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produces Abs that play a homeostatic role (14, 15). Thus, the transition from this natural and benign self-reactivity to a pathological one is likely to involve memory formation and IgG subclass switching and also potential alterations in the AC clearance induced by the Abs.

To address these questions and study autoimmune memory to AC-derived self-antigens, we used wild-type (WT) mice where immune tolerance is broken by multiple injections of syngeneic ACs without adjuvant (9). When the initial response had resolved, we found that a single injection of ACs could recall the immune response, and for some autoantigens even increase it. This autoimmune immune memory was transferable to naive mice and was also responsible for the induction of lupus-like pathology. This is in contrast with the response without end organ involvement observed at the initial break of tolerance. Our findings support the evaluation of the autoreactive immune memory compartment as a possible therapeutic target for treatment of SLE.

Materials and Methods

Mice

C57BL/6 (B6) mice from The Jackson Laboratory were bred and maintained under specific pathogen-free conditions at Karolinska Institutet animal facility in accordance with local ethical guidelines. All animal experiments were approved by the local ethics committee (the North Stockholm District Court).

Apoptosis induction and in vivo administration of ACs and nuclear protein–OVA

For generating ACs, thymocytes from 4- to 5-wk-old sex-matched B6 mice were cultured for 6 h in complete RPMI 1640 media (Invitrogen) plus 1 μM dexamethasone (Sigma-Aldrich) at 37°C and 5% CO2. Annexin V and 7-aminactinomycin D (BD Biosciences) staining confirmed apoptosis (typically ~70% of the thymocytes were apoptotic and 2% necrotic). Approximately 2 × 10^5 ACs per mouse were injected i.v., and 50 μg per mouse on 4-hydroxy-3-nitrophenyl (NP)-OVA (Biosearch Technologies) in aluminum hydroxide (Thermo Fisher Scientific) was injected i.p.

ELISA

Serum Ab levels were measured by ELISA as previously described (16). Briefly, ELISA plates were precoated with methylated BSA and calf thymus DNA (Sigmaid-Aldrich), phosphorylcholine-BSA (provided by Athena Biotechnologies), or NP-BSA (Biotech Sciences). The Ab reactivity in serum was measured with alkaline phosphatase–conjugated anti-mouse IgG/IgM/IgG1/IgG2c/IgG2b/IgG3 mAbs (SouthernBiotech). In some conditions, macrophages were pretreated with 1:200 anti-mouse CD16/CD32 mAbs (BD Biosciences) or mouse sera were pretreated with Protein G Mag Sepharose Xtra and IgG was removed by magnetic capture. All samples were run in duplcates and corrected for background binding.

Immunofluorescence and anti-nuclear Ab test

Cryosections (8 μm) were fixed in acetone and stained with purified or biotinylated primary mAbs, followed by fluorescently labeled secondary Abs or streptavidin. The following anti-mouse mAbs were used: biotin-IgG1/IgG2a/IgG2b/IgG3/IgM, F4/80, FITC-IgG1/IgM (Biologend); anti-T15 (clone Mal5-4); allophycocyanin-PE-B220, PE-IgG2a+2b (BD Biosciences); Alexa Fluor 488–IgG1 (Invitrogen). Staining specificity was confirmed by isotype staining. Anti-nuclear Ab (ANA) tests were performed to control the specificity of the amplification. Relative expression level of a certain gene was calculated with the Pfaffl formula using gpdh as a reference gene.

Fluorescent labeling and serum coating of ACs

For fluorescent labeling of ACs, freshly isolated thymocytes were labeled with lipid dyes pkh26/pkh67 (Sigma-Aldrich) or CFSE (Molecular Probes) according the manufacturers’ instructions before apoptosis induction. For coating of ACs with sera, 10^9/ml ACs were incubated with indicated sera at a dilution of 1:50 in 500 μl of PBS on ice for 30 min and then washed three times with PBS.

