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Lupus-Prone Mice Have an Abnormal Response to Thioglycolate and an Impaired Clearance of Apoptotic Cells

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Deficiency of complement in humans and mice is associated with the development of lupus and with abnormal repair of inflammatory and immune complex-mediated tissue injury. Here we ask whether similar defects in the resolution of inflammation are found in mice prone to spontaneous lupus. We compared the response to an i.p. injection of thioglycolate between two lupus-prone strains (MRL/Mp and NZB/W) and two non lupus-prone strains of mice (C57BL/6 and BALB/c). In all four strains the influx of polymorphonuclear neutrophils (PMN) was similar. However, by 96 h clearance of PMN in the control strains was complete, whereas in the autoimmune-prone strains PMN were still detectable. The number of mononuclear cells recruited was markedly reduced in the lupus-prone strains compared with the controls, and their phenotype was different. The lupus-prone strains had significantly fewer elicited macrophages that were CD11b-high and Ly6C-negative. In lupus-prone mice at 24 h there was a significantly increased number of apoptotic PMN free in the peritoneum, accompanied by a reduced percentage of macrophages containing apoptotic bodies, suggesting a defect in their uptake. An impaired ability of resident peritoneal macrophages from lupus-prone mice to engulf apoptotic cells was demonstrated by in vivo and in vitro cell clearance assays. These observations indicate that lupus-prone strains have an abnormal inflammatory response to thioglycolate and an intrinsic impairment in apoptotic cell uptake. These findings have implications for the initiation of autoimmunity, as lupus autoantigens are expressed on dying cells, and impaired disposal of these could enhance the development of autoimmunity. The Journal of Immunology, 2003, 170: 3223–3232.

Systemic lupus erythematosus (SLE) is a polygenic systemic autoimmune disease characterized by the production of an array of autoantibodies. Interaction of these autoantibodies with their cognate Ags leads to widespread inflammatory injury. Murine models of SLE have been intensively studied; a number of genetic loci have been identified (reviewed in Refs. 1 and 2), and potential pathogenic mechanisms have been proposed (reviewed in Ref. 3). Although the effector mechanisms causing the clinical features of SLE have been studied extensively, the primary defects responsible for the initiation and progression of the disease are still poorly understood.

One clue to the etiology of SLE is the powerful association between hereditary deficiency of complement classical pathway proteins and the development of SLE in humans and mice. Complement deficiency is associated with abnormalities in the resolution of inflammation, particularly the removal of dying cells and immune complexes (4). It has been proposed that defects in these waste disposal mechanisms may predispose to the development of disease by allowing the persistence and presentation of autoantigens in the context of an inflammatory cytokine milieu (5, 6).

Macrophages play a key role in all stages of inflammation from initiation to resolution (reviewed in Refs. 5 and 6). The acute response to an inflammatory insult is usually a rapid infiltration of neutrophils (PMN), which subsequently undergo apoptosis. This occurs within hours and is followed by the recruitment of macrophages that phagocytose the apoptotic PMN (7). Other mechanisms may also play a role in the removal of PMN from the site of inflammation (8). The phagocytosis of apoptotic PMN, unlike the removal of necrotic cells, does not trigger the production of proinflammatory cytokines by macrophages (9–11). On the contrary, it has been suggested that the recognition and engulfment of apoptotic cells by macrophages may actively generate an anti-inflammatory response (12, 13). Thus, defects in the recognition and/or uptake pathways could cause the release of proinflammatory cytokines and toxic substances from inefficiently cleared PMN, resulting in tissue damage.

Macrophages from two murine models of SLE, MRL/Mp and New Zealand Black/White F1 (NZB/W), have previously been shown to have a reduced synthesis of TNF-α, IL-1, IL-12, and IL-6 in response to inflammatory stimuli in vitro (14–19). Results from these studies suggested that the defect in TNF-α production contributed to the reduced IL-1 and, to some extent, IL-6 production (17). A dysregulation of the NF-κB pathway of activation has recently been implicated in determining this abnormal cytokine response (20, 21). In addition, an increased secretion of TGF-β1 has been detected in MRL/Mp (18). The abnormalities in cytokine production appeared to be selective, as a reduction in IL-10 production was not observed (17). It is of note that the aberrant cytokine production in MRL/Mp mice was observed in young pre-symptomatic animals (17) and was independent of the Ifnar mutation (16, 17). The defect was also present in chimeric mice in which MRL/Mp bone marrow-derived cells were transplanted into non-autoimmune A/J hosts (15). Taken together these observations...
would indicate that a dysregulation in cytokine production by macrophages might be a contributory factor to the development of the autoimmune disease.

There is an increasing body of evidence suggesting that apoptotic or necrotic cells are sources of lupus autoantigens in human and murine disease. Many of the autoantigens associated with SLE are expressed in high concentration on apoptotic blebs (22–25), and injection of apoptotic cells into mouse strains not usually susceptible to the development of SLE can induce an autoantibody response (26). Furthermore, findings from our laboratory and others have suggested that an impaired clearance of dying cells may play a pathogenic role in the development of autoimmunity (27–29). In this context, macrophages are the major effector cell type involved in the noninflammatory removal of apoptotic debris. Furthermore, a defect in the ability of macrophages derived from humans with SLE to clear apoptotic cells has been reported (30).

