Requirement for complement in antibody responses is not explained by the classic pathway activator IgM

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Animals lacking complement factors C1q, C2, C3, or C4 have severely impaired Ab responses, suggesting a major role for the classic pathway. The classic pathway is primarily initiated by antigen–Ab complexes. Therefore, its role for primary Ab responses seems paradoxical because only low amounts of specific Abs are present in naive animals. A possible explanation could be that the classic pathway is initiated by IgM from naive mice, binding with sufficient avidity to the antigen. To test this hypothesis, a knock-in mouse strain, Cμ13, with a point mutation in the gene encoding the third constant domain of the μ-heavy chain was constructed. These mice produce IgM in which proline in position 436 is substituted with serine, a mutation previously shown to abrogate the ability of mouse IgM to activate complement. Unexpectedly, the Ab response to sheep erythrocytes and keyhole limpet hemocyanin in Cμ13 mice was similar to that in WT mice. Thus, although secreted IgM and the classic pathway activation are both required for the normal primary Ab response, this does not require that IgM activate C. This led us to test Ab responses in animals lacking one of three other endogenous activators of the classic pathway: specific intracellular adhesion molecule-grabbing nonintegrin R1, serum amyloid P component, and C-reactive protein. Ab responses were also normal in these animals.

IgM mutation | natural IgM

Complement (C) is primarily known as a defense system leading to lysis of pathogens, increased phagocytosis, and inflammation. However, C also plays a crucial role in the generation of Ab responses (reviewed in 1–3). This was first demonstrated by Pepys (4), who found that C3 depletion of mice by treatment with cobra venom factor abrogated Ab responses to sheep erythrocytes (SRBCs). Subsequent studies showed that Ab responses were also severely impaired in C4-deficient humans (5), guinea pigs (6, 7), and mice (8); C2-deficient guinea pigs (7); and C3-deficient dogs (9) and mice (8). Generally, the Ab response to lower antigen (Ag) doses is more dependent on C than responses to higher doses (9, 10). Lack of C5 was compatible with normal Ab responses (11), excluding involvement of the later C factors as well as the lytic pathway. Mice lacking complement receptors 1 and 2 (CR1/2) had severely impaired primary (12–14) as well as secondary (13, 14) Ab responses. In mice, CR1 and CR2 are splice variants encoded by the Cr2 gene. They bind the C3 cleavage products C3b, iC3b, C3dg, and C3d and are expressed on B cells and follicular dendritic cells (FDCs) (reviewed in 15). The phenotype of CR1/2-deficient mice closely resembles that of mice lacking C2, C3, or C4. Therefore, it is generally assumed that the role of C in Ab responses is mediated by CR1/2 and that the importance of C2, C3, and C4 is to generate the C3 split products that are the ligands for these receptors. Several different mechanisms have been suggested to underlie the strong influence of C on Ab responses: increased follicular trapping of immune complexes on $CR1/2^+$ FDCs (14, 16), transport of immune complexes by $CR1/2^+$ marginal zone B cells to the follicles (17, 18), increased Ag presentation by $CR1/2^+$ B cells (19), or increased B-cell signaling attributable to co–cross-linking of the B-cell receptor (BCR) with the CR2/CD19/CD81 complex either by immune complexes or by membrane-bound IgM activating C (20–22).

The C cascade, resulting in cleavage of C3, and thereby in the generation of the ligands for CR1/2, can be activated via three different pathways: the classic, alternative, and lectin pathways. Mice lacking a functional alternative pathway because of deletion of factor B have normal Ab responses to SRBCs (23) as well as to West Nile virus (24), indicating that the alternative pathway is not required. Mannan-binding lectin (MBL) KO mice show a complex phenotype, with normal, moderately higher, or moderately lower Ab responses than WT controls (25–28). The defect in Ab responses occasionally observed in MBL-deficient mice was not nearly as severe as that seen in animals lacking C2, C4, C3, or CR1/2. These observations indicate that the role of C in the generation of normal Ab responses depends primarily on classic pathway activation. The most direct support for this is the reduced HA titers against high doses of SRBCs administered to $C1qA^{-/-}$ mice (29). Responses to malaria parasites or West Nile virus in C1q-deficient mice were much less affected than responses to SRBCs, and were even higher than in WT mice in some cases (24, 30).

The observations that classic pathway activation is crucial not only for secondary but for primary Ab responses to many Ags appear paradoxical (4–6, 12–14, 29). In naive mice, very low levels of specific Abs are present. Therefore, abundant formation of immune complexes that can activate the classic pathway would not be expected. IgM is a very potent activator of the classic pathway, and this property has suggested that IgM might play an important role in the effects of C on the Ab response. Two lines of research support this view. First, Ag-specific IgM, passively administered to mice or humans together with Ag can enhance the Ab response to the Ag via Ab feedback regulation (31–35). This enhancement depends on the ability of IgM to activate C (34, 35). Second, mice lacking secretory IgM have lower Ab responses than WT mice (36–38), and the response can be restored by transfer of IgM from normal mouse serum (36, 38). It seemed plausible that the enhancing effect of natural IgM on Ab responses (36–38) would depend on its ability to activate C, and thereby explain why classic pathway activation plays a crucial role in primary Ab responses. Early primary Ag-specific IgM could

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then further enhance the Ab response via the feedback-enhancing effect (32–35).

We have sought to test this specific explanation by ascertaining whether the ability of natural IgM to activate C is a prerequisite for normal Ab responses. For this purpose, we generated a knock-in mouse strain (Cμ13) producing IgM with a point mutation in the third constant domain of the μ-heavy chain, rendering the IgM unable to activate C. Unexpectedly, the Ab response against several different Ags [SRBCs, keyhole limpet hemocyanin (KLH), and 4-hydroxy-3-nitrophenylacetyl (NP)-KLH] was normal in these mice. This led us to test Ab responses in animals lacking one of three other endogenous activators of the classic pathway: specific intracellular adhesion molecule-grabbing nonintegrin R1 (SIGN-R1) (39), serum amyloid P component (SAP) (40), and C-reactive protein (CRP) (41). Ab responses were also normal in these animals.

Results

Impaired Ab Responses in *C1qA*$^{-/-}$ and *Cr2*$^{-/-}$ Mice. As noted above, different protocols have yielded different effects of C1q deficiency on the immune response (24, 29, 30). For this reason, we directly tested the role of the classic pathway in our experimental system and included *Cr2*$^{-/-}$ mice for comparison. The response to various doses of SRBCs in *C1qA*$^{-/-}$, *Cr2*$^{-/-}$, and WT mice was compared. The IgG anti-SRBC response in *C1qA*$^{-/-}$ and *Cr2*$^{-/-}$ mice was severely impaired (Fig. 1 *E–H*), and the IgM response was lower in *C1qA*$^{-/-}$ and *Cr2*$^{-/-}$ animals (Fig. 1 *A–D*). These observations confirm that the classic pathway is of crucial importance for the generation of normal Ab responses to SRBCs.

