Published in final edited form as:

Immunogenetics. 2011 May ; 63(5): 291–308. doi:10.1007/s00251-010-0507-3.

Macrophages from lupus-prone MRL mice have a conditional signaling abnormality that leads to dysregulated expression of numerous genes

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Abstract

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Electronic supplementary material The online version of this article (doi:10.1007/s00251-010-0507-3) contains supplementary material, which is available to authorized users.

Macrophages (m ϕ) from pre-diseased mice of the major murine inbred models of spontaneous autoimmunity (AI), including multiple lupus-prone strains and the type I diabetes-prone NOD (non-obese diabetic) strain, have identical apoptotic target-dependent abnormalities. This characteristic feature of m ϕ from AI-prone mice suggests that abnormal signaling events induced within m ϕ following their interaction with apoptotic targets may predispose to AI. Such signaling abnormalities would affect predominantly the processing and presentation of self-antigen (i.e., derived from apoptotic targets), while sparing the processing and presentation of foreign antigen (i.e., derived from non-apoptotic sources). Here, we used DNA microarrays to test the hypothesis that m ϕ from AI-prone mice (MRL/MpJ [MRL/+] or MRL/MpJ-*Tnfrsf6^{lpr}* [MRL/lpr]) differentially express multiple genes in comparison to non-AI m ϕ (BALB/c), but do so in a largely apoptotic cell-dependent manner. M ϕ were stimulated with lipopolysaccharide, a potent innate stimulus, in the presence or absence of serum (an experimental surrogate for apoptotic targets). In accord with our hypothesis, the number of genes differentially expressed by MRL m ϕ was significantly increased in the presence vs. the absence of serum, the apoptotic target surrogate ($n=401$ vs. $n=201$). Notably, for genes differentially expressed by MRL m ϕ in the presence of serum, serum-free culture normalized their expression to a level statistically indistinguishable from that by non-AI m ϕ . Comparisons of m ϕ from AI-prone NOD and non-AI C57BL/6 mice corroborated these findings. Together, these data support the hypothesis that m ϕ from MRL and other AI-prone mice are characterized by a conditional abnormality elicited by serum lipids or apoptotic targets.

Keywords

Rodent; Autoimmunity; Lupus; Macrophage; Diabetes; Apoptosis

Introduction

The origins of autoimmunity (AI) remain obscure. Most autoimmune diseases like systemic lupus erythematosus (SLE) are polygenic in nature (Concannon et al. 2005; Harley et al. 2008), with poorly understood environmental triggers and modifiers (Edwards and Cooper 2006; Sarzi-Putini et al. 2005). Analysis of the pathogenesis of autoimmune diseases like SLE is complicated further by the evolving roles of individual cell types during disease progression, from the initiation of AI to the pathologic destruction of organs such as the kidney (Botto et al. 1998; Fairhurst et al. 2006; Liu et al. 2007; Stohl et al. 2008; Takeuchi et al. 2005; Tsubata 2004).

Abnormalities involving nearly all cells of the immune system have been described in SLE. To date, most attention has focused on the role of B and T cells. While multiple abnormalities have been described in these cells and their pivotal role is undisputed (Stohl et al. 2008; Takeuchi et al. 2005; Tsubata 2004), an alternative possibility is that antigen-presenting cells (APC), such as macrophages (m ϕ) and dendritic cells (DC), are inherently defective and contribute to the development of AI. Indeed, several lines of genetic evidence suggest that inherent abnormalities of m ϕ or DC can predispose to AI (Chen et al. 2006; Ludewig et al. 1998; Nath et al. 2008; Zhu et al. 2005). While the mechanism through which these abnormalities predispose to AI remains unclear, APC may promote the emergence of

self-reactive lymphocytes through abnormalities either in the presentation of self-antigen (self-Ag) or in the secondary signals that accompany self-Ag presentation.

Apoptotic cells represent an ongoing and abundant source of self-Ag, and most SLE-related auto-Ag are found on the surface of apoptotic cells or bodies (Casciola-Rosen et al. 1994; Cline and Radic 2004; Levine et al. 2006; Neeli et al. 2007). Interactions between APC and apoptotic target cells are therefore likely to be critical in the development of AI. We propose that AI may occur when abnormal signaling events are induced within the APC following its interaction with apoptotic targets. Such an APC abnormality would affect predominantly the processing and presentation of self-Ag (i.e., derived from apoptotic targets), while sparing the processing and presentation of foreign Ag (i.e., derived from non-apoptotic sources). Indeed, it is a remarkable fact that m ϕ from pre-diseased mice of all the major inbred models of spontaneous AI, including multiple SLE-prone strains (MRL/+, MRL/lpr, NZB, NZW, NZB/W F1, BXSB, and LG), as well as the type I diabetes prone non-obese diabetic (NOD) strain, have identical apoptotic target-dependent abnormalities (Donnelly et al. 1990; Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004). These apoptotic target-dependent abnormalities fall into two major categories: (1) abnormal activity of the cytoplasmic G-protein Rho (a key regulator of the cytoskeleton) resulting in abnormalities of adhesion and cytoskeletal organization (Fan et al. 2006; Longacre et al. 2004) and (2) abnormal expression of multiple soluble mediators of innate and adaptive immunity, including interleukin (IL)-1, IL-6, IL-10, IL-12, and tumor necrosis factor- α (Donnelly et al. 1990; Fan et al. 2004; Koh et al. 2000; Levine et al. 1993). These apoptotic target-dependent abnormalities are present from birth in autoimmune-prone (AI-prone) strains but absent in all non-AI strains evaluated to date (Donnelly et al. 1990; Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004).

The hypothesis that AI-prone m ϕ possess an abnormality triggered by interaction with apoptotic targets leads to the following testable prediction about the pattern of transcribed genes in response to an external stimulus. In the presence of apoptotic targets, gene expression by AI-prone m ϕ should differ strikingly from that by non-AI m ϕ . In contrast, in the absence of apoptotic targets, gene expression by AI-prone and non-AI m ϕ should be similar. To test this hypothesis, we compared gene expression in m ϕ from pre-diseased AI-prone MRL mice and age-matched non-AI BALB/c mice using DNA microarrays. M ϕ were stimulated with endotoxin (lipopolysaccharide [LPS]), a potent innate stimulus, in either the presence or the absence of serum (an experimental surrogate for apoptotic targets). We have previously shown that serum and apoptotic targets are indistinguishable in their effects on m ϕ from AI-prone mice (Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Longacre et al. 2004) and that the critical factor(s) in serum are removed by delipidation (Fan et al. 2004; Koh et al. 2000). In accord with our prediction, the number of genes differentially expressed by MRL m ϕ was significantly increased in the presence vs. the absence of serum. Moreover, for genes differentially expressed in the presence of serum, serum-free culture normalized their expression to a level statistically indistinguishable from that of non-AI m ϕ . Taken together, these data support the hypothesis that m ϕ from MRL and other AI-prone mice are characterized by a conditional abnormality that is elicited by serum lipids or apoptotic targets.

Materials and methods

Mice

MRL/MpJ (MRL/+), MRL/MpJ-*Tnfrsf6^{lpr}* (MRL/lpr), and BALB/cByJ (BALB/c) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice used were between 4 and 7 weeks of age. For any individual experiment, mφ were pooled from 3 to 5 mice of one strain. All comparisons involved mφ from the same or different strains cultured under identical conditions. Replicate experiments were performed on separate days using distinct groups of mice.

Additional confirmatory experiments using NOD/LtJ (NOD) and C57BL/6 mice (also purchased from The Jackson Laboratory) were performed. These additional experiments were identical in methodology to those using MRL and BALB/c mice. For ease of presentation, we describe all aspects of our methods with MRL and BALB/c mice as representative AI-prone and non-AI strains, respectively.

