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Anti-CD40L Immune Complexes Potently Activate Platelets In Vitro and Cause Thrombosis in FCGR2A Transgenic Mice

Liza Robles-Carrillo,* Todd Meyer,* Meghan Hatfield,* Hina Desai,* Mónica Dávila,* Florian Langer,† Mildred Amaya,* Ellen Garber,‡ John L. Francis,* Yan-Ming Hsu,‡ and Ali Amirkhosravi*[

Anti-CD40L immunotherapy in systemic lupus erythematosus patients was associated with thromboembolism of unknown cause. We previously showed that monoclonal anti-CD40L immune complexes (ICs) activated platelets in vitro via the IgG receptor (FcγRIIA). In this study, we examined the prothrombotic effects of anti-CD40L ICs in vivo. Because mouse platelets lack FcγRIIA, we used FCGR2A transgenic mice. FCGR2A mice were injected i.v. with preformed ICs consisting of either anti-human CD40L mAb (M90) plus human CD40L, or a chimeric anti-mouse CD40L mAb (hMR1) plus mouse CD40L. ICs containing an aglycosylated form of hMR1, which does not bind FcγRIIA, were also injected. M90 IC caused shock and thrombocytopenia in FCGR2A but not in wild-type mice. Animals injected with hMR1 IC also experienced these effects, whereas those injected with aglycosylated-hMR1 IC did not, demonstrating that anti-CD40L IC-induced platelet activation in vivo is FcγRIIA-dependent. Sequential injections of individual IC components caused similar effects, suggesting that ICs were able to assemble in circulation. Analysis of IC-injected mice revealed pulmonary thrombi consisting of platelet aggregates and fibrin. Mice pretreated with a thrombin inhibitor became moderately thrombocytopenic in response to anti-CD40L ICs and had pulmonary platelet-thrombi devoid of fibrin. In conclusion, we have shown for the first time that anti-CD40L IC-induced thrombosis can be replicated in mice transgenic for FcγRIIA. This molecular mechanism may be important for understanding thrombosis associated with CD40L immunotherapy. The FCGR2A mouse model may also be useful for assessing the hemostatic safety of other therapeutic Abs.


CD40L is important for the development and production of Abs. Binding of T lymphocyte CD40L with its B lymphocyte receptor, CD40, is indispensable for triggering isotype switching of Abs to T cell-dependent Ags (1). Elevated levels of CD40L in circulation have been observed in patients with autoimmune disorders such as systemic lupus erythematosus (SLE) (2, 3), immune thrombocytopenic purpura (ITP) (4, 5), cystic fibrosis (6), and cancer (7). As aberrant CD40–CD40L interactions are thought to play a pivotal role in these autoimmune diseases, the CD40–CD40L axis is considered one of the most important therapeutic targets for immunological disorders.

Although murine models of monoclonal anti-CD40L therapy showed promising results (8), some clinical trials with anti-CD40L mAbs in patients with SLE and ITP were halted after unexpected fatal thrombotic events (3, 9). Thromboembolism was unexpected because no obvious mechanism directly connected Abs targeting this T cell Ag to the blood coagulation system. Kawai and colleagues (10) reported observing thrombosis in nonhuman primates treated with anti-CD40L mAb. They noted that thrombotic complications were largely eliminated with anticoagulation therapy and implied that vascular endothelial tissue factor “may be relevant to the mechanism for thrombosis induced by mAb against CD40 ligand” (10). However, little progress has been made linking tissue factor to anti-CD40L mAb-induced thrombosis.

Further complicating the matter, CD40L itself appears to be involved in thrombosis (independently of anti-CD40L mAbs). CD40L weakly activates platelets (11, 12) and reportedly promotes thrombus stability (13). CD40L-deficient mice experienced prolonged bleeding and reduced platelet aggregation in vitro (14). Elevated soluble CD40L has been linked to increased risk of atherothrombosis (15).

Given that the majority of circulating CD40L is contained not in T cells but in platelets (16), there is an urgent need to identify the molecular mechanisms that link platelets and CD40L to thrombosis with anti-CD40L mAbs. Only by unraveling these complex interactions can the goal of delivering safe and effective anti-CD40L immunotherapy to patients be fully realized.

