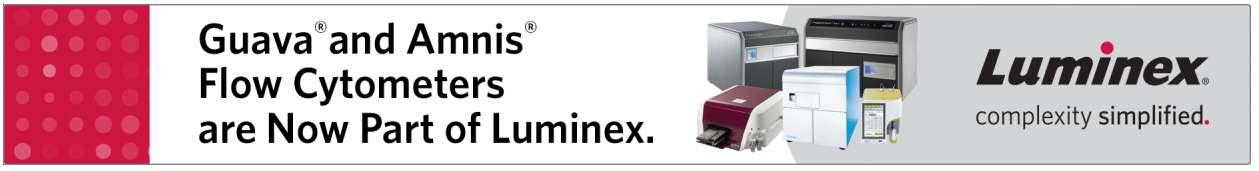

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The Role of the Human Fc Receptor FcγRIIA in the Immune Clearance of Platelets: A Transgenic Mouse Model[1]

Steven E. McKenzie,[2]* Scott M. Taylor,* Padmini Malladi,* Heena Yuhan,* Diana L. Cassel,* Paul Chien,† Elias Schwartz,* Alan D. Schreiber,† Saul Surrey,* and Michael P. Reilly*

In humans, the Fc receptor for IgG, FcγRIIA, is expressed on macrophages and platelets and may play an important role in the pathophysiology of immune-mediated thrombocytopenia. Mice lack the genetic equivalent of human FcγRIIA. To better understand the role of FcγRIIA in vivo, FcγRIIA transgenic mice were generated and characterized. One transgenic mouse line expressed FcγRIIA on platelets and macrophages at levels equivalent to human cells, and cross-linking FcγRIIA on these platelets induced platelet aggregation. Immune-mediated thrombocytopenia in this transgenic line was studied using i.v. and i.p. administration of anti-mouse platelet Ab. In comparison with matched wild-type littermates that are negative for the FcγRIIA transgene, Ab-mediated thrombocytopenia was significantly more severe in the FcγRIIA transgenic mice. In contrast, FcR γ-chain knockout mice that lack functional expression of the Fc receptors FcγRI and FcγRIII on splenic macrophages did not demonstrate Ab-mediated thrombocytopenia. We generated FcγRIIA transgenic × FcR γ-chain knockout mice to examine the role of FcγRIIA in immune clearance in the absence of functional FcγRI and FcγRIII. In FcγRIIA transgenic × FcR γ-chain knockout mice, severe immune thrombocytopenia mediated by FcγRIIA was observed. These results demonstrate that FcγRIIA does not require the FcR γ-chain for expression or function in vivo. Furthermore, taken together, the data suggest that the human Fc receptor FcγRIIA plays a significant role in the immune clearance of platelets in vivo. ***The Journal of Immunology*****, 1999, 162: 4311–4318.**

Immune clearance initiated by autoantibodies occurs in a wide range of autoimmune disorders. Studies of the immune destruction of blood cells, such as platelets in immune thrombocytopenia, have served as a model for exploring the pathophysiology of these disorders. The immune thrombocytopenias represent a diverse group of human platelet disorders that cause considerable morbidity and mortality throughout life. They include autoimmune thrombocytopenic purpura, thrombocytopenia related to HIV infection, thrombocytopenia related to systemic lupus erythematosus, neonatal alloimmune thrombocytopenia, heparin-induced thrombocytopenia with thrombosis, and drug-related thrombocytopenias (1–9). The development of immune thrombocytopenia requires two pathologic steps: 1) formation and sustained production of Ab to a self or neo-Ag and 2) triggering of Ab effector mechanisms that lead to accelerated platelet clearance and/or platelet activation.

Therapy for immune thrombocytopenia (and to some extent other autoimmune disorders) is often directed toward reducing the Ab-mediated clearance by i.v. Ig, glucocorticoids, splenectomy, or other means (2). These seemingly distinct therapies work in large part by inhibiting the splenic macrophage Fc receptor-dependent clearance of these Ab-coated cells.

It has been well established that the Fc receptors for IgG (Fcγ receptors) play a major role in immune clearance, in the accelerated clearance of Ab-coated platelets, and in the therapeutic response (10–18). For example, glucocorticoids diminish thrombocytopenia long before they reduce IgG Ab titers. In fact, glucocorticoids have been demonstrated to down-regulate macrophage and platelet surface Fcγ receptor expression. Intravenous Ig and the administration of anti-D Ig (coating Rh^+ erythrocytes) also inhibit Fc receptors, although additional mechanisms of action for i.v. Ig have been postulated. Removal of the spleen and its Fcγ receptor-laden macrophages is also of major therapeutic benefit in the treatment of immune thrombocytopenia (19). A second line of evidence for the importance of Fcγ receptors in immune clearance comes from studies in mice. Mice deficient in Fcγ receptors do not become thrombocytopenic when treated with anti-platelet Abs (20, 21).

The complexity of the human Fcγ receptor repertoire only recently began to be appreciated (22–28). Human macrophages express on their surface FcγRI, FcγRIIA, and FcγRIIIA. Each is an activating receptor that individually is capable of mediating phagocytosis (29–33). FcγRI and FcγRIIIA require a FcR subunit, or γ-chain, for expression and/or for signaling for phagocytosis in vivo (20, 34). In contrast, FcγRIIA does not require the γ-chain for its expression and phagocytic activity, possessing IgG-binding and signal transduction capabilities in the same molecule (30–32, 35). It is of note that human platelets express FcγRIIA as their sole Fc receptor (29, 36–39). When cross-linked, FcγRIIA fully activates platelets for secretion and aggregation (22–24, 40). The relative contributions of the different Fc receptors to the pathophysiology and therapeutic response in immune thrombocytopenia have not yet been dissected in vivo.