In view of these findings, we decided to compare the induction and resolution of an inflammatory lesion in normal and spontaneously lupus-prone mouse strains. We induced inflammation by an i.p. injection of thioglycolate in mouse strains either permissive (MRL/Mp and NZB/W) or refractory (C57BL/6, BALB/c) to the development of SLE. Using this experimental model of sterile peritonitis we found that the two autoimmune-prone strains analyzed had an aberrant macrophage elicitation. This abnormal response, coupled with an intrinsic defect in the phagocytic uptake of apoptotic cells, resulted in an impaired clearance of PMN from the peritoneum.

Materials and Methods

Animals

MRL/Mp, C57BL/6, BALB/c, CBA, DBA/2, and NZB/W were purchased from Harlan U.K. Ltd. (Bicester, U.K.). Experimental animals were age and sex matched. Female mice were used for all the experiments in this study. Animals were between 8 and 10 wk of age. All care and use of animals were according to institutional guidelines.

Thioglycolate-induced peritonitis

Mice were injected i.p. with 1 ml of sterile 4% Brewer’s thioglycolate solution. At different time points (0, 8, 24, 48, and 96 h) mice were killed, and cells were recovered by peritoneal lavage using 5 ml of ice-cold HBSS/5 mM EDTA. Cells were immediately stored on ice. Peritoneal exudates cells (PEC) were initially counted on a hemocytometer and then differential cell counts were conducted on cytopsins stained with Diff-Quik (Dade-Behring, Munich, Germany). The number of apoptotic PMN was determined by analyzing coded cytopsins. Between 300 and 400 PMN were analyzed in each sample, and PMN were considered apoptotic when they exhibited profound morphological changes, including nuclear fragmentation and condensation, membrane blebbing, and loss of cell volume. Apoptosis in PEC was confirmed by staining the cells with annexin V-FITC (BD PharMingen, San Diego, CA) and biotinylated anti-Gr-1 Ab (BD PharMingen), which specifically recognizes PMN, followed by flow cytometric analysis. Phagocytosis of apoptotic PMN was also scored on coded cytopsins. Between 300 and 400 macrophages were counted, and the phagocytosis was expressed as the percentage of macrophages that contained apoptotic debris and as the phagocytic index (the number of ingested apoptotic cells per 100 macrophages). Macrophages were considered to have phagocytosed an apoptotic body when >50% of it was contained within the body of the macrophage, as previously described (31). In some experiments PEC were analyzed by FACS as well as on cytopsin preparations, as described below.

Abs and FACS analysis

Cells recovered by peritoneal lavage were costained with FITC conjugated F4/80 Ab (Caltag, Burlingame CA) and anti-CD11b-PE Ab (BD PharMingen) after three washes in PBS/1% BSA. Before staining, nonspecific binding of Abs was blocked with the anti-FcR Ab 2.4G2 (BD PharMingen) and 10% normal mouse serum. Samples were then fixed in FACS lysing solution (BD PharMingen) and analyzed on a FACSCalibur (BD Bio sciences, Mountain View, CA). Three-color FACS analysis was conducted with an additional biotinylated Ab that was then detected with a strepta-

vidin-CyChrome conjugate (BD PharMingen). The biotinylated Abs used were anti-Ly6C, anti-Gr-1, anti-B220, anti-Thy1.2 (all from BD PharMingen), and 493 (32) (anti-CD93; a gift from Prof. A. Rolink, Basel, Switzerland). Data were analyzed using WinMDI software (version 2.8; The Scripps Institute, La Jolla, CA).

Phagocytic assays for apoptotic cells

The uptake of apoptotic cells by resident peritoneal macrophages was investigated using in vivo and in vitro assays. The in vivo clearance of apoptotic thymocytes by resident peritoneal macrophages was analyzed as previously described (29). Briefly, mice that received no pretreatment were injected i.p. with 107 apoptotic murine thymocytes, which had been made apoptotic by incubating them in 0.4% BSA/RPMI 1640 medium, supplemented with 1 μM dexamethasone for 3 h. This resulted in a population of cells that was ~30% apoptotic and >95% viable as determined by trypan blue exclusion. Thirty minutes after the injection of apoptotic thymocytes mice were killed, peritoneal cells were recovered in ice-cold buffer, and phagocytosis was scored on coded cytopsins as described above.

To study the in vitro uptake of apoptotic thymocytes resident peritoneal macrophages were plated out in 24-well plates containing coverslips at 7.5 × 104 cells/well in RPMI/10% FCS for 2 h, washed to remove non-adherent cells, and then cultured overnight. Murine thymocytes were made apoptotic as described above, resuspended at 106 cells/ml in RPMI 1640 medium/10% normal mouse serum, and incubated at 37°C for 15 min before adding 1.5 ml to the wells containing 7.5 × 105 macrophages. The tissue culture plates were spun briefly at 50 × g to bring the thymocytes into contact with the macrophages and then were incubated for 30 min at 37°C. Coverslips were removed from the wells and mounted immediately in DePeX (BDH Laboratory Supplies, Poole, U.K.). The uptake of apoptotic cells was determined as described above. Freshly isolated thymocytes, with no induction of apoptosis, were also used as live cell controls in these experiments.