We previously showed that immune complexes (IC) consisting of anti-CD40L mAbs and CD40L (anti-CD40L IC) can activate platelets in vitro, and that this activation is dependent on the IgG Fc receptor present on the platelet surface, FcγRIIa (17). These findings provided a plausible explanation for the incidence of thrombotic complications observed in patients treated with anti-CD40L Ab therapy (18). Although single IgG Ab molecules do not bind (19) or activate (20) platelet FcγRIIa, clustered (i.e., higher order) IgG immune complexes can bind and trigger FcγRIIa signaling. Because platelet activation is intimately involved with blood coagulation, we reasoned that anti-CD40L ICs may induce platelet...
activation in vivo and ultimately lead to the thrombotic complications observed in some patients treated with anti-CD40L immunotherapy. Following our in vitro studies, this report focuses on the robust and rapid platelet activation in vivo by anti-CD40L ICs mediated through the platelet IgG Fc receptor. In this study, we provide compelling evidence that anti-CD40L ICs activate platelets in vivo and cause thrombosis. Because mouse platelets lack FcγRIIa, we used FCGRA2a transgenic mice created by McKenzie and colleagues (21, 22) to show that anti-CD40L ICs can indeed induce thrombosis in mice, suggesting these ICs may have been responsible for mediating thrombosis in patients treated with anti-CD40L Abs.

Materials and Methods

Reagents and mAbs
Hybridoma cells were obtained from American Type Culture Collection (Manassas, VA). M90 (anti-human CD40L IgG1, mAb. ATCC no. HB-12055), control irrelevant IgG (anti-human c-myc 9E10 IgG1, mAb. ATCC no. CRL-1729), and IV3 (anti-human IgG receptor, FcγRIIA, ATCC no. HB-217) were purified into azide-free PBS from conditioned media by protein-G chromatography. M2 (anti-FLAG IgG1, mAb) and 1× FLAG peptide (1.0 kDa monomer) were purchased from Sigma-Aldrich (St. Louis, MO). M2 was dialyzed into PBS to remove glycerol prior to mice injections. Recombinant soluble human CD40L (hCD40L, 18 kDa monomer) and recombinant soluble murine CD40L (mCD40L, 16.4 kDa monomer) were purchased from PeproTech (Rocky Hill, NJ). All reagents were aliquoted and stored at −80°C.

Preparation of chimeric anti-mouse CD40L mAb with human Fc

Construction of chimeric MR1-huIgG1 κ mAb (hMR1) was similar to that of the chimeric hamster-mouse MR1 mAbs described (23). The variable domains of the H chain and L chain of the hamster anti-mouse CD40L mAb (M90) were cloned by RT-PCR from total RNA from the hybridoma. Expression vectors for hamster/human chimeric mAb were constructed by engineering human IgG1 or human κ C region CDNA onto the variable domains of the H chain or L chain, respectively, using standard recombinant DNA techniques. Transiently expressed chimeric MR1 mAb was demonstrated to recapitulate the CD40L binding properties of the hamster mAb by ELISA (Supplemental Fig. 1). Stable expression vectors containing CMV-immediate early promoter-driven transfection cassettes for the Ig L chain and H chain and a dIfrS gene as a selectable marker for the L chain vector were constructed. The expression vectors were transfected into Chinese hamster ovary cells and stable clones were isolated by dual selection. The chimeric Ab was purified from the media of the transfected Chinese hamster ovary cultures by affinity chromatography using the Fast Flow Protein A Sepharose (GE Healthcare, Piscataway, NJ), following the manufacturer’s procedures and buffers exchanged into PBS, pH 7.2. The purified Ab was kept at −80°C and transported with dry ice prior to experimental use.