Generation and Characterization of Cμ13 Knock-In Mice. To test whether C activation by natural IgM explains the requirement for classic pathway activation in primary Ab responses, we generated knock-in (Cμ13) mice using homologous recombination in ES cells. The point mutation introduced is identical to the one previously reported to abrogate the ability of IgM to initiate C-dependent lysis (42) and to bind C1 (43) as well as to enhance Ab responses (34). A codon change resulting in substitution of proline for serine at amino acid position 436 in the third constant domain of the μ-heavy chain was introduced. The vector used for targeting was assembled from the original expression plasmid (pCμ13) reported by Shulman et al. (42) and contains a region of homology to the *Igh*^{a} allele (Fig. 2*A*). The neo cassette was flanked by LoxP sites for excision after targeting (Fig. 2*B*). To remove the neo cassette, targeted ES cells were transfected with a Cre recombinase-expressing plasmid (Fig. 2*D*). ES cells (Bruce 4) derived from C57BL/6 (*Igh*^{b}) mice (44) were transfected, and Southern analysis was used to identify positively transfected clones (Fig. 2*E*). Germline mice were expanded, and peripheral blood was analyzed for the IgM^{a} product indicative of a transfected gene. The mice were backcrossed for 12 generations to BALB/c mice, and unless otherwise stated, Cμ13.BALB/c.N12 mice were used in the experiments.

The B-cell compartment in Cμ13 mice was analyzed and compared with that in BALB/c mice. The numbers of CD19^{+}, B-1a, B-1b, and B-2 B cells in the spleen and peritoneum were similar in the two strains (Table S1). Cμ13 mice also had similar numbers of follicular B cells in the spleen and similar expression of IgM on splenic B cells (Table S2). The number of B220^{+} cells was slightly decreased and that of marginal zone B cells was increased in Cμ13 mice (Table S2).

IgM from Cμ13 Mice Cannot Activate C but Has the Same Size and Half-Life as WT IgM. To confirm that Cμ13 mice did not produce C-activating IgM, the number of single B cells producing IgM anti-SRBCs was tested with two sensitive assays: the direct hemolytic plaque-forming cell (PFC) assay, which only detects B cells producing C-activating IgM, and the enzyme-linked immunospot (ELISPOT) assay, which detects all IgM-producing B cells. When BALB/c and Cμ13 mice were immunized with a high

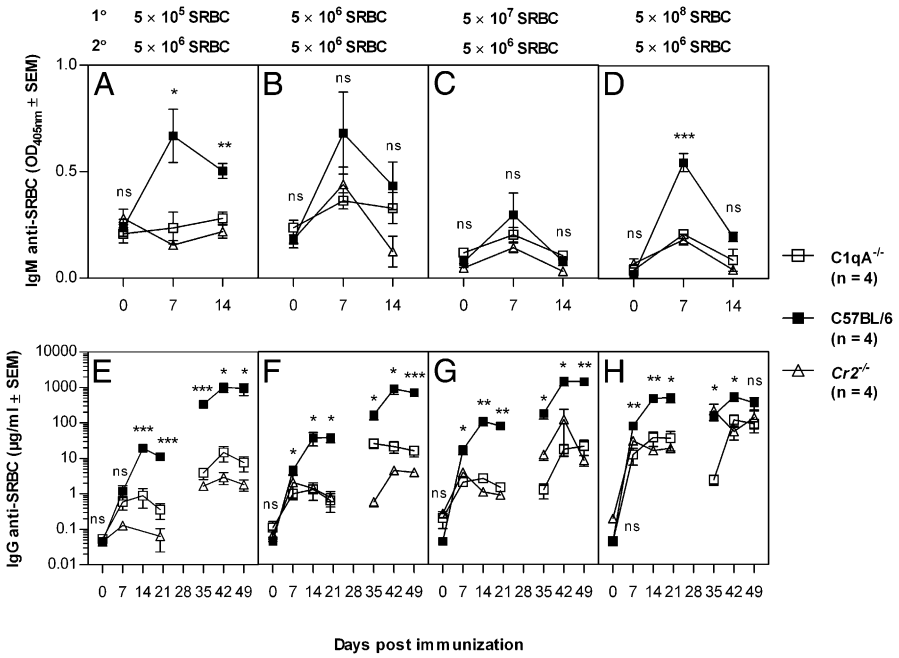

Fig. 1. Impaired Ab responses against SRBCs in *C1qA*$^{-/-}$ and *Cr2*$^{-/-}$ mice. Mice were immunized i.v. with 5×10^5, 5×10^6, 5×10^7, or 5×10^8 SRBCs and boosted i.v. on day 21 with 5×10^6 SRBCs. All groups were prebled and then bled at indicated time points ($n = 4$ per group). (*A–D*) IgM anti-SRBC response. Sera from days 0–21 were diluted 1:125 (*A* and *B*) or 1:625 (*C* and *D*). (*E–H*) IgG anti-SRBC response. The experiment was repeated twice for the dose of 5×10^6 SRBCs and gave similar results. Error bars represent SEM. *P* values represent a comparison between the response in *C1qA*$^{-/-}$ and C57BL/6 mice. ns, not significant ($P > 0.05$); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