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Table I. *Sequence of oligonucleotides used in the PCR and RT-PCR experiments*

Oligonucleotide	Sequence
Human FcγRIIA transgene	
PCR3, sense	5′-CTGGTCAAGGTCACATTCTTC-3′
4INM, antisense	5′-CAATTTTGCTGCTATGGGC-3′
T5S, sense	5′-GGGTAGTTCCTTACAATTTTCCTG-3′
16 M, antisense	5′-CAATGGAAGCAGCCACAGGTT-3′
4.0-7S, sense	5′-GAGAAGAGAATTAGAGAGGTGAGG-3′
4.0-6M, antisense	5′-GACTTACATGATTGCTCT-3′
195M, antisense	5′-ATTGCGACTGCTGTAGCAGCCATT-3′
Mouse FcR γ-chain, wild-type	
gamma1, sense	5′-ACCCTACTCTACTGTCGACTCAAG-3′
gamma2, antisense	5′-TCACGGCTGGCTATAGCTGCCTT-3′
Mouse FcR γ-chain, knock-out locus	
Neo, sense	5′-CTCGTGCTTTACGGTATCGCC-3′
gamma2, antisense	5′-TCACGGCTGGCTATAGCTGCCTT-3′
Mouse whey acidic protein gene	
WAP-S, sense	5′-ATCCATGTCTCCATGCCTTCTTCT-3′
WAP-A, antisense	5′-TGTTGACAGGAGTTTTGCGGGTCC-3′
Mouse β-actin	
mβactinS, sense	5′-ATGGGTCAGAAGGACTCCTA-3′
mβactinA, antisense	5′-CTCGGTCAGGATCTTCATGA-3′

Mouse models have been helpful in examining the pathophysiology of immune clearance. FcR γ-chain knockout (KO)[3] mice have been generated, and in these mice all activating Fcγ receptor functions are abrogated in vivo (20, 34). In addition, there is no evidence of immune thrombocytopenia after injection of anti-platelet Ab in γ-chain KO mice (20). In contrast, matched heterozygous KO mice and wild-type mice develop prominent Ab-mediated thrombocytopenia.

However, major differences exist between the Fcγ receptor repertoire in mouse and humans, notably absence of FcγRIIA on mouse macrophages and absence of any mouse platelet Fcγ receptor (41–47). Therefore, we sought to generate and characterize mice that express FcγRIIA and to test the hypothesis that expression of FcγRIIA plays a role in the pathophysiology of the thrombocytopenia caused by anti-platelet Ab. In this work, we describe the generation of human FcγRIIA transgenic mice and examine the effects of FcγRIIA expression on immune clearance in normal mice and in γ-chain-deficient mice. The FcγRIIA transgenic mice more accurately reflect the human Fcγ receptor repertoire and provide a model for evaluating the pathophysiology of the immune clearance of platelets.

Materials and Methods

Generation and characterization of human FcγRIIA transgenic mice

A P1 clone identified by PCR screening of a human P1 genomic library was used to generate a 72-kb *Sfi*I restriction fragment that included the 20-kb FcγRIIA gene, 45 kb of 5′-flanking region containing the gene promoter that we have previously characterized (48, 49), and 7 kb of 3′-flanking region (Fig. 1*A*). The 20-kb gene includes eight exons: 1) 5′UT (UT, untranslated); 2) 5′UT/S1 (S, signal peptide); 3) S2; 4) EC1 (EC, extracellular domain); 5) EC2; 6) TM (TM, transmembrane domain); 7) C1 (C, cytoplasmic domain); and 8) C2/3′UT. The 72-kb DNA fragment was used to create transgenic mice following established procedures at the Transgenic Mouse Core Facility of The University of Pennsylvania (Philadelphia, PA). All animals were studied under the guidance and approval of the Institutional Animal Care and Use Committees at the University of Pennsylvania and Alfred I. duPont Hospital for Children.

Four offspring were found to contain the human FcγRIIA gene by PCR and Southern blot analyses, as follows. Genomic DNA was isolated from a tail biopsy of 3-wk-old mice and amplified using a primer located in the EC2 exon of the FcγRIIA gene (PCR3) and another located in the following intron (4INM). Primer sequences used for PCR and RT-PCR experiments are shown in Table I. As an internal control, a fragment of the mouse endogenous whey acidic protein (WAP) gene was also amplified using a second set of primers, WAP-S and WAP-A (50, 51). PCR was done for 30 cycles, with each cycle consisting of denaturing at 94°C for 45 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min using a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). PCR products were analyzed by agarose gel electrophoresis for presence of the appropriately sized FcγRIIA band (280 bp). To verify that the transgene contained the full-length FcγRIIA sequence, additional primers were used to amplify the 5′-untranslated region (T5S and 16 M) and the 3′-untranslated region (4.0–7S and 4.0–6 M).

Gene copy number was determined using Southern blot analysis. Genomic DNA (10 μg) was digested with *Bam*HI, separated on a 1% (w/v) agarose gel, and transferred to a Zetabind filter (Cuno, Meridien, CT). *Bam*HI-digested P1 clone DNA was used as a standard for estimating copy number by application of 1, 5, and 10 genome-equivalent copies. The filter was prehybridized in Rapid-hyb buffer (Amersham, Arlington Heights, IL) and then hybridized at 65°C overnight to a radiolabeled probe made with the Random Primers Labeling Kit (Boehringer Mannheim, Indianapolis, IN) using a 800-bp fragment of the FcγRIIA gene as a template (24). The filter was washed in 2× SSC/0.1% (w/v) SDS, 0.2× SSC/0.1% (w/v) SDS, and finally 0.1× SSC/0.1% (w/v) SDS at 65°C, and then exposed to film at −70°C with an intensifying screen. The intensity of bands on film was analyzed by Imagequant PhosphorImager software (Molecular Dynamics, Sunnyvale, CA). This calculation was verified by comparing transgene intensity to known amounts of plasmid DNA on the same Southern blot. Equivalent loading of DNA from genomic samples was verified by hybridization to the labeled endogenous mouse β-actin cDNA (50). Of the four transgenic mouse lines, one (line $F_0$11) contained 10 copies, one (line $F_0$32) 4 copies, and the other two (line $F_0$7 and line $F_0$40) 1 copy of the transgene per haploid genome.