Macrophage culture

Peritoneal exudate cells were harvested by lavage 3 days after i.p. injection of 1.5 ml of 4.05% thioglycollate broth (Donnelly et al. 1990; Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004). Cells were washed twice in RPMI 1640 and plated in 100×15-mm tissue culture dishes at 10×10^6 cells per dish in R.0 culture medium (RPMI 1640 with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin). After incubation for 6 h at 37°C, nonadherent cells were removed by washing twice with RPMI 1640. The remaining adherent cells, >98% mφ as determined by morphologic examination and nonspecific esterase staining (Donnelly et al. 1990; Levine et al. 1993), were cultured in serum-free R.0 medium or serum-containing R.10 medium (R.0 plus 10% fetal bovine serum). Mφ were used after overnight culture in their respective media.

Microarray experimental conditions

After overnight incubation in R.0 or R.10 medium, LPS (*Escherichia coli*-derived, serotype 0111:B4; List Biological, Campbell, CA), was added to a final concentration of 100 ng/ml. RNA was harvested after 0 h (no LPS added), 8 h, or 24 h of stimulation by LPS. We assessed the effect of two experimental variables: (1) the presence or absence of serum and (2) the duration of stimulation (8 or 24 h) with LPS (100 ng/ml). All comparisons were performed in duplicate on separate days. In the presence of serum, separate comparisons were made for MRL/+ vs. BALB/c mφ and for MRL/lpr vs. BALB/c mφ, whereas, in the absence of serum, comparison was limited to MRL/lpr vs. BALB/c mφ (because of the poor quality of cRNA and cDNA obtained in comparisons of MRL/+ and BALB/c under serum-free conditions). An independent preparation of BALB/c mφ was used for each comparison.

RNA isolation and purification

RNA was extracted from mφ using a modified TRIzol protocol (Invitrogen Life Technologies, Carlsbad, CA) that was optimized to obtain high-quality RNA, with little or no DNA contamination, for use in microarray hybridizations. TRIzol reagent (0.1 ml per 10^6

cells) was added to plates of adherent mφ that had been washed once with PBS. Solubilized cells were mixed well, transferred to microfuge tubes, and incubated on ice for 10 min. 0.2 ml of chloroform/isoamyl alcohol (50:1) was added per 1 ml of TRIzol. The mixtures were vortexed and incubated at 4°C for 20 min. After vortexing the tubes again, their contents were transferred to 2-ml Phase-Lock-Gel-Light tubes (Eppendorf, Hamburg, Germany). The Phase-Lock tubes were centrifuged at 14,000×*g* in a microcentrifuge for 10 min at 4°C. Aqueous phases were transferred to Phase-Lock-Gel-Heavy tubes (Eppendorf), and a volume of chloroform/isoamyl alcohol (50:1) equal to the initial TRIzol volume was added to each tube. Tubes were then mixed by inversion and again centrifuged at 14,000×*g* for 10 min at 4°C. Aqueous phases were transferred to new microfuge tubes, and an equal volume of ice-cold iso-propanol was added to each tube. The tubes were mixed by inversion and stored at –20°C until just before use. Centrifugation, washing, and resuspension of RNA were performed according to standard protocols (Ribaud et al. 2002). Each sample of RNA was resuspended in a minimal volume of RNase-free water, generally about 30 µl. RNA derived from thioglycollate-elicited peritoneal mφ is often accompanied by an unknown precipitate that pellets with the RNA and is insoluble in water. Most of the precipitate can be removed by centrifugation after heating the solution of RNA to 65°C for 10 min. As this precipitate interferes with spectrophotometric quantitation of RNA, RNA was quantitated based on initial cell number. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only high-quality RNA was used for hybridization to microarrays. High-quality RNA was indicated by the lack of a DNA signal and by examination of the ratio of 28S to 18S RNA. Additional indications of high-quality RNA, as assessed by gel electrophoresis, included the absence of detectable RNA degradation and the ability to generate long-length cDNA and cRNA.

RNase protection assays

For all array studies, parallel plates of mφ, cultured in either R.0 or R.10, were stimulated with LPS (100 ng/ml) for 0, 8, 24, and 48 h. RNA derived from these plates was used in RNase protection assays to confirm that cytokine profiles were consistent with previously published findings (Fan et al. 2004; Koh et al. 2000; Levine et al. 1993). RNase protection assays were performed using a RiboQuant In Vitro Transcription Kit (Pharmingen, San Diego, CA) according to manufacturer's instructions. All probes were for murine cytokines.

RNA labeling, hybridization to microarrays, and scanning

The University of Chicago Functional Genomics Facility performed labeled cRNA synthesis, hybridization, and scanning of GeneChips according to protocols in the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA) with minor modifications. In brief, first-strand cDNA synthesis was primed from 10 µg of total RNA using a T7-(dT)24 oligonucleotide. From 3 µg of phase-lock-gel-purified double-strand cDNA, biotin-labeled antisense cRNA was synthesized using a BioArray High-Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY). After precipitation with 4 M LiCl, 20 µg of cRNA was fragmented by incubation in fragmentation buffer (40 mM Tris–acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate) for 35 min at 94°C and then hybridized to Affymetrix MG_U74Av2 mouse GeneChip® arrays for 16 h at 45°C in an Affymetrix Hybridization Oven 640. Microarrays were washed and stained with

streptavidin phycoerythrin in an Affymetrix Fluidics Station 400 using the Affymetrix GeneChip protocol. The reaction products from each step were analyzed by electrophoresis, and only high-quality, long-length products were used in subsequent steps.

Microarray data analysis

Microarray Suite Version 5.0 (Affymetrix) was used to analyze all array images. The mouse GeneChip arrays contained 12,488 probe sets, each consisting of 16–20 perfect-match/single-base-mismatch 25-mer oligo pairs. Not all probe sets represent independent genes, as several genes are represented on the array multiple times. Nonetheless, for simplicity, we use the terms “genes” and “probe sets” interchangeably throughout this manuscript, with a preference for the word “genes”. The sequences for each of the 20 perfect-match probes within a given probe set were taken from the sequence of a known mouse gene or expressed sequence tag. The sequence of each mismatch probe is identical to its perfect-match pair except for one nucleotide in the center. Background correction and scaling were performed according to standard Affymetrix protocols. Statistical algorithms, using default parameter values recommended by Affymetrix, were applied after these two adjustments. Specific details of the protocols and statistical algorithms used in Affymetrix MSV 5.0 may be obtained at <http://www.affymetrix.com>.

Signal log ratio

For each of the ~12,000 genes on the microarray, Affymetrix MSV 5.0 generated a signal log ratio (SLR), which represents the logarithm to base two of the relative expression of a given gene on the first array to its expression on the second array. In all comparisons of MRL vs. BALB/c m ϕ , we formulated our data so that the expression level by MRL m ϕ was in the numerator and the expression level by BALB/c m ϕ was in the denominator. Therefore, SLR values <0 indicate relative under-expression by MRL m ϕ , and SLR values >0 indicate relative over-expression by MRL m ϕ . If the SLR equals 0, then the gene is equivalently expressed by MRL and BALB/c m ϕ . The fold-difference in expression between MRL and BALB/c m ϕ can be calculated by the following formula: fold-difference (MRL: BALB/c) = 2^{SLR} . Thus, for example, if the SLR = +1 for a given gene, then the fold-difference in expression is $2^{+1} = 2$, and MRL m ϕ over-express the gene at a 2-fold greater level than do BALB/c m ϕ . Similarly, if the SLR = -1, then the fold-difference in expression is $2^{-1} = 1/2$, and MRL m ϕ under-express the gene by a factor of 2. Finally, if the SLR = 0, then expression is equivalent for MRL and BALB/c m ϕ (fold-difference is $2^0 = 1$).