Measurement of human platelet dense granule release by serotonin release assays

Serotonin release assays (SRAs) was used to measure platelet activation. Human platelets were obtained from whole blood collected into acid citrate dextrose (ACD) tubes (1:9 ratio). Blood was centrifuged at 250 × g for 15 min to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared in a 1.5 ml microfuge tube by mixing the mAb (M90 or hMR1) with its Ag (hCD40L or mCD40L, respectively) in PBS. The mix was incubated for 5 min at RT prior to use. Duplicate reactions (100 μl final volume) of 200 nM 9E10 anti–c-myc and 600 nM hCD40L or 200 nM M2 anti-FLAG and 2.7 μM FLAG peptide. Solutions were incubated for 15 s/ml diluted blood to obtain PRP, and the PRP of mice (n = 10–12) was pooled together. [3H]Serotonin labeling of PRP and washing of platelets in calcium- and albumin-free buffer was performed as described previously, but were calculated with 30 μg/ml PGGL (Sigma-Aldrich) in both steps to prevent spurious activation of platelets during processing. Washed platelets were finally resuspended in albumin and PGL-1-free AFT buffer with 2 mM CaCl2, pH 7.3. Preincubated ICs were prepared in a 1.5 ml microfuge tube by mixing the mAb (M90 or hMR1) with its Ag (hCD40L or mCD40L, respectively) in PBS. The mix was incubated for 5 min at RT prior to use. Duplicate reactions (100 μl final volume) of 200 nM hCD40L or 200 nM mCD40L, whereas the final concentrations of ligands were varied from 0 to 1800 nM hCD40L or 0 to 3600 nM mCD40L. For another set of reactions ligands were kept constant at final concentrations of 200 nM hCD40L or 400 nM mCD40L, whereas the final concentrations of mAbs were varied from 0 to 3600 nM M90 or 0 to 2000 nM hMR1. Molar ratios were calculated as described previously. Negative controls consisted of 200 nM 9E10 anti–c-myc and 600 nM hCD40L or 200 nM M2 anti-FLAG and 600 nM 1× FLAG peptide. PBS and IC components alone were also tested. The remainder of the procedure, including “background” reactions, “total uptake” reactions, and calculations, were carried out as described previously.

Animal studies

Wild-type (WT) mice (B6;SIL) and mice transgenic for human FcγRIIA (B6; SJL-Tg [FCGRA2A]1MKZ/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice genotype was confirmed using The Jackson Laboratory-defined PCR protocol. Mice used in this study were 8–12 wk of age. Animals received tail vein injections (200 μl bolus) containing preformed ICs or complexes in PBS. PRP was obtained by centrifugation of 50 μl final volume of PRP at 300–500 × 103/μl 30 μl agonist (i.e., preformed IC or IC component) to reach the indicated final concentrations. For one set of reactions, M90 was kept constant at a final concentration of 200 nM, whereas hCD40L final concentrations were varied from 0 to 600 nM. Molar ratios were calculated as concentration of hCD40L (mCD40L) to concentration of M90. In another set of reactions, hCD40L was kept constant at a final concentration of 600 nM, whereas M90 final concentrations were varied from 0 to 3200 nM. Molar ratios were calculated as concentration of M90/concentration of hCD40L (mCD40L) in PBS and IC components alone (i.e., 200 nM M90 or 600 nM hCD40L). For another set of reactions mAbs were kept constant at final concentrations of 200 nM M90 or 400 nM hMR1, whereas the final concentrations of ligands were varied from 0 to 1800 nM hCD40L or 0 to 3600 nM mCD40L. For another set of reactions ligands were kept constant at final concentrations of 600 nM hCD40L or 1200 nM mCD40L, whereas the final concentrations of mAbs were varied from 0 to 3600 nM M90 or 0 to 2000 nM hMR1. Molar ratios were calculated as described previously. Negative controls consisted of 200 nM 9E10 anti–c-myc and 600 nM hCD40L or 200 nM M2 anti-FLAG and 600 nM 1× FLAG peptide. PBS and IC components alone were also tested. The remainder of the procedure, including “background” reactions, “total uptake” reactions, and calculations, were carried out as described previously.
platelets. In this differential staining method, fibrin stains bright red, platelets stain gray blue or navy, collagen stains bright blue, and red cells stain orange-yellow. All animal studies were performed according to the Institutional Animal Care and Use Committee guidelines.