The FcR γ-chain KO mice were provided generously by Dr. Jeffrey Ravetch (Rockefeller University, New York, NY). They were analyzed by PCR of genomic DNA using primers specific for the wild-type and KO gene loci. C57BL/6 × SJL F_1 mice (B6SJL F_1) served as wild-type controls (The Jackson Laboratory, Bar Harbor, ME).

The line 11 FcγRIIA transgenic mice were crossed with the FcR γ-chain KO mice and bred to be hemizygous for FcγRIIA transgene and homozygous for the γ-chain KO gene, as assessed by PCR of genomic DNA and analysis of offspring.

For isolation of RNA and analysis of mRNA expression, whole blood (200 μl) was collected from each line of transgenic mice by puncture of the retro-orbital sinus with heparinized hematocrit tubes (Fisher Scientific, Pittsburgh, PA). Platelet-rich plasma was isolated by centrifuging the blood samples at 100 × *g* in a Sorvall RT6000B centrifuge (Sorvall, Newtown, CT). Platelets were then pelleted at 1900 × *g*, and total RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX) using the manufacturer's protocol. RT-PCR was then performed as follows: cDNA was made

[3] Abbreviations used in this paper: KO, knockout; GAM, goat anti-mouse; ^{125}I-IV.3, ^{125}I-labeled IV.3; RT, room temperature; tg, transgenic; WAP, whey acidic protein.

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from total RNA using random hexamers and M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). PCR was then used to amplify a section of the FcγRIIA cDNA using intron-spanning primers to distinguish cDNA from genomic DNA signal. The sense primer chosen was located in the EC2 exon of the FcγRIIA gene (PCR3), and the antisense primer was located in the downstream TM exon (195 M). PCR was done for 35 cycles, with each cycle consisting of denaturing at 94°C for 15 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s, followed by a 72°C hold for 2 min using a GeneAmp PCR System 9600. Products were analyzed by agarose gel electrophoresis for the presence of the appropriately sized bands (300 bp). Primers to mouse β-actin mRNA (mβactinS and mβactinA) were chosen as controls for the integrity of the mRNA through the RT-PCR process.

Transgene-encoded protein was detected on the surface of megakaryocytes and macrophages by immunohistochemical staining of bone marrow aspirates and spleen samples from transgenic mice. Tissues were selected from B6SJL F_1 wild-type mice as negative controls. U937 cells, a monocyte-like cell line that expresses the FcγRIIA receptor, served as a positive control (29). Peritoneal macrophages were induced with thioglycolate following standard procedures (52). Macrophage surface receptor density was quantitated using Scatchard analysis. ^{125}I-IV.3 binding to transgenic mouse macrophages, wild-type mouse macrophages, and human FcγRIIA-positive HEL cell line control was performed to measure FcγRIIA receptor density, as previously reported (53).

Bone marrow aspirates flushed from both femurs and humeri of transgenic or wild-type mice were placed in 1 ml PBS on wet ice. Unfixed samples were placed in a cytospin (Shandon Lipshaw, Pittsburgh, PA) at 150 μl per cyto-funnel and pelleted onto poly(L) slides at 1000 rpm for 10 min. Slides were immediately removed from the cytospin, wrapped in aluminum foil, and placed in a −70°C freezer. Suspended U937 cells were suspended 1:1 (v/v) with PBS. Approximately 100 μl was placed in each funnel, spun at 1000 rpm for 10 min onto poly(L) slides, and stored similarly to the bone marrow slides. Excised spleens from both transgenic and wild-type mice were snap frozen in isopentane chilled in liquid nitrogen, and stored at −70°C. Subsequently, 5-μm sections were cut (Reichert-Jung Frigocut, Leica, Deerfield, IL) and placed on poly(L) slides, which were also stored, wrapped in foil, and frozen at −70°C. Just before staining, all slides were air dried for 10 min and warmed to room temperature (RT).

All slides were briefly rinsed in PBS, then placed in Peroxo-Blocker (Zymed, San Francisco, CA) for 1 min at RT. Slides were rinsed well in PBS, and placed in CAS-Block (Zymed), avidin, and biotin blockers, each for 10 min with several washes of PBS before and after each blocking step. Slides were then placed in the primary Ab IV.3 (monoclonal anti-human FcγRIIA, mIgG2b), at a dilution 1/100 in 2% (v/v) FBS. The sections were incubated (50–100 μl per section) in a humid chamber at 37°C for 30 min, then at RT for an additional 30 min. Negative controls that omitted the primary Ab were incubated similarly in 2% (w/v) FBS and CAS-Block at 1:1. All slides were rinsed well with PBS and placed in the secondary goat anti-mouse (GAM) at 1:500 in CAS-Block in a humid chamber. The samples were incubated at RT for 1 h, rinsed well in PBS and deionized water, and detected with streptavidin-AEC kit (Zymed). Slides were counterstained in Mayer's hematoxylin for 2 min at RT, rinsed in deionized water, and mounted in Advantage aqueous mounting medium (Innovex, Richmond, CA). As a control for identification of mouse macrophages, Ab to mouse FcγRII/III (2.4G2, rat IgG2b; PharMingen, San Diego, CA) was used as primary Ab.

FcγRIIA protein expression and function on platelets

Transgene-encoded protein was detected on the surface of platelets using several methods. In the immunoblotting technique, whole blood was isolated from transgenic mice via cardiac puncture in 3.8% (w/v) sodium citrate to prevent coagulation. Blood samples were spun at 100 × *g* in a Sorvall RT6000B centrifuge to isolate the platelet-rich plasma. Platelet-rich plasma was centrifuged at 1900 × *g* to pellet platelets, and the platelet pellet was washed once with PBS and again pelleted. Platelets were lysed using Triton lysis buffer with protease inhibitors (1% (v/v) Triton X-100, 50 mM Tris 7.6, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Following clarification by centrifugation at 15,000 rpm for 30 min at 4°C, cell lysates were resolved on a 7.5% (w/v) SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and immunoblotted with the anti-FcγRIIA mAb II.1.A.5, provided by Dr. Jurgen Frey (Universitat Bielefeld, Germany). Blots were developed with horseradish peroxidase-conjugated GAM Ab (Bio-Rad, Richmond, CA). Immunoreactivity was detected by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham).