In vitro phagocytosis assay and in vivo clearance of AC

Bone marrow–derived macrophages were generated from B6 mice by culturing bone marrow precursor of 1 × 10^5 cells/ml in complete RPMI 1640 medium (Invitrogen) containing 20% M-CSF–conditioned medium. The phagocytosis assay was performed in serum-free RPMI 1640 medium and analyzed by confocal microscopy or flow cytometry. The phagocytosis index was calculated as: [mean fluorescence intensity [MFI] of pkh26+ in F4/80+ gating] × (frequency of F4/80+ and pkh26+ cells)/100 (here the ACs were labeled with pkh26) as previously described (19). The AC/macrophage ratio was 5:1. All phagocytosis assays were run in triplicates. In some conditions, macrophages were pretreated with 1:200 anti-mouse CD16/CD32 mAbs (BD Biosciences) or mouse sera were pretreated with Protein G Mag Sepharose Xtra and IgG was removed by magnetic separation (GE Healthcare Life Sciences).

Autoantigen microarray

Serum autoantibody profiles were detected by microarray containing 98 autoantigens (University of Texas Southwestern Medical Center). Array and analyses were done by using protocols established in the genomics and microarray core facility in the University of Texas Southwestern Medical Center (20). MeV 4.8.1 software was used for generating heat map and Ap reactivity clusters.

Avidity assay

ELISA plates were precoated with phosphorylcholine-BSA (provided by Athena Biotechnologies). The plates were subsequently incubated with serum samples from either day 7 after the primary, first, or second boost response. The plates were then incubated with different concentrations of sodium thiocyanate (0–3 M) for 15 min. Bound IgG was identified with alkaline phosphatase conjugated anti-mouse IgG mAbs (Southern Biotech). The OD value obtained at 0 M sodium thiocyanate was considered 100% bound IgG.

Statistical analysis

The differences between two groups and among more than two groups were analyzed by Mann–Whitney and one-way ANOVA tests, respectively. The calculations were performed with GraphPad Prism 5 software. A p value <0.05 was considered significant.

Results

Persistent exposure of mice to ACs breaks immune tolerance and induces a memory-like IgG autoantibody response

WT mice are tolerant to ACs, but multiple injections with ACs can break this tolerance and cause an Ab response (9). In this study, we
investigated the potential for development of immune memory to self-antigens in WT mice by injections with syngeneic ACs. The immune responses were investigated at day 7 after the corresponding injections (Fig. 1A).

The serum levels of IgG autoantibody against DNA and phosphorylcholine (PhC), two AC-derived self-antigens relevant for SLE, were transiently increased after the 5 weekly injections and then declined to preimmune (pi) levels after about 1 mo (Fig. 1B). Interestingly, at this stage a single injection could rapidly evoke IgG production, resembling a typical memory Ab response to foreign Ags. The serum IgM of anti-DNA and anti-PhC also showed increased levels of activation compared with the primary response. The secondary boost response also declined and was transient with similar kinetics as the first boost (data not shown). As a control, a separate group of mice also received NP-OVA after apoptotic cell injections and the anti-NP IgG levels were similar between mice with or without prior AC injections (Supplemental Fig. 1A, 1B), showing that the memory-like Ab response was specific to PhC and DNA, and not due to a general alteration of immune reactivity.

To test whether the memory-like boost response had the potential to generate SLE-like manifestations, we examined kidney
Ig deposition, serum ANA, as well as histologic status of the kidneys. The Ig depositions of IgM, IgG1, and IgG2a+2b, but not IgG3, were detected in kidneys of mice at the second boost response, but absent or low in kidneys of mice having primary or the first boost response, respectively (Fig. 1C). Also, serum Abs giving rise to a robust ANA positivity were only detected in sera from mice after the second boost injection. Three ANA patterns were found among seven mice, including nuclear, cytoplasmic, and nuclear plus cytoplasmic patterns (Fig. 1D). When scoring the histologic status of kidneys from unimmunized mice compared with mice that received the second boost of ACs there was a significant difference in the glomerular architecture, where glomerulus in the second boost mice showed evidence for hypertrophy and mesangial thickening (Fig. 1E). A classical adaptive memory response would also include affinity maturation; however, we did not find evidence for this either in the first or second boost response when testing the affinity for anti-PhC in an ELISA assay (Supplemental Fig. 1C). Thus, activation of the memory-like response appears to be more pathogenic than the response of initial break of tolerance but without signs of affinity maturation, at least not to PhC. This shows how the progression of the systemic autoreactive immune response to ACs proceeds with an initial break of tolerance and a rapid secondary response followed by ANA reactivity and deposition of immune complexes in the kidney.

