Brief Definitive Report

Delayed Apoptotic Cell Clearance and Lupus-like Autoimmunity in Mice Lacking the c-mer Membrane Tyrosine Kinase

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Abstract

Mice lacking the membrane tyrosine kinase c-mer have been shown to have altered macrophage cytokine production and defective phagocytosis of apoptotic cells despite normal phagocytosis of other particles. We show here that c-mer–deficient mice have impaired clearance of infused apoptotic cells and that they develop progressive lupus-like autoimmunity, with antibodies to chromatin, DNA, and IgG. The autoimmunity appears to be driven by endogenous antigens, with little polyclonal B cell activation. These mice should be an excellent model for studying the role of apoptotic debris as an immunogenic stimulus for systemic autoimmunity.

Key words: autoimmunity • Lupus Erythematosus, systemic • phagocytosis • apoptosis • macrophages

Introduction

Clearance of apoptotic cells is a critical function of the mononuclear phagocyte system. Macrophages recognize apoptotic cells through an array of surface receptors, among them CD14, CD36, complement receptors, scavenger receptors, and a phosphatidylserine (PS)-specific receptor (1, 2). The phagocytosis and subsequent clearance of apoptotic debris is remarkably rapid and efficient: even in the thymus of young mice, where debris is generated at a great rate, apoptotic bodies can be visualized only with special methods (3). The redundancy of the clearance system is illustrated by apparently normal clearance and by the absence of in vivo apoptotic bodies in most receptor knockout models, with the exceptions of the C1q-deleted mouse (4); and mice with a targeted disruption of the cytoplasmic region of the membrane tyrosine kinase c-mer (5, 6).

The membrane tyrosine kinase c-mer is believed to bind to the protein GAS6, which in turn can bind to PS (7). Mice lacking the intracellular domain of c-mer show increased LPS-induced TNF-α production and suffer increased mortality after LPS administration in vivo (5). They have splenomegaly and identifiable apoptotic bodies within lymphoid organs. We have recently shown that these *mer*^{kd} mice have diminished in vivo clearance of apoptotic lymphocytes generated through corticosteroid administration (6) and that this is due to a macrophage defect. Their macrophages demonstrate impaired in vitro phagocytosis of apoptotic lymphocytes, yet normal phagocytosis of other particles. In our initial report, we indicated that the macrophage defect was accompanied by increased levels of anti native DNA antibodies. We report here that mice lacking the intracellular kinase domain of c-mer show in vivo delayed clearance of exogenously administered apoptotic cells, and spontaneous development of additional serological manifestations of systemic lupus. These include autoantibodies to chromatin, IgG, and DNA, and development of moderate renal pathology. These results and findings from other models of diminished apoptotic clearance give further weight to the notion that apoptotic cells provide an immunogenic stimulus for autoimmunity, and that impaired clearance is an important factor in the pathogenesis of systemic autoimmune disease.

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Materials and Methods

Mice. Mice lacking the intracellular domain of c-mer (mer^{kd}) were generated as described previously (5). The mice used were backcrossed for 10 generations to C57BL/6 (B6) and were maintained in the SPF animal colonies at the University of North Carolina, Chapel Hill; and at the University of Pennsylvania.

Immunoassays. Antibodies to chromatin, IgG and single-stranded DNA (ssDNA) were determined by ELISA as reported previously. Briefly, chromatin was prepared from chicken erythrocytes (8) and ssDNA from calf thymus (9). Plates were coated with 3 μg/ml of antigen and washed. Diluted sera were added, followed by biotin-conjugated developing antisera and then by avidin-alkaline phosphatase (Sigma-Aldrich). Rheumatoid factor (RF) was measured by coating plates with an IgG2b mouse myeloma protein, followed by development with biotin-labeled anti-IgM. Total IgG was measured by ELISA as reported previously (10). IgG and IgM anticardiolipin ELISAs were performed as described previously for human sera (11) with the exception that alkaline phosphatase–conjugated goat anti–mouse IgG and alkaline phosphatase-conjugated goat anti–mouse IgM antibodies (both from Zymed Laboratories) were used as the secondary reagents.

