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Neutrophil apoptosis in autoimmune Fas-defective MRL lpr/lpr mice

Abstract

The apoptosis-defective lpr (fas) mutation in MRL mice causes the early onset of a lupus-like autoimmune disease with concomitant inflammation. In order to analyse the consequences of the impaired Fas-dependent apoptosis on inflammation, the susceptibility to apoptosis of polymorphonuclear leukocytes (PMN), obtained from MRL lpr/lpr mice, has been studied. Peritoneal PMN from lpr/lpr and control (+/+) mice were recruited with a mild inflammatory stimulus. The number of cells collected from the peritoneal cavity of young lpr/lpr mice was comparable to that obtained from age-matched control mice, indicating that PMN homeostasis is maintained regardless of the loss-of-function Fas

mutation. Recruited neutrophils were exposed in culture to apoptosis-inducing stimuli. Treatment with agonist anti-Fas antibody increased apoptosis of +/+ PMN, but did not affect lpr/lpr PMN which do not express Fas on their surface. However, lpr/lpr PMN could undergo both spontaneous and stimulus-induced apoptosis in a fashion comparable to or higher than that of control +/+ mice. Analysis of mRNA expression revealed that lpr/lpr PMN have reduced expression of IL-18, whereas IL-1beta, IFNgamma, caspase 1 and caspase 3 are expressed at levels comparable to those of +/+ cells. However, caspase-3-like activity was higher in PMN from lpr/lpr mice than in +/+ cells, and correlated with enhanced apoptosis. It could be concluded that in young, uncompromised lpr/lpr mice, PMN homeostasis is still fully regulated through the involvement of Fas-independent, compensatory, apoptotic mechanisms. This could include an increased participation of caspase 3 in the apoptotic pathway, consequent to enhanced activation of the enzyme and to the decreased production of IL-18, which acts as a competitive caspase 3 substrate.

Full text

INTRODUCTION

MRL mice bearing the autosomic recessive mutation *lpr* (for lymphoproliferation), develop an early autoimmune syndrome which resembles, in many aspects, human diseases such as systemic lupus erythematosus, rheumatoid arthritis, systemic vasculitis and the autoimmune lymphoproliferative syndrome ALPS. The *lpr* disorder is mainly characterised by massive lymphoproliferation and strong autoantibody production leading to the accumulation of leukocytes and to the onset of severe inflammatory damage with fatal outcome [1-4]. The *lpr* mutation has been located on the *fas* gene and leads to the aberrant transcription and impaired expression of the Fas protein [5]. Fas (CD95/Apo-1) is a cell surface receptor broadly expressed on many cell types, which is able to induce apoptosis when triggered by its natural ligand FasL or by agonist antibodies. In homozygous *lpr/lpr* mice, the presence of mutant Fas leads to defective Fas-dependent lymphocyte apoptosis which is responsible for impaired deletion of peripheral autoreactive lymphocytes leading to lymphoproliferation [6].

Whereas many studies have been performed on *lpr/lpr* mice as regards the regulation of the immune response and their deficient lymphocyte-dependent

functions [4-7], not as much information is presently available on the inflammatory processes active in this Fas-defective model. In fact, besides playing a very important role in the regulation of the immune response by affecting lymphocyte homeostasis, Fas also plays a critical role in limiting inflammation [8, 9]. It has been recently demonstrated that Fas and its ligand FasL are both expressed on polymorphonuclear leukocytes (PMN) and that these cells can undergo apoptosis through the Fas system [10-12].

PMN are the first cells to be recruited to the site of inflammation where they are activated to initiate the defence functions or, in diseased states, act as mediators of tissue damage. During resolution of the reaction, when inflammatory stimuli are no longer present, PMN undergo rapid apoptosis and are readily recognised and engulfed by scavenging cells [13, 14]. Their removal represents a beneficial process for re-esta- blishing normal physiological conditions and avoiding the chronicization of inflammation.

To evaluate the role of defective Fas in causing the late, fatal, chronic inflammatory damage of *lpr/lpr* mice, this study has focusued upon analysing neutrophil apoptosis in these Fas-defective mice. Results obtained indicate that, despite the defect in Fas-mediated apoptosis, PMN homeostasis is normal in *lpr/lpr* mice, possibly as a consequence of compensatory activation of Fas-independent apoptotic mechanisms.

MATERIALS AND METHODS

Animals and cell recovery

Homozygous MRL +/+ and *lpr/lpr* mice, originally obtained from the Jackson Laboratories (Bar Harbor, ME, USA), were bred in the animal facilities of Dompé Research Centre, under specific pathogen- free conditions. Animals aged 4-8 weeks were used throughout the study, unless otherwise indicated. At this age *lpr/lpr* mice had not yet developed the autoimmune symptoms. Three hours after intraperitoneal inoculation of 0.5 ml of sterile 9% casein solution, age- and sex-matched animals were sacrificed by ether hyperanaesthesia and peritoneal exudate cells (PEC) were collected. All procedures were performed in the animal operating rooms in accordance with the ethical guidelines for the conduct of animal research (Authorisation by the Italian Ministry of Health n° 271/95-B, D. Lvo 116/92).

Differential counts performed on cytosmears of PEC stained with Diff-Quik (Baxter, Dudingen, CH) demonstrated that over 75% of the recovered exudate cells were PMN. Routinely, cells were used for experimental purposes without further enrichment. In selected experiments, PMN enrichment was performed on Percoll or dextran gradients to reach 97-99% purity.