The memory-like response is characterized by IgG subclass switching and increased activation of the adaptive immune response

Next, we further characterized the memory-like boost response. We found increased subclass switching to splenic IgG2a+ and IgG2b+ PCs (CD138+B220lo) after both the first and second boost. There was a decrease in IgG1+ plasma cells following the first boost injection, and this population increased again after the second boost (Fig. 2A, Supplemental Fig. 2A). The increase in switching was a decrease in IgG1+ plasma cells following the first boost and uninjected mice were included as controls. One day after transfer, all groups of mice were injected with ACs and the resulting immune responses were compared (Fig. 3A). The results showed that serum anti-DNA IgG was only significantly increased in the M-trsf mice that received a boost injection and not in the N-trsf mice or M-trsf mice that did not receive the boost (Fig. 3B). In more detail we could also show that anti-DNA IgG1/ IgG2a/IgG2b, but not IgM, were rapidly increased and remained until day 27 after injection in M-trsf mice but not in N-trsf mice (Fig. 3C). The anti-PhC Ab responses showed a similar pattern as anti-DNA Ab responses (Supplemental Fig. 3A). Spleens were examined at day 10 after injection with ACs, and increased frequencies of PCs with predominance of IgG2a and IgG2b subclasses (Fig. 3D), GC B cells, and Th cells (Fig. 3E) were found in M-trsf mice, compared with the control groups. Staining of spleen sections with anti-T15 mAb showed large extrafollicular foci in M-trsf mice but not in control mice (Fig. 3F). Similarly, in line with the Ab response, expansion of IgG1+/IgG2b+ extrafollicular foci were found in spleens of M-trsf mice but not in N-trsf mice (Supplemental Fig. 3B). In summary, the transfer experiment demonstrates the induction of memory to self-antigens by persistent exposure to ACs. After transfer into WT mice and upon re-exposure to ACs, these memory B cells generate extrafollicular foci and initiate rapid production of subclass-switched IgG autoantibodies.

The auto-IgG specificity to AC-derived self-antigens in the memory response is selective

ACs are complex Ags carrying different types of epitopes that could activate both specific and polyreactive immune responses. To define the serological spectrum of Ab autoreactivity in this model, we performed an autoantigen microarray (20, 23). Sixty-one auto-IgGs meeting minimal normalized fluorescence intensity requirements were summarized in a heat map (Fig. 4A) (20). An ANOVA test identified 19 auto-IgGs differently expressed among the three groups, shown in five autoantigen reactivity clusters (cluster 1: nucleosome Ag, Smd3, SS-A/SS-B, Sm/RNP, SRP54, gialin, vitronectin; cluster-2: ribo-phospho-protein P1, collagen I, collagen II; cluster 3: M2 Ag, thyroglobulin, TTG; cluster 4: enantactin, ribo-phospho-protein P0; cluster 5: heparan sulfate, laminin, prothrombin protein). The strong responses (normalized fluorescence intensity > 500) were significantly increased in the primary response and further increased in memory response (Fig. 4B). Serum levels of IgM autoantibodies were not significantly different among the three groups (data not shown). Taken together, these data show that the autoreactive memory IgG response is associated with selective self-antigen specificities.

Auto-IgG induced by memory to ACs perform feedback regulation

All established Ab responses result in feedback regulation of the response to subsequent encounters with the same foreign Ags (24). For self-antigens, this is complicated by the fact that there are natural Abs that maintain homeostasis without evoking an immune response (14). To study how the shift in the response altered this balance, ACs were therefore coated with pi, primary, or memory sera and used for in vitro and in vivo assays. We found that the opsonization capacity of IgG in AC-immunized serum was better than IgG in pi serum, as shown by MFI of anti-IgG surface staining on serum-coated ACs. This was also the case for serum from SLE patients, compared with healthy controls (Fig. 5A). We then examined phagocytosis by adding equal numbers of pi-labeled (red, pkh26+) and primary serum (green, pkh67+)–coated ACs into bone marrow–derived macrophage cultures at 37˚C for 1 h. This assay showed that primary serum-coated ACs were preferred targets for phagocytosis (Fig. 5B). To verify that the observed increase in macrophage phagocytosis was caused by specific serum IgGs, we pretreated sera with protein G to remove IgG or macrophages with anti-CD16/32 mAb (Fig. 5C). Flow cytometry analysis showed that
these two treatments significantly decreased phagocytosis, suggesting that the sera targeted ACs to potentially activating FcγRs. We next tested how auto-IgGs contributed to in vivo AC detection and clearance. To do this, four groups of WT mice were injected with ACs weekly for 0, 1, or 4 wk or 4 wk plus a boost injection. At day 7 after the corresponding final injections, the mice were injected i.v. with fluorescently labeled ACs and 45 min later the AC counts per 100 μl of blood were measured by flow cytometry. These results show that AC clearance was most rapid in mice that received the boost injection (Fig. 5D). That the in vivo removal of ACs coated with boost serum was more efficient than in the phagocytosis assay could be due to additional phagocytic cells and receptor systems working in combination, including those of DCs, which have been shown to be able to engulf apoptotic debris in the spleen (25).