Renal Pathology. Kidneys were removed from mice of various ages. One kidney from each mouse was processed for conventional hematoxylin/eosin histology, while the other was snap-frozen in liquid nitrogen. The frozen kidneys were sectioned using a cryostat and stained for IgM, IgG, and C3 using fluoresceinated antibodies. Kidney pathology was scored as reported previously (12).

Immunization with Apoptotic Cells. Spleen and thymus cells were harvested and exposed to 1,500 R of gamma radiation from a ^{137}Cs source. 100×10^6 cells were injected intravenously weekly for 4 wk as described previously (13). Mice were bled as indicated and sera tested for anti-chromatin, anticardiolipin, and anti-DNA antibodies.

Labeling of Apoptotic Cells. Spleen and thymus cell suspensions were exposed to 1,500 of gamma radiation, washed, and incubated with the vital dye tetramethyl rhodamine phosphoramidite (TAMRA; Sigma-Aldrich; reference 14) for 30 min. After washing, the labeled cells were injected intravenously into B6 or mer^{kd} mice and spleens and lymph nodes harvested 14 h later. Cells were examined using flow cytometry to identify TAMRA-stained cells. In parallel to the in vivo studies, aliquots of the irradiated cells were followed for progression of apoptosis in vitro using annexin V binding and DNA quantitation through propidium iodide fluorescence. After 14 h, 100% of the cells were apoptotic by these measures.

Statistical Analysis. Levels of autoantibody production among mouse groups were analyzed with the two-tailed Student's *t* test. $P < 0.05$ was considered significant. Data analysis was performed with Microsoft Excel (1998) software.

Results

In Vivo Clearance of Apoptotic Cells Is Impaired in c-mer^{kd} Mice. Cells from mer^{kd} mice show decreased in vitro phagocytosis of apoptotic cells, yet it was possible that in vivo handling of exogenously administered apoptotic cells was normal. To address this issue, we exposed freshly isolated spleen and thymus cell suspensions to 500 R and labeled them with TAMRA. These labeled preapoptotic cells were infused intravenously into mer^{kd} or B6 mice and their presence was detected by immunofluorescence analysis of their spleen cells after differing intervals. As seen in Fig. 1, in mer^{kd} but not in normal mice there was a small but discrete population of TAMRA-positive cells in the spleen at 14 h. These results are representative of three experiments and in each experiment at least two mice per group were used. In addition to these apoptotic cells, we could also detect TAMRA-labeled debris, indicating the persistence of cells undergoing secondary necrosis. Apoptotic cells were not observed in B6 recipients of apoptotic cells, nor was there evidence in these mice of cells undergoing secondary necrosis. We also looked at mice 2, 3, and 7 d after IV infusion of TAMRA-labeled cells. In neither mer^{kd} nor in B6 mice were there any detectable TAMRA-labeled cells at these later time points, indicating that clearance was eventually accomplished, even in the mer^{kd} animals.

Spontaneous Development of Anti-chromatin Antibodies in mer^{kd} Mice. A cohort of mer^{kd} mice was housed under specific pathogen-free conditions and bled at monthly intervals. As seen in Fig. 2, many of these mice showed progressively increased levels of autoantibodies to chromatin. The autoantibody levels were equivalent to those found in B6/lpr mice housed under similar conditions, but less than levels seen in MRL/lpr mice. Similar results were observed with two other cohorts of mer^{kd} mice examined earlier during the derivation of the B6 congenic strain. It is noteworthy that, while autoantibody levels were highly significant, only 30–50% developed anti-chromatin autoimmunity.

