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Research Article   **Immunology**

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Cell-derived anaphylatoxins as key mediators of antibody-dependent type II autoimmunity in mice

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Complement C5a, a potent anaphylatoxin, is a candidate target molecule for the treatment of inflammatory diseases, such as myocardial ischemia/reperfusion injury, RA, and the antiphospholipid syndrome. In contrast, up until now, no specific contribution of C5a and its receptor, C5aR, was recognized in diseases of antibody-dependent type II autoimmunity. Here we identify C5a as a novel key mediator of autoimmune hemolytic anemia (AIHA) and show that mice lacking C5aR are partially resistant to this IgG autoantibody–induced disease model. Upon administration of anti-erythrocyte antibodies, upregulation of activating Fcγ receptors (FcγRs) on Kupffer cells, as observed in WT mice, was absent in C5aR-deficient mice, and FcγR-mediated in vivo erythrophagocytosis was impaired. Surprisingly, in mice deficient in FcγRI and FcγRIII, anti-erythrocyte antibody–induced C5 and C5a production was abolished, demonstrating the existence of a previously unidentified FcγR-mediated C5a-generating pathway. These results show that the development of a full-blown antibody-dependent autoimmune disease requires C5a — produced by and acting on FcγR — and may suggest therapeutic benefits of C5 and/or C5a/C5aR blockade in AIHA and other diseases closely related to type II autoimmune injury.

Introduction

C5a anaphylatoxin, the smaller proteolytic fragment of complement component 5, induces several biological responses at very low concentrations and is involved in inflammatory and anaphylactic reactions (1). Its receptor, C5aR, has been identified on a variety of immune effector cells, including circulating leukocytes, mast cells, basophils, macrophages, and many others (2). Activation of these cells by C5a results in inflammatory mediator release and granule secretion, which in turn alters vascular permeability, induces smooth muscle contraction, and promotes inflammatory cell migration (1, 2). It is well established that this C5a-triggered cascade of events contributes to the pathogenesis of various diseases in humans, including myocardial ischemia/reperfusion injury and respiratory distress syndrome (3–5). In addition, genetic deletion of C5aR is very effective in preventing inflammation in animal models of erosive arthritis and the neutrophil-dependent antiphospholipid syndrome (6, 7). So far, however, no specific role for C5a is recognized in diseases of antibody-dependent type II autoimmunity.

In the present study we investigated the pathological significance of C5a and C5aR in the development of autoimmune hemolytic anemia (AIHA) in mice. The data suggest a previously unidentified function of C5a for autoantibody-induced cellular destruction through cross-talk of C5aR with activating Fcγ receptors (FcγRs) specifically on liver macrophages but not sinusoidal endothelial

cells (SECs). Moreover, the data also provide the first evidence, to our knowledge, of a specific requirement of Kupffer cell FcγR for C5 and C5a production in anemia. Thus, 2 distinct levels of interactions exist between FcγR and C5a/C5aR, indicating that C5a anaphylatoxins may represent a relevant therapeutic target in the treatment of type II autoimmune injury.

Results

Protection against lethality in AIHA in both FcγRI/III– and C5aR-deficient mice. In a number of autoimmune diseases, autoantibodies are the essential pathogenic factors, e.g., anti-rbc antibodies in AIHA (8). The pathogenicity of the autoantibody can be attributed mainly to the effector functions associated with its Fc region, e.g., interactions with Fc receptor (FcR) and the complement system (9, 10). This has been studied extensively in New Zealand black (NZB) mice, which spontaneously develop anemia as a result of production of autoreactive Coombs anti-rbc antibodies (11). Several cytotoxic antibodies have been derived from NZB mice, and most of them induce anemia by extravascular hemolysis in i.p. injected animals (12). Passive immunization with IgG2b and IgG3 autoantibodies results in a preferential activation of the complement system, leading to complement receptor-3–dependent erythrophagocytosis (13), whereas the pathogenic effects of an anti-rbc IgG1 105-2H antibody are mediated exclusively by FcγRIII on the splenic macrophages and hepatic Kupffer cells (14).

The IgG2a 34-3C autoantibody directed against the anion channel band 3 on erythrocytes (11, 15) is by far the most pathogenic, and a single i.p. injection of 300 μg of the antibody is sufficient to induce lethal AIHA in WT mice. In contrast, NOD mice, which carry mutations at multiple complement and FcγR gene loci (16, 17), were resistant (Figure 1A), as were mice deficient either in the common γ chain of FcRs (FcRγ chain) or in both FcγRI and FcγRIII
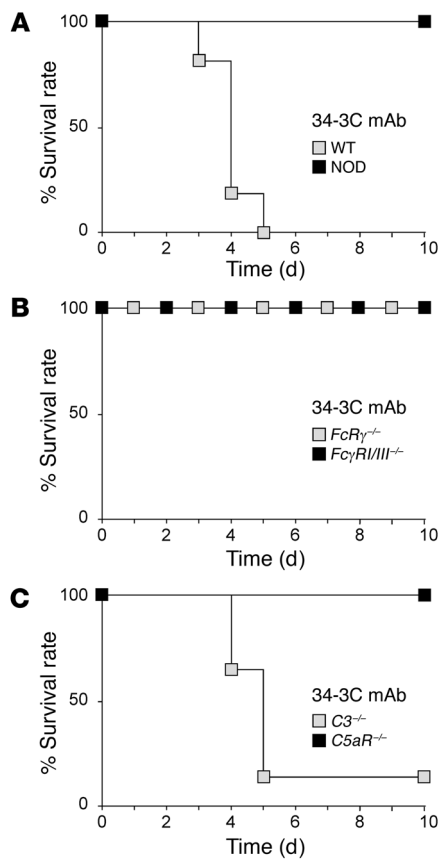
Figure 1
Lethal 34-3C mAb–induced AIHA is FcγRI/III– and C5aR-dependent. (**A**–**C**) Mice (C57BL/6 WT, NOD, *FcRγ*⁻/⁻, *FcγRI/III*⁻/⁻, *C3*⁻/⁻, and *C5aR*⁻/⁻) received 300 μg of anti-rbc 34-3C mAb i.p., and lethality was recorded at the indicated times. Results are expressed as percentage survival rate for groups of 10–24 mice. No lethality developed in mice receiving the isotype-matched W6/32 mAb (data not shown).

phenotype of C5aR deficiency in lethal AIHA, we next determined the cytotoxic response induced by lower doses of the 34-3C autoantibody in *C5aR*⁻/⁻ mice as compared with *FcRγ*⁻/⁻ and *FcγRI/III*⁻/⁻ mice. Anemia occurring in C57BL/6 WT mice injected with 25, 75, and 150 μg 34-3C IgG2a mAb (hematocrit [Ht]: 36.0% ± 0.9%, 27.0% ± 0.8%, and 17.8% ± 1.2%, respectively, at day 3; *n* = 5) was equally prevented or strongly reduced in FcR- and C5aR-deficient mice, with mean Ht levels remaining at 40% or greater (25 and 75 μg) (*P* < 0.001) or having a minor decrease to 31–32% (150 μg) (*P* < 0.001) (Figure 2, A–C). While the *FcRγ*⁻/⁻, *FcγRI/III*⁻/⁻, and *C5aR*⁻/⁻ mice each showed protection against lethality in AIHA induced by 300 μg 34-3C mAb, a persistent though diminished anemia was observed in all the knockouts (Figure 2D). These results suggest that activating FcγR and C5aR act on the same pathway in 34-3C autoimmunity and indicate that, under conditions of high 34-3C mAb titers, additional factors, such as the activation of C3 (13), also play a contributory role.

