Altered Gene Expression in Mice With Lupus Treated With Edratide, a Peptide That Ameliorates the Disease Manifestations

Anat Elmann, Amir Sharabi, Molly Dayan, Heidy Zinger, Ron Ophir, and Edna Mozes

Objective. To identify genes that are differently expressed in (NZB × NZW)F1 mice with established lupus compared with healthy controls, and to determine how gene expression is affected by treatment with hCDR1 (Edratide), a peptide synthesized on the basis of the sequence of the first complementarity-determining region (CDR1) of an autoantibody.

Methods. RNA was extracted from spleen cells of young, disease-free mice and of older mice with systemic lupus erythematosus (SLE) that were treated with hCDR1 or with vehicle alone. Gene expression was assessed using the DNA microarray technique and verified by real-time reverse transcriptase–polymerase chain reaction (RT-PCR).

Results. In mice with SLE, numerous genes showed increased or decreased expression relative to that in the disease-free controls. Treatment with hCDR1 restored the expression of many of these genes to control levels. Real-time RT-PCR verified that in diseased mice RNA transcripts of Tnfsf4, Il5ra, Zbtb20, and Nid1 were up-regulated, while transcripts of Tfpi and S100a8 were down-regulated, and confirmed the effects of hCDR1 on the expression of those genes. Kidney immunostaining demonstrated that the up-regulated expression of OX40 ligand, which is a protein product of the gene tumor necrosis factor (ligand) superfamily member 4, in diseased mice was reduced by hCDR1.

Conclusion. Expression of numerous genes in mice with SLE differs from that in young, disease-free control mice. Treatment with hCDR1 restores the expression of 22% of these genes to levels similar to those in controls. Thus, one of the mechanisms by which hCDR1 exerts its beneficial effects on the clinical symptoms of SLE is through regulation of gene expression.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the increased production of antibodies against several self antigens and by defective T cell–mediated responses. The latter are associated with various clinical manifestations that involve multiple organs and tissues, including immune complex depositions in the kidneys (1). A synthetic peptide, hCDR1 (named Edratide) (2), based on the sequence of the first complementarity-determining region (CDR1) of a human monoclonal anti-DNA antibody that bears the common idiotype 16/6Id (3,4), was shown to be capable of preventing an SLE-like disease or treating an already established disease (5). Beneficial effects of the peptide are manifested in the reduction of autoantibodies and the down-regulation of clinical symptoms, including kidney damage (5). Studies aimed at elucidating the mechanisms that underlie the beneficial effects of hCDR1 demonstrated that treatment of mice with SLE with hCDR1 also resulted in reduced secretion and expression of “pathogenic” cytokines (i.e., interferon-γ [IFNγ], interleukin-1β [IL-1β], tumor necrosis factor α [TNFα], and IL-10), whereas the immunosuppressive cytokine transforming growth factor β was up-regulated (5). Thus, the significant ameliorating effects of hCDR1 are evidently manifested, at least in part, via immunomodulation of the cytokine profile (5–9).

The multiple clinical phenotypes of SLE are influenced by numerous genes. To date, more than 30 chromosomal regions containing genes affecting susceptibility or resistance to lupus have been identified in

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mouse models of SLE. Several of the susceptibility loci map to similar locations across various strains, notably in specific regions of chromosomes 1, 4, 7, and 17 (for review, see ref. 10). Susceptibility genes involved in a mouse model of induced SLE were found to map to chromosomes 6, 7, and 14 (11). A number of studies have documented the contribution of major histocompatibility complex (MHC) (12,13) and non-MHC loci, such as CD22, PD-1, FcγRIIB, and CTLA-4, to lupus susceptibility (for review, see ref. 14). It therefore seems that genes in multiple pathways participate in specific aspects of the disease. In humans, predisposition to SLE was shown to be influenced by the HLA region, complement components, and low-affinity receptors for IgG (for review, see ref. 10).

In attempting to unravel the complexity of SLE, several groups of investigators have employed microarray technology, a powerful tool for investigating differences in gene expression profiles in several diseases and their animal models. To characterize the complexity of immune dysregulation in lupus, some investigators have used complementary DNA (cDNA) arrays to study peripheral blood mononuclear cells (PBMCs) from lupus patients.