Washed platelet aggregation

Human PRP obtained from ACD tubes was incubated for 10 min with aprotinin (2 U/ml) to prevent spurious platelet activation during processing. Platelets were pelleted (900 × g for 10 min) and resuspended in modified Tyrode’s buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM dextrose, 3.3 mM Na2HPO4, 20 mM HEPES, 0.1% BSA, and pH 7.4). Preformed IC was prepared in a 1:5 ml microtube tube by mixing the mAb (M90) with its Ag (hCD40L) in PBS at six times the desired final concentration. The mix was incubated for 5 min at RT prior to use. Reactions (300 μl final volume) consisted of 250 μl washed platelets (250 × 103 μl) and 50 μl agonist (i.e., preformed IC or IC components) to reach a final concentration of 600 nM. Irrelevant monoclonal IgG (0.3 mg/ml) was added to inhibit nonspecific binding. Platelet aggregometry was performed with stirring at 37°C in a four-channel optical platelet aggregometer (Chrono-Log, Havertown, PA). Light transmission was monitored for 14 min after mixing platelets with agonists.

Statistical analysis

Numerical data are listed as the mean ± SD. Data were analyzed using Sigma Plot v.11.0 (Systat, Chicago, IL) by the Student paired t test (for parametric data sets) or the Mann-Whitney rank sum test (for nonparametric data sets). A value of ρ < 0.05 was considered statistically significant.

Results

Analysis of anti-CD40L IC-induced dense granule release from platelets

M90 (a mouse anti-human CD40L mAb) and hCD40L have been shown to form higher-order ICs by Ouchterlony assays (25) and to activate platelets in vitro in an FcγRIIa-dependent manner (17). Because IC concentration and stoichiometry are mutually important in IgG receptor activation, we performed simultaneous analyses of our test reagents (mAbs and Ags) using a quantitative SRA. The objectives were as follows: 1) to further characterize anti-CD40L platelet FcγRIIa activation using human and FCGR2A mouse platelets; and 2) to identify concentration and stoichiometry conditions for maximal platelet activation. In the first approach, M90 was kept constant at 200 nM while varying the concentration of hCD40L (Fig. 1A, 1C). Reactions with hCD40L below 3 nM (molar ratio < 0.015) gave low maximal serotonin releases of <25%. With both human and mouse platelets, concentrations >100 nM with molar ratios from 0.5 through 3 (Ag/IgG) gave values >80% and were therefore maximally active. Similar percent maximal results were obtained when mouse platelets were treated with hMR1 ICs (Fig. 1F). Both hCD40L and mCD40L alone were inactive (Fig. 1B, 1D, 1F). Using a complementary approach, hCD40L was held constant at 600 nM while M90 was varied (Fig. 1B, 1D). When using human platelets in the SRA, M90 below 12 nM (molar ratio < 0.02) gave low maximal serotonin releases of <25%. With mouse platelets, all M90 concentrations tested induced >25% maximal release. With both human and mouse platelets, concentrations >100 nM with molar ratios from 0.17 through 3 (IgG/Ag) gave values >80% and were therefore maximally active. Similar percent maximal results were obtained when mouse platelets were treated with hMR1 ICs (Fig. 1F). Both hCD40L and mCD40L alone were inactive (Fig. 1B, 1D, 1F). Filled circles on y-axis.

Reagent concentration and stoichiometry conditions for subsequent animal studies were based on the SRA results presented in Fig. 1. Using this SRA data, we chose to inject 138 μg IgG and 50 μg CD40L into each mouse. Assuming 2 ml blood volume per animal, this approximates 500 nM IC (1 IgG/3 CD40L stoichiometric ratio). These amounts and ratio were chosen for the following reasons: 1) to ensure that the amounts injected were higher than those causing maximal serotonin release in vitro, taking into account potential clearance mechanisms that exist in vivo (e.g., complement), as well as variations in blood volumes; and 2) the 1:3 (mAb/Ag) ratio was chosen a) because our previous study (17) showed this ratio caused maximal platelet activation as measured by CD62P expression (flow cytometry); and b) because according to our functional assay (SRA), this ratio fell within the maximal range of serotonin release.