Reduced Kupffer cell–mediated destruction of autoantibody-opsonized rbcs in mice lacking either activating FcγR or the C5aR. FcγR-dependent erythrophagocytosis, especially by liver Kupffer cells, had previously been shown to be a major cause of the 34-3C mAb–induced anemic phenotype in mice (10, 12, 14, 19). Because C5aR- and FcR-deficient mice both display an identical pattern of protection/reduction with respect to lethality and Ht levels (Figures 1 and 2), we examined the relevance of C5aR and FcγR to in vivo erythrophagocytosis. The 34-3C mAb–induced cytotoxic response was followed in kinetic studies by quantitative assessment of the percent-

(Figure 1B). These results are in agreement with data of the sublethal AIHA models (14, 18), confirming that the activating FcγRI and FcγRIII are essential for the induction of AIHA. Despite the strong complement-fixing activity of the 34-3C autoantibody, passively induced antibody-dependent AIHA was only affected in part in *C3*⁻/⁻ mice (Figure 1C); this supported the previous conclusion that complement C3 does play a role but not the dominant one in 34-3C autoimmunity (13). However, unexpectedly, we found that *C5aR*⁻/⁻ mice were resistant to 300 μg i.p. injected 34-3C IgG2a mAb (Figure 1C), indicating that 34-3C–induced lethal hemolytic anemia depends not only on FcγR but also on C5aR.

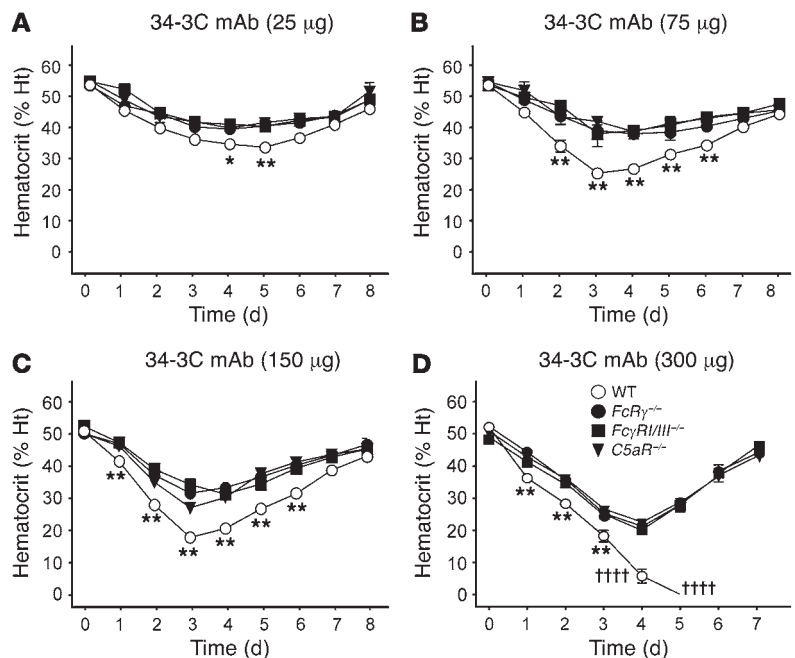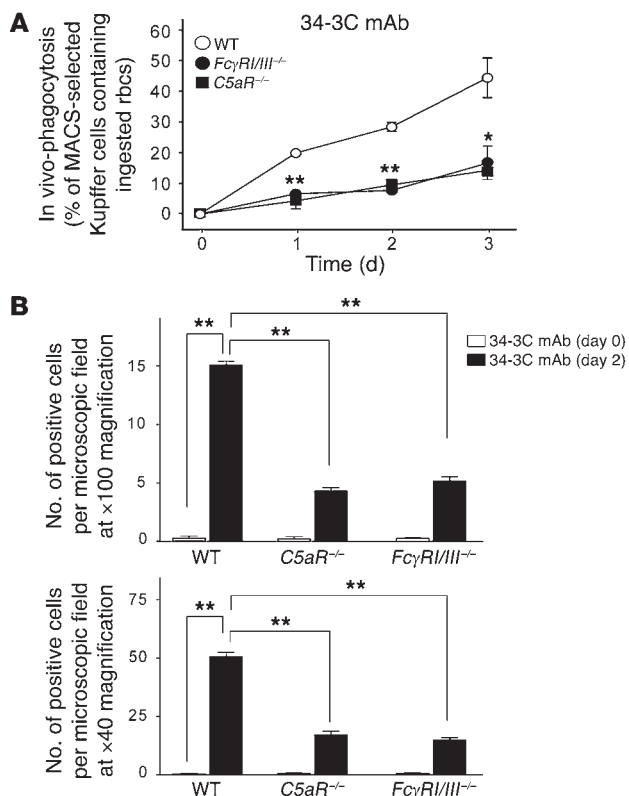*Reduced 34-3C mAb–induced cytotoxic response in FcR- and C5aR-deficient mice.* Given the protective

Figure 2
Reduced cytotoxic response in both FcR- and C5aR-deficient mice during 34-3C mAb–induced AIHA. Daily Ht levels of WT C57BL/6 (open circles), *FcRγ*⁻/⁻ (filled circles), *FcγRI/III*⁻/⁻ (filled squares), and *C5aR*⁻/⁻ (filled triangles) mice injected with increasing dosages of 25 μg (**A**), 75 μg (**B**), 150 μg (**C**), and 300 μg (**D**) of anti-rbc 34-3C mAb. Ht values lower than 40% were considered anemic. Death of a mouse is indicated by a cross. Results are shown as the mean Ht ± SD obtained from 4–9 mice in each group. Significant differences were determined by Student's *t* test (**P* < 0.05; ***P* < 0.001).

Figure 3

Reduced Kupffer cell–mediated rbc destruction in *FcγRI/III−/−* and *C5aR−/−* mice during 34-3C mAb–induced AIHA. (**A**) WT C57BL/6 (open circles), *FcγRII/III−/−* (filled circles), and *C5aR−/−* (filled squares) mice injected with 300 μg of anti-rbc 34-3C mAb were analyzed for Kupffer cell–mediated in vivo phagocytosis at the indicated times. Results are shown as the percentage of Mac-1–selected Kupffer cells containing more than 2 ingested rbcs ± SD obtained from 4–9 mice in each group. (**B**) H&E-stained liver sections obtained from the indicated mice at days 0 and 2 after AIHA induction were analyzed for erythrophagocytosis by light microscopy. Shown is the mean cell count ± SEM of rbc-containing liver cells per microscopic field (original magnifications, ×40 and ×100) obtained from 3–5 mice in each group. (**A** and **B**) Significant differences were determined by Student's *t* test (*P < 0.05; **P < 0.001).

expression by magnetic cell sorting. Mac-1–positive Kupffer cells showed a positive staining with both 1/36 and 2.4G2, while Mac-1–negative SECs stained only with 2.4G2 (Figure 4). So, Kupffer cells, but not SECs, express FcγR as well as C5aR on their surface.

