Extravasations and emigration of neutrophils to the inflammatory site depend on the interaction of immune-complex with Fcγ receptors and can be effectively blocked by decoy Fcγ receptors

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Extravasation and emigration of neutrophils to the site of inflammation are essential early steps in the initiation of many antibody-mediated autoimmune diseases. The Fc domains of cell bound autoantibodies or immune-complexes (IC) are capable of triggering the neutrophil emigration via complement and FcγRs-mediated mechanisms. To define the clinical relevance and the relative contribution of these 2 pathways in IC-mediated neutrophil emigration, we have neutralized the FcγR-binding activity of IC with a recombinant dimeric Fc receptor, CD16A-Ig, and investigated the early events of IC-induced inflammation in mice. Systemic administration of purified CD16A-Ig blocked IC-induced inflammation, mast cell degranulation, and extravasation of neutrophils in a reversed Arthus reaction. Although the binding of CD16A-Ig to IC did not alter the complement-activating properties of IC, no evidence for complement-dependent neutrophil emigration was observed. These results suggest that interaction of IC with cells expressing FcγRs at the inflammatory site results in the secretion of chemottractants, which mediate complement-independent emigration of neutrophils in this cutaneous acute inflammation model. Furthermore, blocking the interaction of IC to FcγRs expressed on inflammatory cells by administering high-avidity Fc fusion dimers of low-affinity FcγRs is an effective way of preventing IC-induced acute inflammation in autoimmune diseases. (Blood. 2008;111:894-904)

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Introduction

Autoimmune diseases are heterogeneous in nature and are the most frequent cause of disability in adults.1 Many autoimmune diseases lead to vital organ damage, disability and are often fatal in humans. Both the cellular and humoral arms of the immune system are involved in the pathogenesis of autoimmune diseases. Abnormal activation of autoantigen specific T and B cells due to molecular mimicry, viral infections, or cytokine dysregulation are among the suggested mechanisms for the initiation of the disease.2 In many autoimmune diseases the binding of autoantibodies to tissues causes tissue injury. The effector functions of the Fc domains of these tissue-antigen bound antibodies depend on their ability to interact with complement components and/or Fc gamma receptors (FcγRs) expressed on inflammatory cells. The relative role and contribution of these 2 effector systems to the initiation and development of antibody mediated inflammatory diseases are still under intense investigation.

Studies have shown that the accumulation of neutrophils at the site of IC deposition is pivotal to the development of antibody-mediated autoimmune inflammation.3 The ICs trigger complement activation and production of chemotactic peptides such as C5a, which directly attract neutrophils to the inflammatory site.4 Apart from attracting neutrophils, C5a also induce degranulation of mast cells resulting in further recruitment of neutrophils to the site of IC deposition.5-7 These studies were further supported by the fact that mice deficient in C5a receptor gene show attenuated autoantibody-induced inflammation.8,9 Thus the complement peptides produced by IC orchestrate accumulation of neutrophils and cause tissue damage. On the other hand, studies using Fcγ knockout mice have demonstrated the pivotal role of FcγRs in neutrophil recruitment7,10 and antibody-mediated autoimmune diseases. The Fcγ subunit is essential for cell-surface expression of activating FcγRs such as CD64, FcγRIV, and CD16A.11-14 These Fcγ or CD16A gene knockout mice did not develop autoimmune diseases under experimental conditions that induced the diseases in wild type mice.15-18

Although these knockout mice studies have demonstrated independent roles of complement and FcγRs, recent observations suggest interdependency of these 2 pathways in the progression of inflammation.19 At the inflammatory site, C5a increases the potency of the FcγR-dependent inflammatory pathway by up-regulating activating CD16A while down-regulating inhibitory CD32B in inflammatory cells.20 Furthermore, the interaction of IC with CD16A upregulates the production of cell-derived C5.21 This suggests that the absence of the complement pathway might influence the effectiveness of the FcγR-mediated pathway of inflammation and vice versa. Because many of these studies used either gene knockout mice or mast cell deficient mice which might have adapted development-mental compensatory mechanisms, the results and interpretations of the relative role of these functionally interdependent pathways may or may not reflect normal conditions. Therefore,
to further understand the IC-induced inflammatory pathways leading to neutrophil emigration in mice with intact complement system and FcγRs, we analyzed acute inflammation induced by IC after administration of novel decoy FcγR dimers. The dimeric CD16A-Ig fusion protein we used22,23 does not block complement activation by IC, but maintains its potential to competitively block the interaction of ICs with FcγR-expressing inflammatory cells. Our data demonstrate that the interaction of IC with FcγR-expressing cells, present at the extravascular site, is critical for the production of complement-independent chemoattractants that trigger the extravasation and emigration of neutrophils during the early phase of the cutaneous Arthus reaction.