FcyR- and C3-dependent phagocytosis assays

One milliliter of a suspension at 7.5 × 104 cells/ml resident macrophages, obtained by peritoneal lavage, was added to each well of a 24-well tissue culture plate containing coated plastic coverslips (Thermanox coverslips; Nunc, Leicestershire, U.K.) and incubated overnight at 37°C in 5% CO2. Murine RBCs were prepared as follows. The RBCs were initially washed three times in PBS and then resuspended to 2% (v/v). For FcγR-dependent uptake experiments 1 ml of murine RBC suspension was incubated in PBS/1% BSA for 1 h at 4°C with 50 μg of the 34-3C Ab (a murine IgG1 anti-mouse RBC) (33), which resulted in a high level of opsonization as assessed by FACS. For complement-mediated phagocytosis, murine RBCs were opsonized for 30 min at 4°C with rat anti-CD24 IgM J11d, which is capable of activating the complement system via the classical pathway, then washed in PBS. Ab-coated RBCs were opsonized in a 10% (v/v) solution of C5-deficient serum (from DBA/2 mice) to allow C3 deposition without cell lysis. The level of C3 opsonisation was checked by FACS using a biotinylated polyclonal anti-mouse C3 Ab (a gift from Prof. S. Izui, Geneva, Switzerland). Opsonized RBC suspensions were then washed with PBS/1% BSA and resuspended to 2% (v/v) in PBS. RBC suspension (250 μl) was added to each well of macrophages, and the plates were spun at 50 × g for 2 min. Plates were then incubated at 37°C for 30 min to allow phagocytosis to occur. Wells were washed twice with ice-cold PBS to prevent further uptake, with a final wash in hypotonic RBC lysis solution to lyse RBC that had bound to macrophages but had not been ingested. The plates were stored on ice while the coverslips were recovered from the wells. Coverslips were mounted on slides and stained with Diff-Quik (Dade-Behring). Phagocytosis was also assessed by light microscopy on coded cytopsins. On cytosin preparations the number of RBCs ingested per 100 macrophages was defined as the phagocytic index, whereas the percentage of effector cells containing at least one erythrocyte was defined as the percentage of phagocytosis. At least 400 macrophages were scored per well, and duplicate wells for each experimental condition were used.

Statistics

The data are presented as the mean ± SEM unless otherwise stated. All statistics were calculated using GraphPad PRISM (version 2.0; GraphPad, San Diego, CA). Nonparametric tests were applied throughout, with differences being considered significant for p < 0.05. One-way ANOVA with Bonferroni multiple comparison test was applied when more than two groups were analyzed.
Results
Lupus-prone strains (MRL/Mp and NZB/W) exhibited an abnormal response to thioglycolate compared with nonautoimmune mice (C57BL/6 and BALB/c), resulting in an impaired resolution of the inflammatory insult. We employed the thioglycolate-induced peritonitis model to explore whether lupus-prone murine strains responded differently to inflammatory insults in comparison with non-lupus-prone strains. In this model an i.p. injection of 1 ml of 4% thioglycolate leads to an acute influx of PMN, followed by a chronic wave of macrophages (34), which are the main effector cells responsible for removing the PMN that die by apoptosis (7).

We initially used C57BL/6 and MRL/Mp mice as examples of a wild-type and an autoimmune-prone strain and analyzed their responses for up to 96 h after thioglycolate injection (Fig. 1). First, a difference in peritoneal resident cell numbers, before the injection of thioglycolate, was observed. In C57BL/6 mice the peritoneal cell number was lower than in MRL/Mp mice ($0.73 \pm 0.05 \times 10^6$ cells/ml compared with $1.17 \pm 0.04 \times 10^6$ cells/ml ($n = 5$; mean ± SEM), respectively; $p = 0.0079$, by Mann-Whitney U test; Fig. 1A). This difference in the number of resident cells may simply reflect the fact that at 6–8 wk of age MRL/Mp mice are generally bigger than C57BL/6 mice. However, the percentages of resident macrophages were similar in the two experimental groups (46.3 ± 2.14% in C57BL/6 mice and 43.0 ± 1.71% in MRL/Mp mice), and PMN represented <1% of the resident cell population. At 24 h after thioglycolate injection many cells had been recruited into the peritoneal cavity in both strains. Despite the difference in resident cell number, the difference in the total number of cells present at 24 h was not significant ($5.57 \pm 0.35 \times 10^6$ cells/ml in the C57BL/6 mice and $4.50 \pm 0.47 \times 10^6$ cells/ml in the MRL/Mp mice ($n = 6$; mean ± SEM); $p = 0.0823$, by Mann-Whitney U test; Fig. 1A). In C57BL/6 mice the number of PEC increased further at 48 h ($6.72 \pm 0.13 \times 10^6$ cells/ml), with no additional increase at 96 h ($6.45 \pm 0.23 \times 10^6$ cells/ml). In C57BL/6 mice the increase in PEC was significant at all time points analyzed compared with that at time zero ($p < 0.001$ at each point, by Bonferroni multiple comparison test). In contrast, in the MRL/Mp mice there was a significant increase in PEC only at 24 h ($p < 0.01$), and PEC numbers fell back to the baseline value at later time points.

Morphology and differential cell counts on cytospin slides were used to define the cell types recruited. In both strains there was a large influx of PMN by 24 h ($2.32 \pm 0.28 \times 10^6$ PMN/ml in the MRL/Mp mice and $2.79 \pm 0.22 \times 10^6$ PMN/ml in the C57BL/6 mice) that was not significantly different between the two strains (Fig. 1B). In the C57BL/6 mice these PMN were rapidly cleared, and by 96 h the PEC consisted almost exclusively of macrophages, with PMN representing ~0.5% ($1.79 \pm 0.36 \times 10^5$/ml) of the total cell population. By contrast, in the MRL/Mp mice, although PMN were cleared between 24 and 48 h, surprisingly at 96 h there was a still a significant number of PMN remaining in the peritoneal lavage ($5.28 \pm 0.70 \times 10^5$/ml), representing ~10% of the total cell number ($p = 0.0087$ compared with the number of PMN present in the C57BL/6 mice at 96 h, by Mann-Whitney U test; Fig. 1B).