Platelet surface receptor density was quantitated using Scatchard analysis. Platelets were prepared from whole blood, as indicated above, counted, and resuspended in 1× Tyrode's buffer. ^{125}I-IV.3 binding to transgenic mouse platelets and human platelet controls was performed to measure FcγRIIA receptor density on the platelet surface, as previously reported (53).

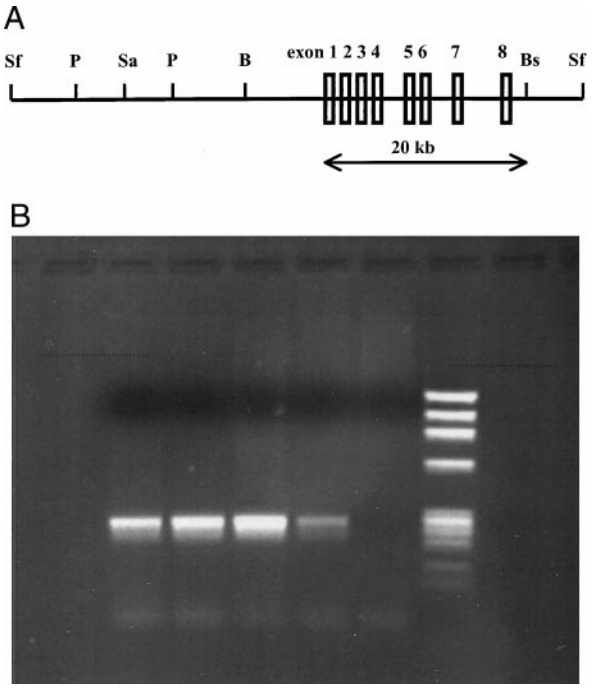

FIGURE 1. Generation and characterization of FcγRIIA transgenic mice. *A*, Map of the 72-kb human genomic DNA fragment containing the 20-kb human FcγRIIA gene used to create the transgenic mice is shown. The relative locations of the eight exons and the sites for digestion by selected restriction endonucleases are shown (Sf = *Sfi*I, *P* = *Pme*I, Sa = *Sal*I, Bs = Bss H II, B = *Bam*HI). *B*, Detection of the human FcγRIIA gene in transgenic mice using PCR primers specific for human FcγRIIA. The expected 280-bp product is seen in *lanes 1–4*, representing mouse $F_0$7, 11, 32, and 40, respectively. *Lane 5* presents the result for a littermate mouse that is negative for the transgene. *Lane 6* contains ϕX-*Hae*III DNA size markers.

Function of the FcγRIIA receptor on the transgenic mouse platelets was tested in aggregation experiments using anti-human FcγRII Ab IV.3 (20 μg/ml) as the primary Ab (22–25). A $F(ab')_2$ GAM Ab (40 μg/ml; Caltag, San Francisco, CA) was added as the secondary Ab to promote cross-linking of the primary Ab. Whole blood was collected via cardiac puncture from wild-type and transgenic mice in 3.8% (w/v) sodium citrate to prevent coagulation. The blood was warmed for several minutes at 37°C and, following addition of primary and secondary Ab or control aggregation reagents (1 U thrombin, 10 μM ADP (final concentration), or 2 μg/ml collagen (final concentration)), aggregation was measured in an aggregometer using measurement of the impedance signal (ChronoLog, Havertown, PA), according to the manufacturer's protocol. Additional aggregation experiments were performed in which 4A5 Ab was added (final concentration 20–40 μg/ml), and aggregation response was measured.

Injection of Abs into mice

Abs or controls were injected either i.p. or i.v., as follows. Mice from each line studied were given a single i.p. injection of 100 μl of PBS containing 70 μg of 4A5, a rat anti-mouse platelet Ab (42). 4A5 is well characterized, and the thrombocytopenia that results from 4A5 injection into wild-type mice has been reported previously by an independent group. The injection time is considered $t = 0$. The 4A5 Ab was purified from the supernatant of a culture of 4A5 hybridoma cells (generously provided by Dr. Sam Burstein of the University of Oklahoma) using a mAb Trap G II kit (Pharmacia, Piscataway, NJ), according to the manufacturer's instructions. A control mouse from each line was injected with 100 μl of PBS containing no Ab. Another group of transgenic mice was injected with 100 μl of PBS containing 70 μg of a rat IgG2a isotype control Ab (PharMingen).

In separate experiments, transgenic mice and wild-type mice were given a single i.v. (tail vein) injection of 70 μg of 4A5 Ab in 50 μl of PBS. A