In 2002, Rus et al (15), using cytokine array membranes to compare gene expression patterns of PBMCs from SLE patients and healthy controls, identified 20 genes that differ significantly between patients and controls and that belong to a variety of families including the IL-1 family, TNF/death receptors, and IL-8 and its receptors. The same investigators subsequently described 29 additional genes that differentiate patients with active disease from those with inactive disease (16) and that belong to various families including adhesion molecules, proteases, the TNF superfamily, and neurotrophic factors. Maas et al (17) reported differences in expression levels of genes encoding proteins that participate in apoptosis, cell-cycle progression, cell differentiation, and migration. Utilizing CD4+ cells from lupus patients, other investigators (18–20) have identified genes related to cellular development, the Ras pathway, CD70 (19), cyclooxygenase 2 (20), and others. Also described were SLE-specific signature genes whose participation in DNA damage/repair pathways resulted in the production of nuclear autoantibodies (21).

In the only reported such study in (NZB × NZW)F1 mice, widely used as a model of SLE, the gene profile of nephritic (NZB × NZW)F1 mice kidneys was compared with that in kidneys of nondiseased NZW control mice (22). The most highly up-regulated gene (EDV; 5.5-fold) in the kidneys (but not in the spleens) of diseased mice corresponded to an endogenous retrovirus related to the Duplan strain (EDV, L08395).

The present study was carried out in an attempt to gain more insight into the molecular mechanisms underlying the previously described effect of hCDR1 on (NZB × NZW)F1 mice with SLE. It was suggested that this protein might exert its beneficial effects by modifying other protein functions or levels or both, as well as through changes in RNA levels (5–9). We used female (NZB × NZW)F1 mice that were treated with hCDR1 or with the vehicle alone, and we employed microarray technology to identify alterations in gene expression profiles in spleen-derived immune cells during SLE development. We were able to demonstrate both genes that were up-regulated and genes that were down-regulated in the diseased mice. Treatment with hCDR1 modified the expression of some of these genes, restoring it to levels similar to those in disease-free controls. Such restoration correlated with amelioration of disease manifestations after treatment with hCDR1. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) of the tested genes confirmed the microarray data. Immunostaining of kidney sections for the protein product of one of the genes, Tnfsf4, further supported the results.

**MATERIALS AND METHODS**

**Mice.** Female (NZB × NZW)F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The study was approved by the Animal Care and Use Committee of The Weizmann Institute of Science, Rehovot, Israel.

**Synthetic peptides.** The peptide used in this study was hCDR1 (Edratide), which is based on the CDR1 of the human monoclonal anti-DNA antibody bearing the common idiotype designated 16/6Id (4). This peptide (GYYWSWIRQPPGK-GEEWIG) was synthesized (solid-phase synthesis by Fmoc chemistry) by PolyPeptide Laboratories (Torrance, CA). Edratide is currently being examined in a phase II clinical trial in SLE patients.

**Vehicle.** The vehicle used was sulfobutylether beta-cyclodextrin (Captisol; CyDex, Lenexa, KS). This solvent is designed to enhance the solubility and stability of drugs. The drug product was developed by Teva Pharmaceutical Industries (Netanya, Israel).

**Treatment of (NZB × NZW)F1 mice with hCDR1.** Female mice age 6–7 months with established SLE were divided into groups (8–10 mice per group) and injected subcutaneously (SC) with hCDR1 (25–50 μg) or vehicle alone once a week for 10 weeks. Proteinuria and anti–double-stranded DNA (anti-dsDNA) autoantibodies were monitored throughout this period.

**Proteinuria.** Proteinuria was measured by a standard semiquantitative test using an Albustix kit (Bayer Diagnostics, Newbury, UK).
Immunohistology. Frozen kidney sections (6 μm) were air-dried and fixed in acetone. Sections were incubated with fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG (γ-chain specific; Jackson ImmunoResearch, Avondale, PA) for 30 minutes and were extensively washed with phosphate buffered saline. The intensity of immune complex deposits was graded on a scale of 0–3 (0 = no deposits; 1 = low-intensity deposits; 2 = moderate-intensity deposits; 3 = high-intensity deposits).