For experimental procedures, cells were cultured for the indicated times in complete medium (RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 muM 2-ME and 50 mug/ml gentamicin sulfate; Life Technologies, Paisley, Scotland), in the absence or in the presence of the following reagents: agonist anti-mouse Fas antibody (Pharmingen; San Diego, CA, USA), Cer-C8 (soluble synthetic ceramide analogue; Sigma Chemical Co., St. Louis, MO, USA), YVAD-CMK (irreversible, cell-permeable, caspase 1 inhibitor; Calbiochem, La Jolla, CA, USA), IL-1beta (hu r IL-1beta, sp. act. 1.0 x 10^8 IU/mg; 15), IL-1ra (hu r IL-1RA; 15), FasL inhibitor (chimeric Fas:Fc; Alexis Biochemicals, San Diego, CA, USA).

Assessment of apoptosis

PMN apoptosis was routinely assessed on the basis of DNA loss measured by propidium iodide staining of nuclei followed by cytofluorimetric analysis. Briefly, 5 x 10^6 cells, in 12 x 75 mm polystyrene tubes, were fixed by adding 5 ml drops of ice-cold 80% ethanol, mixing by pipetting, and incubating for 30 min at 4° C. Cells were then washed once with PBS, resuspended in 1.25 ml of PBS and further incubated for 20 min at room temperature. After addition of an equal volume of propidium iodide (PI) solution (100 mug/ml; Sigma) containing 100 mug/ml of RNase, and overnight incubation in the dark at 4° C, cells were washed with PBS and analysed by flow cytometry (FACScan, Becton-Dickinson, Mountain View, CA, USA). A minimum of 10,000 events per sample was counted and debris was excluded by gating on the basis of physical parameters. Apoptosis is expressed as the percentage of hypodiploid nuclei counted over the total cell population. Estimation of apoptotic cells by flow cytometry was always confirmed by morphological analysis of cytospin smears stained with Diff-Quik (not shown).

In some experiments, apoptosis was assessed by positivity to annexin V staining alone or in double staining with the anti-Gr-1 antibody, a PMN-specific marker. Briefly, cells washed in PBS were resuspended in binding buffer (10 mM

Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$; Sigma) at a concentration of 5 x 10^6 cells/ml. An aliquot of 195 mul of the cell suspension was incubated for 5 min at room temperature with 5 mul of Annexin V-FITC (Boehringer Ingelheim; Heidelberg, Germany). After addition of PI (1 mug/ml), cytofluorimetric analysis of positivity to annexin V was performed on PI-negative cells (viable cells). Counterstaining with a phycoerythrin (PE)-labeled antibody to the PMN marker Gr-1 (Pharmingen) allowed identification of annexin V-positive cells as PMN.

Semi-quantitative RT-PCR

Total RNA was prepared from 1.0-1.5 x 10^7 cells cultured for 18 hours, using the RNeasy Total RNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 mug) was reverse transcribed into cDNA in a total volume of 50 mul, using pdN6 primers (Boehringer-Mannheim, Mannheim, Germany) and MMuLV Reverse Transcriptase (RT; Stratagene, La Jolla, CA, USA). PCR amplification was carried out in a total volume of 25 mul 1 x PCR buffer (Stratagene) containing 2.5 mul of the first strand cDNA, 50 muM of each dNTP (Stratagene), 1 muM of each primer (Endogen, Milano, Italy) and 1 U *Taq* DNA polymerase (Stratagene). The oligonucleotides used as primers were: HPRT 5' (5'-GTT GGA TAC AGG CCA GAC TT T GTT-3'), HPRT 3' (5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'), IL-1beta 5' (5'-TTG AAG AAG AGC CCA TCC TCT G-3'), IL-1beta 3' (5'-GAT CCA CAC TCT CCA GCT GC-3'), IL-18 5' (5'-ATA AAT GAC CAA GTT CTC TTC GT-3'), IL-18 3' (5'-GGA ACA CAA ACC CTC CCC ACC TA-3'), IFNgamma 5' (5'-TGG CTG TTT CTG GCT GTT ACT G-3'), IFNgamma 3' (5-AAT CAG CAG CGA CTC CTT TTC C-3'), caspase 1 5' (5'-AAC ATT ACT GCT ATG GAC AAG GC-3'), caspase 1 3' (5'-CCC AGT CAG TCC TGG AAA TGT GC-3'), caspase 3 5' (5'-AGC ACT GGA ATG TCA TCT CG-3') and caspase 3 3' (5'-GTA GTC GCC TCT GAA GAA GC-3'). Cycle parameters were: annealing 0.5 min at 55° C, elongation 1 min at 72° C, denaturation 0.5 min at 95° C. Resulting PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

For semiquantitative PCR, the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was used as a standard. Cytokines and caspases were amplified out of each cDNA batch with 25, 30, 35 and 40 (or 29, 32, 35 and 38) amplification cycles. After gel analysis, bands were scanned (Personal Densitometer with Image Quant software; Molecular Dynamics, Sunnyvale, CA, USA) and their densities were assessed. For each number of

cycles, the ratio between densities of cytokine or caspase bands and of the corresponding HPRT band was calculated and expressed as relative units.