Preformed anti-CD40L ICs caused thrombocytopenia in FCGR2A mice

To test whether anti-CD40L ICs activate platelets in vivo in an FcγRIIa-dependent manner, FCGR2A mice (transgenic for the human FcγRIIa IgG receptor) and WT mice (which lack FcγRIIa) were injected i.v. with solutions containing ICs that were “preformed” by mixing mAb and Ag in microcentrifuge tubes prior to injection. As determined from our findings in Fig. 1 and in previous studies (17), the IC quantity delivered was 138 μg mAb and 50 μg Ag, approximating 500 nM IC at a 1:3 (mAb/Ag) stoichiometric ratio. Shortly after injection (1–2 min), FCGR2A mice receiving preformed M90 IC (M90 and hCD40L) or hMR1 IC (hMR1 and mCD40L) exhibited prolonged disorientation, shallow breathing, and impaired mobility—behaviors consistent with a thrombotic phenotype. These symptoms persisted throughout the 10 min observation period. WT mice receiving preformed M90 IC and FCGR2A mice receiving preformed aglycosylated hMR1 (Agly-hMR1) IC did not exhibit these symptoms. Control FCGR2A mice injected with irrelevant mAb anti-c–myc 9E10 along with hCD40L, or with preformed irrelevant IC (M2 anti-FLAG mAb and 1× FLAG peptide) or with individual IC components (mAbs alone or ligands alone) were also asymptomatic. No bleeding was observed in any of the animals.

Mice that exhibited the thrombotic phenotype had marked reductions in platelet counts. For the purpose of this study, thrombocytopenia was designated as platelet counts below the 50th percentile of the mean from mice injected with PBS. Although FCGR2A mice injected with M90 IC yielded platelet counts of 253 ± 184 platelets/ml (mean ± SD), WT mice (which lack FcγRIIa) injected with M90 IC had platelet counts of 1028 ± 277 platelets/ml, comparable with those from animals injected with PBS (1143 ± 170 platelets/ml, Fig. 2A). Control FCGR2A mice injected with irrelevant mAb anti-c–myc 9E10 along with hCD40L or irrelevant anti-FLAG IC had mean platelet counts of 1263 ± 24 platelets/ml and 1026 ± 136 platelets/ml, respectively. Similarly, injections of M90 or hCD40L alone did not produce thrombocytopenia (M90 alone 1069 ± 137 platelets/ml; hCD40L alone 893 ± 346 platelets/ml).
As with M90 ICs, injections of hMR1 ICs also caused a marked drop in platelet counts in FCGR2A mice (204 ± 621 platelets/μl), whereas injections of ICs preformed with Agly-hMRI (which does not bind FcgRIIa) had a mean platelet count of 914 ± 118 platelets/μl (Fig. 2B). Mice injected with mCD40L alone were asymptomatic but had a small drop in platelet count (822 ± 210 platelets/μl, p = 0.029) compared with mice injected with PBS (1062 ± 220 platelets/μl). Injections of control mAbs alone (hMR1 alone 1396 ± 345 platelets/μl; and Agly-hMR1 alone 1311 ± 137 platelets/μl). Collectively, the results in Fig. 2 show the correlation between thrombotic symptoms and marked thrombocytopenia is FcgRIIa-dependent.

Preformed anti-CD40L ICs caused thrombosis in FCGR2A mice

H&E-stained mouse lung sections were evaluated for evidence of thrombosis. Lungs from WT mice injected with M90 IC were free of thrombi and had normal cytology (Fig. 3A). In contrast, when M90 IC or hMR1 IC were injected into FCGR2A mice, their lungs revealed thickening of the microvasculature supporting alveoli as well as pervasive intravascular thrombi (M90 IC in Fig. 3B and hMR1 IC in Fig. 3D). Lungs from FCGR2A mice injected with either Agly-hMR1 IC (Fig. 3E) or with anti-c-myc mAb 9E10 and hCD40L (Fig. 3C) showed normal cytological structures comparable to those observed in WT mice. Collectively, the data presented in Fig. 2 and Fig. 3 demonstrate anti-CD40L ICs can activate platelets and cause thrombosis in vivo in an FcgRIIa-dependent manner.

**FIGURE 1.** Characterization of anti-CD40L IC-induced platelet dense granule release. [14C]Serotonin labeled platelets were incubated with preformed ICs at various concentrations and molar ratios using two approaches in the SRA. Human platelets (A) and mouse platelets (C) treated with preformed ICs at constant M90 concentration (200 nM = 30 μg/ml) with varying hCD40L concentrations. Human platelets (B) and mouse platelets (D) treated with preformed ICs at constant hCD40L concentration (600 nM = 10.8 μg/ml) with varying M90 concentrations. Mouse platelets (E) treated with preformed ICs at constant hMR1 concentration (400 nM = 60 μg/ml) with varying mCD40L concentrations. Mouse platelets (F) treated with preformed ICs at constant mCD40L concentration (1200 nM = 19.7 μg/ml) with varying hMR1 concentrations. Open circle (○), at y-axes) represents PBS only (i.e., no IC components). Asterisk (*) represents reactions in which platelets were preincubated with 30 μg/ml IV.3 prior to the addition of preformed ICs at the concentrations indicated in the figure panel. Open triangle (△) adjacent to the asterisk represents ligand alone at the indicated concentration. Serotonin release data were normalized and the mean expressed as percent maximal serotonin release.