The behavior of the two mouse strains was strikingly different with regard to the recruitment of macrophages. First, there was a significant difference in the number of resident macrophages before injection of thioglycolate between the two mouse strains, with C57BL/6 mice having fewer cells than MRL/Mp mice ($0.70 \pm 0.05 \times 10^6$ and $1.12 \pm 0.08 \times 10^6$ macrophages/ml, respectively; $n = 5$; $p = 0.0079$, by Mann-Whitney U test; Fig. 1C). In C57BL/6 mice the thioglycolate injection was followed by an influx of macrophages that, by 96 h, represented ~85% of the cells present in the lavage fluid ($5.92 \pm 0.23 \times 10^6$ cells/ml; $n = 6$; Fig. 1C). In contrast, in MRL/Mp mice a marked impairment in recruitment of macrophages was observed, and at 96 h the macrophages represented only 65% of the total cell population in the
lavage (2.18 ± 0.25 × 10⁶ cells/ml; n = 6; p = 0.0043, by Mann-Whitney U test). At this time point ~10% of the peritoneal cells in MRL/Mp mice were PMN, with the majority of the remaining 25% of cells exhibiting morphology consistent with that of monocytes. This defect in the macrophage influx accounted for the significant difference detected in the total cell counts at the 48 and 96 h points (Fig. 1A). To evaluate whether the reduced number of macrophages present in the peritoneum of MRL/Mp mice at 96 h was the result of a delay in the process of elicitation of macrophages, we extended our analysis to 7 days after the injection of thioglycolate. Even on day 7 the macrophage numbers in the MRL/Mp mice had not reached levels similar to those seen in C57BL/6 mice. On the contrary, macrophage numbers in both strains after 96 h began to decline, showing that the stimulus for monocyte/macrophage influx was ended by this time (data not shown).

In addition to the difference in the number of cells recruited in response to thioglycolate, a striking difference between strains in the morphology of the elicited peritoneal cells on cytospin slides was also observed. There were no morphological differences between resident peritoneal cells from the two strains before thioglycolate injection (Fig. 2). Similarly, there was little difference in the overall morphology of PEC between the two strains at 24 h, despite the reduced recruitment of mononuclear cells in MRL/Mp mice (Fig. 2). However, by 96 h, a clear difference emerged. At this time point PEC from C57BL/6 consisted almost exclusively of cells with the characteristics of macrophages (Fig. 2). In contrast, the thioglycolate-elicited cells in MRL/Mp mice differed from those in C57BL/6 mice, with a great proportion of cells sharing morphological features more typical of monocytes (Fig. 2).

To confirm the findings obtained on cytospin preparations, PEC were stained for the macrophage marker F4/80 and analyzed by flow cytometry. F4/80 is known to be a macrophage differentiation marker and is only expressed at high levels on macrophages (35). The recruitment of F4/80-positive cells mirrored very closely the results obtained by differential cell counts (data not shown).

To characterize further the cell populations observed, PEC were stained for F4/80 and two additional mononuclear cell differentiation markers (CD11b and Ly6C). CD11b is expressed on a range of cell types, including monocytes, macrophages, and PMN. However, it is considered to be a macrophage differentiation marker, because its expression increases upon differentiation of monocytes into macrophages (36, 37). By contrast, Ly6C is a typical monocyte marker and is expressed at high levels on these cells (37–39). Resident macrophages expressed high levels of both CD11b and F4/80 (Fig. 3A and data not shown), and their morphology, as determined by forward and side scatter profiles, was consistent with that of macrophages (data not shown). In addition to the macrophages, resident cells consisted of a large population of cells that

**FIGURE 2.** Representative photomicrographs of cytospin slides of PEC from C57BL/6 and MRL/Mp mice. The cells shown were recovered from untreated animals or from mice 24 or 96 h after thioglycolate-induced inflammation. Cells arrowed in the MRL/Mp slide at 96 h postinjection are mononuclear cells with morphologic features of monocytes that were not observed in the C57BL/6 mice at this time point. Cells were spun onto cytospin slides and stained with Diff-Quik as described in Materials and Methods.
were CD11b-positive but F4/80-negative. The majority of these cells (~65%) were B220-positive B-1 cells, with ~25% being Thy1.2-positive T cells (data not shown). There were no detectable PMN, as defined by Gr-1 expression. The phenotype of the resident peritoneal cells in both experimental groups (MRL/Mp and C57BL/6 mice) appeared similar.

Since we had previously observed that at 96 h lavages from MRL/Mp mice contained a higher number of PMN, which express both CD11b and Ly6C, but are negative for F4/80 (data not shown), we eliminated these cells from our study by analyzing the expression of Ly6C and CD11b only on F4/80-positive cells. The presence of PMN was analyzed by staining with anti-Gr-1 Ab (data not shown). At 96 h after the induction of peritonitis, the majority (74 ± 3.2%) of PEC in C57BL/6 mice expressed F4/80 compared with 62 ± 2.1% (p = 0.055, by Mann-Whitney U test; n = 5) in MRL/Mp mice (Fig. 3A). We quantified macrophage numbers by using these percentages in combination with the total number of PEC in each mouse strain; the resulting number of macrophages derived in this approach correlated closely with that determined by light microscopy. The numbers of macrophages recruited, as determined by cytospin and FACS, respectively, were 5.92 ± 0.23 × 10⁶ and 5.19 ± 0.25 × 10⁶ for C57BL/6, and 1.54 ± 0.25 × 10⁶ and 2.50 ± 0.21 × 10⁶ for MRL/Mp (p > 0.05 in both cases, by Bonferroni multiple comparison test). The level of F4/80 expression observed on MRL/Mp mononuclear cells was also lower than that observed in C57BL/6 mice (Fig. 3A). Furthermore, there was a significant difference in the expression of CD11b between the two strains. In C57BL/6 mice the majority of F4/80-positive cells expressed high levels of CD11b (47 ± 2.2% of the total cell population compared with 23 ± 1.6% in MRL/Mp animals; p = 0.0079, by Mann-Whitney U test). In contrast, in MRL/Mp mice the majority of F4/80-positive cells expressed low levels of CD11b (39 ± 2.5% of the total cell population compared with 27 ± 1.7% in C57BL/6 mice; p = 0.0317, by Mann-Whitney U test).