FIGURE 2. The memory-like boost response is characterized by IgG subclass switch and increased activation of the adaptive immune response. (A–C) Flow cytometry analysis of splenic cells of mice with pi, primary, boost, and second boost responses for the frequencies of IgG2a+2b+ and IgG1+ PCs (CD138+B220+), GC B cells (CD95+GL7+CD19+), and Tfh cells (CD11b+ CD8+ B220− CD4+CD62L+CXCR5+PD1+). (D) Representative images of triple staining with anti-T15 (green), anti-IgG (red), and anti-B220 (blue) mAbs on fixed cryosections of spleen specimens from mice with pi, primary, and boost responses. Original magnification ×20. (E) Flow cytometry analysis (MFI) of MHC class II on plasmacytoid DCs (pDCs; CD11c+CD11b−B220+) and myeloid DCs (mDCs; CD11c+CD11b+B220−) (n = 5–9 per group). *p < 0.05, **p < 0.01 by Mann–Whitney U test.
Next, we investigated the influence of auto-IgG on the immune responses to ACs. Serum-coated ACs from the different groups were injected into WT mice, and their serum anti-DNA IgG levels were followed. We found that the memory serum-coated ACs induced the strongest increase in anti-DNA IgG production compared with the other groups (Fig. 5E). Through the comparison of primary serum coated with and without protein G pretreatment, we observed that this effect was, at least in part, attributed to the...
IgG component. At day 7 after the fourth injection, spleens were examined. Increased MHC class II expression on B cells and increased frequency of GC B cells in AC-immunized serum-coated groups were found compared with the pi serum-coated group (Fig. 5F). Immunofluorescence confirmed the presence of GC structures in the primary and boost serum-coated groups. Additionally, extrafollicular foci structures were present in the boost serum-coated group (Fig. 5G). These results demonstrate a positive feedback mechanism of auto-IgG on autoreactive B cell responses, as has been shown for that of non–self-antibodies (24).

Discussion
The studies of classic spontaneous lupus mouse models have led to identification of susceptibility loci and candidate genes associated with human lupus (2, 26). However, these models have limitations in providing information on the sequential immune mechanisms in lupus pathogenesis and how AC-derived self-antigens drive these changes. In this study, we report that the persistent exposure of WT mice to apoptotic Ags breaks immune tolerance and induces auto-IgG production. This was previously demonstrated by Mevorach et al. (9) for anti-cardiolipin Abs, where they found that a reimmunization gives rise to a second peak in reactivity. They also reported ANA positivity in the primary response, which is something that we did not observe. In this study, with the dose of ACs used, the primary autoantibody response was transient and did not have the potential to induce pathogenicity. This observation resembles the situation in patients where increased autoantibody levels can be detected years before the first symptom appears (2, 6).

However, we found that when the mice were re-exposed to one dose of ACs by boost injections, they responded rapidly with positive ANA tests and kidney pathology. We also detected an increased frequency of GC B cells and Tfh cells, switching to IgG2a and IgG2b subclasses, and serum levels of IgG autoantibodies against selective autoantigens. These phenotypes indicate that in this model, autoantibodies are subjected to somatic hypermutation and class switch recombination, although we did not find any signs of affinity maturation, at least to PhC. It will be interesting for future studies to determine whether this is the case for other AC-derived self-antigens. Importantly, however, our data show that self-antigens alone can induce a classical GC reaction, which gives rise to memory B cells and PCs, similar to the situation observed with foreign Ags (27). More direct evidence for this comes from the...
transfer experiments where ACs were capable of inducing an immune response when memory lymphocytes were transferred. This is also supported by the induction of large extrafollicular foci upon re-exposure to ACs. Also, note that memory T cells that would also be supported by the induction of large extrafollicular foci upon re-exposure to ACs. Also, note that memory T cells that would also be transferred in these experiments, and their contribution to the memory to self-antigens will be interesting to investigate in future studies.