Methods

Cell lines and reagents
The ovalbumin-fluorescein isothiocyanate (FITC-Ova) and rabbit anti-ovalbumin IgG were from Roche Molecular Biochemicals (Indianapolis, IN). PKH-26 labeling kit, mouse IgG subtypes, rabbit anti-DNP IgG, HRP-conjugated anti-human Fc antibody, cobra venom factor, and pepsin were from Sigma (St Louis, MO). Rabbit anti-DNP IgG-Alexa-fluro-448 was from Molecular Probes/Invitrogen (Carlsbad, CA). The Micro BCA-protein assay kit was from Pierce (Rockford, IL) and the horseradish peroxidase (HRP)-substrate from BioRad (Hercules, CA). Fcγ-conjugated F(ab′)2- goat anti–mouse IgG and HRP-conjugated F(ab′)2 goat anti–mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). The Rat-on-Mouse polymer detection kit was from Biocare Medicals (Concord, CA). 2.4G2, an anti-mouse CD16A/CD32B mAb, was from BD Pharmingen. F(ab′)2 of 2.4G2 was generated as described.24 F4/80, rat-anti–mouse CA). 2.4G2, an anti–mouse CD16A/CD32B mAb, was from BD Pharmin

Complement-mediated hemolytic assay
Complement-mediated hemolytic activity of mouse serum was carried out as described.25 Briefly, rabbit red blood cells (rRBCs) were opsonized with rabbit anti-DNP-IgG as described for SRBCs (“EA binding and phagocytosis assay”). The IgG-opsonized rRBCs (100 μL of 2 × 108 cells/mL) in dextrase-gelatin vernal (DGV) buffer were incubated with or without various concentrations of CD16A-Ig for 1 hour at 4°C, after which 100 μL of 1:5 diluted mouse serum was added and the samples incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 0.8 mL of ice-cold DGV buffer. All the samples were centrifuged for 5 minutes at 700g at 4°C. The supernatant was collected and read at 450 nm.

In vivo FcγR-Ig clearance studies
The mice were injected with 100 μg/mouse of dimers intravenously. Subsequently, 3 μL of blood samples were collected at several time points and diluted to 300 μL in phosphate-buffered saline (PBS)/5mM EDTA (ethylene diamino tetra acetic acid) and plasma was separated by centrifugation. A sandwich enzyme-linked immunosorbent assay (ELISA) was carried out to detect the presence of dimers in the plasma using FcγR-specific mAbs as capture antibodies and HRP-conjugated anti-human Fc–specific antibody as the detection antibody.

Reversed passive Arthus reaction (RPA)
RPA was carried out as described.16 To determine the effect of locally administered CD16A-Ig, mice were injected intradermally in the dorsal skin with 25 μL of PBS (control), 20 μg of anti-ovalbumin rabbit IgG (anti-Ova), or various concentrations of CD16A-Ig or CD32A-Ig mixed with 20 μg anti-Ova at defined sites. To determine the effect of systemically administered CD16A-Ig, CD16A-Ig was administered intravenously to the mice (n = 3) at concentrations of 5, 25, and 50 μg/mL of blood. The blood volume in milliliters was estimated to be 7% of mouse body weight in grams.27 After 1 hour, mice were injected with 12.5 or 25 μg per site of anti-Ova intradermally in a total volume of 25 μL of PBS at different sites. At 5 minutes after injection of anti-Ova, RPA was initiated by injecting 100 μL PBS containing 500 μg of ovalbumin and 1% Evan blue through the tail vein. The mice were killed after 3 hours and the injection sites on the reverse side of the skin were examined for extravasation of the blue dye. Photographs were taken immediately and used to quantitate the degree of inflammation elicited by RPA reaction. The intensity of each dermal lesion, shown in blue in the photographs, was quantitated using ImageJ software (National Institutes of Health, Bethesda) and KaleidaGraph (Synergy Software, Reading, PA). After the photographs, skin biopsies taken from injection areas were subjected to histologic analysis. The skin biopsies were sliced horizontally, fixed in 4% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Immunohistochromistry of paraffin-embedded tissue was performed to detect the accumulation of macrophage using rat anti-mouse F4/80 antibody. The antibodies were detected using Rat-on-Mouse polymer detection kit.