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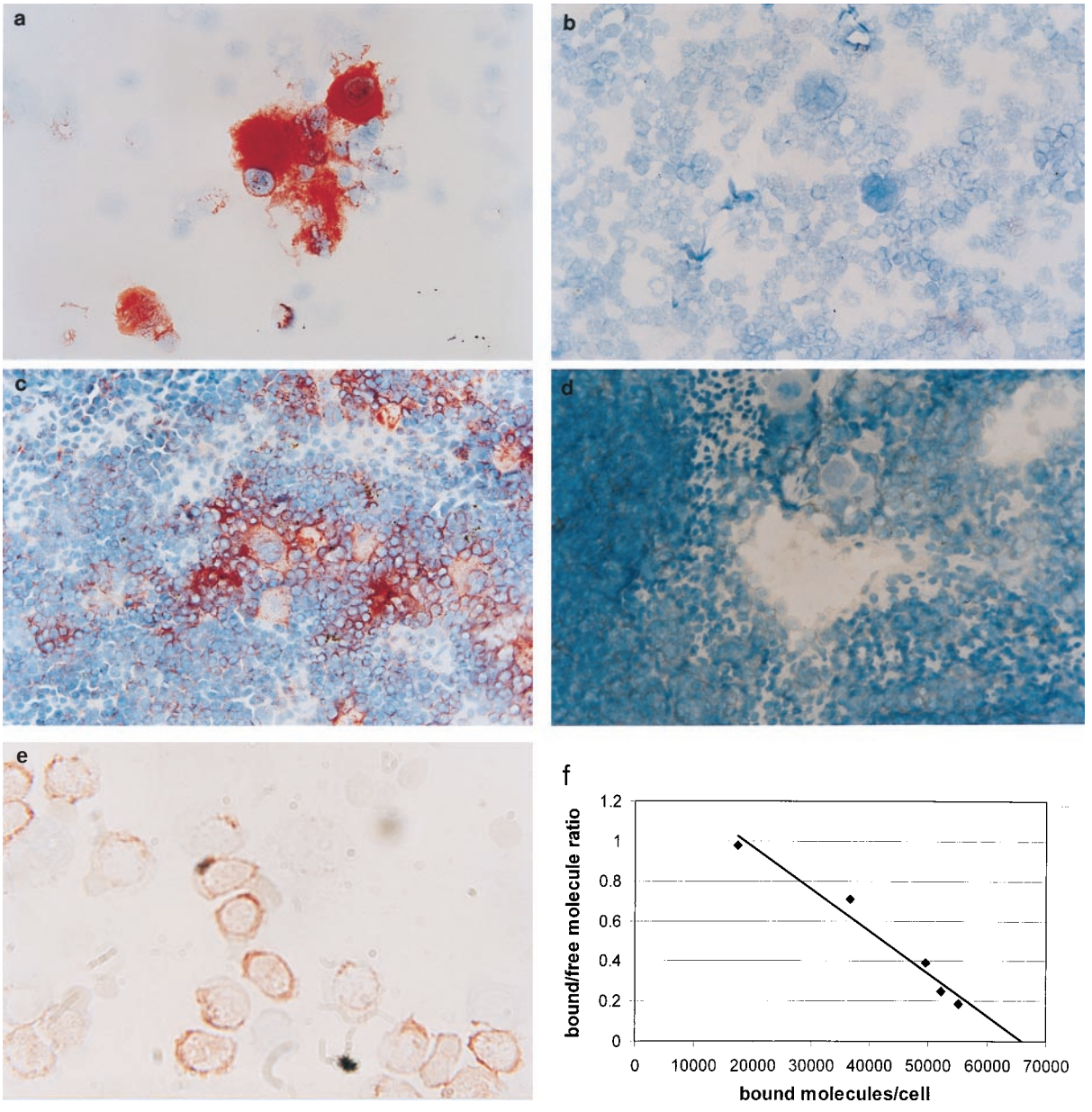

FIGURE 2. Immunohistochemical analysis and Scatchard analysis of human FcγRIIA expression in transgenic mice. *a*, Bone marrow of line 11 IIA tg mouse. *b*, Bone marrow of nontransgenic control. *c*, Spleen of line 11 IIA tg mouse. *d*, Spleen of nontransgenic control. Primary staining with IV.3 was followed by alkaline phosphatase-conjugated secondary Ab and developed with substrate to give the red color for positive cells. All are ×400 magnification. Only the line 11 IIA tg samples (*a* and *c*) stained for human FcγRIIA on megakaryocytes and macrophages in marrow and spleen. *e*, 2.4G2 staining (anti-murine FcγRII/III) of thioglycolate-induced peritoneal macrophages (×1000 magnification), and *f*, colocalization of human FcγRIIA on the same set of macrophages by ^{125}I-IV.3 binding. The Scatchard analysis indicates ~65,000 IV.3 binding sites on the surface of line 11 transgenic mouse macrophages, equivalent to the receptor level on human macrophages (55).

control mouse from each line was given an i.v. injection with 50 μl of PBS containing no Ab.

Platelet counts

Platelet counts were obtained before and at timed intervals after injection of anti-platelet Abs. Whole blood (200 μl) was collected by puncture of the retro-orbital sinus of anesthetized mice using heparinized hematocrit tubes (Fisher Scientific). Platelet-rich plasma was isolated, and platelet counts were obtained using a Coulter Z1 counter set for a mouse platelet aperture (Coulter, Miami, FL). The platelet counts are reported in number/μl.

Statistical analysis

The nadir platelet count following anti-platelet Ab injection was the basis for comparison. Platelet counts from groups of 6 to 12 FcγRIIA transgenic, γ-chain KO, (FcγRIIA transgenic × γ-chain KO), and wild-type mice each were compared using ANOVA analysis, with two-sided $p < 0.05$ considered significantly different.

Results

Generation and characterization of human FcγRIIA transgenic mice

The FcγRIIA gene encompasses 20 kb and includes eight exons (48) (Fig. 1*A*). A 72-kb P1 genomic clone fragment was used for creation of transgenic mice. This genomic clone encodes the R^{131} polymorphic form of human FcγRIIA, which binds the widest range of mouse IgG subclasses, specifically binding mouse IgG1 and rat IgG2a (the 4A5 isotype) well (54). We produced four FcγRIIA transgenic mouse founders (B6SJL genetic background)