Analysis of caspase activity

Cell lysates were obtained by resuspending PMN at a concentration of 1 x 10^8 cells/ml in hypotonic buffer containing HEPES 25 mM pH 7.5, $MgCl_2$ 5 mM, EDTA 1 mM and the protease inhibitors PMSF (1 mM), pepstatin (10 mug/ml) and leupeptin (10 mug/ml) (all from Calbiochem). Nuclei were removed by centrifugation at 2,000 x g for 20 min at 4° C. Supernatant was recovered and centrifuged again at 18,000 x g for 20 min at 4° C. On this final supernatant preparation (containing cytoplasm and membranes), caspase 1-like activity (caspase 1, 4 and 14) was measured by kinetic detection of fluorescent cleavage product using the selective substrate tetrapeptide YVAD-7-amino-4-methylcoumarin (YVAD-AMC; Calbiochem). Caspase 3-like activity (caspase 3, 6, 7, 8 and 10) was similarly assessed on the selective substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC; Calbiochem). Specificity controls were run by adding the protease inhibitors YVAD-CMK (caspase 1 inhibitor) and DEVD-CMK (caspase 3 inhibitor), together with the substrate.

RESULTS

PMN recruitment is not altered in MRL lpr/lpr *mice*

In order to obtain the amount of PMN sufficient for experimental studies, cells of MRL */pr/lpr* mice were recruited to the peritoneal cavity by elicitation with an inflammatory stimulus. However, since strong inflammatory activation can make PMN resistant to apoptosis [16, 17], PMN were recruited by intraperitoneal inoculation of casein 9%, 3 hours before cell collection. This proved to be a mild inflammatory stimulus, able to recall cells to the inflammatory site but unable to block spontaneous apoptosis. As shown in Figure 1, the influx of inflammatory cells into the peritoneal cavity is equally vigorous both in normal MRL +/+ mice and in the Fas-defective */pr/lpr* mice, being comparable to that of unrelated C3H mice. Moreover, no significant difference between very young, disease-free animals (4-8 weeks of age) and mice at the beginning of the overt pathology (12-15 weeks of age) was observed in terms of cell recruitment. Among peritoneal exudate cells, PMN accounted for 75-85% of the total recovered population both in normal and in Fas-defective animals (Figure 1).

PMN from MRL lpr/lpr *mice are resistant to apoptotic triggering by Fas*

Since the genetic defect in the *fas* gene does not allow the expression of functional Fas protein on cells from MRL */pr/lpr* mice, these cells are expected to be resistant to apoptosis induced by Fas triggering. Indeed, peritoneal exudate PMN from */pr/lpr* mice do not express Fas on their surface (data not shown) and could not undergo accelerated apoptosis following exposure to an anti-Fas agonist antibody (Figure 2, lower left panel). In contrast, PMN from age-matched Fas-expressing +/+ mice are significantly stimulated to apoptosis by anti-Fas antibody.

Spontaneous and induced apoptosis is not impaired in PMN from MRL lpr/lpr *mice*

Despite their inability to undergo apoptosis in response to Fas triggering, PMN from */pr/lpr* mice did not appear to be otherwise defective in their apoptotic mechanisms. Indeed, as shown in Figure 2 (upper left panel), spontaneous apoptosis of */pr/lpr* PMN in culture, takes place with completely normal kinetics and reaches even higher levels than those of +/+ cells. With the use of a specific inhibitor of FasL, spontaneous apoptosis turned out to be only partially dependent on Fas in cells from +/+ mice, but completely Fas-independent in */pr/lpr* PMN (Figure 2, upper right panel). In the Fas-independent system of apoptosis in response to ceramide, apoptosis was greatly increased in Fas-defective */pr/lpr* PMN as well as in normal +/+ cells (Figure 2, lower right panel). Apoptosis was routinely measured in the whole peritoneal exudate cell population, which could include up to 20% of cells other than PMN. Parallel experiments with highly purified PMN preparations (> 99%; data not shown) and of double labelling with annexin V (to measure apoptosis) and antibodies to Gr-1 (to identify PMN), confirmed the above data as relating to PMN apoptosis. As an example, the cytofluorimetric analysis of a representative experiment of spontaneous apoptosis after 18 hours of culture is shown in Figure 3.

Cytokines and caspases in PMN from MRL lpr/lpr *mice*

In order to investigate the mechanisms of apoptosis in Fas-defective */pr/lpr* PMN, the role of cytokines and caspases that could regulate the apoptotic pathway, has been analysed. Since it has been reported that IL-1beta could down-regulate PMN apoptosis [16] and that PMN can produce IL-1beta [18, 19],

