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*J Immunol* 2003; 170:3223-3232; doi: 10.4049/jimmunol.170.6.3223

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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 intensely 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 ifpr 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 (PEC) were initially counted on a hemocytometer and then differential cell counts were conducted on cytocentrifuge preparations stained with Diff-Quik (Dade-Behring, Munich, Germany). The number of apoptotic PMN was determined by analyzing coded cytospins. 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 cytospins. 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 cytoospin 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 Biosciences, Mountain View, CA). Three-color FACS analysis was conducted with an additional biotinylated Ab that was then detected with a strepta-

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### 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 ± 0.05 × 10^6/ml compared with 1.17 ± 0.04 × 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 ± 0.35 × 10^6 cells/ml in the C57BL/6 mice and 4.50 ± 0.47 × 10^6/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 ± 0.13 × 10^6 cells/ml), with no additional increase at 96 h (6.45 ± 0.23 × 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 ± 0.28 × 10^6 PMN/ml in the MRL/Mp mice and 2.79 ± 0.22 × 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 ± 0.36 × 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 still a significant number of PMN remaining in the peritoneal lavage (5.28 ± 0.70 × 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 ± 0.05 × 10^6 and 1.12 ± 0.08 × 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 ± 0.23 × 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.
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.

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, 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 by this approach correlated closely with that determined by light microscopy. The numbers of macrophages recruited, as determined by cytoospin and FACS, respectively, were 5.92 ± 0.23 × 10^6 and 5.19 ± 0.25 × 10^6 for C57BL/6, and 1.54 ± 0.25 × 10^6 and 2.50 ± 0.21 × 10^6 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 proportion 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 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 mice vs 14.0 ± 0.65 and 20.0 ± 3.23% in C57BL/6 and BALB/c mice, respectively; C57BL/6 or BALB/c vs MRL/Mp or NZB/W, p < 0.05, by Bonferroni multiple comparison test; Fig. 5B). Thus, there was a mismatch between the reduced prevalence of macrophages containing apoptotic debris in the autoimmune strains of mice and

**FIGURE 4.** Recruitment of F4/80-positive cells expressing high levels of CD11b 96 h after thioglycolate injection in lupus-prone (MRL/Mp and NZB/W) and nonautoimmune (C57BL/6 and BALB/c) mice. PEC were subjected to FACS analysis as described in Materials and Methods. The number of fully differentiated macrophages, defined by their positivity for F4/80 and high expression of CD11b, was markedly reduced in the lupus-prone strains (MRL/Mp and NZB/W).

**FIGURE 5.** Clearance of PMN 24 h after thioglycolate injection. A, Graph showing the percentage of uningested apoptotic PMN remaining free in the peritoneal lavages from autoimmune-prone (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 thioglycolate 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 cells in the two autoimmune-prone strains (C57BL/6, 4.72 ± 0.36 × 10⁵; BALB/c, 4.40 ± 0.18 × 10⁵; MRL/Mp, 8.41 ± 0.22 × 10⁵; NZB/W, 8.87 ± 0.55 × 10⁵) (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 with autoimmune-prone 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. 6A). 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-opsonized 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 thioglycolate. 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 < 0.0079, by Mann-Whitney U test). The cells from MRL/Mp exhibited markedly impaired uptake compared with C57BL/6 macrophages (p = 0.00093, 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 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 = 0.00093, by Mann-Whitney U test). Horizontal bars denote means.

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 C1qRp; data not shown) (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 cytoplasm 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 (H2b). 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), 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.

In conclusion, we have shown impairment in the phagocytic clearance to thiglycolate 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.

Acknowledgments

We thank all of the staff of the animal facility for their technical assistance.

References