ConSTRUCTION, expression, and purification of recombinant soluble CD16A-Ig and CD32A-Ig
The construction and expression of the dimeric form of CD16A-Ig by ligating the extracellular domain of human CD16A to the Fc domain of the human IgG1 heavy chain has already reported by us.22 The Fc domain has mutations that abolish the FcγR-binding activity of IgG1 molecules.25-27 A similar strategy was used to construct and express the R-allele of human CD32A-Ig. The recombinant molecules were purified from CHO cell transfectants using a protein-G GammaBind Plus (Pharmacia Biotech, Piscataway, NJ) Sepharose column. The purified CD16A-Ig and CD32A-Ig were analyzed using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions, and the protein bands were visualized by silver staining and Western blot. Protein concentration was measured using the Micro BCA protein assay kit (Pierce) with BSA as a standard.

Soluble IC binding assay
Soluble immune complex (sIC) was prepared by mixing FITC-Ova with rabbit anti-Ova IgG (1:1 molar ratio) for 4 hours at 4°C.28,29 The complex was centrifuged at 355g for 30 minutes at 4°C and the supernatant was used for the FITC-IC binding assay. The macrophages (50 μL of 5 × 106) were preincubated with blocking agents for 30 minutes at 4°C and then incubated with FITC-IC (20 μg/mL) in binding buffer (pH 7.4) for 1 hour at 4°C. The cells were then washed and analyzed by flow cytometry.

EA binding and phagocytosis assays
Analysis of rabbit anti-DNP IgG–opsonized sheep erythrocytes (EA) binding to macrophages was carried out by flow cytometry as described earlier28,29 after labeling EA with PKH fluorescent dye. Briefly, sheep red blood cells (SRBC) were coated with DNP as described10 and opsonised with rabbit anti-DNP IgG. The phagocytosis assay was carried out using flow cytometry as described by Zhang et al11 using sheep erythrocytes with Alexa-flura-448–labeled rabbit anti-DNP antibodies (Alexa-EA) at a ratio of 1:50 (macrophages to Alexa-EA) in the presence or absence of 2.4G2 or CD16A-Ig.
In some experiments, hypocomplementemia was induced by injecting the mice twice intraperitoneally with 150 U cobra venom factor (CVF) at 8-hour intervals beginning 24 hours before the initiation of RPA reaction. The complement-depleting activity of CVF was determined by assaying the hemolysis of rRBC by mouse serum as described.

Microscopes and image acquisition

Images shown in Figures 2A and 3A were taken using digital Sony cyber-shot DSC-P200, 7.2 mega pixel camera (Sony Style, Atlanta, GA). Images shown in Figures 4A,C, and 5A,C were acquired using a Nikon Eclipse 80i microscope with 40×/0.95 DICM/N2 objectives. Neutrophils and macrophages were counted in the slides at 10 random sites using 63×/1.4 DIC N2 objectives (Nikon Instruments, Melville, NY). Mast cell degranulation (Figure 4 insets) images were acquired using Olympus BX40F microscope (Olympus, Melville, NY) with 100×/1.4 NA objectives and photographs were taken with a digital Nikon-D100 6.1 mega pixel camera. All the photos were saved as JPEG files. Images were processed using Microsoft Photo Editor software (Microsoft, Redmond, WA).