the role of IL-1beta in the apoptotic mechanism of *lpr/lpr* PMN has been assessed. As already shown for human PMN, exogenously added IL-1beta may partially protect mouse peritoneal PMN from spontaneous apoptosis. This protection was of similar extent in +/+ and *lpr/lpr* cells and ranged between 20 and 35% (Table 1). On the other hand, data obtained with the use of IL-1RA indicated that, in *lpr/lpr* PMN, endogenous IL-1beta has no significant role in modulating apoptosis. In fact, apoptosis of both +/+ and *lpr/lpr* PMN was fully comparable in the presence or in the absence of the IL-1 inhibitor (Table 1). Analysis of expression of IL-1beta was assessed, together with that of IL-18 and IFNgamma, and of caspase 1 and caspase 3, two enzymes involved both in the apoptotic mechanism and in the maturation/degradation of cytokines. In particular, immature pro-IL-1beta and pro-IL-18 are substrates for caspase 1 [20-22], whereas mature IL-18 is degraded by caspase 3 [22]. As shown in Figure 4, no significant differences could be found in the expression of cytokines and caspases between +/+ and *lpr/lpr* PMN, except for IL-18. Indeed, IL-18 mRNA was significantly less expressed in *lpr/lpr* PMN as compared to +/+ cells. The IL-18 protein was below limits of detection both in +/+ and in *lpr/lpr* PMN supernatants (data not shown). Since caspase expression at the mRNA level does not allow prediction of the presence of the active enzyme, the enzymatic activity of apoptotic caspases within *lpr/lpr* PMN was analysed and compared to that of +/+ cells. Caspase 1-like activity was essentially undetectable both in *lpr/lpr* and in +/+ fresh PMN and was not increased upon time in culture (the activity was always below 1 pmole/min/mg and therefore not significantly different from assay background; data not shown). In addition, PMN apoptosis was not significantly affected in the presence of a caspase 1 inhibitor (YVAD-CMK; Table 1). Thus, despite the presence of mRNA, caspase 1 activity was undetectable and apparently not relevant for apoptosis of PMN of either strain. On the other hand, the activity of caspase 3-like proteases was significantly increased by time in culture and was present at higher levels in PMN from *lpr/lpr* mice, thus strictly correlating with apoptosis (Figure 5).

DISCUSSION

Mice homozygous for the *lpr* gene *(lpr/lpr)* develop a severe, progressive, autoimmune syndrome characterised by defective lymphocyte apoptosis, the presence of autoreactive T cells, massive lymphoproliferation and autoantibody production. The disease presents with lupus-like pathological manifestations such as arthritis, vasculitis and nephritis [1-4, 23, 24], with establishment of a

severe inflammatory status responsible of tissue damage and fatal outcome. Polymorphonuclear neutrophils (PMN) play a major effector role in inflammation. PMN spontaneously and rapidly undergo apoptosis, an event of critical importance for the resolution of inflammatory reactions [12-14]. Thus, PMN apoptosis is a crucial event for homeostasis of inflammatory processes and is subjected to tight regulation to avoid pathological derangement. Inflammatory cytokines, *e.g.* IL-1 and GM-CSF, can prolong PMN survival and promote PMN-induced tissue damage during the pathological chronicisation of inflammation [16, 17].

One of the main events at the basis of the *lpr/lpr* autoimmune syndrome is a defect in the expression and function of the Fas protein, a membrane receptor responsible for apoptosis in many cell types [5, 25]. PMN express Fas on their surface and also produce the Fas ligand, FasL. Spontaneous PMN apoptosis could be at least in part due to autocrine Fas stimulation [10-12], although studies in *lpr/lpr* (lacking functional Fas) and *gld/gld* mice (lacking functional FasL) suggest that the Fas/FasL system is not involved in spontaneous PMN apoptosis in the mouse [26, 27]. The role of the spontaneous apoptosis of PMN, is the rapid removal of inflammatory cells when their presence might be detrimental. During inflammation, cytokines, bacteria and bacterial products prolong the life span of PMN and allow the development of their defensive functions [16, 28]. Fas-dependent, stimulus-triggered PMN apoptosis may thus intervene as a late mechanism of PMN removal to resolve the inflammatory response, once the specific immune response has been initiated. In fact, Fas-mediated apoptosis is one of the main mechanisms of the immune system for silencing potentially harmful immune reactions (*e.g.* autoimmunity or immune reactions in vital organs). Activated T cells are the main effectors of Fas-dependent cell death through their capacity to produce FasL [29]. During resolution of the inflammatory reaction Fas-positive PMN can be rapidly disposed of by activation of their Fas-dependent apoptotic program induced by FasL-producing immune cells.

The present study addresses the issue of PMN apoptosis *versus* survival and, more generally, PMN homeostasis in Fas-defective MRL *lpr/lpr* mice. Despite the competence of *lpr/lpr* PMN to undergo spontaneous apoptosis, the severe inflammatory status of aged *lpr/lpr* mice would suggest a defective stimulus-induced PMN apoptosis at later stages, eventually leading to the fatal outcome. Investigation of PMN functions in young *lpr/lpr* mice, before the onset of

pathological signs, would allow us to understand whether the abnormal inflammatory reaction in old mice is due to intrinsic PMN abnormalities or is rather a consequence of altered, disease-dependent PMN stimulation. Some reports describe altered PMN functions in old *lpr/lpr* mice, *e.g.* decreased or increased extravasation, depending on the stimulus [30, 31] and increased numbers of circulating PMN [31]. These however might not be due to intrinsic PMN defects could be the consequence of altered cytokine production (*e.g.* hyperproduction of TGFbeta which inhibits PMN extravasation) [31, 32].

Data reported in this paper indicate that PMN homeostasis is normal in MRL *lpr/lpr* mice, despite their inability to undergo Fas-dependent apoptosis. PMN recruitment following *in vivo* inflammatory stimulation is apparently normal. Spontaneous or stimulus-induced apoptosis is also not reduced, but rather enhanced, as compared to cells from +/+ mice. The unimpaired levels of apoptosis observed in *lpr/lpr* PMN, apparently confirm the hypothesis that spontaneous apoptosis is not Fas-dependent in mouse PMN. However, data reported here indicate that inhibition of Fas/FasL interaction can at least partially decrease spontaneous apoptosis of +/+ PMN. This would indicate that the Fas/FasL system takes part in the mechanism of spontaneous apoptosis of murine PMN, and that apoptosis of *lpr/lpr* PMN is quantitatively normal because of compensatory Fas-independent mechanisms. Analysis of stimulus-induced apoptosis showed that *lpr/lpr* PMN are fully able to undergo apoptosis, at levels even higher than in their normal counterparts. The consistently higher levels of apoptosis in *lpr/lpr* PMN again suggest that their defective Fas-dependent apoptosis is compensated for through amplification of alternate mechanisms.