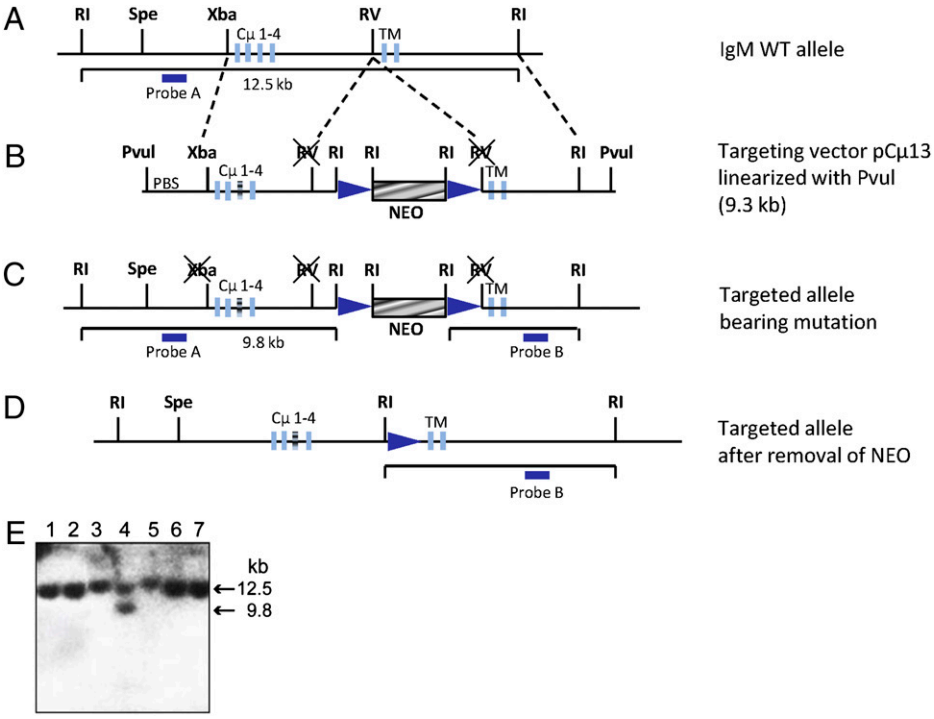

Fig. 2. Construction of gene targeting vector to introduce a mutation in IgH Cμ region. (*A*) Map of (WT) IgH Cμ region. (*B*) Targeting vector pCμ13-neo, including a "floxed" neocassette and mutant Cμ domain. (*C*) Map of targeted allele. (*D*) Targeted allele after removal of neo. (*E*) Southern analysis of neoresistant ES cells transfected with pCμ13 identified one clone (no. 4) as heterozygous for the WT and Cμ13 alleles. Genomic DNA was digested with EcoRI, separated on a 0.7% agarose gel, and blotted to nitrocellulose. Hybridization with probe A revealed WT (12.5 kb) and mutant (9.8 kb) alleles.

dose of SRBCs, they responded with high numbers of B cells producing IgM anti-SRBCs (40,714 and 69,033 by ELISPOT assay per spleen, respectively) (Table 1). When the same spleen cell suspensions were tested in the PFC assay, 39,800 BALB/c B cells produced C-activating IgM anti-SRBCs, thus corresponding well to the 40,714 that the ELISPOT assay detected. In contrast, Cμ13 B cells did not produce any C-activating IgM above background levels (Table 1). Thus, the B cells in the Cμ13 mouse spleens produced IgM anti-SRBCs, as detected by ELISPOT assay, but this IgM could not activate C, as shown by the inability to form hemolytic plaques in the PFC assay.

In the PFC assay, guinea pig serum was used as the source of C because murine C is too fragile. It was important to establish that IgM from Cμ13 mice was also unable to activate mouse C. We tested whether SRBC-specific IgM derived from BALB/c or Cμ13 mice was able to initiate deposition of C3 on the surface of SRBCs when mouse plasma was the source of C. Incubation of SRBCs with IgM anti-SRBCs isolated from BALB/c mice, together with plasma from C57BL/6, BALB/c, or Cμ13 mice, led to deposition of mouse C3 (Fig. 3 *A*, *C*, and *D*; green curves), whereas incubation with $C1qA^{-/-}$ plasma resulted in very little C3 deposition (Fig. 3*B*, green curve). IgM anti-SRBCs from Cμ13 mice were unable to initiate C3 deposition in all situations (Fig. 3 *A–D*, orange curve). No C3 deposition was seen after incubation with plasma alone, indicating that SRBCs by themselves do not activate C (Fig. 3 *A–D*, blue curve).

Next, sera from SRBC-immunized Cμ13 and WT mice were size-fractionated by passage over a Sepharose CL-6B column. This column separates proteins ranging in molecular mass between 1×10^5 and 4×10^6 Da, and is therefore well suited to detect pentameric IgM (molecular mass = 9×10^5 Da) and monomeric IgM (molecular mass = 1.8×10^5 Da). Separation of IgM and IgG (molecular mass = 1.5×10^5 Da) peaks was obtained, ascertaining that monomeric IgM would have been

Table 1. B cells from Cμ13 mice produce IgM that cannot activate C

		PFC anti-SRBCs per spleen†		ELISPOT/spleen‡	
Strain	Immunization*	Log_{10} ± SD (geometric mean)		Log_{10} ± SD (geometric mean)	
BALB/c (*n* = 4)	1×10^8 SRBCs	4.6 ± 0.2	(39,800)	4.6 ± 0.2	(40,714)
Cμ13 (*n* = 6)	1×10^8 SRBCs	2.1 ± 0.3***	(115)	4.8 ± 0.1^{ns}	(69,033)
BALB/c (*n* = 4)	Nil	2.5 ± 0.3	(291)	3.2 ± 0.3	(1,479)
Cμ13 (*n* = 2)	Nil	1.9 ± 0.3	(87)	3.2 ± 0.1	(1,543)

Cells tested in PFC and ELISPOT assays were from the same spleens. *P* values represent comparisons between the response in BALB/c mice and Cμ13 mice. ns, not significant ($P > 0.05$); ***$P < 0.001$. Data are representative of two experiments with Cμ13.N12 and two experiments with Cμ13.N8.

*Mice were immunized i.v. with 1×10^8 SRBCs per mouse in 0.2 mL of PBS.

†Direct PFC assay (measures single B cells producing C-activating IgM anti-SRBCs).

‡ELISPOT assay (measures single B cells producing IgM anti-SRBCs, regardless of C activation).

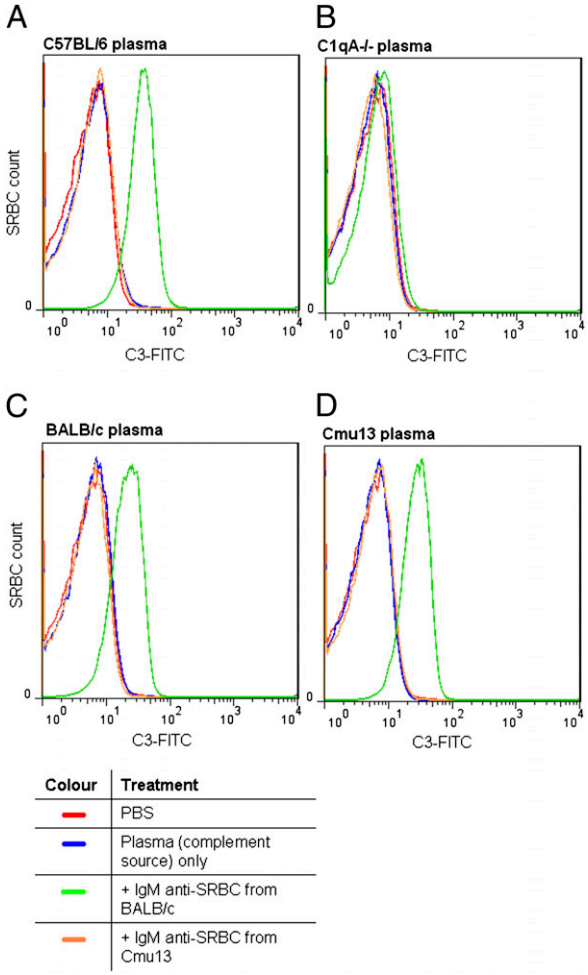

Fig. 3. IgM produced by Cμ13 mice cannot initiate C3 deposition on SRBCs. SRBCs were incubated with fresh plasma from C57BL/6 (*A*), *C1qA*$^{-/-}$ (*B*), BALB/c (*C*), or Cμ13 (*D*) mice either alone (blue curves) or together with IgM anti-SRBCs from BALB/c (green curve) or Cμ13 (orange curve) mice. SRBCs incubated with PBS (red curve) were used as a negative control. The C3 deposition on SRBCs was determined by flow cytometry after incubation with anti-mouse C3 Abs. Data are representative of eight (*A* and *B*) or three (*C* and *D*) experiments.

detected (Fig. S1*A*). IgM from the two strains had a similar profile after passage over the column, indicating that they have the same size and are assembled in a similar way (Fig. S1*A*), thus confirming previous observations (43, 45). Moreover, IgM from both Cμ13 and BALB/c mice had an HA titer of 1:128 compared at the same IgM concentrations (~0.87 mg/mL as measured by absorbance at 280 nm).