C5aR-dependent upregulation of Kupffer cell FcγRI/III expression in AIHA. It has been shown recently that, in different in vivo mouse models of antibody-dependent inflammatory diseases, C5a positively regulates expression of FcγR (23–26). Also, Kupffer cells isolated from anemic WT mice displayed strongly increased levels of FcγRI (Figure 5A), FcγRIII (Figure 5B), and FcRγ (Figure 5C) mRNA at 24 hours after 34-3C mAb injection. This activation of the transcription of the FcγR genes was completely absent in *C5aR−/−* mice (Figure 5), demonstrating that also in AIHA C5a/C5aR mediates upregulation of activating FcγR.

age of Mac-1–positive liver Kupffer cells that contained ingested erythrocytes. As shown in Figure 3, WT C57BL/6 mice displayed an excessive Kupffer cell–mediated clearance of rbcs within 3 days after 34-3C mAb treatment. *C5aR−/−* and *FcγRI/III−/−* mice equally exhibited diminished pathology with only minor hepatic erythrophagocytosis (Figure 3A). These findings were confirmed by qualitative (see Supplemental Figure 1; available online with this article; doi:10.1172/JCI25536DS1) and quantitative (Figure 3B) histological studies on liver sections. The cell count of WT phagocytic liver cells per microscopic field (original magnifications, ×40 and ×100) was markedly reduced in both C5aR- and FcγR-deficient mice (*P* < 0.001; Figure 3B).
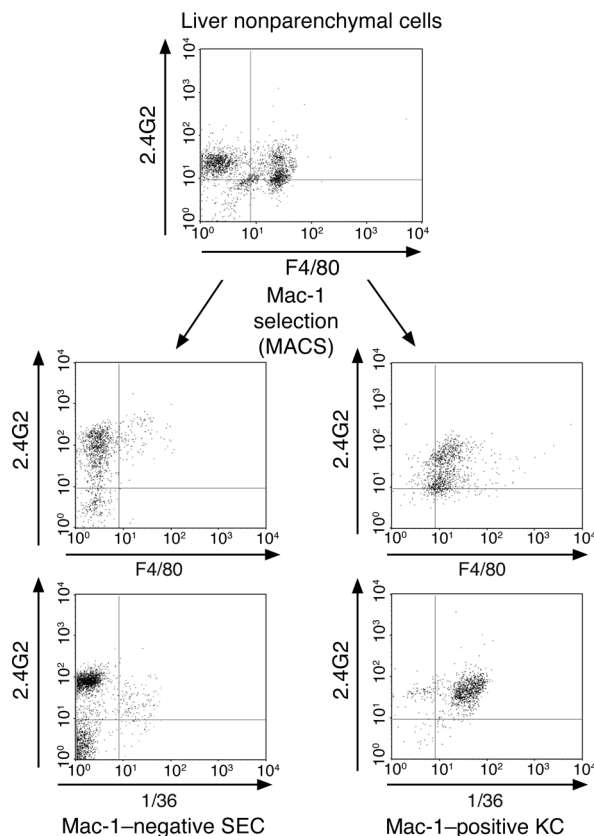
Coexpression of C5aR and FcγR on Kupffer cells. The liver consists mainly of hepatocytes, SECs, and Kupffer cells. In 2 independent studies (20, 21), it was previously shown that the anti–FcγRII/III mAb 2.4G2 interacts with both SECs (Mac-1–negative cells) and macrophages (Mac-1–positive Kupffer cells). C5aR expression on these 2 different FcR-positive liver cell populations was analyzed using rat anti–mouse C5aR mAb 1/36 (22) in flow cytometry of subsets of liver cells, which were isolated by the collagenase perfusion method, separated from hepatocytes by density centrifugation, and selected for the presence or absence of Mac-1

Figure 4

Coexpression of C5aR and FcγR on Mac-1–positive liver Kupffer cells. Liver nonparenchymal cells were separated into Mac-1–positive Kupffer cells (KC) and Mac-1–negative SECs by magnetic activated cell sorting (MACS). All cell fractions were stained with PE–anti–FcγRII/RIII 2.4G2 mAb in combination with FITC-conjugated anti–F4/80 or anti-C5aR 1/36 mAbs and analyzed on a FACScan. Representative results of individual mice are shown.
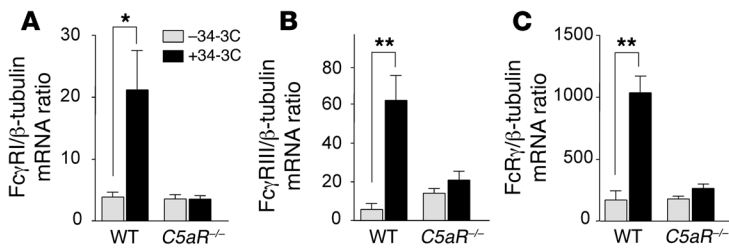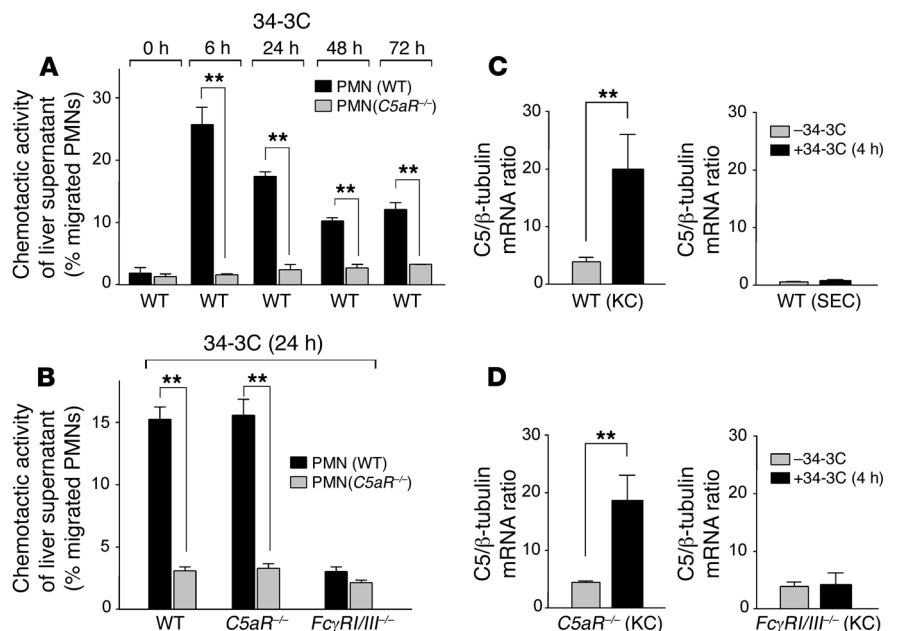
Figure 5
C5aR-dependent upregulation of FcγRI/RIII mRNA in Kupffer cells during AIHA. mRNA expression of FcγRI (**A**), FcγRIII (**B**), and FcRγ (**C**) was determined by TaqMan RT-PCR analysis of liver Kupffer cells derived from C57BL/6 WT and C5aR⁻/⁻ mice at 24 hours after injection of 34-3C mAb (black bars) as compared with mice not receiving 34-3C mAb (gray bars). Results are expressed as mean values ± SD obtained from 3–5 mice in each group. Significant differences were determined by Student's t test (*P < 0.05; **P < 0.001).