Sensitivity and specificity

Differentially expressed genes were identified using a strategy of recurrence (Longacre et al. 2005) and a threshold absolute value for SLR of 0.8 (1.74-fold induction). A strategy of recurrence requires that the SLR of a differentially expressed gene exceed a preset threshold on all individual comparisons. We have previously shown that a strategy of recurrence increases the number of linearly independent comparisons that can be derived from a given number of experimental replicates (Longacre et al. 2005). Moreover, for any given threshold, a strategy of recurrence is more specific than a strategy of means (Longacre et al. 2005). The theoretical basis for this method of analysis has been proven mathematically, and its

practical application has been validated by both computer simulation and examination of real data (Longacre et al. 2005).

In the case of duplicate experiments, a strategy of recurrence yields one additional linearly independent comparison, or a total of three independent SLR values per experimental condition. To avoid the arbitrariness involved in selecting three out of the four possible comparisons, all four comparisons were utilized. The fourth non-linearly independent comparison provides additional specificity, although less than that from the first three linearly independent comparisons (Longacre et al. 2005). Hence, differentially expressed genes were defined as those whose SLR was either ≥ 0.8 on all three linearly independent comparisons (plus a fourth non-linearly independent comparison) or ≤ -0.8 on all three linearly independent comparisons (plus a fourth non-linearly independent comparison). The expected specificity for this strategy and threshold is $>99.9\%$; this is equivalent to a false-positive rate of ~ 0.5 –1 gene per 1,000 examined. The expected sensitivity depends upon the degree of differential expression. For a gene whose level of induction is 2-fold ($\text{SLR} = \pm 1.0$), the expected sensitivity is 0.33, whereas for a gene whose level of induction is 4-fold ($\text{SLR} = \pm 2.0$), the expected sensitivity is >0.96 . An example of the step-by-step application of our algorithm is given in Fig. 1 and discussed in the Results.

Thus, our method of analysis is highly specific but only moderately sensitive, especially for genes whose differential expression is ~ 2 -fold or less. It should be emphasized that a trade-off inevitably exists between specificity and sensitivity, in that an increase of one always leads to a decrease of the other. To determine whether MRL $m\phi$ have a global defect in gene expression triggered by serum and/or apoptotic cells, specificity is a critical issue. We therefore chose a strategy that minimized the inclusion of false-positive genes, even at the expense of failing to detect a proportion of differentially expressed genes. Additional issues of sensitivity, specificity, internal consistency, and potential sources of bias are detailed elsewhere (Longacre et al. 2005).

Statistics

Data are presented as mean $\pm$ SD or mean $\pm$ SE. Statistical significance was determined by ANOVA or a two-tailed Student's *t* test.

Results

MRL $m\phi$ differentially express multiple genes when cultured in the presence of serum

Given the complexity of the data generated by gene arrays, we limited our analysis to a single representative SLE-prone background (MRL/+ or MRL/lpr) and a single representative non-AI control strain (BALB/c). To minimize the contribution of disease, we used only young, pre-diseased MRL mice (<7 weeks of age) as a source of $m\phi$. Differentially expressed genes in this case are more likely to reflect intrinsic differences, including susceptibility to AI, rather than manifestations of disease. Finally, since partially degraded RNA within apoptotic targets interferes with the performance of DNA microarrays (unpublished observations), we used fetal bovine serum rather than apoptotic targets to elicit abnormal behavior by MRL $m\phi$. We have previously demonstrated that serum and apoptotic

target cells are indistinguishable in their effects on m ϕ from SLE-prone mice (Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Longacre et al. 2004). Since the critical factor(s) in serum are removed by delipidation (Fan et al. 2004; Koh et al. 2000), we speculate that apoptotic bodies and/or oxidized lipids and lipoproteins, which engage many of the same m ϕ receptors as do apoptotic cells, are the responsible components (Chang et al. 1999; de Almeida and Linden 2005; Erwig and Henson 2008; Fukasawa et al. 1996; Krysko et al. 2006). Thus, for the purposes of these studies, serum is an experimental surrogate for apoptotic cells.

MRL/+ mice are AI-prone, but develop SLE-like disease late in life. In contrast, MRL/lpr mice, which are genetically identical to MRL/+ mice except for a mutation in Fas, develop SLE early on and usually die by 6 to 7 months of age. We first compared gene expression by m ϕ from MRL/+ vs. BALB/c mice in the presence of serum following 8 or 24 h of LPS stimulation. We identified 217 and 109 genes that were differentially expressed following 8 and 24 h of LPS stimulation, respectively. The union of these two sets yielded 270 genes. We also compared MRL/lpr vs. BALB/c m ϕ and found 131 and 151 genes that were differentially expressed following 8 and 24 h of LPS stimulation, respectively. The union of these two sets yielded 223 genes. Combination of these two sets (i.e., 270 genes and 223 genes, respectively) yielded a final set of 401 genes differentially expressed by SLE-prone MRL vs. non-AI BALB/c m ϕ .

Of the 401 differentially expressed genes, we identified only 92 in comparisons of both MRL/+ vs. BALB/c m ϕ and MRL/lpr vs. BALB/c m ϕ . On the surface, this limited concordance seems to suggest differences between congenic MRL/+ and MRL/lpr mice. However, a more likely explanation relates to the stringency of our algorithm for identifying differentially expressed genes. This issue, and its relationship to the unavoidable trade-off between specificity and sensitivity, is discussed in depth in the Online Resource.

Figure 1 provides an example of the step-by-step application of our algorithm for determining differential expression, in this case for MRL/+ vs. BALB/c m ϕ stimulated for 8 h with LPS. A summary of our analysis of differentially expressed genes for all other comparisons is given in Table S1 (see Online Resource). Of the ~12,000 genes represented on an array, only ~5,000 were expressed by m ϕ (i.e., deemed “present” by Affymetrix MSV 5.0) and therefore subjected to comparative analysis. Hence, in the presence of serum, SLE-prone MRL m ϕ differentially expressed ~8% of all transcribed genes (401 out of ~5,000). For these 401 genes, the average difference in expression was ~4-fold (SLR=±2; Table S2, Online Resource). This value is well above the inherent “noise” of measurement, corresponding to a 1.35-fold difference of expression (SLR =±0.43). These issues are discussed in greater depth in the Online Resource.

We set our threshold for differential expression to achieve a specificity of >99.9% (false-positive rate of ~0.5–1 in 1,000) (see Material and methods). Analysis of our data suggests that the actual specificity may have been slightly lower, at 99.8% (false-positive rate of ~2 in 1,000; Table S1, Online Resource). This means that as many as 20 of the 401 differentially expressed genes (or ~5%) may represent false-positives. Details of our experimental

estimation of the false-positive rate, as well as a discussion of the effects of varying the threshold for differential expression, are provided in the Online Resource.

Serum-free culture reduces the number of differentially expressed genes in MRL m ϕ

The number of genes differentially expressed by MRL m ϕ was fewer in the absence, than the presence, of serum. Comparison of gene expression by m ϕ from MRL/lpr vs. BALB/c mice in the absence of serum revealed 149 and 121 differentially expressed genes following 8 and 24 h of LPS stimulation, respectively (Table S1, Online Resource). The union of these two sets yielded 201 genes that were differentially expressed by MRL/lpr m ϕ in serum-free culture.