Statistical analysis

A statistical comparison of the control and treated samples was performed using student t test; \( P < .01 \) (*) considered as significant and \( P < .001 \) (**) considered as highly significant.

Results

The human CD16A-Ig dimer blocks IC binding and phagocytosis by mouse macrophage FcγRs

Previously, we reported that purified human CD16A-Ig was able to compete with cell surface expressed human FcγRs for binding to ICs. It is known that human FcγRs are known to bind IgG from other species, such as mouse and rabbit. However, before administering human dimeric CD16A-Ig and CD32A-Ig in mice, we assessed whether the purified dimeric molecules could block IC binding functions of mouse FcγRs in vitro. We used the mouse macrophage cell line P388D1, which expresses all 3 types of
FcγRs: CD16A, CD32B, and CD64. Coincubation of the P388D1 cells with CD16A-Ig blocked the binding of the soluble IC (sIC) in a dose dependent manner (Figure 1A). At 25 μg/mL, CD16A-Ig blocked up to 70% of sIC binding. The combination of CD16A-Ig and mIgG2a was able to block up to 80%, whereas mIgG2a alone blocked only 20% of sIC binding (Figure 1B). Because monomeric mIgG2a binds specifically to CD64,34 the results suggest that CD64 contributes minimally to IC binding by macrophages in our system. This is not due to lack of CD64 functional activity. Flow cytometry analysis showed that the macrophage cell line that we used binds to free IgG2a (Figure S1A, available on the Blood website; see the Supplemental Materials link at the top of the online article), suggesting that either some or all of the CD64 is functionally available to bind IgG. The results obtained using the F(ab')2 fragment of 2.4G2, which specifically blocks CD16A/CD32B molecules, also demonstrate that 80% of the IC binding is mediated by CD16A/CD32B-dependent mechanisms (Figure S1B). At present we do not know the reason for the low IC binding activity of CD64. It is possible that the high affinity CD64 molecule may be partially occupied by IgG from the serum used for culturing cells or has lower affinity for rabbit IgG. The combination of CD16A-Ig and CD32A-Ig blocked up to 70% of sIC binding, which is not more effective than CD16A-Ig alone; suggesting that CD32A-Ig is not an efficient blocker of mouse FcγRs in vitro. This is further supported by the minimal blockage observed with CD32A-Ig (Figure 1B). Some IC, such as autoantibody-coated target cells, are particulate in nature and therefore we assessed whether the soluble dimeric receptors can block the binding of particulate IC (antibody-coated sheep erythrocytes; EA) to mouse macrophage cells. Nearly 70% of EA binding to P388D1 was blocked by CD16A-Ig at 25 μg/mL, whereas CD32A-Ig was unable to block EA binding (Figure 1C).

Results obtained using mIgG2a and mAb specific to CD16A/CD32B showed that CD64 plays little or no role in the EA binding (Figure 1C), which is consistent with the results from the sIC-binding studies described above (Figure 1B). CD16A-Ig also blocked EA binding to macrophages isolated from mouse peritoneal cavity in a dose dependent fashion (Figure S2A). At 25 μg/mL, CD16A-Ig was able to block up to 70% of EA binding, whereas CD32A-Ig was unable to block at the doses tested (Figure S2B). Phagocytosis of antibody-coated target cells by macrophages and neutrophils is one of the major mechanisms by which autoantibodies lead to tissue injury in many acute and chronic inflammatory autoimmune diseases. Therefore, we determined whether CD16A-Ig could block phagocytosis mediated by FcγRs expressed on mouse macrophages. Flow cytometric analysis of phagocytosed Alexa-EA showed that pretreatment of EA with 50 μg/mL of CD16A-Ig blocked up to 60% of the EA phagocytosis by the P388D1 cells (Figure 1D). A similar result was observed using primary cells such as the thioglycollate-elicited peritoneal macrophages (Figure S2C). Taken together, this data demonstrates that dimeric CD16A-Ig is capable of competing with FcγRs expressed on mouse inflammatory cells for binding to IC.