The reduced percentage of F4/80-positive cells expressing high levels of CD11b suggested that fewer recruited mononuclear cells were differentiating into macrophages in MRL/Mp mice. This was confirmed by the analysis of Ly6C expression. Cells that were positive for F4/80 were gated, and their expression of CD11b and Ly6C was analyzed by FACS (Fig. 3, B and C). At 96 h the F4/80-positive peritoneal cells from C57BL/6 mice contained a high proportion of cells expressing high levels of CD11b (63 ± 1.4%) and a small proportion of cells that were positive for Ly6C (23.9 ± 2.9%). In contrast, the F4/80-positive cells from MRL/Mp mice contained a lower percentage of cells expressing high levels of CD11b (32 ± 2.1%) and a significantly higher proportion of cells that were positive for Ly6C (59.4 ± 1.9%). Thus, the majority of mononuclear cells in the C57BL/6 lavages 96 h after thioglycolate injection were positive for F4/80, expressed CD11b at high levels, and were negative for Ly6C. In contrast, in the lavages from MRL/Mp mice the majority of mononuclear cells that were F4/80 positive showed significant levels of expression of CD11b, which was not present in C57BL/6 mice. This suggested that the CD11b expression in MRL/Mp mice was not due to the presence of F4/80-positive cells, but rather to the presence of Ly6C-positive cells that were recruited as monocytes and then differentiated into macrophages.

FIGURE 3. FACS analysis of PEC from C57BL/6 (left panels) or MRL/Mp (right panels) mice costained with F4/80-FITC, CD11b-PE, and Ly6C-biotin. A. Representative example of F4/80-positive cells at 0 and 96 h after thioglycolate injection. The phenotypes of the resident peritoneal cells in the experimental groups (MRL/Mp and C57BL/6 mice) were similar. However, at 96 h the percentage of PEC positive for F4/80 and the level of F4/80 expression observed in the MRL/Mp mononuclear cells were lower than those in C57BL/6 mice. B. Representative analysis of CD11b expression on cells gated as F4/80-positive. At 96 h after the induction of peritonitis in C57BL/6 mice the majority of F4/80-positive cells expressed high levels of CD11b (64% of the gated cell population in the example shown and 63 ± 1.4% (mean ± SEM) in a group of five mice). In contrast, the majority of the F4/80-positive cells in MRL/Mp mice expressed low levels of CD11b (67% of the gated cell population in the example shown and 62 ± 2.9% (mean ± SEM) in a group of five mice). C. Representative analysis of Ly6C expression on cells gated as F4/80-positive. At 96 h after the induction of peritonitis in C57BL/6 mice the minority of F4/80-positive cells expressed Ly6C (18% of the gated cell population in the example shown and 23.9 ± 2.9% (mean ± SEM) in a group of five mice). In contrast, the majority of F4/80-positive cells in MRL/Mp mice expressed Ly6C (63% of the gated cell population in the example shown and 59.4 ± 1.9% (mean ± SEM) in a group of five mice). The data shown are representative of three independent experiments.
positive expressed CD11b at low levels and were also positive for Ly6C. Thus, the FACS analysis of PEC 96 h after thioglycolate injection showed that MRL/Mp mice exhibited reduced recruitment and impaired differentiation of monocytes into fully activated macrophages compared with C57BL/6 mice.

Following these findings we extended these investigations to include two additional mouse strains, one autoimmune-prone (NZB/W) and the other nonautoimmune (BALB/c). Results from a representative experiment are shown in Fig. 4. In both autoimmune-prone strains at 96 h postinjection there was a significantly reduced elicitation of cells positive for F4/80 expressing CD11b at high levels compared with the two nonautoimmune strains (Fig. 4; C57BL/6, 2.066 ± 0.21 × 10^6/ml; MRL/Mp, 0.79 ± 0.13 × 10^6/ml; NZB/W, 0.89 ± 0.15 × 10^6/ml; BALB/c, 2.5 ± 0.46 × 10^6/ml; mean ± SEM; n = 4–6; p < 0.01 for both lupus-prone strains compared with the nonautoimmune-prone strains, by Bonferroni multiple comparison test). Analysis of cytospin preparations supported these findings and confirmed the difference in the morphologic features of PEC between the lupus-prone and non-lupus-prone strains (data not shown).