**Anti-CD40L ICs produced fibrin-rich thrombi in FCGR2A mice**

FCGR2A mice were injected with preformed M90 IC with or without bivalirudin pretreatment and lung sections were analyzed by a modified Carstairs staining method to identify the composition of thrombi previously observed. Shortly after M90 IC injection, animals without bivalirudin (same conditions as Fig. 3B) exhibited thrombotic symptoms that lasted for the duration of the observation period (60 min) and showed an 80% drop in platelet count (data not shown). Carstairs stain analysis of lung tissues identified both platelet aggregates and fibrin clots in pulmonary thrombi (Fig. 4A). Platelet poor plasma from these mice had a 2-fold increase in circulating thrombin-antithrombin complexes (a marker of coagulation activation) compared with control mice (data not shown). Mice pretreated with bivalirudin showed a 50% drop in platelet count (data not shown) and experienced a transient mild shock-like phenotype, but quickly regained normal activity. Platelet aggregates were observed in the pulmonary vasculature of bivalirudin-treated mice, but no evidence of fibrin deposition was observed (Fig. 4B).

**Sequential injection of anti-CD40L IC components caused thrombocytopenia and thrombosis in FCGR2A mice**

The results described previously suggest anti-CD40L ICs, preformed ex vivo, can trigger platelet FcgRIIa activation in vivo. If platelets activated by anti-CD40L ICs contributed to thrombosis in patients receiving immunotherapy, such ICs could reasonably be expected to assemble in the circulation. We therefore sought to determine whether separate injection of mAb and Ag into FCGR2A mice would produce effects similar to those observed...
Having preformed ICs, FCGR2A mice were sequentially injected with IC components (mAb, followed by Ag, or vice versa) and their effect on platelet activation was compared with that of preformed ICs. Mice exhibited the thrombotic phenotype described previously shortly after sequential injection of M90 and hCD40L. Injection of M90 followed by hCD40L yielded a mean platelet count of 253 ± 184 platelets/nl and PBS yielded 1277 ± 253 platelets/nl. Thrombocytopenia was thus detected regardless of the order of injection of the IC components.

Mice injected sequentially with hMR1 and mCD40L experienced a delayed onset of milder symptoms (7–8 min postinjection). Sequential injections of hMR1 and mCD40L (Fig. 5B) resulted in a drop in platelet counts: 667 ± 146 platelets/nl with mAb injected first, and 663 ± 120 platelets/nl with ligand injected first. In comparison, PBS yielded 1260 ± 231 platelets/nl and preformed hMR1 IC caused a very low platelet count (204 ± 212 platelets/nl). H&E analysis of lungs from all sequentially injected animals (Fig. 5) revealed evidence of intravascular thrombi similar to that presented in Fig. 3A, 3D (data not shown).

Consistent with in vivo results, washed human platelets aggregated in response to sequential addition of M90 and hCD40L (Fig. 5C). These aggregation profiles were comparable to those produced by preformed M90 IC.

**Discussion**

Using FCGR2A transgenic mice generated by McKenzie and colleagues (21) and anti-CD40L ICs, we attempted to better understand the molecular mechanisms underlying the thrombotic complications observed in patients treated with anti-CD40L mAb. We report in the study that higher-order anti-CD40L ICs robustly activate platelets in vitro and trigger thrombosis in vivo. Importantly, both of these effects require a productive interaction between IgG-Fc and FcγRIIa—effects that were not observed in WT mice or when an aglycosylated variant of anti-CD40L mAb was used. In addition, we present data linking anti-CD40L IC-induced platelet activation with coagulation activation in vivo and thrombosis. These findings expand our understanding of the mechanistic pathways that can lead to anti-CD40L–induced thrombosis.