CD16A-Ig inhibits antibody-mediated acute inflammation in vivo in a murine reversed passive Arthus reaction (RPA) model

Next, we determined whether the in vivo administration of purified CD16A-Ig dimer could block the antibody mediated acute inflammation induced in a murine RPA model.35 RPA has been extensively studied as an in vivo model of immune complex-mediated acute tissue injury and inflammation. The reaction is induced by...
injecting an antigen (chicken ovalbumin; Ova) intravenously along with a blue dye followed by intradermal administration of polyclonal antibodies (rabbit antiovalbumin IgG; anti-Ova) specific to the antigen. Vascular permeability and extravasation of neutrophils, a hallmark of the IC-induced immunopathologic cascade, can be visualized by extravasation of intravenously injected Evan blue dye and standard histologic analysis.

First, we determined the optimal concentration of anti-Ova required to induce inflammation by injecting various concentrations of anti-Ova (0-50 μg/site) intradermally. We found that Arthus reaction was induced optimally with 20 μg/site of rabbit anti-Ova (data not shown). We then determined the ability of locally administered CD16A-Ig and CD32A-Ig to inhibit the induction of RPA. The results show that the administration of CD16A-Ig at the site of IC-formation significantly (P < .001) decreased the size and intensity of the inflammation compared with the sites injected with rabbit anti-Ova alone (Figure 2A left panels). The degree of inflammation was not reduced at CD32A-Ig injected sites, suggesting that CD32A-Ig is unable to block IC binding to mouse FcγRs in vivo.

The IC-mediated Arthus reaction may be triggered by either of two pathways: the complement system and the FcγRs. To determine the contribution of complement-mediated RPA in our system, hypocomplementemia was induced in mice using cobra venom factor (CVF) as described by Hazenbos et al. The depletion of complement was confirmed by estimating the hemolytic activity of the serum obtained from CVF-treated mice and normal mice (Figure S3). We then studied the effectiveness of CD16A-Ig and CD32A-Ig administration in inhibiting the RPA. No decrease in the intensity of RPA was observed in complement-depleted mice (Figure 2A right panels). CD16A-Ig blocked RPA in a dose dependent manner (Figure 2B). At

![Figure 3. Systemic administration of CD16A-Ig efficiently blocked the reversed passive Arthus reaction in vivo in mice without complement depletion.](image-url)
50 μg/mL it was able to block up to 70% of RPA, whereas CD32A-Ig had no detectable effect under similar conditions (Figure 2B). Moreover, the combination of CD16A-Ig and CD32A-Ig was able to block only 70% of RPA, which is not more effective than CD16A-Ig alone; suggesting that CD32A-Ig is not competing with murine FcγRs in vivo (data not shown).

We then determined the efficacy of these dimers in blocking the IC-mediated inflammation when administered systemically. As observed with locally administered CD16A-Ig, the systemically administered CD16A-Ig also blocked RPA in a dose dependent manner (Figure 3Aiv-vi). A dose of 50 μg/mL blood CD16A-Ig blocks more than 70% of the inflammation as measured by the intensity of the Evan blue extravasation (Figure 3B). Under similar conditions, as seen in the previous experiments (Figure 2), CD32A-Ig was unable to block RPA (Figure 3B), demonstrating the specificity of the inhibition of RPA by CD16A-Ig. Similar experiments with systemically administered molecules conducted in CVF-treated mice showed that complement depletion did not have any effect on the intensity of RPA (data not shown). These data show that systemic administration of recombinant soluble dimeric CD16A-Ig effectively blocks the IC-mediated inflammation in vivo.

As specificity controls for RPA, groups (n = 3) of mice were injected intradermally at different sites with either PBS or PBS containing various concentrations of anti-Ova antibody or FcγRs.
dimers, and immediately 100 µl of 1% Evan blue in PBS intravenously. These control experiments show that the intradermally injected FcγR-Ig dimers or anti-Ova do not cause inflammation per se (data not shown). We have also determined the effect of systemically administered anti–mouse CD16A mAb on the RPA. The results show that IC-induced RPA can be effectively blocked by anti-CD16A/CD32B mAb (Figure 3B); demonstrating IC-induced inflammation in our experiments is mediated by FcγRs.