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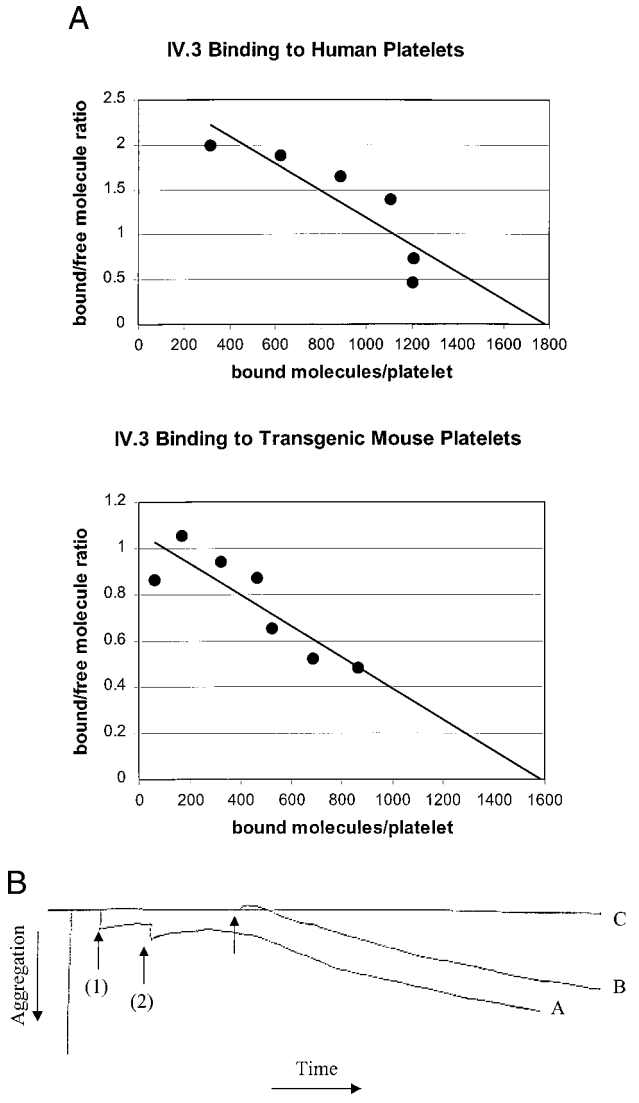

FIGURE 3. Platelet FcγRIIA expression and function in transgenic mouse line 11. *A*, Measurement of FcγRIIA platelet surface receptor density. Scatchard plots for human platelets and line 11 FcγRIIA transgenic hemizygous mouse platelets binding ^{125}I-IV.3 are shown, as described (53). The number of sites/platelet is comparable (1800, human; 1550, FcγRIIA transgenic mouse). *B*, Platelet aggregation was stimulated by human FcγRIIA using anti-FcγRIIA Ab IV.3 (arrow 1) followed by GAM (arrow 2), and plotted as extent of platelet aggregation versus time. Aggregation occurred only when GAM was added after prior addition of anti-FcγRIIA Ab. The extent of FcγRIIA-mediated platelet aggregation (curve A) was comparable with that of the positive control, thrombin (curve B). Platelet aggregation in response to 4A5 (curve C), as well as spontaneous aggregation, was negligible.

containing the entire FcγRIIA gene, as assessed by positive PCR reactions with three different sets of primers, from the middle of the gene (Fig. 1*B*) as well as the most 5′ and 3′ ends. Southern blot analysis confirmed the presence of the transgene, the absence of any major gene rearrangement, and estimation of transgene copy numbers ranging from 10 ($F_0$11) to 4 ($F_0$32) to 1 ($F_0$7 and 40) (data not shown). Each FcγRIIA transgenic mouse founder demonstrated germ-line transmission of the transgene.

FcγRIIA mRNA expression was studied in each of the four transgenic mouse lines using RT-PCR. Platelets were isolated from whole blood, RNA was prepared from the platelets, and RT-PCR with primers specific for human FcγRIIA was performed. All four transgenic mouse lines expressed human FcγRIIA mRNA in their platelets (data not shown).

Tissue and cellular distribution of the FcγRIIA protein were studied by immunohistochemistry using IV.3 Ab. Controls included nontransgenic mice and studies in which the primary Ab was omitted. FcγRIIA expression was found in marrow megakaryocytes and leukocytes (identified in other sections with 2.4G2 Ab to the endogenous mouse FcγRII/III) in our transgenic animals (Fig. 2*a*). FcγRIIA expression in megakaryocytes and macrophages was also detected in the spleen (Fig. 2*c*), while the lymphoid cells in the follicles showed no expression. Colocalization of 2.4G2 staining (anti-murine FcγRII/III) and human FcγRIIA (^{125}I-IV.3 binding) was shown on the same set of thioglycolate-induced peritoneal macrophages from line 11 transgenic mice (Fig. 2, *e* and *f*). The Scatchard analysis indicates ~65,000 IV.3 binding sites on the surface of line 11 transgenic mouse macrophages, equivalent to the receptor level on human macrophages (55).

Platelets were analyzed for FcγRIIA protein expression with several assays. Mice from the transgenic lines were positive for platelet membrane FcγRIIA expression, as assessed by immunoprecipitation of platelet lysates with the anti-human FcγRIIA mAb IV.3, followed by immunoblot with a second anti-FcγRIIA Ab (II.1A.5). Immunoprecipitates from the transgenic lines contained a protein whose size was identical to the human FcγRIIA receptor, ~40 kDa (data not shown). Platelet FcγRIIA surface receptor density measured using ^{125}I-IV.3 binding showed 1550 sites per platelet in the hemizygous line 11, which is in the normal range for human platelets (Fig. 3*A*).

Line 11 contained the greatest FcγRIIA gene copy number, produced the highest qualitative RT-PCR signal in RNA analysis, and had the highest protein signal in whole platelet ELISA and immunoprecipitation/immunoblot experiments. We also demonstrated by Scatchard analysis that the FcγRIIA protein level on line 11 platelets is within the normal range for human platelets. This line was chosen as the major FcγRIIA transgenic mouse line for further studies in vitro and in vivo.

Platelet aggregation studies

We examined platelet aggregation to determine function of the FcγRIIA receptor on platelets of transgenic mice. Human platelets,

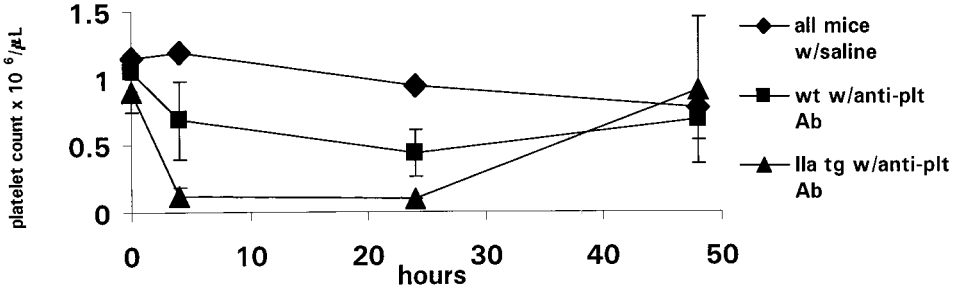

FIGURE 4. Immune thrombocytopenia following i.v. injection of anti-platelet Ab 4A5. The mean platelet counts (+/−SEM) over time are shown. Thrombocytopenia is more severe in IIA tg than wild-type mice, and the difference is significant ($p < 0.05$ by ANOVA, $n = 6$ in each group).