Inflammatory cytokines such as IL-1 can prolong PMN survival by protecting cells from apoptosis [16, 33]. Thus, the role of the IL-1 system in the apoptotic mechanism of *lpr/lpr* PMN was investigated. As already observed for human PMN, exogenous IL-1beta could partially but significantly prolong murine PMN survival in culture. Endogenous IL-1 produced by PMN does not play any significant role in modulating PMN apoptosis, since addition of the IL-1 inhibitor IL-1RA has no effect on PMN apoptosis. It can be hypothesized that murine PMN do not control their own apoptosis through autocrine IL-1, possibly because they are unable to synthesize sufficient amounts of the active cytokine. By examining IL-1beta mRNA expression in PMN of +/+ and *lpr/lpr* mice, which was significantly and comparably expressed in both strains, it may be suggested that the difference in the apoptotic pathway of *lpr/lpr versus* +/+ PMN does not involve

synthesis or activity of endogenous IL-1beta. The fact that no autocrine IL-1-dependent regulation of survival was observed, despite the presence of significant IL-1beta mRNA levels, could be explained by examining the availability of the IL-1beta-converting enzyme caspase 1. Caspase 1 mRNA was present at comparable levels in +/+ and in *lpr/lpr* PMN. However, caspase 1 enzymatic activity was undetectable, suggesting that the post-translational mechanism of enzyme maturation and activation is not operative. Thus, despite expression of IL-1beta mRNA, the unavailability of active caspase 1 may limit cytokine maturation.

The compensatory apoptotic mechanism of *lpr/lpr* PMN could be based on the enhanced activation of caspase 3, another proteolytic enzyme involved both in Fas-dependent and independent PMN apoptosis [34-36]. At the mRNA level, caspase 3 is similarly expressed in +/+ and in *lpr/lpr* PMN. However, the caspase 3-like enzymatic activity is significantly higher in *lpr/lpr* cells, suggesting that caspase 3 matures and is activated more efficiently. In addition, *lpr/lpr* PMN express significantly lower levels of mRNA for IL-18, a cytokine which is inactivated and degraded by caspase 3 [22]. It is tempting to speculate that a decrease in one of its substrates (IL-18) may shift caspase 3 towards a more consistent participation in the apoptotic pathway. It is thus proposed that Fas-defective *lpr/lpr* PMN may undergo apoptosis through Fas-independent, caspase 3-dependent compensatory mechanisms, possibly involving enhanced enzyme activation and decreased presence of competitive substrates.

This study thus suggests that activation of the inflammatory response in MRL *lpr/lpr* mice is more likely a consequence rather than a concomitant cause of the pathology. Although *lpr/lpr* mice develop severe reactions against self-antigens due to impaired lymphocyte apoptosis, their response to foreign antigens is apparently normal, indicating the persistence of an effective inflammatory response. Thus, chronic inflammation observed in the later stage of the disease could not be attributed to PMN resistance to apoptosis, but appears to be primarily dependent on other phenomena such as the persistent activation of autoreactive lymphocytes.

CONCLUSION

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Figures

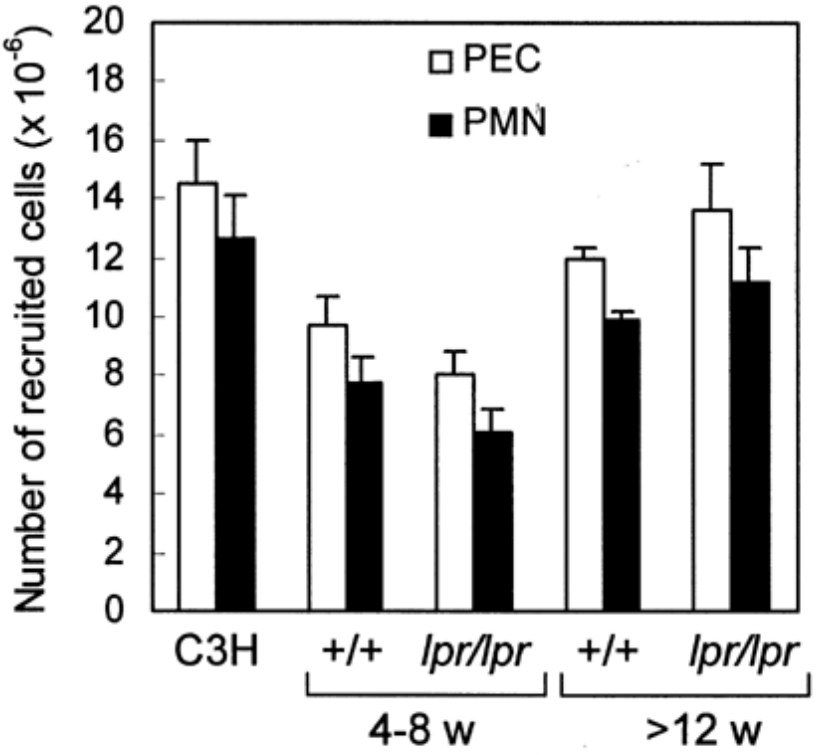


Figure 1
Recruitment of inflammatory cells in the peritoneal cavity of MRL *lpr/lpr* mice at different ages.