Previously, we reported (17) that anti-CD40L ICs caused platelet CD62P (P-selectin) exposure detectable by flow cytometry in a narrow stoichiometric range, maximal at a 1:3 ratio of Ab to Ag (M90/hCD40L) and dependent on FcγRIIA. In the current study, using a quantitative platelet function assay (SRA), we corroborated maximal activity at this ratio and also found anti-CD40L ICs exhibited activity across considerably wider stoichiometries than measured by flow cytometry.

To identify concentration and stoichiometry conditions for maximal platelet activation, we compared activity profiles of M90 and hMR1 ICs in dose response SRAs (using human or FCGR2A mouse platelets), where one IC component was held constant, and the other was varied (Fig. 1). The activation profiles observed with constant mAb and varying ligand concentrations.

**FIGURE 2.** Preformed anti-CD40L ICs caused thrombocytopenia in mice transgenic for the human FcγRIIa IgG receptor but not in WT mice. Mice were injected (i.v.) with preformed ICs or individual IC components. Platelets were counted 10 min postinjection. Dashed lines indicate the 50th percentile of mean platelet count of mice injected with PBS. A, Platelet counts from WT and FCGR2A transgenic mice injected with preformed anti-human IC (138 μg M90 and 50 μg hCD40L) or individual components. Platelet counts from FCGR2A transgenic mice injected with irrelevant IgG control (138 μg 9E10 anti-c-myc and 50 μg hCD40L) or irrelevant preformed IC control (138 μg M2 anti-FLAG and 2.7 μg 1× FLAG) are also shown. IC or Ab/ligand components are listed below animal genotype. B, Platelet counts from FCGR2A transgenic mice injected with preformed anti-mouse IC (138 μg hMR1 or Agly-hMR1 and 50 μg mCD40L) or individual components. Data from groups marked with p values were compared with the corresponding PBS group using the Student’s t test or, in the case of mCD40L, the Mann-Whitney rank sum test.

**FIGURE 3.** IC-induced thrombosis occurred only in mice transgenic for the human FcγRIIa gene. H&E-stained lung sections from WT mice (A) and FCGR2A mice (B) injected with preformed M90 IC (138 μg M90 and 50 μg hCD40L). C, FCGR2A mice injected with 138 μg irrelevant IgG control 9E10 anti-c-myc and hCD40L 50 μg. D, FCGR2A mice injected with preformed IC138 μg hMR1 and 50 μg mCD40L. E, FCGR2A mice injected with 138 μg Agly-hMR1 and 50 μg mCD40L. Arrow identifies normal vasculature supporting alveoli. Arrowhead identifies occluded vessel. Asterisks (*) identify blood vessels adjacent to pulmonary bronchiole. Images were captured at ×400 magnification.
Having demonstrated that anti-CD40L ICs directly activate mouse platelets in vitro through FcγRIIa, we used two unique tools to study the platelet FcγRIIa-dependent effects of anti-CD40L ICs in vivo. By comparing genetically modified animals (WT versus FCGR2A mice injected with M90 IC; Fig. 2A) and by comparing ICs prepared with modified mAbs (glycosylated versus nonglycosylated human IgG1 mAbs), we showed that anti-CD40L ICs cause thrombocytopenia. For example, M90 IC activated human and mouse platelets (in vitro) with similar potency through FcγRIIa as evidenced by a complete blockade with anti-FcγRIIa mAb, IV.3 (Fig. 1). This observation was expected because: 1) IgG1 (whether human or mouse) when clustered by an Ag can activate FcγRIIa (19, 26); and 2) both human and transgenic mouse platelets express FcγRIIa at similar levels (21). Furthermore, because both M90 and hCD40L and human mMr1 and mCD40L ICs caused strong serotonin release from mouse platelets and because platelet activation by both ICs was dependent on CD40L, it appears that in these experiments species differences in ligand did not affect IC-induced platelet activation. Although CD40L alone can weakly activate platelets (e.g., via CD40), it appears when complexed with anti-CD40L, this ligand serves mainly to localize the IC to the platelet surface and enhance activation through FcγRIIa (25). Although human CD40L reportedly does not bind mouse CD40 (mCD40) (27), we found that hCD40L localizes labeled M90 to mouse platelet surfaces in a manner independent of FcγRIIa (L. Robles-Carrillo, unpublished data). The identities of the binding sites by which hCD40L localizes labeled M90 to mouse platelet surfaces (except that it is not via FcγRIIa) are not known at this point. Interspecies interactions between CD40L and CD40 or certain platelet integrins (αIIbβ3, α5β1, αM) remains a topic of ongoing research (28).