Mer^{kd} Mice Develop Autoantibodies to Single- and Double-stranded DNA. Antibodies to DNA are hallmarks of SLE and antibodies to double-stranded (ds)DNA are consid-

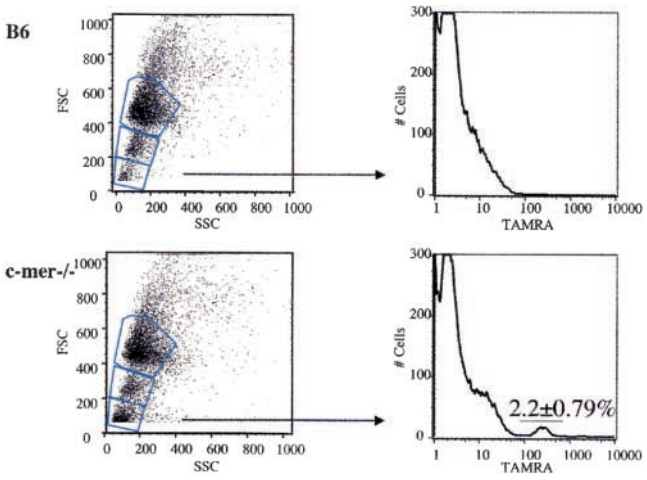

Figure 1. Detection of apoptotic cells after infusion. $75–100 \times 10^6$ TAMRA-labeled apoptotic cells were given IV to mer^{kd} or B6 mice and spleens removed 14 h later and analyzed using flow cytometry. Apoptotic cells were identified by gating using forward and side scattering and are denoted in the middle and bottom boxes (early and late apoptotic cells). Red fluorescence of these populations is shown on the right, and depicts TAMRA-labeled cells. This figure is representative of three experiments. In each experiment at least two mice per group were used. Mean ± SD of all three experiments is shown.

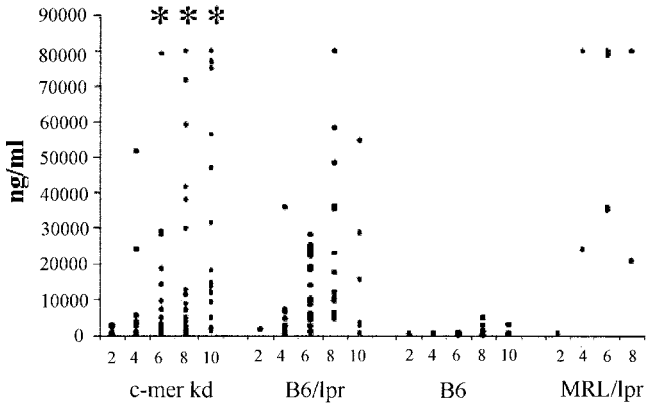

Figure 2. Anti-chromatin in *mer*^{kd} and control mice. Mice aged from 2–10 mo were bled and serum analyzed by ELISA for anti-chromatin. Results indicated ng/ml of specific antibody. Similar results were observed in two other cohorts of mice. Asterisks indicate $P < 0.05$ when *mer*^{kd} mice were compared with B6 mice.

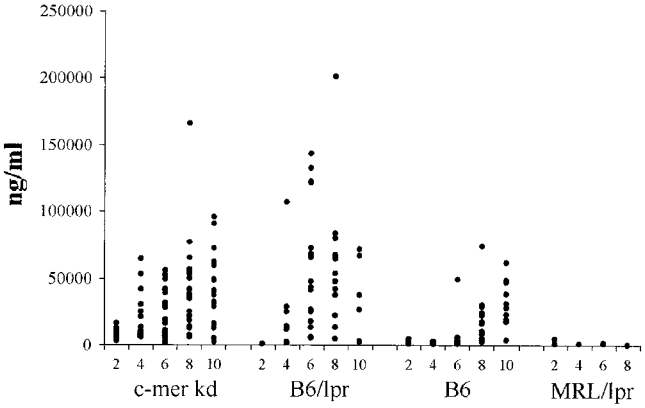

Figure 4. Rheumatoid factor in *mer*^{kd} and control mice. The same sera were tested for IgM antibody to IgG2^{b} by ELISA. Results are representative of three experiments.

ered nearly diagnostic of this disorder. As seen in Fig. 3, *mer*^{kd} mice developed anti-ssDNA as they grew older. Antibodies to dsDNA were also observed in substantial numbers of mice (14/24 at 5 mo by *Crithidia luciliae* immunofluorescence assay; reference 5). There was little difference in levels observed in females vs males for either specificity (not shown).