Inflammatory peritoneal exudate cells (PEC) of C3H, MRL +/+ and young or old MRL *lpr/lpr* mice were collected and examined 3 hours after an i.p. inoculum of 0.5 ml casein 9% in PBS. Open columns indicate the total cell number; filled columns represent PMN numbers. Data are the mean ± SEM of 3-22 experiments, for a total of 10-75 mice/group.

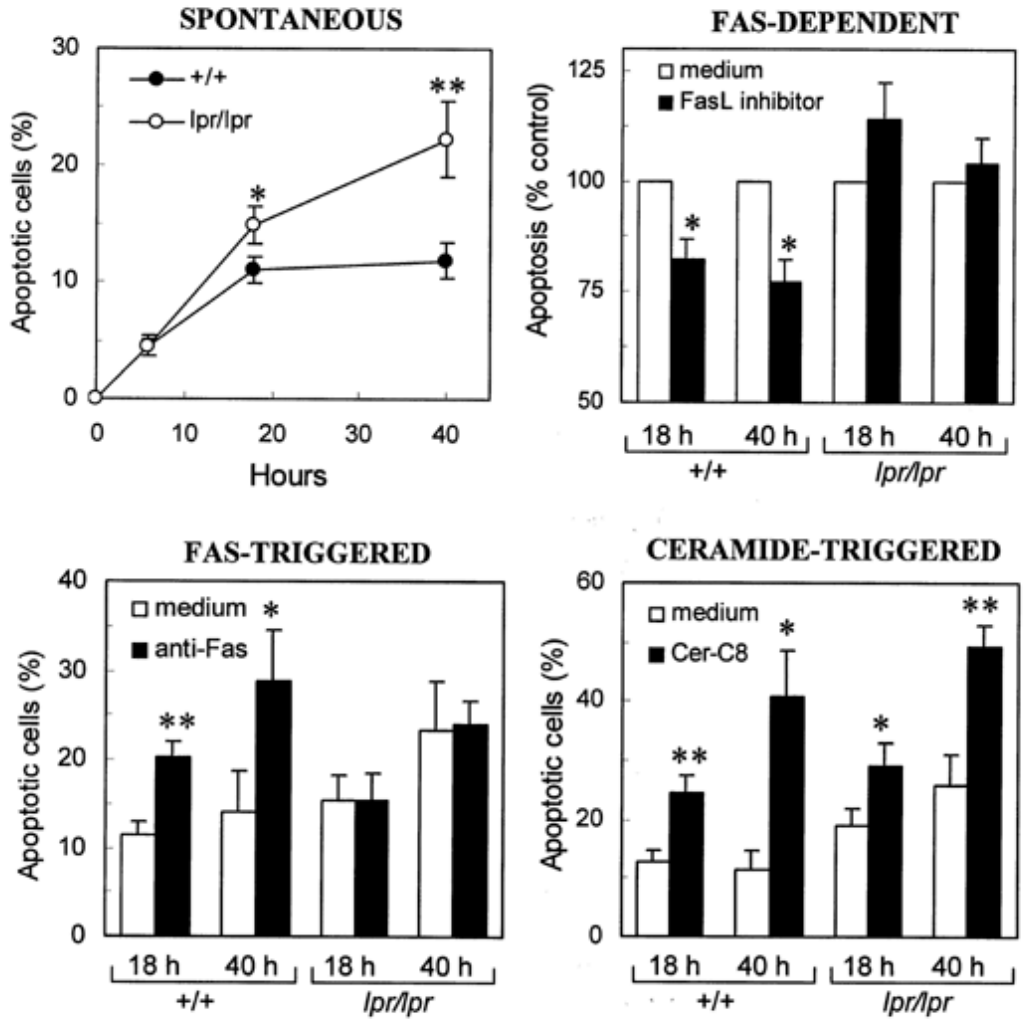


Figure 2
Spontaneous and stimulus-induced apoptosis of peritoneal exudate cells from MRL *lpr/lpr* mice.

Apoptosis of PEC (75-85% PMN) from MRL *lpr/lpr* mice was measured cytofluorimetrically after propidium iodide staining and compared with that of cells from MRL +/+ mice.

Upper left panel: spontaneous apoptosis of cultured cells from *lpr/lpr* (o) and +/+ mice (▒). Data are the mean ± SEM of results from 6-28 separate experiments.

* p < 0.05 and ** p < 0.005 *versus* MRL +/+ cells.

Upper right panel: spontaneous apoptosis of cultured cells from *lpr/lpr* and +/+ mice was assessed in the absence (medium; open columns) or in the presence of 1-10 mug/ml of FasL inhibitor (filled column). Data are the mean ± SEM of results from 8-14 separate determinations and are presented as % of control apoptosis (measured as number of apoptotic cells) in the absence of FasL inhibitor (taken as 100%).

* p < 0.005 *versus* MRL +/+ cells.

Lower left panel: apoptosis of +/+ and *lpr/lpr* cells cultured for 18 or 40 hours in medium alone (medium; open columns) or medium containing 300 ng/ml of an agonist antibody against murine Fas (anti-Fas; filled columns). Data are the mean ± SEM of 3-11 separate experiments.