**Histologic analyses show that CD16A-Ig blocks IC-induced accumulation of inflammatory cells and tissue damage mediated by mouse FcγRs**

To visualize the intensity and character of the inflammation in RPA, the skin biopsies taken from antibody-injected areas of untreated, CD16A-Ig–treated and CD32A-Ig–treated mice, shown in Figure 3A, were subjected to histologic analysis. As a control, a PBS-injected area of untreated mice was used. Tissue sections from the anti-Ova injected area of untreated mice revealed edema and an inflammatory infiltrate composed predominantly of neutrophils as characterized by their multilobed nuclei (Figure 4Ai,B) and degranulated mast cell (Figure 4Ai top inset). Histologic sections of anti-Ova–injected areas of skin taken from the mice treated with CD16A-Ig showed a marked reduction in neutrophil accumulation, margination around venules (Figure 4Aiii,B), and degranulation of mast cells (Figure 4Aiii top inset) compared with the untreated mice (Figure 4Ai), whereas the skin sections of CD32A-Ig–treated mice (Figure 4Aiv) showed features that were similar to those observed in untreated mice (Figure 4Ai). PBS-injected area of untreated mice did not show any evidence of inflammation (Figure 4Aii).

Because complement peptides such as C5a are known chemoattractants of neutrophils, we have also analyzed the tissue sections of mice treated with CVF to determine the role of complement in inducing cellular infiltration in RPA. The biopsies taken from antibody-injected and PBS-injected areas of the skin of untreated, CD16A-Ig–treated, and CD32A-Ig–treated mice were subjected to histologic analysis. As seen in Figure 4C,D, the neutrophil accumulation, margination around venules, and mast cell degranulation (top insets) in the anti-Ova injected areas of untreated (Figure 4Ci), CD16A-Ig treated (Figure 4Ciii), and CD32A-Ig–treated (Figure 4Civ) mice were similar to those of mice without...
complement depletion (Figure 4Ai-Aiv). These data suggest that the interaction of IC with FcγRs expressed on the cells reside at the site of IC-formation, not the complement pathway, play a major role in induction of RPA and neutrophil emigration from the circulation. These histologic data further confirm the results obtained using dye extravasation method (Figures 2, 3), and show that recombinant CD16A-Ig dimer blocks IC-induced tissue damage when administered in vivo.

We have also carried out immunohistochemistry to determine the accumulation of macrophages in RPA (Figure 5A,B). Resident macrophages were found in the PBS injected area of the skin (Figure 5Ai), while accumulation of macrophages was observed in antibody-injected area of skin in untreated mice (Figure 5Ai). The results from the dimer treated mice show that like neutrophils, the IC-induced accumulation of macrophages was also inhibited by CD16A-Ig administration (Figure 5Aii,B). CD32A-Ig treatment did not inhibit macrophage accumulation (Figure 5Aiv). Analysis of slides from complement-depleted mice demonstrated that the accumulation of macrophages is also independent of the complement system (Figure 5C,D). Interestingly, unlike neutrophils, we did not observe margination of macrophages around venules, suggesting that most of these macrophages may have migrated from the tissues surrounding the IC injected area (data not shown). This suggests that the mechanism of accumulation of these two types of inflammatory cell populations may be distinct.

**CD16A-Ig binding to IC does not block the complement-mediated hemolytic activity of IgG molecule**

The Fc domain of IgG IC is capable of inducing inflammation using both FcγRs and complement pathways. Although complement- and FcγR-binding sites on the Fc domain of IgG are distinct, it is a possibility that the binding of CD16A-Ig molecule to the Fc domain of IC may hinder the binding of Clq and thus prevent complement activation. To rule out whether CD16A-Ig binding altered the complement activating property of IC, we determined the hemolysis of rabbit anti-DNP IgG opsonized rRBCs by mouse serum in the presence and absence of CD16A-Ig. Because mouse complement does not lyse IgG-opsonized sheep or mouse RBCs, we used rabbit anti-DNP IgG opsonized rRBCs for these assays.21 Titration of mouse serum showed that 1:5 dilution caused 50% hemolysis of rRBC (Figure S4). At the same serum dilution, the complement-mediated hemolysis of rRBCs was unaffected by the presence of CD16A-Ig (Figure 6A). This suggests that the lack of inflammation induced by IC during CD16A-Ig administration is not due to any alteration in complement activating property of IC that might have resulted from binding of Fc domain of IgG by CD16A-Ig.