Comparison of the set of 401 genes differentially expressed in the presence of serum with the set of 201 genes differentially expressed in the absence of serum allowed us to sort genes into three distinct categories: (1) genes differentially expressed only in the presence of serum ("SERUM ONLY", $n=280$); (2) genes differentially expressed only in the absence of serum ("SERUM-FREE ONLY", $n=80$); and (3) genes differentially expressed in both the presence and the absence of serum ("SERUM & SERUM-FREE", $n=121$). "SERUM ONLY" genes, which are the most numerous, followed the pattern we have previously observed for multiple soluble mediators of innate and adaptive immunity (Donnelly et al. 1990; Fan et al. 2004; Koh et al. 2000; Levine et al. 1993). Differential expression by genes in this category is consistent with an inherent abnormality in MRL m ϕ that is triggered by serum lipids or apoptotic targets (Fan et al. 2004; Koh et al. 2000). "SERUM-FREE ONLY" genes, which are fewer in number, are not accounted for by our hypothesis. Genes in this category may represent errors of assignment, an unavoidable byproduct of the stringency of our algorithm (elaborated below and in the Online Resource). Finally, "SERUM & SERUM-FREE" genes presumably reflect fixed strain-specific differences between MRL and BALB/c mice.

To test this interpretation, we compared the degree of differential expression in the presence vs. the absence of serum for genes in each of the three categories. For each gene, we calculated the ratio of the absolute value of its SLR in the presence of serum (SLR_{SERUM}) to that in the absence of serum ($SLR_{SERUM-FREE}$). For genes differentially expressed only in the presence of serum ("SERUM ONLY"), the ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ should be >1 . For genes differentially expressed only in the absence of serum ("SERUM-FREE ONLY"), the ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ should be <1 . Finally, for genes differentially expressed in both the presence and absence of serum ("SERUM & SERUM-FREE"), the ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ should be ~ 1 .

The results of this analysis are shown in Fig. 2. The differences in $SLR_{SERUM}/SLR_{SERUM-FREE}$ among the three categories of genes were highly significant ($p < 10^{-10}$ by ANOVA at both 8 and 24 h). With respect to classification, genes in the "SERUM ONLY" category appear to be sorted correctly. The mean ($\pm$ SE) ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ for these genes was 2.7 ± 0.2 and 2.6 ± 0.2 after 8 and 24 h of LPS stimulation, respectively. This is consistent with ~ 6 -fold greater differential expression in the presence vs. the absence of serum. Genes in the "SERUM & SERUM-FREE" category also appear to be classified correctly. The mean ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ for these genes was ~ 1.0 (1.1 ± 0.1 and 1.2 ± 0.1 at 8 and 24 h, respectively). This is consistent with a fixed degree of differential

expression, irrespective of the presence or absence of serum. These differences in $SLR_{SERUM}/SLR_{SERUM-FREE}$ for “SERUM ONLY” genes vs. “SERUM & SERUM-FREE” genes were highly significant ($p < 10^{-13}$ by two-tailed t test).

In contrast, genes in the “SERUM-FREE ONLY” category seem to be incorrectly classified and may belong more properly to “SERUM & SERUM-FREE” category. Thus, their mean ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ was close to 1.0 (0.8 ± 0.1 and 0.8 ± 0.1 at 8 and 24 h, respectively), indicating that their differential expression was similar, irrespective of the presence or absence of serum. The basis for this misclassification most likely lies in the stringency of our algorithm to identify differentially expressed genes. This issue is discussed in depth in the Online Resource.

To address this issue further, we performed individual pair-wise statistical comparisons. Notably, at both 8 and 24 h, the mean $SLR_{SERUM}/SLR_{SERUM-FREE}$ for “SERUM-FREE ONLY” genes did not differ from “SERUM & SERUM-FREE” genes ($p > 0.05$ by two-tailed t test), but did differ significantly from “SERUM ONLY” genes ($p < 10^{-13}$ by two-tailed t test). Together, these data suggest that errors in assignment may have affected predominantly genes in the “SERUM-FREE ONLY” category.

We conclude that differentially expressed genes could be classified into two predominant categories: (1) those differentially expressed only in the presence of serum ($n = 280$) and (2) those differentially expressed in a more or less constitutive manner, irrespective of the presence or the absence of serum ($n = 121$). These results are consistent with the hypothesis that $m\phi$ possess a signaling abnormality, affecting gene transcription only in the presence of apoptotic cells (or their experimental surrogate, serum).

Genes differentially expressed by MRL $m\phi$ in the presence of serum are expressed normally in the absence of serum

We next looked in greater detail at the individual expression patterns for the 280 “SERUM ONLY” genes by superimposing the frequency distributions of their SLR's determined in the presence vs. the absence of serum (Fig. 3). As shown, there is a marked separation between the distributions of SLR in the presence vs. absence of serum. The histograms in the presence of serum are biphasic, representing genes that are either relatively over-expressed ($SLR > 0$) or under-expressed ($SLR < 0$) by MRL $m\phi$. In marked contrast, the histograms in the absence of serum are well centered about the origin.

To determine whether the degree of correction that occurred in the absence of serum was complete or only partial, we restricted our analysis to a single strain comparison (MRL/lpr vs. BALB/c) and generated scatter plots in which each of the 123 “SERUM ONLY” genes differentially expressed by MRL/lpr $m\phi$ at 8 and/or 24 h is depicted by a single point (Fig. 4). The X -axis and Y -axis represent the SLR at 8 and 24 h, respectively. Examination of the scatter plot for culture in the presence of serum reveals a preponderance of genes in the first and third quadrants (Fig. 4a). This indicates that the sign (positive or negative) of the SLR for most genes was the same at 8 and at 24 h, that is, genes under-expressed at 8 h were also under-expressed at 24 h, and vice versa. Genes that were differentially expressed to a greater extent at 8 h lie closer to the X -axis, whereas genes that were differentially expressed to a

greater extent at 24 h lie closer to the *Y*-axis. Points lying on or close to the diagonal represent genes that were differentially expressed to a similar extent at 8 and 24 h. Regression analysis reveals that there is a significant correlation between the SLR values at 8 and 24 h ($r^2=0.59$, $p<10^{-26}$). Moreover, the slope of the best-fitting line, 0.78, is close to 1, indicating that the degree of differential expression at 8 h is very close to, but slightly greater than that at 24 h.

The scatter plot for these same 123 genes in the absence of serum reveals several striking differences (Fig. 4b). First, the genes are more closely clustered about the origin. To quantify this observation, we calculated the distance from the origin for each gene in the two scatter plots. In the absence of serum, the mean SLR distance was 0.8 ± 0.6 , as compared with 1.6 ± 0.6 in the presence of serum ($p<10^{-30}$ by two-tailed paired *t* test). Second, the genes were no longer exclusively clustered in the first and third quadrants but were more evenly distributed throughout all four quadrants. This suggests a more random distribution of SLR, consistent with a greater contribution of noise. Regression analysis confirms a decreased, though still significant, correlation between the SLR values at 8 and 24 h ($r^2=0.35$, $p<10^{-13}$). Third, for comparison, we also generated the scatter plots for these same 123 genes in same-vs.-same comparisons of BALB/c vs. BALB/c (Fig. 4c) and MRL vs. MRL (Fig. 4d). The mean distances from the origin for these same-vs.-same scatter plots were 0.7 ± 0.1 and 0.8 ± 0.1 , respectively, and, remarkably, statistically not different from that for serum-free culture ($p>0.05$ by two-tailed paired *t* test). Thus, serum-free culture reduces the differential expression of “SERUM ONLY” genes to a level statistically indistinguishable from that found in same-vs.-same comparisons. In other words, differences in the expression of these 123 genes were completely corrected by the removal of serum, with the average differential expression reduced to a level indistinguishable from identity. We conclude that culture in the absence of serum corrects the differential expression of genes in the “SERUM ONLY” category, at least to the limit of detectability for the microarrays we used.