Defective clearance of apoptotic PMN in autoimmune-prone strains

In the thioglycolate-induced peritonitis model the elicited PMN rapidly undergo apoptosis; macrophages are the primary cell type responsible for the clearance of these cells in vivo (7). We therefore explored whether the reduced recruitment of mononuclear cells in the lupus-prone strains caused defective clearance of PMN, which might lead to an increased number of apoptotic PMN remaining in the peritoneum. At 24 h the peritoneal lavages from MRL/Mp and NZB/W mice contained a significantly higher percentage of late apoptotic PMN (7.35 ± 0.38 and 5.83 ± 0.38%, respectively) compared with C57BL/6 and BALB/c mice (2.67 ± 0.25 and 2.56 ± 0.66%, respectively; C57BL/6 or BALB/c vs MRL/Mp or NZB/W, p < 0.001, by Bonferroni multiple comparison test; Fig. 5A). In parallel with this observation, the percentage of macrophages containing apoptotic debris in autoimmune mice was significantly lower than the percentage in control mice (5.38 ± 0.87 and 9.60 ± 0.088% in MRL/Mp and NZB/W) and normal (C57BL/6 and BALB/c) mice 24 h after thioglycolate injection. The lupus-prone strains had a significant increase in the number of late apoptotic cells present in the peritoneum compared with the normal strains (p < 0.001, by Bonferroni multiple comparison test). Horizontal bars denote means. B, Scatter plot showing the percentage of macrophages containing apoptotic bodies at the same time point as in A. Phagocytic uptake was impaired in the autoimmune-prone strains compared with the normal animals (p < 0.05, by Bonferroni multiple comparison test). Horizontal bars denote means. The data shown are representative of three independent experiments.
the increased number of free apoptotic PMN present in the peritoneal cavity in these mice compared with the control strains.

**Defective in vivo clearance of apoptotic cells by resident peritoneal macrophages from autoimmune-prone mice**

The lower percentage of macrophages containing apoptotic debris suggested that the macrophages from lupus-prone mouse strains had an intrinsic defect in the removal of apoptotic cells. We investigated this using a model of apoptotic cell clearance in which murine apoptotic cells, prepared in culture, are injected into the peritoneum (29). We analyzed the uptake of apoptotic cells by the resident peritoneal macrophages to avoid the confounding factor of the different number and phenotype of mononuclear cells recruited by thiglycolate in the autoimmune-prone strains compared with control strains. The resident populations appeared similar in morphology in all strains, except that there were higher numbers of resident peritoneal macrophages in the two autoimmune-prone strains (C57BL/6, 4.72 ± 0.36 × 10^5; BALB/c, 4.40 ± 0.18 × 10^5; MRL/Mp, 8.41 ± 0.22 × 10^5; NZB/W, 8.87 ± 0.55 × 10^5 (mean macrophages/ml ± SEM); p < 0.001 when comparing control strains with autoimmune-prone mice; n = 5/group). FACS analysis of cell surface markers did not reveal any significant difference in the expression of F4/80, CD11b, Ly6C, and CD93 between strains (Fig. 3A and data not shown). The uptake of apoptotic cells was examined by FACS and by counting ingested cells on coded cytopsins; the results from both these analyses correlated very closely. In C57BL/6 and BALB/c mice, 30 min after the injection of murine apoptotic thymocytes, the percentages of macrophages containing apoptotic bodies were 34.4 ± 4.1 and 30.8 ± 3.7%, respectively (mean ± SEM). These figures were significantly lower in the two lupus-prone strains (12.7 ± 1.4% for MRL/Mp/+/+ mice and 14.5 ± 1.1% for NZB/W mice; p < 0.01, by Bonferroni multiple comparison test; Fig. 6). As we had observed a higher number of resident peritoneal macrophages in the lupus-prone strains compared with the nonautoimmune strains, we hypothesized that the clearance of apoptotic cells may have been affected by the different ratios of apoptotic cells to macrophages. We therefore compensated for this difference in further in vivo clearance experiments by injecting a proportionally higher number of apoptotic thymocytes into lupus-prone mice (1.8 × 10^7 in MRL/Mp in contrast to 10^7 in C57BL/6 mice). Increasing the number of apoptotic cells injected into MRL/Mp mice had no effect on the uptake of these cells by resident peritoneal macrophages. A higher percentage of macrophages from C57BL/6 mice ingested thymocytes than from MRL/Mp mice (47.6 ± 4.9 vs 28.1 ± 6.5%; p < 0.05).

**Defective in vitro uptake of apoptotic cells by resident peritoneal macrophages from autoimmune-prone mice**

The results from our in vivo studies demonstrated a reduced clearance of apoptotic cells in the two autoimmune-prone strains investigated. To determine whether this was due to an intrinsic defect in the macrophages or to the potential autoimmune milieu present in the lupus-prone mice, we conducted in vitro phagocytosis studies. Resident macrophages were plated out overnight before being exposed to apoptotic thymocytes for 30 min. After overnight culture there was no difference in the growth or morphology of the resident macrophages from the different strains investigated (data not shown). The viabilities of the macrophages, assessed by annexin V binding, were also similar (data not shown). The uptake of apoptotic thymocytes was significantly reduced in MRL/Mp macrophages compared with macrophages from C57BL/6 mice (69.2 ± 3.9 vs 30.8 ± 3.9%; p = 0.0079, by Mann-Whitney U test; Fig. 7A). A similar phagocytic defect was observed when MRL/Mp macrophages were compared with macrophages from CBA animals, an H2 haplotype-matched strain (37.6 ± 1.3 vs 51.3 ± 1.9%, MRL/Mp and CBA, respectively; mean ± SEM; p < 0.001, by Mann-Whitney U test).