**In vivo half-life of CD16A-Ig**

Analysis of the kinetics of clearance from the circulation shows that the plasma level of both the administered dimers dropped quickly (nearly 23%) until 8 hours and then started declining rapidly. The reason for the sudden initial drop is not clear. It is possible that the dimers might be diffusing into extracellular space from the circulation. The FcγR-Ig dimers were detected in mouse blood up to 160 hours after their administration. The in vivo half-life in the circulation for both dimeric CD16A-Ig and CD32A-Ig was estimated to be 120 hours (Figure 6B). This suggests that it is possible to maintain a plasma concentration of the systemically administered decoy receptors high enough to block of IC-mediated inflammation for several days. The high half-life of Fc fusion proteins has been attributed to the Fc domain; the Fc domain of IgG binds to neonatal Fc receptor (FcRn), which results in protection from degradation by the endocytic machinery of the cells.22 The Fc domain that we used here to make FcγR-Ig fusion proteins has mutations that affect the FcRn binding.23-27 Because FcRn and FcγR bind to distinct sites on IgG, we do not anticipate that the mutations in the Fc domain have any effect on the FcRn binding and consequently the half-life of the mutated IgG-Fc fusion molecules.

**Discussion**

Autoantibody-antigen IC formed in many autoimmune diseases amplify tissue damage by attracting inflammatory cells, such as...
neutrophils using both complement and FcγR-dependent mechanisms. Dissecting the precise roles of these two pathways in recruitment of neutrophils to the inflammatory site will lead to a better understanding of the mechanisms and treatment of the disease. We have used an experimental strategy that selectively neutralizes the FcγR-binding activity of the ICs without manipulating the expression of the FcγRs or complement receptors on the inflammatory cells. The CD16A-Ig dimer that we used competitively blocked the binding of IC to inflammatory cells in vitro assays and blocked IC-induced inflammation in vivo at circulating concentrations as low as 25 to 50 μg/mL. Accumulation of neutrophils is a hallmark of IC-induced RPA. Therefore, the relative roles of complement-derived chemotactic peptides and FcγRs in recruiting neutrophils to the extravascular sites of IC formation were investigated by analyzing neutrophil infiltration in normal and dimer administered mice before or after complement depletion. The absence of neutrophil infiltration in the dimer-treated animals (Figure 4D) and the presence of neutrophil infiltration at the site of IC formation despite the depletion of complements (Figure 4Ci) suggest that the chemotactic complement peptides are not involved in the recruitment of neutrophils to the site of IC deposition in the model system used here. A similar complement-independent but FcγR-dependent accumulation of macrophages was observed in RPA. Complement peptides such as C5a have been shown not only to recruit neutrophils via chemotaxis, but also activate mast cell degranulation. Thus, it is intriguing that complement activation that might have been triggered by IC in CD16A-Ig–administered mice did not cause mast-cell degranulation. The abrogation of mast-cell degranulation by neutralizing ICs with CD16A-Ig in the cutaneous Arthus reaction model presented here suggests that the complement activation by IC alone may not be sufficient for mast-cell degranulation. This suggests that initiation of mast-cell degranulation depends essentially on IC-FcγRs interaction but not on the complement activating property of the ICs. While this is contrary to observations made in other systems that show that mast cells degranulate in response to C5a, it is in agreement with studies using FcγR-knockout mice that show that FcγRs on mast cells are necessary for initiation of IC-induced inflammation. Activated mast cells are known to produce compounds that are chemotactic to neutrophils, and therefore it is possible that absence of neutrophil infiltration in the dimer-treated mice may be a consequence of lack of the mast-cell degranulation.