FcγR-dependent C5 and C5a production in AIHA. Bioactive C5a in homogenate supernatants from liver samples taken at various time points after 34-3C–induced AIHA was determined by measurement of C5a-mediated C5aR-triggered neutrophil chemotactic activity in a Transwell chemotaxis chamber. In WT and C5aR⁻/⁻ mice, C5a was produced in liver early during the onset of anemia, reaching maximal levels at 6 hours with a sustained plateau between 24 and 72 hours (Figure 6, A and B). In marked contrast, FcγRI/III⁻/⁻ mice showed strongly diminished levels (by more than 90%; P < 0.001 as compared with WT C57BL/6 mice) of C5a in the liver 24 hours after AIHA (Figure 6B). Moreover, in FcR-positive liver cells of all WT mice analyzed, mRNA expression of C5 was markedly upregulated in Kupffer cells but not SECs after 4 hours of 34-3C treatment (Figure 6C). This 34-3C–induced activation of C5 mRNA synthesis, which also occurred in C5aR⁻/⁻ mice, was completely abrogated in FcγRI/III⁻/⁻ mice (Figure 6D), suggesting that, in antibody-dependent hemolytic anemia, engagement of activating FcγR by 34-3C–opsonized erythrocytes strongly triggers C5 and C5a production.

Generation of C5a by ex vivo–stimulated Kupffer cells but not SECs. Because Kupffer cells showed an FcγR-dependent increase of C5 mRNA in AIHA, we speculated that activation of these effector cells may be directly involved in the generation of C5a. To test this hypothesis, we analyzed the C5a production in primary cultures of both Kupffer cells and SECs. In line with the data on C5 mRNA regulation in anemia, 3-hour incubation with 34-3C IgG2a–opsonized rbcs (20 μg of the mAb was used for opsonization) was associated with an induction of bioactive C5a in culture supernatants of Kupffer cells (Figure 7A) but not SECs (Figure 7B). C5a production mediated by 34-3C was not observed in controls when non-opsonized rbcs were used (Figure 7, A and B).

Effects of C5 and C5aR inhibition and FcγRI/III deficiency on C5a production and phagocytosis by macrophages in vitro. To extend the results from the in vivo and ex vivo studies, we also conducted both in vitro phagocytosis and C5a production experiments using peritoneal macrophages (PMs) from healthy WT C57BL/6 mice. First, series of various doses (0, 5, 10, 20, and 30 μg) of the 34-3C mAb were used for IgG opsonization of rbcs. There was a significant increase in phagocytic activity with increasing dosage and a concomitant increase of bioactive C5a in the culture supernatant of PMs (Figure 8, A and B). A concentration of 20 μg 34-3C mAb was then used for opsonization in all subsequent experiments. In contrast with PMs from WT mice, FcγRI/III–negative PMs failed to produce C5a upon stimulation, and, as expected, in vitro phagocytosis was markedly decreased (Figure 8, C and D). When PMs were preincubated with the neutralizing anti-C5aR mAb 1/36 (22), the capacity of these cells to phagocytose 34-3C–bound rbcs was also substantially reduced (Figure 8E). Similar results were obtained after treatment with an anti-C5 mAb (Figure 8E). PMs treated with anti-C5 mAbs, but not those treated with anti-C5aR mAbs, showed strongly diminished C5a production (Figure 8F), confirming the reported specificity of the anti-C5 mAb BB5.1 in blocking the cleavage site of C5 (27). Collectively, these data demonstrate that engagement of activat-

Figure 6
FcγR-dependent C5a production and induction of C5 mRNA synthesis in Kupffer cells but not SECs in anemia. (A and B) Supernatants of liver homogenates from WT mice collected at different time points (**A**) or from WT, C5aR⁻/⁻, and FcγRI/III⁻/⁻ mice collected 24 hours after AIHA induction (**B**) were assayed for C5a-dependent chemotactic activity on PMNs derived from WT mice (black bars) or C5aR⁻/⁻ mice (negative control, gray bars). (**C** and **D**) Kupffer cell and SEC C5 mRNA levels of the indicated WT, FcγRI/III⁻/⁻, and C5aR⁻/⁻ mice at 4 hours after injection of 34-3C mAb (black bars) as compared with mice not receiving 34-3C mAb (gray bars). (**A–D**) Results are expressed as mean values ± SD obtained from 3–5 mice in each group. Significant differences were determined by Student's t test (**P < 0.001).
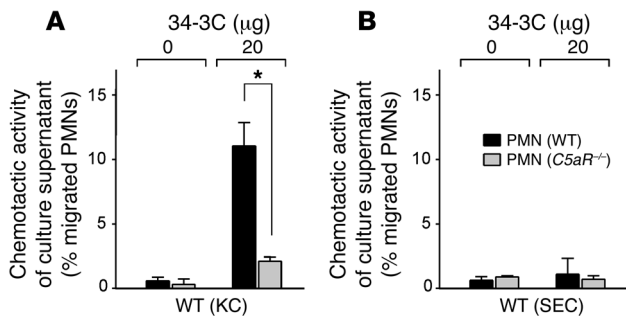
Figure 7
34-3C mAb–induced C5a production in primary cultures of Kupffer cells but not SECs. Cultured Kupffer cells (**A**) and SECs (**B**) from WT mice were incubated with rbcs opsonized with 34-3C mAb (20 μg) or with rbcs alone (0 μg). After 3 hours of incubation, culture supernatants were assayed for C5a-dependent chemotactic activity on PMNs derived from WT mice (black bars) or $C5aR^{-/-}$ mice (gray bars). (**A** and **B**) Results are expressed as mean values ± SD obtained from 2 individual experiments performed in duplicate. Significant differences were determined by Student's t test (*$P < 0.05$).

ing FcγR on 2 different macrophage populations, the Kupffer cells (Figures 6 and 7) and PMs (Figure 8), can trigger the production of C5 and C5a that is integral for FcγR-mediated erythrophagocytosis both in vitro and in vivo.

