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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,* Yen-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 chimerized 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 antibodies. The Journal of Immunology, 2010, 185: 000–000.

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 FCG2R2A 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, FcγRIIIa, 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 mouse anti-CD40L mAb with human Fc

Construction of chimeric MR1-hlgG1 × 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 (MR1) were cloned by RT-PCR from total RNA from the hybridoma. Expression vectors for hamster/human chimeric mAbs were constructed by engineering human IgG1 or human κ C region CDNAS 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 transcription cassettes for the Ig L chain and H chain and a dihfr gene as a selectable marker for the H chain vector or a neomycin resistance gene 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) (1:9 ratio). Blood was mixed for 15 min to obtain platelet rich plasma (PRP). PRP was incubated with [14C]serotonin (0.1 μCi/ml, [14C]-HT, GE Healthcare) for 45 min at 37°C. Platelets were washed in calcium- and albumin-free Tyrode’s buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.42 mM Na2HPO4, 2 mM MgCl2, 5 mM HEPES, 5.6 mM dextrose, 1.5 U/ml aspartate, pH 6.2). Washed platelets were resuspended in albumin- and apyrase-free (AFT) Tyrode’s buffer with 2 mM CaCl2, pH 7.3. Preformed 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 media in flat-bottomed wells were counted using a Coulter AcT diff cell counter (Beckman Coulter, Brea, CA). Lungs were harvested, preserved in formalin, and processed and stained with HE staining for the visualization of intravascular thrombi. A modified Carstairs method (24) was used for the specific detection of fibrin and thrombi.
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 apyrase (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 NaH₂PO₄, 20 mM HEPES, 0.1% BSA, and pH 7.4). Preformed IC was prepared in a 1:5-microtube fugue 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 × 10⁶/µl) and 50 µg α-ligand (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 aggreometry 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; filled circles on y-axes).

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 “pre-formed” 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/µl (mean ± SD), WT mice (which lack FcγRIIa) injected with M90 IC had platelet counts of 1028 ± 277 platelets/µl, comparable with those from animals injected with PBS (1143 ± 170 platelets/µl, 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/µl and 1026 ± 136 platelets/µl, respectively. Similarly, injections of M90 or hCD40L alone did not produce thrombocytopenia (M90 alone 1069 ± 137 platelets/µl; hCD40L alone 893 ± 346 platelets/µl).
As with M90 ICs, injections of hMR1 ICs also caused a marked drop in platelet counts in FCGR2A mice (204 ± 6212 platelets/nl), whereas injections of ICs preformed with Agly-hMRI (which does not bind FcgRIIa) had a mean platelet count of 914 ± 6118 platelets/nl (Fig. 2B). Mice injected with mCD40L alone were asymptomatic but had a small drop in platelet count (822 ± 6210 platelets/nl, p = 0.029) compared with mice injected with PBS (1062 ± 220 platelets/nl). Injections of control mAbs alone (hMR1 alone 1396 ± 345 platelets/nl; and Agly-hMR1 alone 1311 ± 137 platelets/nl). 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 thrombosis (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.

**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...
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 * were compared with the corresponding PBS group using the Student t test or, in the case of mCD40L, the Mann-Whitney rank sum test.

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.
Platelet types were fully activated with CD40L concentrations prior to M90 IC injection. Section from FCGR2A mice pretreated with bivalirudin (78 mg/kg) 2 min prior to M90 IC injection. Section from FCGR2A mice injected with preformed M90 IC. Lung section from FCGR2A mice pretreated with bivalirudin (78 mg/kg) 2 min prior to M90 IC injection.

CD40L ICs in vivo. By comparing genetically modified animals (Fig. 1), followed a sigmoidal profile in which both platelet types were fully activated with CD40L concentrations >100 nM and molar ratios >0.5 (Ag/IgG). The absence of activity observed at low CD40L concentrations could be due to inadequate IC formation. With constant ligand and varying mAb concentrations (Fig. 1B, 1D, 1F), the profiles were also sigmoidal. Maximal activity was observed at mAb concentration >100 nM with molar ratios >0.17 (IgG/Ag). In this case, the absence of activity with low amounts of mAb could be attributed either to IgG-Fc concentration insufficient to activate FcγRIIa or to inadequate IC formation.

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 hMR1 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 M90 to mouse platelets (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 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...
References