However, the participation of cell-derived C5a in neutrophil recruitment to inflammatory site cannot be ruled out. Recently, it has been shown that engagement of activating FcγRs up-regulated the production of cell-derived C5 and C5a, and, subsequently, C5a up-regulated the expression of activating FcγRs while down-regulating inhibitory FcγRs, suggesting cell-derived C5a could play a role in IC-induced inflammation by altering the level of expression of FcγRs. Thus the IC can produce the neutrophil chemoattractant C5a using two different pathways; directly by triggering classical pathway of complement activation, or indirectly by inducing production of cell-derived C5a by engaging FcγRs. The complement depletion studies described here used CVF, which blocks the three major pathways of C5a production by binding to C3 but not the cell-derived pathway. Therefore, although the results presented here show that complement activation pathways directly triggered by IC do not recruit neutrophils, they do not rule out the role of cell-derived C5a that are produced as a result of IC interaction with the inflammatory cells in neutrophil recruitment. At present, the relative contribution of the direct and the cell-derived pathways of complement activation in the recruitment of neutrophils are not known.

The results show that the extravasation and emigration of neutrophils that occur during the early phase of IC-induced cutaneous inflammation depend on the ability of IC to interact with FcγRs and are independent of the complement activation pathways that can be blocked by CVF. The administration of CD16A-Ig also blocked mast-cell degranulation at the inflammatory site. Based on these observations, we hypothesize that the administration of decoy CD16A-Ig dimer prevented IC from binding to mast-cell FcγRs by occupying the Fc domain of the IC (Figure 7) and consequently prevented mast cell degranulation, neutrophil recruitment, and inflammation. This is in agreement with the previous observations from FcγR knockout mice and mast cell deficient mice, which suggested that the Arthus reaction depends on FcγRs-triggered mast-cell degranulation. In contrast to our observations with recombinant soluble CD16A-Ig dimer presented here, an earlier study that
used a recombinant soluble CD32A has concluded that parenter-
ally administered soluble FcγRs is not a practical approach to
block IC-mediated inflammation.52 Their conclusion was based
on the observation that the systemically administered CD32A
has a half-life of only 25 minutes and is unable to block Arthus
reaction. This report also showed that soluble CD32A could
block Arthus reaction only when administered locally along with
the IC at a concentration of 5mg/mL. It is possible that the re-
quirement for such a high concentration and very low in vivo
half-life may be due to the monomeric nature of soluble CD32A
that was used in their study. Interestingly, however, our results
with CD32A-Ig dimer suggest other possibilities. We found that
CD32A is not able to block the Arthus reaction in mice even after
dimerization. This is not due to loss of function of CD32A after
dimerization; the purified CD32A-Ig is able to competi-
tively block binding of CHO cells expressing cell surface
CD32A to IC (data not shown). Our results suggest that
CD32A-Ig is not able to compete with mouse FcγRs for binding
to IC either because the affinity of CD32A-Ig may be too low, or
mouse FcγRs and human CD32A may be binding to distinct regions of rabbit IgG. Thus our results, although supporting the
observation reported by Ireino et al52 that CD32A cannot prac-
tically block the IC-mediated inflammation, do, however,
demonstrate the feasibility of using systemic administration of
CD16A-Ig to block acute inflammation mediated by IC.

In summary, our results suggest that the interaction of IC
with the FcγRs expressed on cells residing in the extravascular
site triggers a pathway of neutrophil recruitment that is indepen-
dent of IC-induced complement activation. Our results empha-
size that the engagement of FcγRs by ICs is a crucial event in
initiating the inflammatory cascade. The observations reported
here may be of clinical relevance. The interaction of FcγR-
bearing inflammatory immune cells with autoantigen-antibody
ICs is implicated in the pathogenesis of several autoimmune
diseases such as systemic lupus erythematosus, glomerulonephri-
tis, and arthritis.53,54 Neutrophil infiltration plays an important
role in the initiation of inflammation in these autoimmune
diseases. Because the inflammation and recruitment of neutro-
phils can be blocked by systemically administered decoy
FcγR-Ig dimers, this strategy could be applied to treat antibody-
mediated inflammatory and autoimmune diseases.

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Authorship

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and drafting the paper; R.A.H., S.F., P.P., T.L., and M.S., performed
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