Effect of inhibitory FcγRII deficiency on phagocytosis and C5/C5a production in vitro and during anemia in vivo. In $FcγRII^{-/-}$ mice (28), immune inflammation critically depends on the balance of activating FcγR and inhibitory FcγRII signals (29, 30), and amelioration of the type II autoimmune response in immune thrombocytopenic purpura by intravenous Igs correlates with high expression levels of FcγRII (31). Given the critical importance of both C5aR and FcγR in anemia, we thus finally evaluated the role of FcγRII in C5a production, phagocytosis, and 34-3C type II autoimmunity. $FcγRII^{-/-}$ PMs, when compared with WT PMs, exhibited a significantly enhanced phagocytosis (Figure 9A) and C5a production (Figure 9B), indicating that FcγRII can negatively regulate FcγRI- and FcγRIII-triggered activation of PMs. However, the genetic deletion of FcγRII had no substantial influence on the 34-3C–induced anemic phenotype for all parameters analyzed, including C5 gene induction on Kupffer cells (Figure 10A), liver C5a production (Figure 10B), and the time- and dose-dependent clearance of rbcs when measured for both Ht levels and the percentage of Mac-1–selected Kupffer cells containing
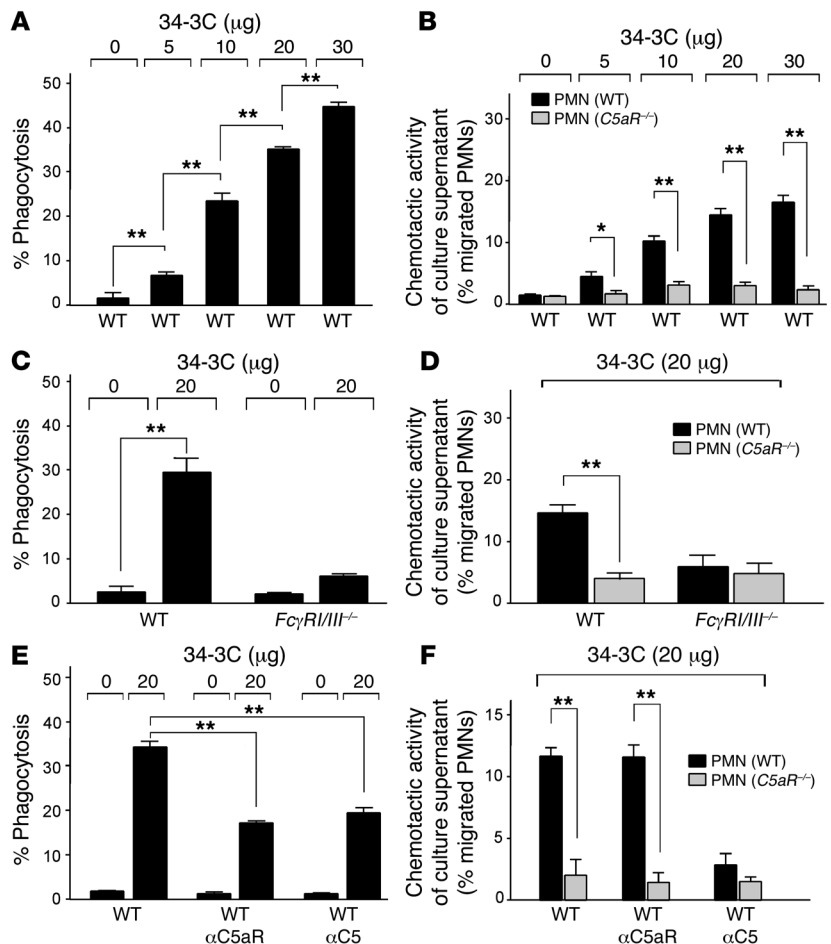
ingested rbcs (Figure 10, C and D). Importantly, FcγRII expression differed between liver and PMs as revealed by both FACS and real-time RT-PCR analysis, showing highly abundant FcγRII levels in PM cells that were limited in Kupffer cells (Figure 11). Moreover, the recently reported FcγRII expression in the liver (20) could be mainly attributed to the SECs but not Kupffer cells (Figure 11), giving a rationale for why FcγRII, although expressed in the liver, is not the expected negative regulator in anemia.
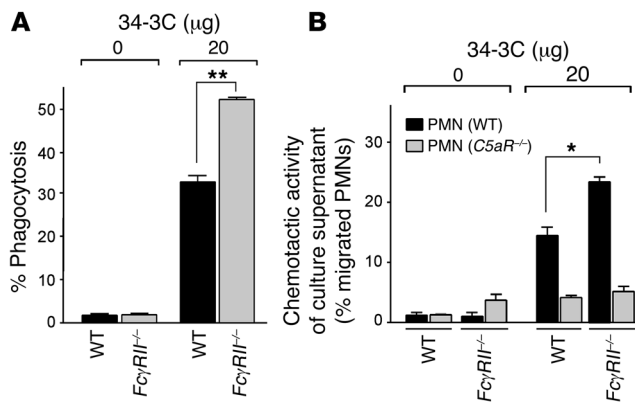
Discussion

In the present study, we identified a crucial role of C5a in IgG-dependent AIHA, independent from the chemotactic function of this anaphylatoxin. To our knowledge, this result provides the first direct in vivo evidence for the activating role of C5a and C5aR in

Figure 8
FcγR-dependent C5a production and C5/C5a/C5aR–dependent phagocytosis of 34-3C IgG–opsonized rbcs in peritoneal macrophages (PMs) in vitro. (**A–F**) Adherent PM cells from WT or $FcγRI/III^{-/-}$ mice were incubated with rbcs opsonized with the indicated doses of the 34-3C mAb in the absence or presence of blocking anti-C5 (αC5) or anti-C5aR (αC5aR) mAbs. (**A**, **C**, and **E**) After 4 hours of incubation, extracellular rbcs were lysed by a hypotonic shock, and the percentage of PM cells that had ingested more than 1 rbc was determined by light microscopy as described in Methods. (**B**, **D**, and **F**) After 3 hours of incubation at 37°C, culture supernatants were assayed for C5a-dependent chemotactic activity on PMNs derived from WT mice (black bars) or $C5aR^{-/-}$ mice (gray bars). (**A–F**) Results are expressed as the mean values ± SD from 2–3 individual experiments performed in duplicate. Significant differences were determined by Student's t test (*$P < 0.05$; **$P < 0.001$).
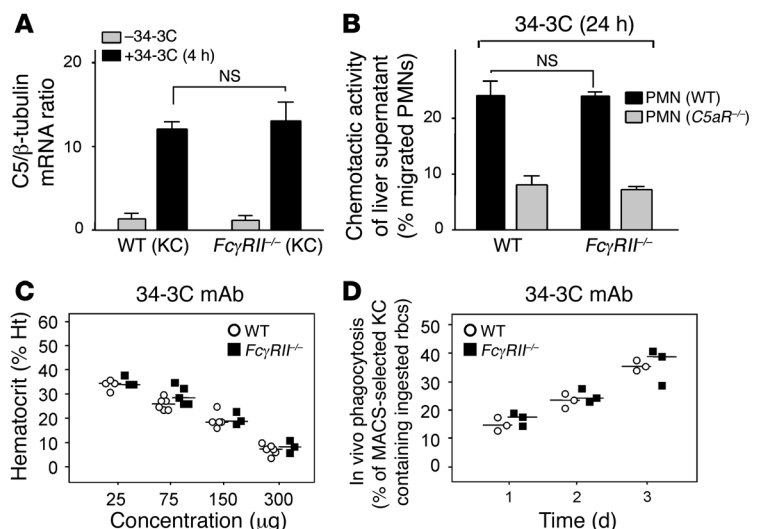
Figure 9
Enhanced phagocytosis and C5a production in PMs from FcγRII-deficient mice. (**A** and **B**) PM cells from WT or *FcγRII*^{-/-} mice were incubated with rbcs opsonized with 34-3C mAb (20 μg) or with rbcs alone (0 μg). Quantitative assessment of phagocytosis (**A**) and C5a production (**B**) was performed as described in the legend to Figure 8. Results are expressed as mean ± SEM from 2 individual experiments performed in triplicate (**A**) or duplicate (**B**). Significant differences were determined by Student's *t* test (*$P < 0.05$; **$P < 0.001$).

antibody-mediated type II autoimmunity, which was not expected before. Studies with macrophage-depleted mice demonstrated that Kupffer cells are one of the crucial effector cell types in murine AIHA (32). Our results clearly show that in vivo phagocytosis of 34-3C–opsonized erythrocytes by Kupffer cells is strongly impaired in *C5aR*^{-/-} mice, indicating that this process requires C5aR, which, along with activating FcγR, is expressed on liver Kupffer cells. Recently, it was reported that C5a positively regulates FcγR in IgG-mediated lung inflammation and peritonitis (23–26). These data, combined with our observations that the release of C5a was rapidly induced in hemolytic anemia and C5aR was required for 34-3C–induced increase of FcγRI and FcγRIII expression in Kupffer cells, indicate that C5a acts as a general regulator of FcγR expression at different tissue sites in both antibody-dependent inflammation and autoimmune disease.