* p < 0.05 and ** p < 0.001 *versus* unstimulated cells.

Lower right panel: apoptosis of +/+ and *lpr/lpr* cells cultured for 18 or 40 hours in medium alone (medium; open columns) or containing the synthetic ceramide analogue C8 at a concentration of 50 muM (Cer-C8; filled columns). Data are the mean ± SEM of 5-8 separate experiments.

* p < 0.01 and ** p < 0.005 *versus* unstimulated cells.

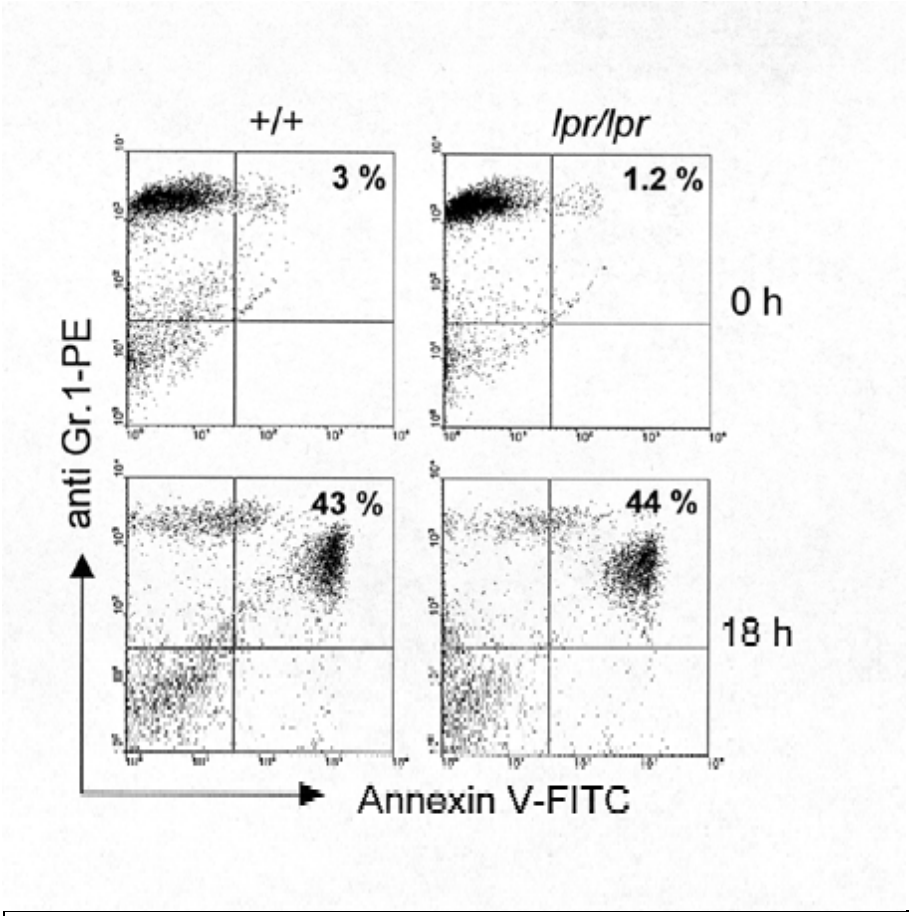


Figure 3
Spontaneous apoptosis of PMN from MRL *lpr/lpr* mice.

Apoptosis of PMN was measured by cytofluorimetric analysis of peritoneal exudate cells from MRL +/+ (left panels) and *lpr/lpr* mice (right panels) after double staining with annexin V (horizontal axis) and with a PE-labelled antibody to the PMN marker Gr-1 (vertical axis). Apoptosis was evaluated on fresh cells (upper panels) or after 18 hours of culture (lower panels). The percentage of PMN ($Gr\text{-}1^+$) undergoing apoptosis (annexin V^+) is indicated in the upper right quadrant of each panel. Results are from a single, representative experiment.

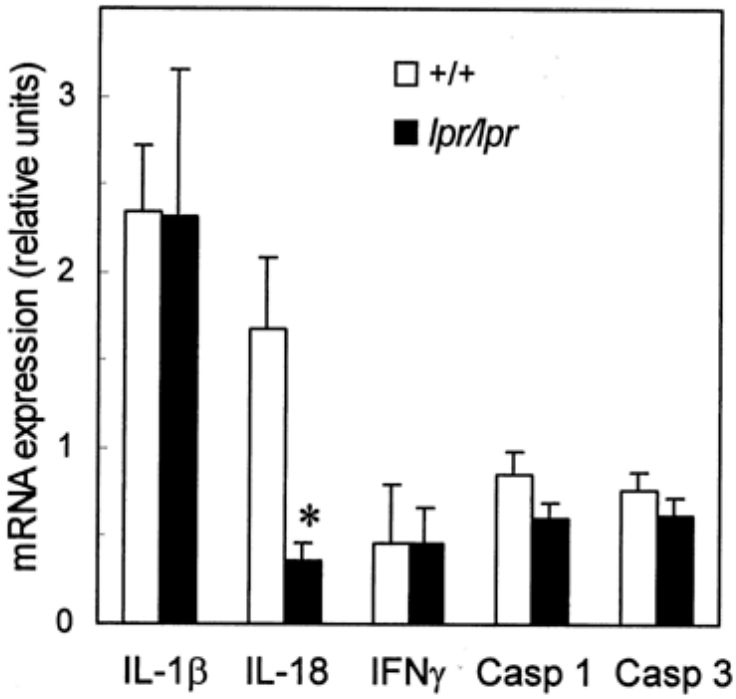


Figure 4
Expression of cytokine and caspase mRNA in PMN from MRL *lpr/lpr* mice.