Having demonstrated that anti-CD40L ICs directly activate mouse platelets in vitro through FcγRIIa, we used two unique tools to study the platelet FcγRIIa-dependent effects of anti-CD40L ICs in vivo. By comparing genetically modified animals (WT versus FCGR2A mice injected with M90 IC; Fig. 2A) and by comparing ICs prepared with modified mAbs (glycosylated versus nonglycosylated Agly-hMr1; Fig. 2B) we showed anti-CD40L ICs cause thrombotic thrombocytopenia in an FcγRIIa-dependent manner.

These observations correlated with pulmonary thrombosis (Fig. 3) and elevated thrombin-antithrombin complexes levels. Because platelets and fibrin within a thrombus cannot be differentiated by H&E staining, we used a modified Carstairs staining method to identify platelets and fibrin in pulmonary thrombi. Anti-CD40L–ICs caused the formation of thrombi that contain fibrin (Fig. 4A), indicating concomitant coagulation activation, which is clearly important in Ab-mediated thrombosis as reflected by the treatment of heparin-induced thrombocytopenia patients with thrombin inhibitors. When animals were pretreated with bivalirudin (a direct thrombin inhibitor), platelet aggregates but not fibrin were observed (Fig. 4B). In summary, direct activation of mouse platelets by anti-CD40L ICs in vitro (SRA) together with the observed IC-induced thrombocytopenia, shock, and pulmonary thrombosis indicate that the injection of anti-CD40L ICs causes direct platelet activation in vivo.

Having demonstrated that preformed ICs caused thrombocytopenia leading to thrombosis, we sought to determine whether thrombocytopenia could also occur when IC components were introduced separately. M90 ICs, whether preformed or sequentially
directly activated by anti-CD40L ICs via Fc signaling, unless it is cross-linked into higher-order ICs by anti-mouse antibodies. This suggests FcR signaling, as well as antibody signaling, may be involved in the activation of platelets and induction of thrombosis in vivo. Furthermore, human immune complex studies with antisera against endothelial growth factor (30) and the anti-hCD40L (M90 IC) studies presented in this paper suggest FCGR2A mice may prove valuable for future drug development.

Overall, the findings of the current study provide evidence of anti-CD40L-mediated platelet thrombosis. By using an FCGR2A transgenic mouse model, we identified a molecular mechanism linking anti-CD40L ICs to thrombocytopenia and thrombosis in vivo. A similar anti-CD40L IC with an aglycosylated Ab was inactive. These findings may be useful in understanding thrombosis in anti-CD40L immunotherapy. Furthermore, human immune complex studies with antisera against endothelial growth factor (30) and the anti-hCD40L (M90 IC) studies presented in this paper suggest FCGR2A mice may prove valuable for future drug development with anti-CD40L and other Abs intended for therapeutic application, because these animals carry a human platelet IgG receptor not present in mice previously used for preclinical analysis.

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Disclosures

The authors have no financial conflicts of interest.

References


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SUPPLEMENTAL FIGURE 1: Binding analysis of hMR1 to mouse CD40L. ELISA plates were coated with recombinant soluble mouse CD40L at 1 μg/ml in 0.1 M sodium carbonate, pH 9.0, over night at 4 °C. Plate was blocked with 1% BSA/PBS for 1 hr at room temperature and washed three times with 0.1% TWEEN-20/PBS. Serially (1:3) diluted chimeric hMR1 antibody was added and incubated at room temperature for 1 hr. Washed three times with 0.1% TWEEN-20/PBS. Diluted (1:2000) goat-anti-human IgG antibody conjugated with horse radish peroxidase (Jackson ImmunoResearch Laboratory, Inc.) was added and incubated for 1 hr at room temperature. After washing off the unbound secondary antibody, the plate was developed with peroxidase substrate, BD OptEIA (BD Biosciences). The development was terminated within 10 minutes using 0.2 N sulfuric acid. The bound hMR1 was quantified by reading at 450 nm.