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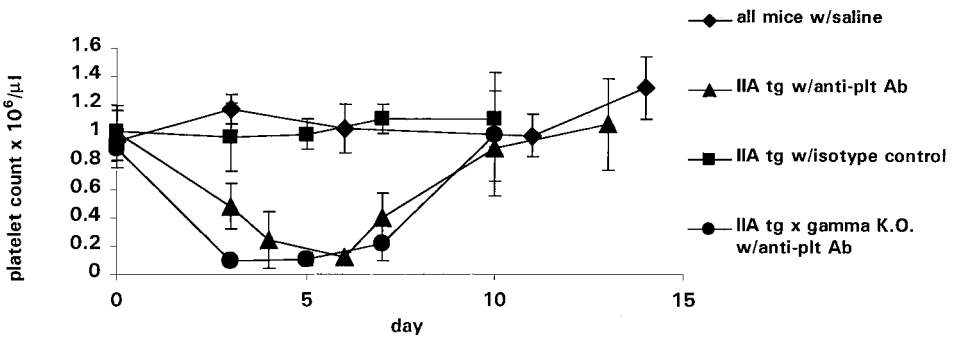

FIGURE 5. Immune thrombocytopenia following i.p. injection of anti-platelet Ab 4A5. Shown is the time course of mean platelet counts (+/−SEM) for line 11 IIA tg mice and IIA tg × γ-chain KO mice following injection of anti-platelet Ab. Saline and Ab isotype controls are as indicated. Both line 11 IIA tg mice and IIA tg × γ-chain KO mice demonstrate statistically significantly lower nadir platelet counts ($p < 0.05$ by ANOVA) than controls.

but not wild-type mouse platelets, aggregated in response to treatment with anti-human FcγRIIA mAb IV.3, followed by cross-linking with $F(ab')_2$ GAM Ab. As a positive control, platelets from both humans and wild-type mice aggregated in response to thrombin, ADP, or collagen stimulation, as described in *Materials and Methods*. Platelets from line 11 were reproducibly activated in the aggregation assay with IV.3 + GAM cross-linking (Fig. 3*B*). Additional aggregation experiments were performed in which 4A5 Ab was added (final concentration 20–40 μg/ml), and the aggregation response was measured. There was no aggregation of platelets from wild-type or FcγRIIA transgenic mice (Fig. 3*B*). This agrees with the original description of the 4A5 Ab by another group, in which the Ab did not cause platelet aggregation/activation of platelets of wild-type mice (42). Addition of ADP following the absence of activation in response to 4A5 confirmed the ability of those platelets to be activated.

Immune clearance of platelets following injection of anti-platelet Abs

As a model for immune clearance, we determined the clearance of platelets following injection of anti-platelet Abs. Wild-type and transgenic mice were treated with anti-platelet Ab, 4A5, which is known to induce moderate thrombocytopenia in wild-type mice (42). Wild-type mice had moderate thrombocytopenia, reaching platelet counts in the 600,000/μl range, as previously reported. In contrast, FcγRIIA transgenic line 11 mice exhibited significantly more severe thrombocytopenia in vivo than wild-type mice following i.v. Ab injection ($p < 0.05$, Fig. 4). Saline-injected controls showed no significant change in platelet count.

Intravenous and intraperitoneal injections of anti-platelet Ab showed comparable results, differing only in the kinetics of clearance. Intravenous injection led to a more rapid platelet count decrease and to a more rapid recovery, measured in hours (Fig. 4). The rebound rise in platelet count is in agreement with earlier studies of Ab-mediated thrombocytopenia (41). Intraperitoneal injections of anti-platelet Ab showed a similar decrease in platelet counts, with platelet clearance over days rather than over hours. As with i.v. injections, i.p. injections of anti-platelet Ab caused immune thrombocytopenia that was more profound in the FcγRIIA transgenic animals ($p < 0.05$, Fig. 5). Neither saline injection nor injection of an isotype control Ab caused thrombocytopenia. Transgenic line 11 mice demonstrated the most severe thrombocytopenia. The thrombocytopenia in FcγRIIA line 32 mice was also significantly more severe than that in wild-type mice ($p < 0.05$), indicating that the immune clearance of platelets mediated by FcγRIIA is evident in two independent transgenic lines. There was no statistically significant difference in the nadir platelet counts induced by 4A5 between FcγRIIA transgenic line 11 and line 32.

As expected, no significant Ab-induced thrombocytopenia was observed in FcR γ-chain KO mice, extending published observations with another anti-platelet Ab and confirming that the mechanism of thrombocytopenia is through Fcγ receptor-mediated clearance (20).