To test whether the half-life of mutant IgM was different from that of WT IgM, immune sera from Cμ13 or BALB/c mice were passively administered to naive BALB/c mice and the decline in IgM anti-SRBC titers was followed. The two IgM types had a similar half-life (Fig. S1*B*).

Lack of C-Activating IgM Does Not Abrogate Ab Responses to SRBCs or KLH. Next, the importance of C-activating endogenous IgM for primary and secondary Ab responses was investigated. Cμ13 and BALB/c mice were immunized with four different doses of SRBCs and boosted on day 21 with 5×10^6 SRBCs. For comparison, *Cr2*$^{-/-}$ mice were included. Whereas the IgG anti-SRBC response in *Cr2*$^{-/-}$ mice was severely impaired, both Cμ13 and BALB/c mice produced high levels of IgG anti-SRBCs (Fig. 4 *E*–*H*). We performed three experiments with the same setup as the one shown in Fig. 4 and one additional experiment in which only the primary response was studied. Occasionally, Cμ13 mice produced less IgG anti-SRBCs than BALB/c mice (e.g., day 49; Fig. 4*E*). However, the differences were inconsistent, usually not significant, and far from the severely impaired responses seen in the *Cr2*$^{-/-}$ mice. Data presented in Fig. 4 are from one of the two experiments in which the difference between Cμ13 and BALB/c mice was most pronounced. Analogous to what was seen in the ELISPOT assay (Table 1), the serum IgM response was slightly higher in Cμ13 mice than in BALB/c mice, particularly when lower Ag doses were used. The IgM response in *Cr2*$^{-/-}$ mice was usually slightly lower than the response in both BALB/c and Cμ13 mice (Fig. 4 *A*–*D*). Thus, lack of C-activating IgM had only a minor and inconsistent effect on the IgG response to SRBCs, whereas, as expected from previous findings (10, 12, 13), lack of CR1/2 led to severely impaired IgG responses.

Specific IgM can enhance Ab responses in a C-dependent fashion not only to SRBCs but to KLH (35). Moreover, the observations of low Ab responses in mice lacking secretory IgM, which suggested that C activation by natural IgM may explain the requirement for classic C activation, were made after immunization with hapten-conjugated KLH (36, 37). Hence, it was of interest to determine whether Ab responses to KLH were affected in Cμ13 mice. However, IgG responses to KLH were not impaired in Cμ13 mice, which, instead, had a slightly higher response than WT animals at a few time points (Fig. 5). In analogy with previous findings, the KLH response in *Cr2*$^{-/-}$ animals was severely impaired (Fig. 5). One of the most clearcut examples of an impaired response in mice lacking secretory IgM was observed after i.v. administration of 1 μg of NP-KLH (37). When we repeated this experiment and immunized Cμ13, BALB/c, and *Cr2*$^{-/-}$ mice with 1 μg of NP-KLH administered i.v., the IgG response to anti–NP-KLH was similar in Cμ13 and BALB/c mice (Fig. S1*C*).

Altogether, these observations suggest that C-activation by IgM in naive mice cannot explain the requirement for classic pathway activation for normal Ab responses to SRBCs or KLH.

Lack of SIGN-R1, SAP, or CRP Does Not Abrogate Ab Responses to SRBCs. Apart from immune complexes, proteins suggested to activate C1q in mice are CRP (41), SAP (40) and SIGN-R1 (39). Because IgM-mediated activation did not explain the C1q dependence of Ab responses, we asked whether any of the other classic activators were important. SIGN-R1 was conditionally deleted by treating BALB/c or Cμ13 mice with the SIGN-R1–specific mAb 22D1, previously shown to ablate SIGN-R1 expression in vivo (39). The treatment completely prevented localization of FITC-dextran on marginal zone macrophages known to depend on SIGN-R1 (46), thus confirming that the mAbs down-regulated SIGN-R1 (Fig. S1*D*). On immunization with SRBCs, mice in which SIGN-R1 was conditionally deleted responded equally well as the controls (Fig. 6 *A* and *B*). Again, *Cr2*$^{-/-}$ mice included for comparison responded poorly (Fig. 6*A*). Next, SAP$^{-/-}$, CRP$^{-/-}$, and WT controls were immunized with SRBCs, and their specific IgG responses were analyzed. Mice lacking CRP or SAP responded equally well to SRBCs as WT controls (Fig. 6*C*). Thus, SIGN-R1, CRP, and SAP are not the factors necessary for activating C1q and allowing a normal Ab response.

Discussion

At the onset of the current study, several reports directly or indirectly pointed toward a role for classic pathway activation for the generation of normal Ab responses (5–8, 23, 26, 27, 29). However, lack of C1q was compatible with a normal or even higher Ab response against certain Ags in some situations (24, 30), and to ascertain that C1q was important in our system, we first analyzed the Ab responses to a wide range of SRBC doses