Rheumatoid Factor Appears Late in Mer^{kd} *Sera.* RF accompanies other serological features of SLE in some patients, and is known to be prominent in B6/lpr mice. As shown in Fig. 4, *mer*^{kd} mice developed RF by 6–8 mo. As has been reported for B6/lpr mice (15), RF levels were greater in females. This autoantibody was primarily directed at IgG2b; little reactivity was found against IgG1 (data not shown).

Autoimmunity in mer^{kd} *Mice Is Not Accompanied by Polyclonal B Cell Activation.* Diffuse B cell activation accompanies SLE-like autoantibody formation in several models of murine systemic autoimmunity. To assess polyclonal antibody production, we measured total IgG levels by ELISA. As shown in Fig. 5, there was a gradual increase with age of IgG levels in both normal and *mer*^{kd} mice. The latter did not show evidence of diffuse B cell activation. We also quantitated polyclonal B cell activation using an enzyme-linked immunospot (ELIspot) assay to enumerate splenic B cells secreting IgG. The mean number of antibody-secreting cells per 10^6 spleen cells of four 5-mo-old *mer*^{kd} mice was 3,230 ± 710, compared with 1,030 ± 430 in four age-matched B6 mice. This difference was of borderline statistical significance ($P = 0.051$). Thus, the cell-based assay may have detected a small degree of polyclonal B cell activation in the *mer*^{kd} mice, though this may reflect the increased amount of autoantibody secretion.

C-mer^{kd} *Mice Develop Mild Renal Pathology Late in Life.* Our initial observations on *mer*^{kd} mice before extensive backcrossing to B6 indicated that they developed severe lupus-like focal membranoproliferative glomerulonephritis, particularly females. After extensive backcrossing to B6, we no longer observed severe renal disease. In contrast, *mer*^{kd} mice on the B6 background developed mesangial lesions

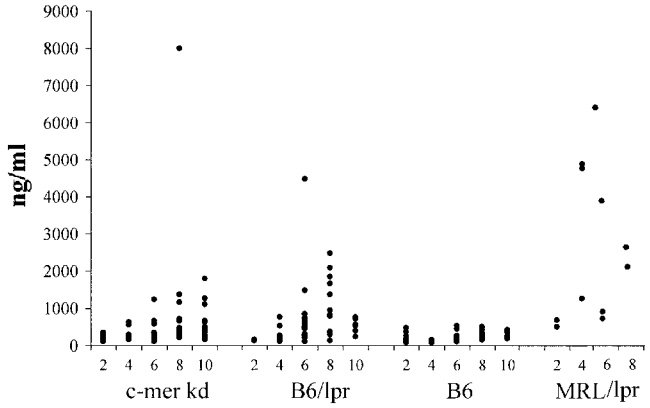

Figure 3. Anti-ssDNA in *mer*^{kd} and control mice. The same cohort of mice shown in Fig. 2 was tested for anti-ssDNA by ELISA. Results are reported as ng/ml of specific antibody.

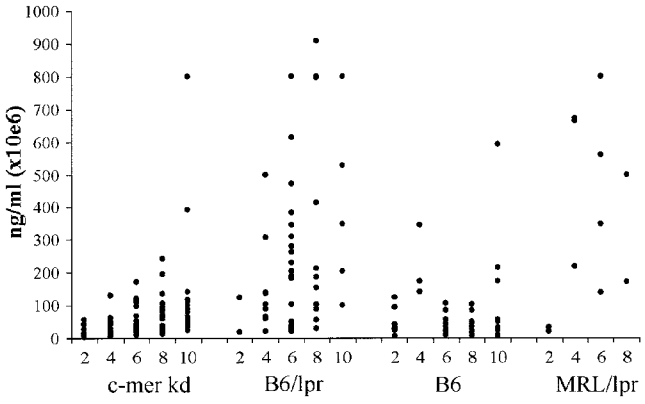

Figure 5. Total IgG in *mer*^{kd} and control mice. Total IgG was measured by ELISA.

with deposition of modest amounts of C3, IgM, and IgG. Kidneys from 18 6-mo-old *mer*^{kd} were examined microscopically and immunofluorescence staining and blindly graded from 0 to 4+. Only one mouse developed 3+ or greater IgG staining. This was in a mesangial pattern, consistent with light microscopic observations. This animal also had 4+ IgM mesangial deposition. The mean IgG staining for these 18 mice was 0.5+; IgA was 0.5+; IgM 1.3+; and C3 0.8+. Of 138 4 mo and older mice examined by dipstick, 19 had 2+ or greater proteinuria. These findings are consistent with their apparent normal lifespan and fecundity in our animal colony. It is likely that the 129 background genes contributed to the autoimmunity observed before backcrossing.