In addition, our results concerning the source of C5a production in vitro and in vivo unexpectedly revealed that FcγRs on Kupffer cells are critically required for C5a production. *FcγRI/III*^{-/-} mice enabled us to identify the activating FcγRs as dominant mediators of autoantibody-induced release of C5a in AIHA. Since *FcγRI/III*^{-/-} mice are resistant to lethal hemolytic anemia, the classical and alternative pathways of complement activation appear insufficient to compensate for the lack of cellular FcγR in the Kupffer cell–mediated induction of the release of C5a by the liver. Moreover,

cell culture experiments demonstrate these cells to be fully competent for serum complement-independent C5 and C5a production that, as further documented in PMs, requires the presence of FcγR. Our data are thus consistent with a pathogenic mechanism of antibody-dependent hemolytic anemia in which IgG-opsonized erythrocytes, perhaps through a C5 cleavage enzyme from Kupffer cells, trigger FcγR-mediated C5 synthesis and C5a release, which in turn activate a positive cellular feedback signal upon engagement of C5aR, resulting in increased FcγRI and FcγRIII expression required for effective erythrophagocytosis. These results not only show the relevance of threshold activation of FcγR regulated by C5a activity in AIHA but also underscore the importance of FcγRs as cellular mediators of a novel discrete C5a-generating pathway. Exploiting this regulatory loop of C5a and FcγR offers new insights into the cellular mechanisms that determine the pathogenic effects of autoantibodies.

Currently, it is thought that immune inflammation and antibody-induced autoimmunity are regulated by the balance of activating FcγRI/III and inhibitory FcγRIIB signals (29). Indeed, our results in cell culture systems show that autoantibody-induced generation of C5a and in vitro phagocytosis of 34-3C–opsonized erythrocytes are negatively influenced by FcγRII in PMs. However, *FcγRII*^{-/-} mice exhibit no enhanced Kupffer cell–dependent C5 synthesis and cellular clearance. High FcγRII levels are detectable in both PM cells and liver SECs but are limited in Kupffer cells. This may explain why the condition of FcγRII deficiency results in a normal, not enhanced, autoantibody-triggered anemic phenotype and may also indicate that FcγRII, while considered a potential therapeutic target in other autoimmune diseases (33), is not a likely candidate target molecule for the treatment of anemia.

Figure 10
34-3C mAb–induced AIHA is FcγRII-independent. (**A–D**) Experimental AIHA was induced in WT or *FcγRII*^{-/-} mice by i.p. injection of 300 μg (**A**, **B**, and **D**) or the indicated doses (**C**) of the pathogenic 34-3C mAb, and the mice were examined at 4 hours for C5 mRNA levels in Kupffer cells (**A**), at 24 hours for C5a production in liver supernatants (**B**), at 96 hours for Ht levels (**C**), and at day 1–3 for in vivo erythrophagocytosis by Mac-1–selected Kupffer cells (**D**). Results are expressed either as mean values ± SD obtained from 3–6 mice in each group (**A** and **B**) or as values of individual mice (**C** and **D**). No significant differences were observed between WT and *FcγRII*^{-/-} mice for all parameters.
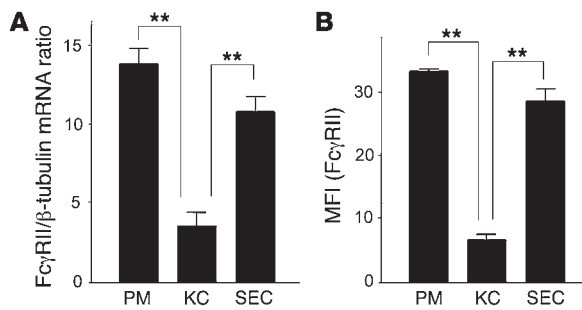
Figure 11
Expression analysis of FcγRII in Kupffer cells and SECs as compared with PM cells. (**A** and **B**) The indicated cells were MACS-isolated from the peritoneum and liver of C57BL/6 WT mice and analyzed for production of FcγRII mRNA (**A**) by real-time RT-PCR and FcγRII protein (**B**) by flow cytometry using the anti-FcγRII Ly17.2 mAb conjugated to FITC. Results are expressed as mean ± SEM from 3–4 mice. Significant differences were determined by Student's t test (**$P < 0.001$). MFI, mean fluorescence intensity.

Our work significantly extends previous reports that describe a dominant contribution of activating Fcγ receptors in experimental models of type II autoimmune diseases (23, 29, 33). The results presented here provide evidence that C5a, which is produced by and acts on FcγR, can serve as a critical sensor of autoantibody-mediated effects in AIHA. We also note that inhibition of C5 and inhibition of C5aR are each effective in reducing Fc-mediated phagocytosis, indicating an important role of C5a rather than C5b in the lysis of sensitized rbcs in vitro. The physiological relevance of this finding has yet to be determined in vivo. It may well be that C5b-induced formation of the membrane attack complex accounts for the residual anemia at high autoantibody titers in C5aR deficiency. The identification of the requirement of cell-derived C5 and C5a for immune elimination of erythrocytes by FcγR defines a novel mechanism of cell lysis in anemia and may suggest the potential use of anaphylatoxins as therapeutic targets for the control of AIHA. Anti-C5 biologicals, such as eculizumab (34), have already shown beneficial effects of C5 inhibition to reduce hemolysis in patients with paroxysmal nocturnal hemoglobinuria (PNH) (35). However, clinical studies in which AIHA has been treated in similar ways are not realized. Despite the fact that the molecular events of rbc destruction are quite different in AIHA and PNH (a disease that arises via somatic gene mutations and subsequent production of blood cells with a deficiency of surface proteins that protect the cells against attack by the complement system), our findings in mice may support the view that blockade of C5 cleavage into C5a and C5b would be a reasonable approach for the treatment of not only PNH but also other hematological disorders, as suggested here for AIHA.

Methods

Mice. The generation of *FcγRI−/−* and *FcγRIII−/−* mice derived from ES cells of 129 origin and backcrossed for more than 4 generations with C57BL/6 mice has been described previously (18, 36). These mice were then intercrossed to obtain mice doubly deficient in FcγRI and FcγRIII. The homozygous *FcγRI/III−/−* mice were selected, and *FcγRI/III+/+* WT C57BL/6 littermates were used for all comparisons. *FcγRII−/−*, *C5aR−/−*, and *C3−/−* C57BL/6 mice were kindly provided by T. Takai (Tohoku University, Sendai, Japan) (28), U. Höpken (Max-Delbrück-Center for Molecular Medicine, Berlin,

Germany) (37), and M. Carroll (Harvard University, Boston, Massachusetts, USA) (38). *FcRγ−/−* C57BL/6 mice were purchased from Taconic. NOD mice were obtained from Charles River Laboratories. All these mice were used at 8–14 weeks of age. Experiments were performed with approval from the Bezirksregierung Hannover.