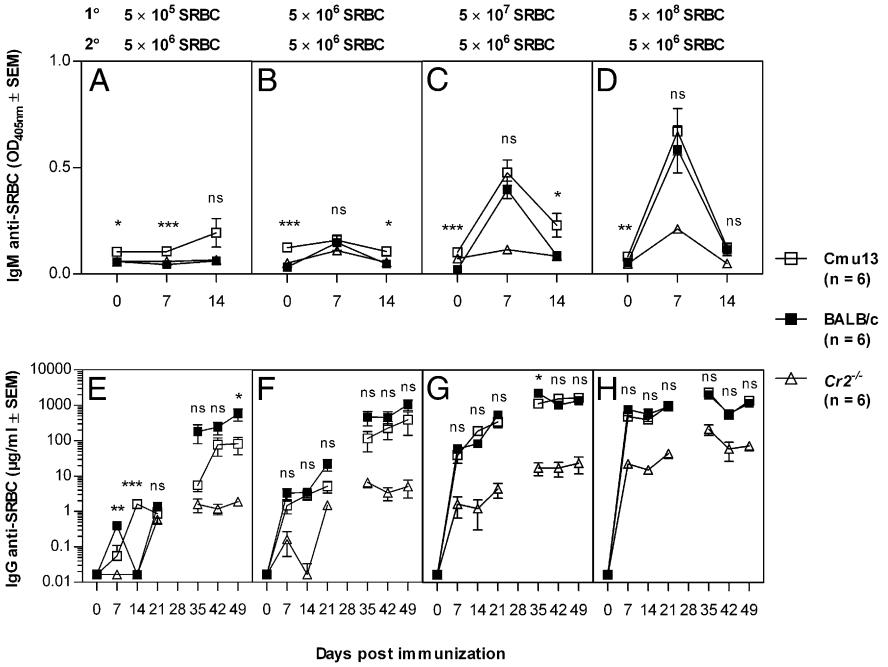

Fig. 4. Lack of C-activating IgM does not abrogate Ab responses against SRBCs. Mice were immunized i.v. with 5×10^5, 5×10^6, 5×10^7, or 5×10^8 SRBCs and boosted i.v. on day 21 with 5×10^6 SRBCs. All groups were prebled and then bled at indicated time points, and sera were screened by ELISA ($n = 6$ mice per group). (*A–D*) IgM anti-SRBC response on days 0–14. Sera were diluted 1:625. (*E–H*) Primary and secondary IgG anti-SRBC responses. Data are representative of three to four experiments. Error bars represent SEM. *P* values represent a comparison between the response in Cμ13 and BALB/c mice. ns, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

administered i.v. to *C1qA*$^{-/-}$ mice. The results confirmed and extended the observations by Cutler et al. (29) that primary and secondary IgG responses against SRBCs were indeed severely impaired in *C1qA*$^{-/-}$ mice (Fig. 1). Puzzled by the requirement of the classic pathway in primary Ab responses, wherein only low amounts of specific Ab are present to start the activation, we sought an explanation.

Based on previous observations, one possibility seemed to be that natural IgM could activate C in naive animals (i.e., in a primary response) because (*i*) one of the most potent activators of C1q is IgM; (*ii*) mice lacking secretory IgM have lower Ab responses than WT mice and can be rescued by transfer of IgM from normal mouse serum (36, 37); and (*iii*) specific IgM is known to feedback-enhance Ab responses, and this process is C-dependent (34, 35). To test the hypothesis, the Cμ13 knock-in mouse strain with a point mutation in the gene encoding the CH3 domain of the μ-heavy chain, making its IgM unable to activate C, was generated. Cμ13 mice were unable to form direct PFCs and could not induce deposition of C3 on SRBCs (Fig. 3 and Table 1). These findings agree well with earlier studies of IgM with the same proline-to-serine exchange at residue 436 of the μ-chain: IgM had a 50-fold decrease in affinity for human C1 at physiological ionic strength (43) and was unable to induce hemolysis of erythrocytes (42, 43). Thus, we conclude that the point mutation introduced in Cμ13 mice resulted in non–C-activating IgM.

Analysis of IgM with the same point mutation as the one introduced into the genome of the Cμ13 mice showed that the light chain and the μ-chain in mutant and WT IgM had the same size (45) and that >90% of both types of IgM was assembled in the

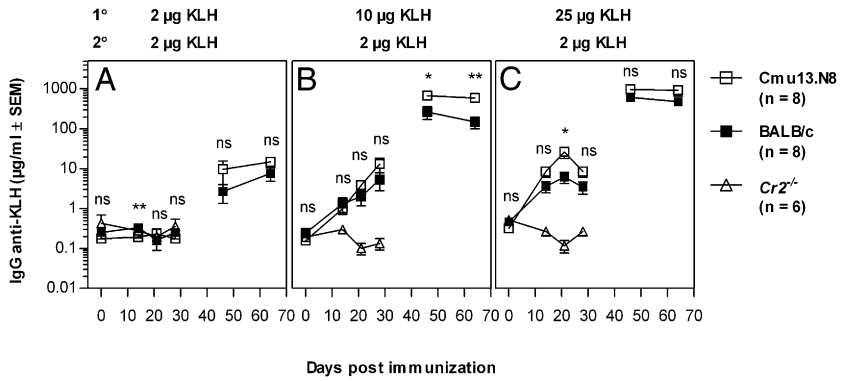

Fig. 5. Cμ13 mice do not have impaired Ab responses against KLH. Mice were immunized i.v. with 2 (*A*), 10 (*B*), or 25 (*C*) μg of KLH and boosted i.v. on day 35 with 2 μg of KLH. All groups were prebled and then bled at indicated time points ($n = 6$–8 mice per group). Sera were screened for IgG anti-KLH by ELISA. Cμ13 mice used in this experiment were backcrossed for eight generations to BALB/c mice. Data are representative of one experiment. Error bars represent SEM. *P* values represent a comparison between the response in Cμ13 and BALB/c mice. ns, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$.