Repeated Immunization of mer^{kd} *Mice Accelerates Anti-cardiolipin But Not Anti-DNA Autoantibody Production.* It has been reported that transient autoantibody production to nuclear antigens and to phospholipids can be achieved upon immunization of normal mice with apoptotic cells. Because we hypothesized that *mer*^{kd} mice undergo self-immunization with apoptotic cells, we asked whether the infusion of exogenous apoptotic cells might lead to earlier or to great amounts of autoantibody production. *Mer*^{kd} and B6 mice were given 10^7 irradiated thymocytes, following the protocol used by Mevorach and colleagues (13). Mice were so immunized at 3 mo of age, and were bled at monthly intervals. 5 of 10 apoptotic cell-immunized *mer*^{kd} mice showed an increase in IgG anti-cardiolipin antibodies (doubling or more of ELISA optical density [O.D.]), compared with two of ten B6 controls. The mean baseline anti-cardiolipin ELISA O.D. of the five *mer*^{kd} mice which evinced an increase in O.D. was 0.128 $\pm$ 0.21; the peak response, one month later was 0.347 $\pm$ 0.132. IgM anti-cardiolipin levels did not increase in any of the experimental groups. 2 of 10 apoptotic cell-immunized B6 mice showed an increase at one month in IgG anti-cardiolipin antibody levels (0.089 $\pm$ 0.27 to 0.194 $\pm$ 0.43). Neither anti-chromatin nor anti-DNA antibody production was accelerated in immunized *mer*^{kd} mice compared with saline-injected controls by 8 wk after immunization. In B6 recipients of apoptotic cells, we noted no anti-DNA or anti-chromatin autoantibody production at any point sampled, including serum collected as late as 12 wk after immunization.

Discussion

The principal findings of our study were that *mer*^{kd} mice developed autoimmunity to nuclear antigens, accompanied later on by rheumatoid factor. A defect in the rate of apoptotic cell clearance was apparent in vivo, yet only antiphospholipid autoantibody production was accelerated upon administration of apoptotic cells in vivo, indicating that endogenous sources of apoptotic cells were sufficient to provide the stimulus for most autoantibodies. It was noteworthy that polyclonal B cell activation was not a feature of the autoimmune syndrome of the c-mer–deficient mice. This may reflect the specific stimulus of autoantigen in provoking autoantibodies, without concurrent stimulation of nonautoreactive B cells.

Vast numbers of apoptotic cells are generated through cell senescence, maturation, and turnover. Multiple macrophage scavenger receptors serve the important function of recognizing and promoting engulfment and removal of apoptotic cells (2). Several recent reports have emphasized that defective macrophage clearance of apoptotic debris may lead to autoimmunity. For instance, absence of C1q or of the CR2 receptor lead to autoimmunity, as does knocking out the gene encoding serum amyloid protein and deletion of DNase I (16). It has been proposed that these genetic manipulations, by altering the efficiency of clearance of apoptotic cells, promote self-immunization with nuclear antigens exposed in blebs on the surface of dying cells (17). While direct proof of such self-immunization is not available, the hypothesis is appealing and has been widely disseminated.

In support of the notion that apoptotic cells represent a vehicle for potential autoimmunization, investigators have documented modest transient antibody production in animals immunized with apoptotic cells (13). In other systems, T cell reactivity to transfected antigens contained within apoptotic cells in vivo and in vitro has been observed, and is enhanced when appropriate costimulatory molecules and cytokines are present (18, 19). The present findings are consistent with the hypothesis that apoptotic cells represent a potential source of autoantigens.