To obtain a full spectrum of the evolving memory to self-antigens, we performed an autoantigen microarray. The analysis detected serum auto-IgGs specific to 19 autoantigens enriched in sera of memory response mice. The Ag with the highest reactivity was anti–Sm/RNP IgG. Importantly, anti–Sm/RNP IgG is one of the anti-nuclear IgGs with the most clinical significance and thus is included in serological diagnostic criteria (28). Another hypothesis is that this is due to that affinity maturation reached a threshold level before tissue pathology can be observed. As a part of this study we also wanted to determine how the autoantibody response feedback regulates subsequent responses to self-antigens. IgGs against foreign Ags play protective roles in facilitating removal of invading microbes, as well as feedback on subsequent B cell responses through binding of IgG subclasses to corresponding FcγRs on B cells and innate immune cells (24, 35). Alternatively, auto-IgGs against myosin, laminin, and heparan sulfate, which have been reported to have pathogenic significance in lupus nephritis (32–34), were only increased to very low levels in the first boost response and not in the primary response. Taken together, these differences possibly explain the lack of kidney pathology in the primary and first boost response. Because IgG deposits in kidney as well as alterations in glomerular tissue architecture started to become apparent in the second boost response, it is possible that the production of these auto-IgGs must reach a threshold level before tissue pathology can be observed. Another hypothesis is that this is due to that affinity maturation taking a longer time to develop in self-responses to ACs.

FIGURE 5. Feedback regulation of IgG autoantibodies in response to ACs after memory induction. (A) The binding capacity of serum IgG to ACs was measured by flow cytometry, shown as MFI of anti-IgG surface staining on ACs (n = 7–9 per mouse group, n = 5–15 per human group). HC, healthy control. (B) Representative confocal image of macrophage phagocytosis of ACs. Equal numbers of pi serum-coated (pkh26+ in red) and primary serum-coated (pkh67+ in green) ACs were mixed and added to macrophage (F4/80+ in blue) cultures at 37°C for 1 h. (C) Macrophage phagocytosis index of ACs, analyzed by flow cytometry. Dotted line indicates phagocytosis index of ACs; white bars indicate phagocytosis index of ACs coated with indicated serum; gray bars indicate phagocytosis index of serum-coated ACs, where sera were pretreated with protein G to remove IgG components (p< 0.001). At day 7 after the fourth injection in (E), spleens were examined for B cell responses by flow cytometry analysis for MFI of MHC class II on B cells and frequency of GC B cells (n = 3 per group). *p < 0.05, **p < 0.01, ***p < 0.001 by Mann–Whitney U test.
whereas IgG1 preferentially engages the inhibitory FcγRIIB. We show that similar to IgGs against foreign Ags, the auto-IgGs also facilitate the removal of ACs from the circulation, at least in part through FcγR-mediated mechanisms. In this regard, we speculate that increased switching to IgG2a and IgG2b subclasses in the memory response could contribute to the increased response to ACs by binding to and activating FcγR. Indeed, when we performed the same injection scheme but with serum-coated ACs, we found that compared with pi serum-coated ACs, memory serum-coated ACs exacerbated in vivo auto-IgG production and give rise to expansion of extral follicular foci in the spleen. These effects are partially abrogated by protein G pretreatment, indicating the contribution of IgG components. Increased switching to IgG2a and IgG2b subclasses in memory responses probably plays a role in these effects by positive feedback regulation of the autoreactive B cell response.

Taken together, our findings shed light on the development of memory to AC-derived self-antigens. We show that the response of initial break of tolerance is benign, whereas the autoreactive memory response is linked to pathogenicity. We demonstrate that it is likely due to selective memory for certain Ag specificities and IgG subclass switch. We suggest using this lupus model to test how to steer the response to self-antigens away from the pathogenic memory. Also, this model can be used to study flare courses of the disease as well as how existing and novel treatments directly affect these.

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Disclosures

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