Genes differentially expressed in both the presence and absence of serum have features of strain-specific differences

We generated similar scatter plots for the 121 genes differentially expressed both in the presence and in the absence of serum (Fig. 5). As expected, the scatter plots for genes in this category look remarkably similar in the presence (Fig. 5a) and absence (Fig. 5b) of serum. For both conditions, genes are clustered tightly about the diagonal, indicating not only a high degree of correlation between the SLR at 8 and 24 h, but also approximately equal degrees of differential expression at these two time points. These data suggest that genes in this category represent fixed strain-specific differences between MRL and BALB/c m ϕ and that their degree of differential expression is unaffected by serum. Regression analysis is consistent with this interpretation (serum present, $r^2=0.87$, $p<10^{-30}$, slope=0.80; serum absent, $r^2=0.89$, $p<10^{-30}$, slope=0.91).

As further evidence that these genes represent fixed strain-specific differences, we also performed linear regression comparing their SLR in the presence of serum to their SLR in the absence of serum at each of the two time points (Figure S1, Online Resource). At both 8 and 24 h, the SLR in the presence vs. absence of serum were not only highly correlated but

also approximately equal as indicated by slopes close to 1.0 (at 8 h, $r^2=0.90$, $p<10^{-61}$, slope=1.04; at 24 h, $r^2=0.89$, $p<10^{-57}$, slope=0.91).

Relative expression of cytokines, chemokines, and their receptors by m ϕ from MRL vs. BALB/c mice

We have previously published data generated by RNase protection assay, in which we compared the expression of multiple cytokine genes by m ϕ from MRL vs. BALB/c mice cultured in the presence and absence of serum (Fan et al. 2004; Koh et al. 2000). In all cases where comparison could be performed, the results of microarray analysis agreed with those of RNase protection assays (Table S3, Online Resource).

Our microarray analysis allowed us to identify additional cytokine-related genes that are differentially expressed by m ϕ from pre-diseased MRL mice (Table S4, Online Resource). Combined with previous results (Fan et al. 2004; Koh et al. 2000), 19 cytokine-related genes (10 identified before and 9 from this study) showed serum-dependent dysregulation. Of these, 17 were under-expressed by MRL m ϕ in the presence of serum, and 2 were over-expressed. All 19 had normal or nearly normal expression when MRL m ϕ were cultured in the absence of serum. An additional 5 genes were differentially expressed both in the presence and in the absence of serum. All were under-expressed by MRL m ϕ . 34 genes (29 from this study) were expressed equivalently by MRL and BALB/c m ϕ , irrespective of the presence or absence of serum. Finally, the expression level for 74 genes fell below the limits of detection by DNA microarrays for all experimental conditions.

The nature of genes differentially expressed by MRL vs. BALB/c m ϕ in the presence of serum

Notably, cytokines, chemokines, and their receptors comprised a relatively small proportion of the 401 genes differentially expressed by MRL vs. BALB/c m ϕ in the presence of serum. A complete summary of these genes is given in Table S5 (see Online Resource) and is grouped into categories based on the relevant biological function or system involving the gene. The differentially expressed genes affect a broad spectrum of immunologic and biologic systems, including genes in the following categories: (1) cytoskeleton, microtubular network, and regulators of adhesion/migration; (2) apoptotic regulation and apoptotic cell clearance; (3) cell cycle regulation; (4) antigen processing; (5) transcriptional regulation; (6) mediators and receptors of innate immunity; (7) signal transduction (immune and non-immune specific); and (8) metabolism. In all categories, some genes were over-expressed, while others were under-expressed, by MRL (AI-prone) vs. BALB/c (non-AI) m ϕ in the presence of serum.

Serum-dependent differential expression of genes extends to m ϕ from NOD mice, a model of autoimmune diabetes mellitus

To this point, our results have involved only a single strain comparison, namely, pre-diseased AI-prone MRL mice vs. age-matched control BALB/c m ϕ . We wished to determine whether serum-dependent differences in the overall pattern of gene expression can also be observed in comparisons of other AI-prone and control m ϕ . We chose NOD and C57BL/6 mice as alternative examples of AI-prone and control mice, respectively. NOD mice spontaneously

develop type I diabetes mellitus. These data permitted three new comparisons: MRL/lpr vs. C57BL/6, NOD vs. BALB/c, and NOD vs. C57BL/6.

For each of these three comparisons, genes were sorted into the same three categories: “SERUM ONLY”, “SERUM-FREE ONLY”, and “SERUM & SERUM-FREE”. After sorting, as in Fig. 2, we compared the degree of differential expression in the presence vs. the absence of serum for genes in each of these three categories by calculating for each gene the ratio of the absolute value of its SLR in the presence of serum (SLR_{SERUM}) to that in the absence of serum ($SLR_{SERUM-FREE}$). The results of this analysis are shown in Fig. 6. As in the comparison of MRL vs. BALB/c mice, the differences in $SLR_{SERUM}/SLR_{SERUM-FREE}$ among the three categories of genes were highly significant at both 8 and 24 h (MRL/lpr vs. C57BL/6: $p < 10^{-13}$ by ANOVA; NOD vs. BALB/c: $p < 10^{-7}$; and NOD vs. C57BL/6: $p < 10^{-4}$). Importantly, all three new comparisons replicated the essential features observed in the comparison of MRL vs. BALB/c m ϕ . Thus, genes in the “SERUM ONLY” and “SERUM & SERUM-FREE” categories appeared to be sorted correctly, whereas those in the “SERUM-FREE ONLY” category appeared to be misclassified.

Genes in the “SERUM ONLY” category were correctly classified in that they showed a high degree of serum-dependent differential expression. The mean ($\pm$ SE) ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ for these genes was: MRL/lpr vs. C57BL/6, 1.9 ± 0.1 and 3.7 ± 0.5 at 8 and 24 h; NOD vs. BALB/c, 3.8 ± 0.7 and 3.3 ± 0.6 at 8 and 24 h; and NOD vs. C57BL/6, 2.8 ± 0.6 and 3.7 ± 0.5 at 8 and 24 h. These differences are consistent with ~4 to 12-fold greater differential expression in the presence vs. the absence of serum. Genes in the “SERUM & SERUM-FREE” category also appear to be classified correctly. The mean ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ for these genes was ~1.0 in all three comparisons: MRL/lpr vs. C57BL/6, 1.0 ± 0.1 and 1.0 ± 0.1 at 8 and 24 h; NOD vs. BALB/c, 1.1 ± 0.1 and 1.2 ± 0.1 at 8 and 24 h; and NOD vs. C57BL/6, 1.1 ± 0.1 and 1.3 ± 0.1 at 8 and 24 h. These ratios are consistent with a fixed degree of differential expression, irrespective of the presence or absence of serum.

In contrast, just as for the MRL vs. BALB/c comparison, genes in the “SERUM-FREE ONLY” category appear to be incorrectly classified, belonging more properly to the “SERUM & SERUM-FREE” category. Thus, their mean $SLR_{SERUM}/SLR_{SERUM-FREE}$ ratio was close to 1.0 (MRL/lpr vs. C57BL/6 are 0.8 ± 0.1 and 0.8 ± 0.1 at 8 and 24 h; NOD vs. BALB/c are 0.8 ± 0.1 and 0.9 ± 0.1 at 8 and 24 h; and NOD vs. C57BL/6 are 0.9 ± 0.1 and 1.2 ± 0.4 at 8 and 24 h), indicative of a similar degree of differential expression in the presence vs. the absence of serum. As previously discussed in reference to Fig. 2, the basis for this misclassification most likely lies in the stringency of our algorithm to identify differentially expressed genes and is discussed in depth in the Online Resource.