It was of interest that the effect of immunization with apoptotic cells was evident only in increased anti-phospholipid antibody titers, with little effect on anti-DNA or anti-chromatin. This finding suggests that the continuous burden of endogenous apoptotic cells is sufficient to drive the immunization process in *mer*^{kd} mice; additional apoptotic cells may add little to the immunization process. The increase in anti-phospholipid antibodies may indicate a special role for mer in the clearance of debris containing phospholipids, perhaps via binding by mer (via GAS6) of phospholipid-rich apoptotic bodies as has been suggested (20).

While absence of certain scavenger receptors results in autoimmunity, mice without other receptors (SRS A, CD36) do not develop an autoimmune phenotype despite in vitro defects. What accounts for the autoimmunity in *mer*^{kd} mice? One possibility is that the c-mer receptor is more relevant for certain autoantigens than some of the others. This hypothesis is supported by the observation of accumulated apoptotic cells in *mer*^{kd} mice but not in SRS-A–deficient animals in vivo. Another possibility is that c-mer binds to apoptotic cells at a stage at which they are more immunogenic. The expression of phosphatidylserine on apoptotic cells is an early event, and is believed to mediate binding via growth arrest specific protein 6 (GAS 6; reference 21) to c-mer. A failure to eliminate apoptotic cells at this very early stage may lead to accumulation of late apoptotic cells and of cells undergoing secondary necrosis. These cells may present a more immunogenic challenge and may thus provoke autoantibody formation.

Another consideration for understanding autoimmunity in *mer*^{kd} mice concerns the nature of our knockout mouse.

Because only the cytoplasmic domain of c-mer has been removed, it is possible that *mer*^{kd} macrophages retain the ability to bind apoptotic cells but not to internalize them. Macrophage-bound apoptotic cells adherent to c-mer may lead to cross priming and self-immunization. We think this possibility unlikely because we have seen little if any c-mer surface expression in *mer*^{kd} mice (unpublished data).

A final explanation for the autoimmune phenotype of *mer*^{kd} mice deserves serious consideration. As initially reported, c-mer serves an important role in regulating the cytokine profile of macrophages. Macrophages from *mer*^{kd} mice secrete larger amounts of TNFα than do control macrophages, leading to increased LPS sensitivity (5). Others have emphasized the key role of cytokine expression in macrophages ingesting apoptotic cells. In contrast to their secretion of inflammatory cytokines when confronted with necrotic cells, macrophages ingesting apoptotic cells produce a panel of noninflammatory cytokines such as TGFβ and IL-10, thereby reducing the immunogenicity of apoptotic cells (22). *Mer*^{kd} macrophages, in addition to their impaired clearance of apoptotic cells, may generate an inflammatory pattern of macrophage cytokine production. The mice may therefore have both an increased burden of apoptotic cells and a macrophage environment conducive to the induction of T cell immunity to apoptotic cell antigens. The combination of macrophage activation in an inflammatory mode, together with persistence of late apoptotic cells and possibly cells undergoing secondary necrosis, may present a sufficient autoimmunogenic stimulus so that tolerance to nuclear antigens is broken and autoimmunity ensues.

In support of the idea that macrophage activation is crucial in promoting autoimmunity to apoptotic debris is a recent report emphasizing the lymphoproliferative and autoimmune phenotype of mice lacking c-mer, axl, and tyro-3. These animals develop apparently higher levels of autoantibodies to DNA than c-mer single knockouts, together with other autoantibody specificities (23). The more severe autoimmunity in these animals may reflect their intense macrophage activation, or may be due to a more severe apoptotic clearance defect in the absence of all three members of this receptor tyrosine kinase family.

The finding of autoimmunity in *mer*^{kd} mice opens the way to new avenues to study the role of apoptotic debris as an immunogenic stimulus in autoimmune disease. Macrophages from these mice, because of their delayed phagocytosis of apoptotic cells, may make available nuclear antigens to dendritic cells, as has been described in the MHC class I pathway (24), and thereby generate immunogenic self-peptide complexes in the context of MHC class II molecules. We are currently devising model systems to assess disposition of apoptotic cell derived antigens in *mer*^{kd} mice. These experiments should allow deduction of the role of apoptotic cell uptake and macrophage cytokine production on the presentation of nuclear autoantigens relevant to SLE.

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