Pouns, O., Laroche-Traineau, J., Nurden, A., Peter, K., Little, M., and Clofent-Sanchez, G. (2003) Clin. Immunol (Orlando). 108, 199–210
21. Bhatt, D. L., and Topol, E. J. (2003) Nat. Rev. Drug Discov. 2, 15–28
22. Sanz, L., Kristensen, P., Blanco, B., Facteau, S., Russell, S. J., Winter, G., and Alvarez-Vallina, L. (2002) Gene Ther. 9, 1049–1053

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