Experimental AIHA. Mouse anti–mouse rbc IgG2 monoclonal autoantibody 34-3C (a non-agglutinating mAb) (12) was purified from tissue culture supernatant by protein G affinity chromatography. Mice were injected i.p. with different dosages of 25–300 μg of 34-3C or the same amounts of isotype-matched control antibody W6/32 (14). Survival was monitored, and daily Ht levels were determined with heparinized microhematocrit capillary tubes in a microfuge (5 minutes at 13,900 g) using blood samples obtained from the retroorbital plexus. H&E-stained formalin-fixed sections of major organs were prepared from mice sacrificed at days 0 and 2 after 34-3C injection and examined for histopathological changes. Livers obtained after perfusion were processed for examinations of C5a bioactivity, erythrophagocytosis, and both Kupffer cell and SEC FcγR, C5, and C5aR mRNA/protein. Perfused liver was excised, minced into small pieces, and treated with HBSS plus 0.025% collagenase V (Sigma-Aldrich); this was followed by passages through stainless steel gauze (mesh, 500 and 100 μm) to obtain single-cell suspensions. Parenchymal hepatocytes were discarded by sedimentation at 50 g. The remaining nonparenchymal cell fraction was centrifuged at 800 g for 10 minutes and immediately taken for SEC and Kupffer cell isolation and analysis as described below, while the obtained supernatant was stored at –80°C for later determination of C5a bioactivity.

SEC and Kupffer cell isolation. The resuspended nonparenchymal cells were layered on Percoll gradient (50% and 25%; density, 1.07 g/ml and 1.03 g/ml, respectively) and centrifuged at 1,000 g for 20 minutes. The interphase containing mainly SECs and Kupffer cells was again centrifuged and resuspended in 80 μl of modified PBS buffer containing 0.5% BSA plus 0.2 mM EDTA. Twenty microliters of Mac-1 magnetic beads (Miltenyi Biotec) were added and incubated for 15 minutes, followed by 2 washes and a final resuspension in 2 ml of modified PBS buffer prior to magnetic activated cell sorting (MACS). AutoMACS separation of Kupffer cells was done using the Posseld program for a double selection of Mac-1–positive cells to attain maximum purity (>97%, as assessed by flow cytometry using FITC–F4/80 mAb staining). Both the obtained Mac-1–positive Kupffer cells and the Mac-1–negative SECs were used for comparative analysis.

Determination of erythrophagocytosis by Kupffer cells. Mac-1–selected Kupffer cells were isolated from mice sacrificed 1–3 days after 34-3C injection as described before and examined for erythrophagocytosis by light microscopy. Cells containing more than 2 rbcs were considered phagocytic. A total of 200 Kupffer cells were counted by 2 blinded reviewers in 4–6 C57BL/6, *C5aR−/−*, and *FcγRI/III−/−* mice, and the percentage of Mac-1–positive cells showing ingested rbcs was determined for each group of mice.

C5, C5aR, and FcγR expression and transcription analysis. Expression of C5aR and FcγR on liver cells (2 × 10⁴) before and after MACS separation was measured by flow cytometry, using a FACScan flow cytometer (BD). The following FITC- and PE-conjugated antibodies were used: anti-FcγRII (Ly17.2) (20); anti–FcγRII/III (clone 2.4G2) and anti–Mac-1 (clone M1/70.15) (both from BD Biosciences — Pharmingen); anti–F4/80 (Serotec); and anti-C5aR (clone 1/36) (22). In some experiments, total RNA was prepared from both SEC and Kupffer cell preparations of both anemic and nonanemic mice using RNAzol reagent (WAK–Chemie Medical GmbH) and analyzed for FcγRI, FcγRII, FcγRIII, FcRγ, and C5aR transcription levels normalized to tubulin by both TaqMan (Applied Biosystems) and real-time RT-PCR using published primers and probes (24, 26, 39). For detection of C5 transcripts, the following C5-specific primers were used: sense, 5′-GCATTTCTGACAC-CAGGCTTC-3′; antisense, 5′-AGCGCACAGTCAGCTTCCA-3′ (40).

Determination of C5a bioactivity. Liver supernatants collected from mice sacrificed 1–3 days after 34-3C injection were used for the determination of autoantibody-induced C5a-mediated neutrophil chemotactic activity in anemia. In addition, culture supernatants of SECs, Kupffer cells, and PM cells obtained in vitro at 3 hours after incubation with 34-3C–opsonized rbcs were also used. Bone marrow cells (consisting of 64–68% neutrophils) from C57BL/6 and *C5aR*−/− mice were suspended at 7.5×10^6 cells per milliliter RPMI 1640 medium and 0.5% BSA. One hundred microliters of the bone marrow cell suspension was placed into the insert of a Transwell chemotaxis chamber, and the bottom well was filled with 600 μl of liver and culture supernatants diluted 1:2 in RPMI/1% BSA. Inserts were combined to the lower chambers and incubated at 37°C and 6% CO_2 for 2 hours. After the incubation, 50 μl of 70 mM EDTA solution was added into the lower chambers to release adherent cells from the lower surface of the membrane and from the bottom of the well. Plates were further incubated 30 minutes at 4°C, inserts were removed, and the transmigrated neutrophils were vigorously suspended and counted with a FACSCalibur (BD Biosciences) for 1 minute at 60 μl/min with gating on forward and side scatter. Migration of polymorphonuclear leukocytes (PMNs) from the insert to the bottom well was quantitated as percentage of total PMNs loaded into the upper chamber.

34-3C mAb activation of PMs in vitro. Resident PM cells were flushed out of the peritoneal cavity of WT, *FcγRI/III*−/−, or *FcγRII*−/− mice, washed twice with PBS, and suspended in RPMI 1640 medium and 1% BSA. The PM cells were allowed to adhere for 4 hours on chamber slides (Nunc) at a density of 3×10^5 cells per well, followed by the removal of nonadherent cells and overnight incubation. Freshly isolated rbcs from WT mice were washed twice with ice-cold PBS by centrifugation at 550 *g* and processed for opsonization. Hereby, 10 μl of pelleted rbcs were incubated at 4°C for 1 hour with 10 μl of 34-3C mAb at subsaturating to saturating concentrations (5–30 μg/ml). Aliquots of 200 μl of 1% opsonized rbc suspension

were added to each well and incubated at 37°C for 3 or 4 hours. For some experiments, PM cells were first incubated for 1 hour at 37°C with the blocking 1/36 anti-C5aR (22) and BB5.1 anti-C5 (27) (provided by Roche) antibodies. Culture supernatants were collected after 3 hours of incubation and analyzed for bioactive C5a as described before. After 4 hours of incubation, noningested extracellular rbcs were lysed by hypotonic shock, immediately followed by 2 washes with PBS. PM cells were fixed with 4% paraformaldehyde and conventionally stained with Giemsa/H&E, and phagocytosis was determined by light microscopy.

Statistics. Statistical analysis was performed using the Prism 4 statistical software package (GraphPad Software). To analyze differences in mean values, a 2-tailed unpaired Student's *t* test was used. $P < 0.05$ was considered statistically significant, and $P < 0.001$ was considered highly significant.