**Increased in vitro uptake of opsonized RBC by resident macrophages from autoimmune mice**

To confirm the specificity for apoptotic cells of the phagocytic defect observed in lupus-prone mice we investigated FcγR- and complement-mediated phagocytic mechanisms. Resident peritoneal macrophages were incubated in vitro with murine RBCs that had been opsonized with IgG1 or C3, as described in Materials and Methods, and uptake was assessed after 30 min. In contrast to what we observed with apoptotic cells, but in agreement with previous reports (40, 41), macrophages from MRL/Mp mice exhibited a marked increase in FcγR-dependent phagocytosis (81.5 ± 3.3% of MRL/Mp macrophages containing ingested RBC vs 43.2 ± 3.8% for C57BL/6; p = 0.0093, by Mann-Whitney U test; Fig. 7B). In addition, as previously shown (42), the resident macrophages from both experimental groups failed to engulf C3-opsonised RBCs (data not shown).

**Discussion**

Macrophages are the primary cell type responsible for the resolution of inflammation and the clearance of apoptotic debris in most tissues (5, 6). Here we have described an aberrant response of peritoneal macrophages from two autoimmune-prone strains (MRL/Mp and NZB/W) to a sterile injection of thiglycolate. This defect resulted in quantitative and qualitative differences in the recruitment of mononuclear cells and a delay in the resolution of the inflammatory insult, and was associated with delayed resolution of inflammation characterized by increased numbers of neutrophil apoptotic bodies remaining in the peritoneal cavity.

In two lupus-prone autoimmune strains of mice we observed an increased number of total resident peritoneal cells compared with...
mice exhibited a marked increased in phagocytic uptake (uptake was assessed after 30 min. Resident macrophages from MRL/Mp and C57BL/6 mice were incubated with IgG-coated RBC. The Mann-Whitney test compared with C57BL/6 macrophages (p = 0.0093, by Mann-Whitney U test). Horizontal bars denote means.

FIGURE 7. A. Uptake of apoptotic thymocytes by resident peritoneal macrophages in vitro. Resident macrophages from MRL/Mp and C57BL/6 mice were kept in 24-well plates overnight at a concentration of 7.5 × 10⁶ macrophages/well. Apoptotic thymocytes (1.5 × 10⁶) were then added, and uptake was assessed after 30 min by counting coded cytospin slides. Resident macrophages from MRL/Mp exhibited markedly impaired uptake compared with C57BL/6 macrophages (p = 0.0079, by Mann-Whitney U test). B. Uptake by resident peritoneal macrophages in vitro of opsonized RBC by FcγR-dependent mechanisms. Resident macrophages from MRL/Mp and C57BL/6 mice were incubated with IgG-coated RBC. The uptake was assessed after 30 min. Resident macrophages from MRL/Mp mice exhibited a marked increased in phagocytic uptake (p = 0.0093, by Mann-Whitney U test). Horizontal bars denote means.

Those in two control strains. Russell and colleagues (41) have previously reported this difference and showed that the number of resident peritoneal cells in autoimmune-prone strains increased with aging. Here we detected a significant difference in the numbers of resident peritoneal macrophages even in young prediseased mice. However, the resident cell populations were indistinguishable in number and morphology when analyzed on cytospins and by FACS. Following the administration of thioglycolate, the recruitment of PMN was similar in all four strains investigated. However, a striking difference in the mononuclear cell response was observed between lupus-prone and control strains of mice. The two autoimmune-prone strains, MRL/Mp and NZB/W, had a significantly reduced influx of mononuclear cells compared with the nonautoimmune strains, C57BL/6 and BALB/c. This confirms and extends the findings of Hamilton et al. (43), who also noted a reduced yield of macrophages from MRL/Mp mice, compared with C57BL/6 mice, in response to thioglycolate injection.

In addition to the reduced number of mononuclear cells elicited, we observed qualitative differences in the cells recruited in response to thioglycolate. In the C57BL/6 mice, 4 days after thioglycolate injection the majority of elicited cells expressed F4/80, had a high level of CD11b expression, and were negative for Ly6C, all hallmarks of elicited macrophages. In contrast, the cells with this phenotype were the minority in MRL/Mp mice. In these mice many of the elicited mononuclear cells, despite being positive for F4/80, expressed CD11b at low levels and were positive for Ly6C, markers for monocytes rather than macrophages. These flow cytometric data supported the morphological findings from the cytospins that showed that in the autoimmune-prone strains the morphology of many of the elicited cells was that of monocytes. These findings taken together would indicate that even though mononuclear cells were recruited into the peritoneum in response to thioglycolate in MRL/Mp mice, they were not able to fully differentiate into macrophages. In MRL/Mp mice, in addition to the increased proportion of cells expressing Ly6C, we observed a concomitant reduction in the expression of CD93 (also known as C1qR) (32). We have recently shown that thioglycolate-induced inflammation leads to a greatly increased expression of CD93 on elicited macrophages compared with resident peritoneal macrophages (unpublished observations). The reduced expression of this marker on peritoneal mononuclear cells from the autoimmune strains of mice provides additional evidence that they failed to mature into macrophages.

Thioglycolate-elicited macrophages from lupus-prone mice have previously been shown to display an in vitro dysregulation of cytokine production (14–19, 43). In MRL/Mp and NZB/W strains a reduced synthesis of TNF-α associated with an impaired production of IL-1 and IL-6 has been reported (17). This abnormal cytokine secretion was detected in macrophages from young prediseased mice, suggesting the possibility of an intrinsic defect (15). Recently, a common defect in the NF-κB pathway of activation has been shown to contribute to the aberrant levels of cytokines detected in MRL/Mp and NZB/W strains (20, 21). In MRL/Mp mice the reduced secretion of IL-1 may be explained by the increased expression of TGF-β1 reported in this strain (18). A recent study has also shown that pre-exposure to apoptotic cells leads to a reduced production of IL-1 by macrophages in response to LPS and is a shared characteristic of macrophages from lupus-prone strains (44). Further studies will be needed to determine whether this dysregulation of proinflammatory cytokines detected in vitro contributed to the in vivo phenotype observed in our experimental model.