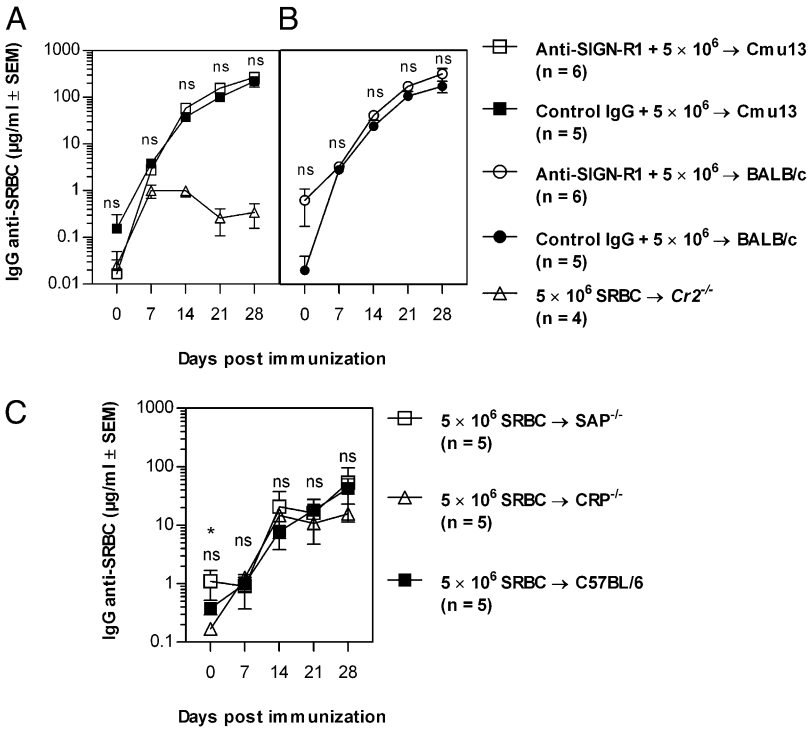

Fig. 6. Lack of SIGN-R1, CRP, or SAP does not impair the Ab response against SRBCs. Cμ13 (*A*) or BALB/c (*B*) mice were pretreated with 100 μg of 22D1 (anti–SIGN-R1) or 100 μg of hamster-IgG (control Ab) and were immunized i.v. 24 h later with 5×10^6 SRBCs in 0.2 mL of PBS. (*C*) $SAP^{-/-}$, $CRP^{-/-}$, and C57BL/6 mice were immunized i.v. with 5×10^6 SRBCs in 0.2 mL of PBS. Sera were screened for IgG anti-SRBCs by ELISA (n = 4–6 mice per group). Data in *A* and *B* are representative of four experiments, and data in *C* are representative of one experiment. Error bars represent SEM. *P* values represent comparisons between 22D1 and hamster-IgG pretreated mice (*A* and *B*) or between C57BL/6 and $CRP^{-/-}$ or $SAP^{-/-}$ mice (*C*). ns, not significant ($P > 0.05$); *$P < 0.05$.

form of pentamers (43). We show here that IgM in sera of Cμ13 and WT mice has the same size, the same ability to agglutinate SRBCs, and a similar half-life. Taken together, these observations strongly suggest that the mutant IgM does not have major structural defects.

To test the Ab response in Cμ13 mice, we chose to use SRBCs and KLH as Ags, because their dependence on C to generate normal Ab responses was previously well-studied. Additionally, the ability of IgM to enhance Ab responses has primarily been described for large Ags like KLH, SRBCs, and malaria parasites, whereas enhancement of responses to small proteins is very difficult to achieve. Hypothetically, this is explained by the requirement for IgM to activate C to be able to enhance Ab responses (34, 35): Only when the large IgM molecule binds to an Ag allowing attachment of several of its subunits (i.e., a large Ag) can it achieve the conformation change necessary to bind C1q. Surprisingly, the primary and secondary Ab responses to a wide range of concentrations of SRBCs and KLH were largely normal in Cμ13 mice (Figs. 4 and 5), whereas both $C1qA^{-/-}$ and $Cr2^{-/-}$animals had severely and consistently impaired Ab responses. These results unequivocally demonstrate that C1q is required for the generation of normal primary and secondary Ab responses but that IgM is not the major C1q activator in this situation. At some of the time points analyzed, Cμ13 mice had a significantly lower IgG anti-SRBC response than WT animals, although the levels were far from as low as those consistently seen in $C1qA^{-/-}$ and $Cr2^{-/-}$ mice. Therefore, we cannot exclude the possibility that C-activating IgM in the WT mice plays a minor role in enhancing the Ab responses, according to the original hypothesis. In our system, both membrane-bound IgM (forming part of the BCR) and secreted IgM lack the ability to activate C. The occasionally impaired Ab responses may thus also be explained by lack of C activation by the BCR, which has been shown to be important for some Ab responses (22). Another possibility is that the slightly lower numbers of $B220^+$ B cells in the Cμ13 mice would explain the tendency for a lower Ab response.

An important pillar of the hypothesis was the observation that mice lacking secretory IgM had impaired Ab responses (36–38) and that transfer of IgM from normal mouse serum rescued the response (36, 37). In these studies, the researchers did not study responses to SRBCs but to influenza virus or hapten-conjugated proteins, and this is a possible reason for the different results. In one study, 1 μg (but not 10 or 100 μg) of NP-KLH given i.v. induced a lower response in animals lacking secretory IgM than in WT animals (37). We did not find a significantly lower Ab response to 2 μg of KLH (Fig. 5) or to 1 μg of NP-KLH (Fig. S1*C*) in the Cμ13 mice. Therefore, it is unlikely that the use of different Ags explains the different results. Another pillar of the hypothesis was that specific IgM has the ability to enhance Ab responses to low doses of, for example, SRBCs and that this feedback enhancement is dependent on the ability of IgM to activate C (34, 35). It was therefore inferred that natural IgM may use this circuit to enhance very early Ab responses. Obviously, this was not the case, because Cμ13 and WT mice have similar Ab responses. This suggests that the amount of natural IgM binding with high enough affinity to SRBCs was insufficient to induce the level of classic pathway activation that can be induced by specific IgM and results in feedback enhancement (34, 35).

Although we originally considered a unified hypothesis to explain why the immune response would depend on both the requirement for C and for secreted IgM, it is also possible that two independent mechanisms are at work (i.e., that the role of natural IgM does not involve C and that C is activated by something other than IgM). For example, the role of IgM might be linked to its binding to a putative Fc receptor (47–49) or perhaps attributable to simple aggregation of the Ag, thereby making it more

immunogenic. Moreover, mice lacking secretory IgM have developmental defects in their B-cell compartment secondary to the lack of secretory IgM, and it is conceivable that this explains their impaired Ab responses (37, 50).

The classic pathway can be activated not only by IgM but by IgG. A few early studies reported that IgG could sometimes act to enhance Ab responses to SRBCs. However, investigators using monoclonal IgG (excluding the risk of contaminating IgM) find that IgG Abs of all subclasses suppress Ab responses to SRBCs (51–53). These data suggest that any presence of IgG anti-SRBCs in naive mice would lead to a lower rather than higher Ab response. Moreover, two or more IgG molecules are required to create high enough avidity to bind C1q and initiate the classic pathway activation (54). The probability that naive mouse serum would contain enough IgG anti-SRBCs to result in such high density of IgG on the SRBC surface appears small. Thus, we find it extremely unlikely that IgG would be involved in the generation of primary Ab responses through C activation.

Because IgM-mediated C activation was dispensable for the generation of normal Ab responses, we tested whether other endogenous proteins known to activate C1q (i.e., CRP, SAP, SIGN-R1) (39–41) played a role. KO mice lacking CRP or SAP had normal Ab responses. Likewise, WT or Cμ13 mice in which SIGN-R1 was conditionally deleted by treatment with a SIGN-R1–specific mAb had normal Ab responses. Thus, the role of the classic pathway in generating Ab responses could not be explained by CRP, SAP, SIGN-R1, or SIGN-R1 in combination with C-activating IgM. In addition, we have excluded the possibility that SRBCs by themselves would activate C in the presence of mouse plasma (Fig. 3, blue curve).