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1. Ember, J.A., Jagels, M.A., and Hugli, T.E. 1998. Characterization of complement anaphylatoxins and their biological responses. In *The human complement system in health and disease*. J.E. Volankis and M.M. Frank, editors. Marcel Dekker Inc. New York, New York, USA. 241–284.
2. Gerard, C., and Gerard, N.P. 1994. C5a anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* 12:775–808.
3. Vakeva, A.P., Agah, A., Rollins, S.A., Matis, L.A., and Stahl, G.L. 1998. Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation.* 97:2259–2267.
4. Fitch, J.C., et al. 1999. Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. *Circulation.* 100:2499–2506.
5. Stevens, J.H., et al. 1986. Effects of anti-C5a antibodies on the adult respiratory distress syndrome in septic primates. *J. Clin. Invest.* 77:1812–1816.
6. Ji, H., et al. 2002. Arthritis critically dependent on innate immune system players. *Immunity.* 16:157–168.
7. Girardi, G., et al. 2003. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J. Clin. Invest.* 112:1644–1654. doi:10.1172/JCI200318817.
8. Gehrs, B.C., and Friedberg, R.C. 2002. Autoimmune hemolytic anemia. *Am. J. Hematol.* 69:258–271.
9. Schreiber, A.D., and Frank, M.M. 1972. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. *J. Clin. Invest.* 51:575–582.

10. Clynes, R., and Ravetch, J.V. 1995. Cytotoxic antibodies trigger inflammation through Fc receptors. *Immunity.* 3:21–26.
11. Izui, S. 1994. Autoimmune hemolytic anemia. *Curr. Opin. Immunol.* 6:926–930.
12. Shibata, T., et al. 1990. Monoclonal anti-erythrocyte autoantibodies derived from NZB mice cause autoimmune hemolytic anemia by two distinct pathogenic mechanisms. *Int. Immunol.* 2:1133–1141.
13. Azaredo da Silveira, S., et al. 2003. Complement activation selectively potentiates the pathogenicity of the IgG2b and IgG3 isotypes of a high affinity anti-erythrocyte autoantibody. *J. Exp. Med.* 195:665–672.
14. Meyer, D., et al. 1998. FcγRIII (CD16)-deficient mice show IgG isotype-dependent protection to experimental autoimmune hemolytic anemia. *Blood.* 92:3997–4002.
15. Leddy, J.P., Falany, J.L., Kissel, G.E., Passador, S.T., and Rosenfeld, S.I. 1993. Erythrocyte membrane proteins reactive with human (warm-reacting) anti-red cell autoantibodies. *J. Clin. Invest.* 91:1672–1680.
16. Prins, J.B., et al. 1993. Linkage on chromosome 3 of autoimmune diabetes and defective Fc receptor for IgG in NOD mice. *Science.* 260:695–698.
17. Johansson, A.C., et al. 2001. Genetic control of collagen-induced arthritis in a cross with NOD and C57BL/10 mice is dependent on gene regions encoding complement factor 5 and FcgammaRIIb and is not associated with loci controlling diabetes. *Eur. J. Immunol.* 31:1847–1856.
18. Ioan-Facsinay, A., et al. 2002. FcγRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity.* 16:391–402.
19. Fossati-Jimack, L., et al. 2000. Markedly different pathogenicity of four immunoglobulin G isotype-switch variants of an antierythrocyte autoantibody

is based on their capacity to interact in vivo with the low-affinity Fcgamma receptor III. *J. Exp. Med.* 191:1293–1302.
20. Schiller, C., et al. 2000. Mouse FcγRII is a negative regulator of FcγRIII in IgG immune complex-triggered inflammation but not in autoantibody-induced hemolysis. *Eur. J. Immunol.* 30:481–490.
21. Ahmed, S.S., et al. 1995. Fc receptors in liver sinusoidal endothelial cells in NZB/W F1 lupus mice: a histological analysis using soluble immunoglobulin G-immune complexes and a monoclonal antibody (2.4G2). *Hepatology.* 22:316–324.
22. Soruri, A., Kim, S., Kiafard, Z., and Zwirner, J. 2003. Characterization of C5aR expression on murine myeloid and lymphoid cells by the use of a novel monoclonal antibody. *Immunol. Lett.* 88:47–52.
23. Schmidt, R.E., and Gessner, J.E. 2005. Fc receptors and their interaction with complement in autoimmunity. *Immunol. Lett.* 100:56–67.
24. Shushakova, N., et al. 2002. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcγRs in immune complex-induced lung disease. *J. Clin. Invest.* 110:1823–1830. doi:10.1172/JCI200318817.
25. Godau, J., et al. 2004. C5a initiates the inflammatory cascade in immune complex peritonitis. *J. Immunol.* 173:3437–3445.
26. Skokowa, J., et al. 2005. Macrophages induce the inflammatory response in the pulmonary Arthus reaction through Gα₂ activation that controls C5aR and Fc receptor cooperation. *J. Immunol.* 174:3041–3050.
27. Frei, Y., Lambris, J.D., and Stockinger, B. 1987. Generation of a monoclonal antibody to mouse C5 application in an ELISA assay for detection of anti-C5 antibodies. *Mol. Cell. Probes.* 1:141–149.
28. Takai, T., Ono, M., Hikida, M., Ohmori, H., and

Ravetch, J.V. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature.* **379**:346–349.

29. Ravetch, J.V., and Bolland, S. 2001. IgG Fc receptors. *Annu. Rev. Immunol.* **19**:275–290.

30. Nieswandt, B., et al. 2003. Targeting of platelet integrin α$_{IIb}$β$_3$ determines systemic reaction and bleeding in murine thrombocytopenia regulated by activating and inhibitory FcγR. *Int. Immunol.* **15**:341–349.

31. Samuelsson, A., Towers, T.L., and Ravetch, J.V. 2001. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science.* **291**:484–486.

32. Jordan, M.B., van Rooijen, N., Izui, S., Kappler, J., and Marrack, P. 2003. Liposomal clodronate as a novel agent for treating autoimmune hemolytic anemia in a mouse model. *Blood.* **101**:594–601.

33. Takai, T. 2002. Roles of Fc receptors in autoimmunity. *Nat. Rev. Immunol.* **2**:580–592.

34. Hillmen, P., et al. 2004. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* **350**:552–559.

35. Young, N.S. 2005. Paroxysmal nocturnal hemoglobinuria: current issues in pathophysiology and treatment. *Curr. Hematol. Rep.* **4**:103–109.

36. Hazenbos, W.L., et al. 1996. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII (CD16) deficient mice. *Immunity.* **5**:181–188.

37. Höpken, U.E., Lu, B., Gerard, N.P., and Gerard, C. 1997. Impaired inflammatory responses in the reverse Arthus reaction through genetic deletion of the C5a receptor. *J. Exp. Med.* **186**:749–756.

38. Wessels, M.R., et al. 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc. Natl. Acad. Sci. U. S. A.* **92**:11490–11494.

39. Radeke, H.H., et al. 2002. Opposite regulation of type II and III receptors for immunoglobulin G in mouse glomerular mesangial cells and in the induction of anti-glomerular basement membrane (GBM) nephritis. *J. Biol. Chem.* **277**:27535–27544.

40. Haviland, D.L., Haviland, J.C., Fleischer, D.T., and Wetsel, R.A. 1991. Structure of the murine fifth complement component (C5) gene. *J. Biol. Chem.* **266**:11818–11825.