Expression of mRNA for IL-1beta, IL-18, IFNgamma, caspase 1 and caspase 3 was assessed in PMN from +/+ and *lpr/lpr* mice by semi-quantitative RT-PCR and reported in arbitrary units, calculated as the ratio with expression of the housekeeping gene HPRT. Results are the mean ± SD of 2-7 separate determinations.

* $p < 0.05$ *versus* MRL +/+ cells.

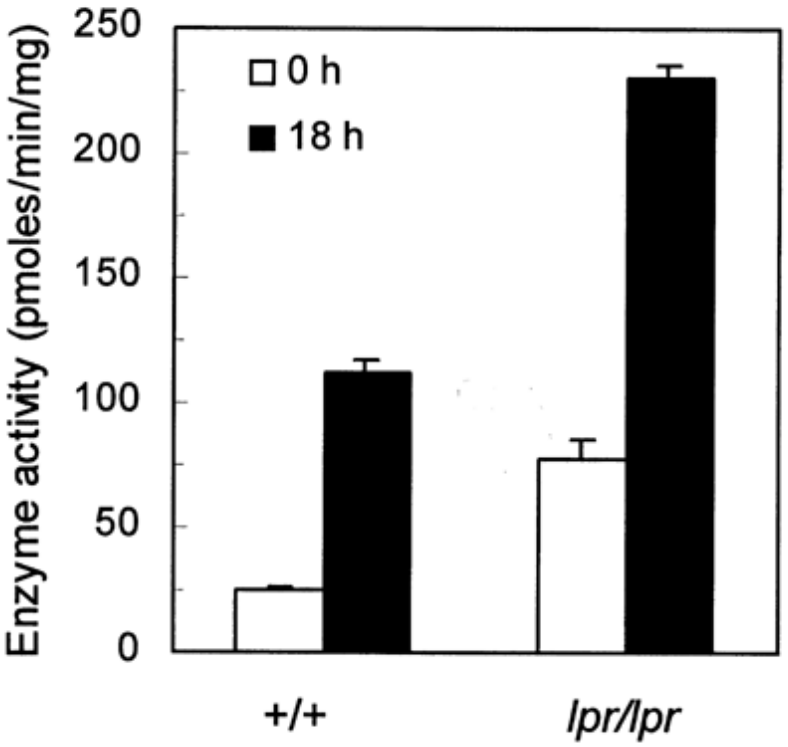


Figure 5
Caspase 3-like activity in PMN from MRL *lpr/lpr* mice.

Caspase 3-like activity was measured in homogenate preparations of either fresh (0 hour; open columns) or cultured (18 hours; filled columns) PMN from MRL +/+ and *lpr/lpr* mice. Enzymatic activity was assessed by cleavage of the selective substrate DEVD-AMC and expressed as pmoles of degraded substrate/min/mg of cell protein. The caspase 3-like activity was completely inhibited by the specific inhibitor DEVD-CMK (data not shown).

Results are the mean ± SD from duplicate determinations within a single experiment representative of 7 performed.

Caspase 3-like activity in *lpr/lpr* PMN was significantly higher ($p < 0.01$) than that of +/+ PMN.

Table 1
IL-1beta and caspase 1 in apoptosis of PMN from *lpr/lpr* mice

Treatment[b]	Apoptosis (% control spontaneous death)[a]	
	+/+	*lpr/lpr*
IL-1beta	64.6 ± 6.5*	77.5 ± 3.6*
IL-1ra	101.3 ± 15.9	92.0 ± 4.5
YVAD-CMK	97.0 ± 8.8	94.8 ± 2.9

[a] Apoptosis is expressed as percentage *versus* the apoptosis value for PMN exposed to culture medium alone (taken as 100%). Data are the mean ± SD of 2-17 separate determinations.
[b] PMN were exposed for 18 hours to culture medium alone or containing IL-1beta (100 ng/ml), IL-1ra (100 ng/ml) or the caspase 1 inhibitor YVAD-CMK (3-30 muM), before cytofluorimetric determination of apoptosis.
* $p < 0.05$ *versus* untreated control.

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type 1 diabetes, islets of Langerhans, beta-cells, cytokines, interleukin-1beta, interleukin-1beta receptor type I, nitric oxide, inducible nitric oxide synthase, heat shock protein 70, interferon regulating factor 1.

Neutrophil apoptosis in autoimmune Fas-defective MRL lpr/lpr mice

E. Del Giudice, A. Ciaramella, N. Balestro, D. Neumann, P. G. Romano, M. P. Cesaroni, G. Maurizi, P. Ruggiero, D. Boraschi, P. Bossù
neutrophils, apoptosis, autoimmunity.

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D. Arsenijevic, F. de Bilbao, P. Giannakopoulos, L. Girardier, S. Samec, D. Richard
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P. Pal Singh, S. Singh
Plasmodium berghei, protection, rmGM-CSF, met-enkephalin.

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D. Kube, D. Schmidt, M. Mörmann, A.C. Uhlemann, J. Tomiuk, H. Tesch, P.G. Kremsner
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