These results are again consistent with the hypothesis that m ϕ possess a signaling abnormality, affecting gene transcription only in the presence of apoptotic cells (or their experimental surrogate, serum). Thus, differentially expressed genes in these three new comparisons between AI-prone and control m ϕ could again be classified into two predominant categories: (1) those differentially expressed only in the presence of serum and

(2) those differentially expressed in a more or less constitutive manner, irrespective of the presence or the absence of serum.

Next, as in the comparison of MRL/lpr vs. BALB/c mφ (see Fig. 4), we generated scatter plots for each of the sets of “SERUM ONLY” genes, as derived from the three new comparisons (Fig. 7). Paired scatter plots in the presence and absence of serum for each of the three comparisons replicated the essential features observed in the comparison of MRL vs. BALB/c mφ. Thus, in the presence of serum, a preponderance of genes lies in the first and third quadrants (Fig. 7a, c, e), indicating that genes under-expressed at 8 h were also under-expressed at 24 h, and vice versa. Regression analysis confirms that there is a significant correlation between the SLR values at 8 and 24 h (MRL/lpr vs. C57BL/6 are $r^2=0.58$, $p<10^{-55}$; NOD vs. BALB/c are $r^2=0.70$, $p<10^{-50}$; NOD vs. C57BL/6 are $r^2=0.66$, $p<10^{-30}$). The slopes of the best-fitting lines—0.62, 0.88, and 0.71, respectively—indicate that the degree of differential expression at 8 h is close to, but slightly greater than that at 24 h.

As in the comparison of MRL vs. BALB/c mφ, the scatter plots for these same genes in the absence of serum revealed several striking differences (Fig. 7b, d, f). First, the genes are more closely clustered about the origin. Thus, the mean SLR distance was significantly decreased in the presence vs. absence of serum for all three new comparisons (MRL/lpr vs. C57BL/6 are 1.8 ± 0.1 vs. 1.1 ± 0.1 , $p<10^{-39}$ by two-tailed paired *t* test; NOD vs. BALB/c are 1.7 ± 0.1 vs. 1.1 ± 0.1 , $p<10^{-23}$; NOD vs. C57BL/6 are 1.7 ± 0.1 vs. 1.0 ± 0.1 , $p<10^{-21}$). Second, the genes were no longer exclusively clustered in the first and third quadrants but were more evenly distributed throughout all four quadrants, suggestive of a more random distribution of SLR and consistent with a greater contribution of noise. Third, for comparison, we generated scatter plots for these same genes in same-vs.-same comparisons of C57BL/6 vs. C57BL/6 and NOD vs. NOD (not shown). The mean distances from the origin for these same-vs.-same scatter plots were 0.7 ± 0.1 and 1.0 ± 0.1 , respectively, and, remarkably, statistically not different from that for serum-free culture ($p>0.05$ by two-tailed paired *t* test). Thus, serum-free culture reduced the differential expression of “SERUM ONLY” genes to a level statistically indistinguishable from that found in same-vs.-same comparisons.

Finally, as in the comparison of MRL/lpr vs. BALB/c (see Fig. 5), we generated scatter plots for each of the sets of “SERUM & SERUM-FREE” genes, as derived from the three new comparisons (Fig. 8). As expected, the scatter plots for genes in this category look remarkably similar in the presence (Fig. 8a, c, e) vs. the absence (Fig. 8b, d, f) of serum. Irrespective of the presence or absence of serum, genes from all three new comparisons were clustered tightly about the diagonal. This indicates a high degree of correlation between the SLR at 8 and 24 h, as well as an approximately equal degree of differential expression at these two time points. Genes in this category therefore most likely represent fixed strain-specific differences. Regression analysis confirms that their degree of differential expression is largely unaffected by serum: MRL/lpr vs. C57BL/6 (serum present, $r^2=0.86$, $p<10^{-132}$, slope=0.91; serum absent, $r^2=0.89$, $p<10^{-148}$, slope=0.94); NOD vs. BALB/c (serum present, $r^2=0.89$, $p<10^{-98}$, slope=1.02; serum absent, $r^2=0.88$, $p<10^{-93}$, slope=0.97); NOD vs. C57BL/6 (serum present, $r^2=0.90$, $p<10^{-74}$, slope=0.92; serum absent, $r^2=0.89$, $p<10^{-70}$, slope=0.99).

Discussion

We have previously shown that m ϕ from pre-diseased mice of all the major inbred models of spontaneous AI, including SLE and autoimmune diabetes, have an identical abnormality that leads to the dysregulated expression of multiple cytokines and chemokines [Donnelly et al. 1990; Fan et al. 2004; Koh et al. 2000; Levine et al. 1993] and the diminished activity of Rho, a key regulator of the cytoskeleton (Fan et al. 2006; Longacre et al. 2004). This abnormality is conditional, as it occurs only when m ϕ are cultured in the presence of serum lipids or apoptotic target cells. We have shown that serum lipids and apoptotic targets are indistinguishable in their effects on m ϕ from AI-prone mice (Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Longacre et al. 2004). Thus, in the presence of serum lipids or apoptotic targets, the abnormality is fully manifest. In contrast, in the absence of serum lipids and/or apoptotic targets, the behavior of m ϕ from AI-prone mice is fully comparable to that of m ϕ from non-AI strains.

Here, we used DNA microarrays to test our hypothesis that m ϕ from AI-prone mice possess a signaling abnormality triggered by interaction with apoptotic targets. A strong prediction of this hypothesis is that gene expression by AI-prone m ϕ should differ from that by non-AI m ϕ only when expression is assessed in the presence of apoptotic targets and/or serum lipids. In the absence of apoptotic targets and/or serum lipids, gene expression by AI-prone and non-AI m ϕ should be closely comparable. We focused our analysis on a single representative SLE-prone background (MRL/+ or MRL/lpr) and a single representative non-AI control strain (BALB/c). Additional comparisons with AI-prone NOD and non-AI C57BL/6 mice were made for corroboration. Since partially degraded RNA within apoptotic cells can interfere with the performance of DNA microarrays (unpublished observations), we used serum rather than apoptotic cells to elicit abnormal behavior by MRL m ϕ . Thus, in this study, we did not demonstrate directly the effect of apoptotic targets and/or serum lipids on AI-prone m ϕ . While follow-up work will address this point, it should be noted that, in our previous studies (Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Longacre et al. 2004), we have shown clearly that serum lipids are fully comparable to apoptotic targets in terms of eliciting all abnormalities of AI-prone m ϕ .

Our analysis revealed that genes differentially expressed by MRL vs. BALB/c m ϕ could be sorted into one of two major categories. Genes in the first category ($n=121$) were differentially expressed in a fixed manner, irrespective whether m ϕ were cultured in the presence or absence of serum. The degree of differential expression was essentially the same in the presence and absence of serum, consistent with the notion that genes in this category represent fixed genetic differences between the two strains. From a functional perspective, these genes may be either incidental to disease (e.g., related to coat color) or tentatively associated with AI (e.g., MHC-related).

Genes in the second category ($n=280$) conformed to our hypothesis. Differential expression was seen only when m ϕ were cultured in the presence of serum. Remarkably, the expression of these same genes by MRL vs. BALB/c m ϕ was normalized when m ϕ were cultured in the absence of serum. The mean difference in expression of these genes by MRL vs. BALB/c m ϕ was reduced from ~4-fold in the presence of serum to a level statistically

indistinguishable from identity in the absence of serum. Taken together, our data are consistent with the hypothesis that m ϕ from AI-prone mice possess a conditional abnormality, which affects gene expression only when m ϕ are cultured in the presence of serum or apoptotic cells.