The impaired response of the macrophages from the two lupus-prone strains was accompanied by impaired removal of dying
PMN. Although the PMN were initially cleared at a similar rate, perhaps reflecting the effects of other mechanisms operating in the initial phase (8, 9), a significant delay in the removal of PMN in MRL/Mp and NZB/W mice at 96 h was observed. Approximately 10% of the cell population at 96 h in the two autoimmune-prone strains were PMN, whereas this figure was <1% in the two control strains. In addition, in the autoimmune-prone strains there was a significant increase in the amount of apoptotic PMN remaining free in the peritoneal cavity. The reduced number of phagocytic cells recruited to the peritoneum in the autoimmune strains could only partially explain this inefficient removal of apoptotic PMN.

The finding that the percentage of macrophages ingesting apoptotic debris was significantly lower despite an increased ratio of apoptotic PMN to macrophages suggested an inherent phagocytic defect in lupus-prone mice. Evidence in support of this hypothesis was provided by in vivo and in vitro assays examining the phagocytic uptake of apoptotic murine thymocytes by resident peritoneal macrophages. In both assays the engulfment of apoptotic thymocytes by MRL/Mp or NZB/W resident macrophages was significantly impaired compared with that of C57BL/6 or BALB/c resident macrophages. It is of note that resident peritoneal macrophages from all strains examined appeared similar in morphology on cytospin preparations and showed similar expression of cell surface markers by FACs. In this context it is also relevant to note that monocyte-derived macrophages from humans with SLE also exhibit impaired phagocytosis of apoptotic cells in vitro (30, 45) and in vivo (46). In contrast to our results, two previous in vitro studies have been unable to demonstrate a difference in the uptake of apoptotic cells by MRL/Mp macrophages compared with nonautoimmune strains (47, 48). In these previous reports the control strains were H2 haplotype-matched to the MRL/Mp mice (H2β). However, we still detected a significantly impaired uptake of apoptotic thymocytes by MRL/Mp macrophages compared with that by macrophages from haplotype-matched CBA mice. The explanation for these contradictory findings may lay in the differences in the time selected to compare the uptake or in the ratio of apoptotic cells to macrophages used in the study described here compared with previous investigations. The time course of the uptake of apoptotic cells by macrophages has previously been shown to be critical when investigating differences in the clearance of apoptotic cells (29), and in our study we analyzed the uptake at an earlier time point (30 min) compared with the previous reports (47, 48). Notably, in previous in vitro studies it was shown that prior exposure to apoptotic cells can down-modulate the ability of the macrophages to ingest apoptotic cells and that MRL/Mp macrophages were hypersensitive to this inhibitory effect (47, 49). It is thus possible that previous exposure of the mononuclear cells to apoptotic debris in circulation may contribute to the impaired disposal of apoptotic cells in lupus-prone strains. An increased rate of spontaneous cell apoptosis has been described in SLE patients and mouse models of SLE (50–52), and this may account for the presence of elevated concentrations of nucleosomes found in the circulation of lupus patients (53). However, it is also of note that, as in previous studies, we have used young (8- to 10-wk-old), pre diseased mice. In addition, previous studies have detected higher levels of apoptosis in monocytes and macrophages from SLE patients, which would be consistent with the reduced uptake of apoptotic cells (45, 54) observed in our assays. However, in the present study there was no detectable difference in annexin V binding to resident macrophages from MRL/Mp mice and the control strains either ex vivo or in vitro (data not shown).

In MRL/Mp mice the phagocytic defect for apoptotic cells was not accompanied by a generalized impairment in the engulfment of cells mediated by another pathway. Indeed, the uptake of IgG-coated murine RBCs, via FcγR, in MRL/Mp was greater than that in C57BL/6 mice (Fig. 7B). This is in accordance with two previous reports (40, 41).

The defect reported here in resolving an inflammatory lesion, characterized by impaired clearance of apoptotic neutrophils, may have important implications for the initiation and perpetuation of autoimmune responses in lupus-prone strains. Accumulating evidence suggests that apoptotic cells may be a source of the autoantigens targeted by the autoantibodies in SLE. A reduced ability of macrophages to clear apoptotic debris may cause a persistent elevation of the autoantigenic material derived from apoptotic and necrotic cells. Failure to efficiently clear apoptotic cells may lead to the persistence of late apoptotic and necrotic cells, and this may be especially harmful in the context of an inflammatory response. Under some circumstances, an overload of apoptotic cells has been shown to lead to a specific immune response against these cells (55, 56). Thus, an impairment in the disposal mechanisms may divert the clearance pathways of autoantigens at sites of inflammation toward pathways that may lead to the breakdown of tolerance to the autoantigens contained in injured tissues (56, 57). In particular, the presence of a milieu of inflammatory cytokines may favor the clearance of apoptotic debris by dendritic cells, resulting in the maturation of these cells and the initiation and perpetuation of an autoimmune response.

In conclusion, we have shown impairment in the inflammatory response to thioglycolate in two murine models of SLE. We have provided in vivo and in vitro evidence for a functional intrinsic defect of the macrophages from lupus-prone strains in the phagocytosis of apoptotic cells, a potential source of autoantigens. The presence of a shared phenotype in the murine SLE models examined provides support for the hypothesis of a common phagocytic defect (or separate defects within a common signaling pathway) predisposing to the development of SLE.

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References