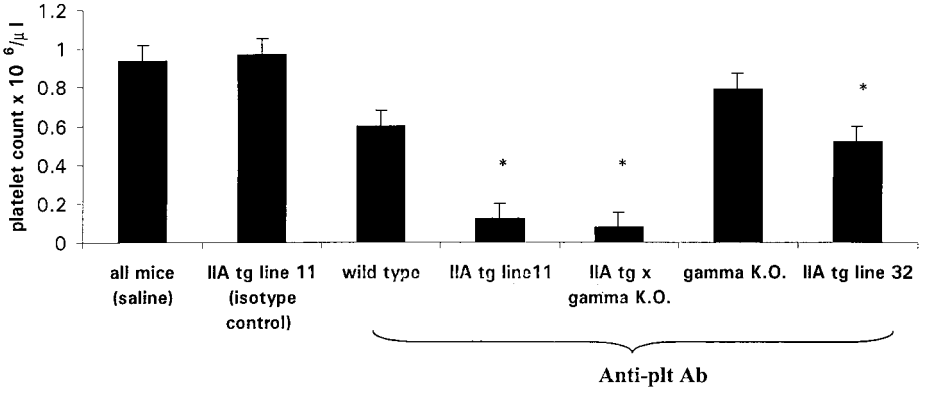

FIGURE 6. Nadir platelet counts for the mouse genetic lines following i.p. injection of anti-platelet Ab 4A5. IIA tg 11 mice reach thrombocytopenic nadirs that are significantly different from the thrombocytopenia in wild-type animals, as indicated by the asterisk ($n = 6$ in all groups; shown is mean ± SEM; $p < 0.05$ by ANOVA). IIA tg line 32 also demonstrated thrombocytopenia more severe than the thrombocytopenia in wild-type animals, as indicated by the asterisk ($p < 0.05$). There was no statistically significant difference in the nadir platelet counts induced by 4A5 between FcγRIIA transgenic lines 11 and 32. The IIA tg × γ-chain KO genetically crossed mice demonstrated significantly lower platelet nadir counts than wild-type mice and γ-chain KO mice ($p < 0.05$). Saline or isotype controls are as indicated.

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Immune clearance in FcγRIIA transgenic × FcR γ-chain KO mice

FcγRIIA transgenic mice were bred to FcR γ-chain KO mice to examine the role of FcγRIIA in immune clearance in vivo in the absence of FcγRI and FcγRIII. As described in *Materials and Methods*, the mice tested were homozygous for the γ-chain KO (γ-chain null or −/−) and hemizygous for the FcγRIIA transgene. In contrast to the γ-chain KO mice, these IIA tg × γ-chain KO mice experienced severe thrombocytopenia when injected with anti-platelet Ab. The nadir platelet counts averaged 73,000/μl ($n = 6$, range = 37,000–114,000/μl), which is significantly different from γ-chain KO mice treated with anti-platelet Ab ($p < 0.05$; Figs. 5 and 6). The nadir platelet counts were comparable with that of the FcγRIIA transgenic mice that coexpress FcγRI/γ and III/γ. Since FcγRI and FcγRIIIA are deficient in FcR γ-chain KO mice, these experiments provide evidence for a critical role for FcγRIIA in the immune clearance of platelets in vivo.

Discussion

We report the creation and characterization of human FcγRIIA transgenic mice. We generated a transgenic mouse line in which the expression of the FcγRIIA receptor in spleen macrophages and platelets more closely recapitulates the expression in humans. These mice allowed us to test the hypothesis that expression of FcγRIIA plays a role in immune clearance.

In our study, we examined thrombocytopenia due to anti-platelet Abs as a model of immune thrombocytopenia. Although there are a number of reported murine models of immune thrombocytopenia (20, 21, 41–46), none of them possesses the Ab effector mechanisms found in humans. We have demonstrated that thrombocytopenia due to anti-platelet Ab is more profound in the presence of FcγRIIA expression. This was observed for both i.v. and i.p. Ab administration in our primary transgenic line. A second independent FcγRIIA transgenic line also demonstrated more severe thrombocytopenia due to anti-platelet Ab. We found the same moderate thrombocytopenia induced by anti-platelet Ab in wild-type mice as originally reported (42), and observed no anti-platelet Ab-induced thrombocytopenia in FcR γ-chain KO mice.

Our creation of the genetic cross of the FcγRIIA transgenic mice with the FcR γ-chain KO mice further provided the opportunity to study the contribution of FcγRIIA as an important Fcγ receptor in Ab-induced thrombocytopenia. These genetically crossed mice developed immune thrombocytopenia as profound as FcγRIIA transgenic mice in which murine FcγRI and FcγRIII were coexpressed. FcγRI and FcγRIII are deficient in FcR γ-chain KO mice, while murine FcγRIIB is expressed. Murine FcγRIIB is a regulatory Fcγ receptor that has an inhibitory function (28, 32, 56). Thus, in the FcγRIIA transgenic × FcR γ-chain KO mice, FcγRIIA is the major activating Fcγ receptor. Our data provide the first evidence of the significant role for FcγRIIA in immune clearance in vivo and independent of FcγRI, FcγRIII, and the FcR γ-chain.

Involvement of FcγRIIA with the γ-chain has been proposed as a result of experiments suggesting functional interaction in transfected B cells (57), although FcγRIIA does not require the γ-chain for phagocytosis in vitro (30–32). Our studies in vivo clearly demonstrate that the γ-chain is not necessary for the effect of FcγRIIA in immune clearance.

The FcγRIIA transgenic mice represent an advance in mouse models of human immune thrombocytopenia. Our data suggest that therapies to diminish Ab effector mechanisms in human immune thrombocytopenia should be directed at the expression or function of FcγRIIA as well as the other Fcγ receptors. Our FcγRIIA transgenic mouse model provides a mechanism for testing potential therapeutic modalities. Since these mice express the activating platelet Fcγ receptor, they may also be useful in studies of immune thrombocytopenias accompanied by thrombosis, such as heparin-induced thrombocytopenia with thrombosis in which the platelet Fc receptor is known to play a pathophysiologic role (5, 6, 58). We cannot preclude any 4A5-induced platelet activation in vivo, but that is unlikely given the result that 4A5 did not activate platelets of wild-type or transgenic mice in vitro.

While we have demonstrated the importance of FcγRIIA in vivo in the immune clearance of platelets, the relative contribution of platelet and/or macrophage FcγRIIA expression to the pathophysiology of the thrombocytopenia is uncertain. It is likely that molecular genetic approaches will help dissect the comparative roles of these two lineages. We recently identified sequences that mediate megakaryocyte-specific expression in transgenic mice in vivo using the PF4 gene promoter (50), as well as the promoter sequences responsible for the regulation of FcγRIIA transcription in vitro in megakaryocytic and myelomonocytic cell lines (Cassel et al., manuscript in preparation). Application of this knowledge to achieve selective expression of FcγRIIA in the platelet or spleen macrophage in vivo should help address the role of these lineages in immune thrombocytopenia.

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