In light of these data, we propose an alternate explanation for the association between impaired clearance of apoptotic cells and the development of systemic AI (Botto et al. 1998; Cohen et al. 2002; GaipI et al. 2005). Discussions of this association often neglect the fact that impaired clearance of apoptotic targets has two important consequences, whose roles in the development of AI have not been properly distinguished: (1) persistence of the apoptotic corpses and (2) diminution or loss of apoptotic target-initiated signaling events (Cocco and Ucker 2001). We suggest that it is the second consequence, namely, the diminution or loss of apoptotic cell-dependent proximal signaling events in professional APC, which plays the larger role in the development of AI. According to this model, loss of self-tolerance with resultant AI may occur through multiple abnormalities, either acquired or genetic, that interfere with the set of signaling events induced in APC by the recognition and/or engulfment of apoptotic targets.

In this regard, we and others have shown that the recognition and/or uptake of apoptotic targets by phagocytes is associated with a number of early signaling events (Chung et al. 2007; Cocco and Ucker 2001; Cvetanovic and Ucker 2004; Cvetanovic et al. 2006; Mitchell et al. 2006; Patel et al. 2006; Patel et al. 2007; Reddy et al. 2002). For example, receptor-mediated discrimination of apoptotic targets leads to profound inhibition of NF κ B-dependent transcriptional activity and the signaling kinase ERK1/2, but potent activation of JNK1/2 and p38 (Cocco and Ucker 2001; Cvetanovic and Ucker 2004; Patel et al. 2006; Reddy et al. 2002). In addition, signaling events induced by phagocytosis of apoptotic targets include the activation of Akt, a major regulator of cell survival (Patel et al. 2007; Reddy et al. 2002).

Abnormalities that interfere with apoptotic target-dependent signaling events and thereby predispose to AI can conceivably occur at multiple steps. These abnormalities may affect not only the recognition and/or engulfment of apoptotic targets (e.g., as in the targeted deletion of C1q and MER (Botto et al. 1998; Cohen et al. 2002; GaipI et al. 2005)), but also any of the signaling events or cascades elicited by apoptotic targets (Cocco and Ucker 2001; Cvetanovic and Ucker 2004; Patel et al. 2006; Patel et al. 2007; Reddy et al. 2002). Many of these signaling events or cascades are likely to be activated uniquely by the specific recognition of apoptotic targets. Once activated, these signaling events can modulate the function of responding cells, such as m ϕ , through their effects on other signaling pathways and gene transcription (Fig. 9). For example, the potent anti-inflammatory activity of apoptotic targets, their most defining characteristic, is well documented (Chung et al. 2007; Cocco and Ucker 2001; Cvetanovic et al. 2006; Cvetanovic and Ucker 2004; Fadok et al. 1998; Mitchell et al. 2006; Voll et al. 1997). We refer collectively to the set of unique signaling events induced in m ϕ by interaction with apoptotic targets as the “apoptotic response module” and speculate that one or more of the signaling elements within this module functions abnormally in m ϕ from MRL and other AI-prone mice. In the absence of apoptotic targets, the “apoptotic response module” remains inactive, and gene transcription

in response to innate stimuli such as LPS occurs normally. In contrast, in the presence of apoptotic targets (or their experimental surrogate, serum), the “apoptotic response module” is activated, and gene transcription by m ϕ from AI-prone mice is abnormal.

The differentially expressed genes identified in this study represent the downstream effects of a putative abnormality within the “apoptotic response module” of MRL m ϕ (Tables S3, S4, and S5, Online Resource). These genes can contribute in important ways to the initiation, maintenance, and/or manifestations of AI. For example, included among these genes are multiple cytokines, chemokines, and related proteins that play important roles in the regulation of innate and adaptive immunity (Tables S3 and S4, Online Resource). Also included are genes that contribute to other vital aspects of innate and adaptive immunity, including antigen processing, adhesion and motility, recognition and clearance of dead cells, co-stimulation of lymphocytes, and the signaling response to various extracellular mediators of immunity (Table S5, Online Resource). The selective dysregulation of these genes, occurring only in the presence of apoptotic targets or serum lipids, has broad potential to upset the delicate balance between tolerance and AI.

Several observations suggest that the apoptotic target-dependent abnormalities, described in this and previous publications (Donnelly et al. 1990; Fan et al. 2004; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004), may play an important role in the earliest stages of disease, perhaps during the initiation and/or maintenance of AI. First, the abnormality is found in all of the major inbred murine models of SLE and is absent in all tested non-AI strains (Donnelly et al. 1990; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004). Second, the same abnormality also occurs in the NOD mouse, which develops autoimmune diabetes, a distinct model of spontaneous AI that is organ-specific rather than systemic (Fan et al. 2004; Fan et al. 2006). Third, the abnormality is present in MRL mice as young as 1 week of age (Levine et al. 1993) and shows unaltered expression throughout the lifespan of MRL mice, independent of disease and inflammatory sequelae (Levine et al. 1993; Koh et al. 2000). Finally, the abnormality is present to an equivalent degree in MRL/lpr and MRL/+ mice (Donnelly et al. 1990; Fan et al. 2006; Koh et al. 2000; Levine et al. 1993; Longacre et al. 2004). The first develops an early and severe form of SLE-like disease, while the second shows a more moderate and slowly progressing form of AI. Together, these findings support an early and critical role for apoptotic cell-dependent dysregulation of gene expression in m ϕ from MRL and other AI-prone strains in the initiation and development of AI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by: National Institutes of Health grants DK59793 (to JSL) and HL69722 (to JSL); National Institutes of Health training grant T32DK07510 (to AA); Canadian Institute of Health Research grants MOP-42391 (to JR) and MOP-67101 (to JR); and a Genzyme Renal Innovation Program award from Genzyme, Inc (to JSL). The authors thank the University of Chicago Functional Genomics Facility, headed by Richard Quigg, and especially Xinmin Li, Jaejung Kim, Jamie Zhou, Chris Dyanov, and Miglena Petkova, for cRNA labeling and hybridization.

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Fig. 1.

Comparison of genes differentially expressed by MRL/+ vs. BALB/c mice. A representative example of the results obtained using our algorithm for determining differentially expressed genes is shown. RNA from MRL/+ and BALB/c m ϕ stimulated for 8 h with LPS (100 ng/ml) was compared by DNA microarray analysis. Replicate experiments were performed on two separate days. “*Within*” comparisons refer to RNA samples obtained from m ϕ cultured on the same day, whereas “*across*” comparisons refer to RNA samples obtained from m ϕ cultured on different days as parts of replicate experiments. Differentially expressed genes were defined as those whose SLR was ≥ 0.8 on both “*within*” as well as on both “*across*” comparisons, or ≤ -0.8 on both “*within*” as well as on both “*across*” comparisons. Although the final set of differentially expressed genes is based upon four comparisons (two “*within*” and two “*across*”), only three of these four comparisons are linearly independent (Longacre et al. 2005)

Fig. 2.

Comparison of genes differentially expressed in the presence and/or absence of serum. RNA from MRL/lpr and BALB/c m ϕ stimulated for 8 or 24 h with LPS (100 ng/ml) were compared by DNA microarray analysis. Genes at each time point were separated into three categories—those differentially expressed only in the presence of serum (*SERUM ONLY*), those differentially expressed only in the absence of serum (*SERUM-FREE ONLY*), and those differentially expressed in both the presence and the absence of serum (*SERUM & SERUM-FREE*). We determined the ratio of the SLR magnitude in the presence of serum, SLR_{SERUM} , to that in the absence of serum, $SLR_{SERUM-FREE}$, for all genes in each of the three categories. The figure depicts the mean ($\pm$ SE) ratio of $SLR_{SERUM}/SLR_{SERUM-FREE}$ for each category at each time point. Differences among the three categories were significant at both 8 and 24 h ($p < 10^{-10}$ by ANOVA). Genes in *SERUM ONLY* differed significantly from those in both *SERUM-FREE ONLY* and *SERUM & SERUM-FREE* at 8 and 24 h ($p < 10^{-13}$ by two-tailed *t* test), whereas genes in *SERUM-FREE ONLY* did not differ from those in *SERUM & SERUM-FREE* ($p = NS$ by two-tailed *t* test).