The most surprising finding in this report was that C activation by IgM did not play a significant role in Ab responses despite the fact that C1q was indeed required. In addition, none of the other endogenous classic activators tested here influenced the Ab response. Lack of CR1 and CR2, which are receptors for C3 fragments, leads to similar impairment of Ab responses as lack of C1q, C3, C2, and C4. Therefore, the possibility that C1q, per se, is the effector molecule central for Ab responses seems unlikely, and its role is probably to start the cascade generating the C3 split products that are ligands for CR1/2. Several mechanisms have been suggested to explain the role of these receptors in Ab responses, including the following: (*i*) increased B-cell signaling by co–cross-linking of the BCR and CR2/CD19 coreceptor complex by Ag–C complexes (20, 21), (*ii*) enhanced Ag presentation to T-helper cells via increased CR1/2-mediated uptake of Ag–C complexes by B cells (19), or (*iii*) more efficient localization of Ag–C complexes on the $CR1/2^+$ FDC in B-cell follicles (14, 16–18). The latter could be a combination of transport of Ag-C from the marginal zone to the follicles by $CR1/2^+$ marginal zone B cells and subsequent focusing of Ag on the FDC. Regardless of which mechanism is operational, activation of the classic pathway obviously must take place to generate the C3 split fragments required for Ab responses. However, in the present study, we were unable to define this classic pathway activator. One possibility is that a combined defect of two or more of the tested classic activators would prove to have a more dramatic effect than the isolated lack of any one factor. Another possibility is that the spontaneous low-level activation of C1, primarily mediated by soluble IgG, is sufficient to activate the classic pathway to the level required for normal Ab responses (55). Finally, a hitherto unknown murine classic pathway activator may be involved.

Materials and Methods

Mice. BALB/c mice were obtained from Bommice, and C57BL/6 mice were obtained from Jackson Laboratories. Mice lacking CR1/2 ($Cr2^{-/-}$) (13) were backcrossed for 10 generations to BALB/c mice (56). $C1qA^{-/-}$ mice (57) were obtained from Marina Botto (Imperial College London, United Kingdom), and $SAP^{-/-}$ (58) and $CRP^{-/-}$ (59) mice were obtained from Mark Pepys (Imperial College London, United Kingdom). All animal experiments were approved by Uppsala Animal Research Ethics Committee. The mice were bred and maintained in the animal facilities at the National Veterinary Institute (Uppsala, Sweden). Animals were age- and sex-matched within each experiment.

Cμ13 PCR Assay. Cμ13 mice were constructed as described in *Results* and were backcrossed to BALB/c mice for 12 generations. Mice carrying the mutation were screened by PCR assay. Tail DNA was obtained using a tissue genomic kit (Viogene, Inc.), and the PCR assay was done with the following primers: CU13F (5′-AGGAGCCTCTGTAAGGAGTC-3′) and CU13R (5′-TGGGTCTTGGTACCAAGAGA-3′). Using reagents from Applied Biosystems, the gene amplification was performed in a 25-μL PCR mixture containing 10× PCR buffer, 4 mM $MgCl_2$, 20 mM dNTP, 10 μM each primer, 0.06 U/mL AmpliTaq DNA polymerase, and 1 μL of DNA (4 min at 94 °C, 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C; repeat previous steps for 35 cycles; and 8 min at 72 °C). PCR products were run in electrophoresis on a 1.5% (wt/wt) agarose gel in 1× Tris-acetate-EDTA buffer at 100 V. The Cμ13 fragment gave a 360-bp band, and the WT fragment gave a 200-bp band.

Ags. SRBCs were purchased from the National Veterinary Institute (Håtunaholm, Sweden) and stored in sterile Alsever's solution at 4 °C. Before use, SRBCs were washed in PBS three times. KLH and FITC-dextran were obtained from Sigma–Aldrich, and NP-KLH was obtained from Biosearch Technologies.

Immunizations and Blood Sampling. All injections were in one of the lateral tail veins, with the indicated Ag and Ab doses diluted in 0.2 mL of PBS unless otherwise stated. Mice were bled from their tails or retroorbital plexa at indicated time points, and sera were stored at −18 °C before analysis.

Abs. For flow cytometry, the following mAbs were used: phycoerythrin (PE)-Cyanine 5 (Cy5)–labeled rat IgG2a anti-CD45R (B220, clone RA3-6B2), PE-Cy5–labeled rat IgG2b anti-CD11b (Mac-1α, clone M1/70), and allophycocyanin-labeled rat IgG2a anti-CD5 (Ly-1, clone 53-7.3) (all from eBioscience) as well as PE-labeled rat IgG2a anti-CD23 (FcεRII, clone B3B4), FITC-labeled rat IgG2b anti-CD1d (CD1.1, Ly-38, clone 1B1), FITC-labeled rat IgG2a anti-CD19 (1D3), biotinylated rat IgG2a anti-IgM (clone R6-60.2), and rat IgG2b anti-CD16/CD32 (clone 2.4G2) (all from BD Pharmingen). FITC-labeled sheep anti-mouse C3 (The Binding Site) was used to detect C3 deposition on SRBCs. For ELISA and ELISPOT assay, sheep anti-mouse IgG and goat anti-mouse IgM, both conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories), were used.

Anti–SIGN-R1–specific mAbs were derived from 22D1 hybridoma cells (a kind gift from Chae Gyu Park, The Rockefeller University, New York) (46) cultured in DMEM (Sigma–Aldrich) with 5% (vol/vol) FCS (Sigma–Aldrich). Supernatant was collected, and IgG Abs were affinity-purified on a protein A Sepharose column according to the manufacturer's instructions (Pharmacia, Inc.). Control IgG was derived from normal Syrian hamster sera (Jackson ImmunoResearch Laboratories) affinity-purified on protein A Sepharose. Ab concentrations were measured as absorbance at 280 nm, where OD 1.5 = 1 mg/mL.

IgM Anti-SRBC Purification. IgM precipitates as euglobulin in distilled water (32, 60). Five milliliters of serum taken on day 5 from either BALB/c or Cμ13 mice immunized i.v. with 0.2 mL of 10% (vol/vol) SRBC suspension was dialyzed against 5 L of demineralized water (pH 5.5) overnight at 4 °C. The serum was then centrifuged at 22,000 × *g* for 30 min, resuspended in 10 mL of demineralized water, and washed twice by means of centrifugation as above. The pellet was dissolved in 5 mL of PBS with 0.04% NaN_3 and centrifuged at 15,700 × *g* for 1 min. The supernatant was collected and adjusted to 5 mL with PBS with 0.04% NaN_3 and run over a protein A Sepharose column to remove remaining IgG. Fractions passing the column were collected, and those containing protein (IgM), as measured by OD at 280 nm, were pooled and concentrated by spin columns (Macrosep 50K; Pall Filtron), dialyzed against PBS, sterile-filtered, and stored at 4 °C until use. The IgM preparations contained no detectable IgG anti-SRBCs as measured by ELISA.

Sepharose CL-6B Chromatography. BALB/c and Cμ13 mice were immunized with 10% (vol/vol) SRBCs and bled 5 d later. Five milliliter of sera from each strain was centrifuged for 5 min at 1,224 × *g* and filtered through a 0.45-μm filter (VWR). Serum was loaded onto a 1,000-mL Sepharose CL-6B column (GE Healthcare) equilibrated with PBS and 0.04% NaN_3 (Sigma–Aldrich). The

column was adjusted to the recommended flow rate 0.3 mL/min, and fractions of 9 mL were collected. Each fraction was then analyzed for IgM and IgG anti-SRBCs in ELISA. Fractions containing IgM were pooled and treated as above.

HA Assay. Fifty microliters of purified IgM (collected after passage over Sepharose CL-6B) was serially diluted in PBS in V-bottomed microtiter plates (Greiner Bio-One GmbH). A 1% (vol/vol) SRBC suspension (25 μL) in PBS and 25 μL of PBS were added to each well, and plates were incubated at 37 °C for 1 h. The HA titer was defined as the highest dilution in which HA of SRBCs was still detected.

Flow Cytometry. Spleens from BALB/c and Cμ13 mice were meshed through a nylon screen, and cells were suspended in 10 mL of PBS. After centrifugation, erythrocytes were lysed in hypotonic buffer [0.15 M NH_4Cl (Merck), 1.0 mM $KHCO_3$ (Sigma–Aldrich), and 0.1 mM Na_2EDTA (Sigma–Aldrich), pH 7.3] for 5 min at 4 °C. For preparation of peritoneal cells, the peritoneum of mice was filled with 5 mL of ice-cold PBS and 3–4 mL was extracted back. Cells were washed once and resuspended in 3 mL of PBS containing 2% (vol/vol) FCS, and 100 μL (~1×10^6 cells) was transferred into tubes for flow cytometry (BD Falcon; BD Biosciences). To block Fc receptors, cells were incubated with anti-CD16/CD32 (0.05 μg/mL) at 4 °C for 5 min. Thereafter, cells were stained with a mixture of anti-B220 (2 μg/mL), anti-CD23 (2 μg/mL), and anti-CD1d (5 μg/mL) or with a mixture of anti-CD11b (2 μg/mL), anti-CD5 (2 μg/mL), and anti-CD19 (5 μg/mL) for 30 min at 4 °C. For detection of surface IgM, biotinylated anti-IgM (5 μg/mL) was used together with streptavidin-PE (2 μg/mL; Pharmingen). After incubation, cells were washed once in PBS containing 2% FCS. The cells were counted on an LSRII flow cytometer (BD Biosciences) and analyzed with Flow Jo software (Tree Star, Inc.).

C3 Deposition on SRBCs. To obtain mouse plasma as a C source, 1.5 mL of blood from retroorbital plexa of unimmunized mice was collected into tubes containing 1.5 μL of lepirudin (50 mg/mL, Refludan; Celgene AB) and immediately put on ice (61). Lepirudin is an anticoagulant reported not to activate C (62). Tubes were centrifuged at 11,200 × *g* for 5 min at 4 °C; plasma was then removed and kept on ice. All experiments with plasma were performed the same day as it was collected. Packed SRBCs (10 μL) were incubated for 30 min at 37 °C, together with 45 μL of plasma and 45 μL of PBS or 45 μL of plasma and 45 μL of IgM anti-SRBCs. IgM anti-SRBCs were from BALB/c or Cμ13 mice and were adjusted to an HA titer of 1:4. The erythrocytes were washed three times in PBS with 2% (vol/vol) FCS and put on ice. One microliter of SRBC pellet was transferred to FACS tubes (BD Biosciences) and incubated with anti-mouse C3 on ice for 30 min. SRBCs were then washed three times, counted on a FACScan (BD Biosciences), and analyzed with Flow Jo software.

PFC Assay. A modified version (63) of the Jerne hemolytic PFC assay (64) was used. Briefly, agarose, SRBCs, guinea pig serum (as a source of C), and spleen cells were plated on a glass slide and incubated at 37 °C for 3 h. Subsequently, hemolytic plaques were counted blindly.

ELISPOT Assay. Microtiter plates (Immulon 2HB; Thermo Electron Corporation) were coated with SRBCs as described earlier (65) and blocked with cell culture medium (DMEM) with 5% (vol/vol) FCS overnight at 4 °C. Spleen cells were washed twice in PBS and resuspended in DMEM with 5% (vol/vol) FCS. Appropriately diluted cell suspension in 100 μL DMEM with 5% (vol/vol) FCS was added to the plates and incubated at 37 °C for 3 h in 5% (vol/vol) CO_2. Plates were washed in PBS three times, and 50 μL of anti-mouse IgM-alkaline phosphatase diluted 1:500 in PBS containing 0.05% Tween-20 (VWR), 0.25% dry milk (Semper AB), and 0.04% NaN_3 was added and incubated overnight at 4 °C. Following three PBS washes, 50 μL of 5-bromo-4-chloro-3-inodyl phosphate substrate (BCIP/NBT; Sigma–Aldrich) was added and incubated for 60 min at room temperature in a dark chamber. Finally, the plates were washed once in dH_2O and left to dry, and the number of spots was counted blindly under a stereomicroscope.

ELISA. The IgG anti-SRBC and anti-KLH ELISAs were performed as described earlier (65) with the following modifications: microtiter plates were used (Immulon 2HB); plates were blocked with 200 μL of 5% (wt/wt) dry milk in PBS at room temperature 2 h or overnight at 4 °C before use; and sera or Abs were diluted in PBS containing 0.05% Tween-20, 0.25% dry milk, and 0.04% NaN_3. IgM anti-SRBC ELISAs were performed as above but using goat anti-mouse IgM-alkaline phosphatase. For IgG anti–NP-KLH ELISAs, plates were coated with 100 μL of 10 μg/mL NP-KLH at 4 °C overnight and the assay was performed in the same way as the IgG assay described above. The absorbance at 405 nm was read after 30 (IgG anti-SRBCs) or 60 (IgM anti-SRBCs) min. Results are given either as absorbance or μg/mL after calibration against a standard curve with hyperimmune polyclonal IgG anti-SRBCs or anti-KLH, purified over a protein A Sepharose column (Pharmacia, Inc.). Data were analyzed with SOFTmax (Molecular Devices) or Magellan (Tecan Group) software. When data are shown as absorbances, dilutions were chosen such that the OD values did not reach plateau levels.

Confocal Microscopy. Mice were given 100 μg of 22D1 or hamster IgG administered i.v. and were immunized with 100 μg of FITC-labeled dextran 24 h later. One hour after immunization, spleens were removed and flash-frozen in optimal cutting temperature (OCT) embedding media (Sakura Finetek). Eight-micrometer sections were cut with a cryostat, thaw-mounted on Super frost plus glass slides (Menzel-Gläser), and air-dried for 2 h. The slides were mounted in Fluoromount G (Southern Biotech) and analyzed with an LSM 510 META confocal microscope (Carl Zeiss).

Statistical Analysis. Statistical differences among groups were determined by the Student *t* test. Statistical significance levels were set as follows: not significant, $P > 0.05$; $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

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