Fig. 3.

Expression characteristics of genes differentially expressed only in the presence of serum (*SERUM ONLY*). Genes differentially expressed only in the presence of serum were divided into four groups, according to whether they were detected in comparisons using MRL/+ (*left*) or MRL/lpr (*right*) m ϕ and following 8 h (*top*) or 24 h (*bottom*) of LPS stimulation. For each group of genes, the frequency distribution of individual SLR values is plotted for culture in the presence (*filled*) or absence (*unfilled*) of serum

Fig. 4.

Individually plotted expression patterns of genes differentially expressed only in the presence of serum (*SERUM ONLY*). RNA from MRL/lpr and BALB/c m ϕ , stimulated for 8 or 24 h with LPS (100 ng/ml), were compared by DNA microarray analysis. Shown are scatter plots for genes differentially expressed in the presence, but not absence, of serum at either 8 or 24 h ($n=123$). The *X*-axis represents the SLR at 8 h, whereas the *Y*-axis represents the SLR at 24 h. It should be noted that both axes are $\log_2$ scales. The *upper plots* show comparisons of MRL/lpr vs. BALB/c m ϕ for culture in the presence (a) or absence (b) of serum. The *lower plots* show control same-vs.-same comparisons for these same genes in the presence of serum for m ϕ from BALB/c (c) or MRL/lpr (d) mice. The mean distance from the origin, as calculated by the Pythagorean Theorem, is shown for each scatter plot

Fig. 5.

Individually plotted expression patterns of genes differentially expressed in both the presence and the absence of serum (*SERUM & SERUM-FREE*). RNA from MRL/lpr and BALB/c m ϕ stimulated for 8 or 24 h with LPS (100 ng/ml) were compared by DNA microarray analysis. Shown are scatter plots for genes differentially expressed in both the presence and the absence of serum at either 8 or 24 h ($n=121$). The X-axis represents the SLR at 8 h, whereas the Y-axis represents the SLR at 24 h. It should be noted that both axes are $\log_2$ scales. Plots represent comparisons of these genes for culture in the presence (a) or absence (b) of serum. The mean distance from the origin, as calculated by the Pythagorean Theorem, is shown for each scatter plot. Consistent with the notion that these genes represent fixed genetic differences between the two strains, the mean distances for culture in the presence vs. absence of serum do not differ ($p=NS$ by two-tailed paired t test)

Fig. 6.

Comparison of genes differentially expressed in the presence and/or absence of serum. RNA from $m\phi$ from the indicated AI-prone (MRL/lpr and NOD) and control (BALB/c and C57BL/6) strains were stimulated for 8 or 24 h with LPS (100 ng/ml) and compared by DNA microarray analysis. Genes at each time point were separated into three categories—those differentially expressed only in the presence of serum (*SERUM ONLY*), those differentially expressed only in the absence of serum (*SERUM-FREE ONLY*), and those differentially expressed in both the presence and the absence of serum (*SERUM & SERUM-FREE*). We determined the ratio of the SLR magnitude in the presence of serum, SLR_{SERUM} , to that in the absence of serum, $SLR_{SERUM-FREE}$, for all genes in each of the three categories. The figure depicts the mean ($\pm SE$) ratio of $SLR_{SERUM} / SLR_{SERUM-FREE}$ for each category at each time point. Differences among the three categories were significant at both 8 and 24 h for each of the three strain comparisons (MRL/lpr vs. C57BL/6, $p < 10^{-13}$ by ANOVA; NOD vs. BALB/c, $p < 10^{-7}$; and NOD vs. C57BL/6, $p < 10^{-4}$). Genes in *SERUM ONLY* differed significantly from those in both *SERUM-FREE ONLY* and *SERUM & SERUM-FREE* at 8 and 24 h (MRL/lpr vs. C57BL/6, $p < 10^{-7}$ by two-tailed *t* test; NOD vs. BALB/c, $p < 10^{-3}$; and

NOD vs. C57BL/6, $p < 10^{-3}$), whereas genes in *SERUM-FREE ONLY* did not differ from those in *SERUM & SERUM-FREE* ($p = \text{NS}$ by two-tailed t test)

Fig. 7. Individually plotted expression patterns of genes differentially expressed only in the presence of serum (*SERUM ONLY*). RNA from mφ from the indicated strains were stimulated for 8 or 24 h with LPS (100 ng/ml) and compared by DNA microarray analysis. Shown are scatter plots for genes differentially expressed in the presence, but not absence, of serum at either 8 or 24 h (MRL/lpr vs. C57BL/6, $n=284$; NOD vs. BALB/c, $n=193$; NOD vs. C57BL/6, $n=129$). The *X*-axis represents the SLR at 8 h, whereas the *Y*-axis represents the SLR at 24 h. It should be noted that both axes are $\log_2$ scales. The *left-hand* plots show comparisons of AI-prone (MRL/lpr or NOD) vs. control (BALB/c or C57BL/6) mφ in the presence of serum. The *right-hand* plots show comparisons in the absence of serum. The mean distance from the origin, as calculated by the Pythagorean Theorem, is shown for each scatter plot

Fig. 8.

Individually plotted expression patterns of genes differentially expressed in both the presence and the absence of serum (*SERUM & SERUM-FREE*). RNA from mφ from the indicated strains were stimulated for 8 or 24 h with LPS (100 ng/ml) and compared by DNA microarray analysis. Shown are scatter plots for genes differentially expressed in the presence, but not absence, of serum at either 8 or 24 h (MRL/lpr vs. C57BL/6, $n=308$; NOD vs. BALB/c, $n=203$; NOD vs. C57BL/6, $n=146$). The *X*-axis represents the SLR at 8 h, whereas the *Y*-axis represents the SLR at 24 h. It should be noted that both axes are $\log_2$ scales. The *left-hand* plots show comparisons of AI-prone (MRL/lpr or NOD) vs. control (BALB/c or C57BL/6) mφ in the presence of serum. The *right-hand* plots show comparisons in the absence of serum. The mean distance from the origin, as calculated by the Pythagorean Theorem, is shown for each scatter plot. Consistent with the notion that these genes represent fixed genetic differences between the two strains, the mean distances for culture in the presence vs. absence of serum do not differ ($p=NS$ by two-tailed paired *t* test)

Fig. 9.

Model for the conditional signaling abnormality that leads to the dysregulated expression of multiple genes in $m\phi$ from pre-diseased AI-prone MRL mice. The clearance of apoptotic cells by $m\phi$ is associated with a number of early signaling events, depicted collectively as an Apoptotic Response Module (ARM). The ARM includes signaling elements that are activated uniquely in response to receptor-mediated recognition of apoptotic cells. While the major known effects of the ARM entail the modulation of signaling events and gene transcription in response to innate stimuli, the range of ARM's effects is likely to be broader. We speculate that one or more of the signaling elements within this module functions abnormally in $m\phi$ from MRL mice. In the absence of apoptotic targets, the ARM is inactive, and gene transcription in response to innate stimuli such as LPS occurs normally. In contrast, in the presence of apoptotic targets, the ARM is activated, and gene transcription by $m\phi$ from MRL and other AI-prone mice is abnormal