Immunohistochemistry. Frozen kidney sections (6 μm) were air-dried and fixed in acetone. For detection of OX40 ligand (OX40L) expression, sections were incubated for 16 hours at room temperature with anti-OX40L monoclonal antibody (M-20; Santa Cruz Biotechnology, Santa Cruz, CA). FITC-conjugated mouse anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) was then added. The intensity of fluorescence was graded on a scale of 0–2 (0 = no staining; 1 = low or intermediate intensity; 2 = high intensity). A double-blinded analysis was performed.

Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-dsDNA antibodies. Anti-dsDNA antibodies were detected using λ phage dsDNA, as previously described (5).

Isolation of splenocytes and preparation of RNA. Spleens of the different experimental groups were either pooled or processed individually. Splenocytes were treated with 0.8% NH4Cl and 0.1% KHCOr in water to deplete red blood cells. Total RNA was isolated from 80–100 × 106 spleen cells using the RNA/DNA/protein isolation reagent (TRI Reagent; Molecular Research Center, Cincinnati, OH), and samples of 15 μg of RNA were further processed before hybridization.

Microarray and statistical analysis. RNA samples were hybridized to the mouse genome Affymetrix GeneChip 430A2 array (Affymetrix, Santa Clara, CA). We used R packages from the Bioconductor project (23). Initially, probe signal summarization, normalization, and background subtraction were performed using robust multichip analysis (24) in “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance. We then performed the statistical test for differentially expressed genes using the Statistical test for differentially expressed genes using the “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance. We then performed the statistical test for differentially expressed genes using the “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance.

Real-time RT-PCR. Messenger RNA was analyzed by quantitative real-time RT-PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). RNA samples were hybridized to the mouse genome Affymetrix GeneChip 430A2 array (Affymetrix, Santa Clara, CA). We used R packages from the Bioconductor project (23). Initially, probe signal summarization, normalization, and background subtraction were performed using robust multichip analysis (24) in “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance. We then performed the statistical test for differentially expressed genes using the “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance. We then performed the statistical test for differentially expressed genes using the “affy” package with default parameters. We then performed the statistical test for differentially expressed genes using the Linear Models for Microarray package (25), which allows a better variance estimation by calculating the moderated estimate of variance.

RESULTS

Table 1. Effect of treatment with hCDR1 on clinical manifestations of SLE in (NZB × NZW)F1 mice*

<table>
<thead>
<tr>
<th>Anti-dsDNA antibodies, OD†</th>
<th>Proteinuria, g/litre‡</th>
<th>Intensity of ICDs§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 dilution</td>
<td>1:250 dilution</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.10 ± 0.19</td>
<td>1.64 ± 0.48</td>
</tr>
<tr>
<td>hCDR1</td>
<td>1.46 ± 0.32</td>
<td>0.48 ± 0.21</td>
</tr>
<tr>
<td>P</td>
<td>0.0009</td>
<td>0.0062</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD. Data are from 1 experiment representative of 3 performed. Mice (n = 8–10 per group) were injected subcutaneously once a week for 10 weeks. hCDR1 is a peptide synthesized on the basis of the sequence of the first complementarity-determining region (CDR1) of an autoantibody; SLE = systemic lupus erythematosus; anti-dsDNA = anti–double-stranded DNA; OD = optical density; ICDs = immune complex deposits.
† Measured in sera from mice that were bled after termination of treatment.
‡ Determined at the end of the experiment.
§ Graded on a scale of 0–3 (0 = no deposits; 1 = low-intensity deposits; 2 = moderate-intensity deposits; 3 = high-intensity deposits).

Amelioration of disease manifestations in (NZB × NZW)F1 mice with established SLE by treatment with hCDR1. Female mice age 6–7 months with SLE were treated with 10 weekly SC injections of hCDR1 or vehicle and followed up for disease manifestations. One week after the treatment ended, the mice were killed and their kidneys were evaluated for immune complex deposits. The results of a representative experiment are presented in Table 1, which summarizes some clinical manifestations of the experimental mice. It can be seen in the table that mice treated with vehicle alone exhibited high levels of anti-dsDNA autoantibodies. In mice treated with hCDR1, however, these levels were significantly reduced. Treatment with hCDR1 also ameliorated kidney disease, as indicated by a decrease both
in proteinuria and in the intensity of immune complex deposits in the kidneys relative to mice treated with vehicle alone. Proteinuria, anti-dsDNA autoantibodies, and immune complex deposits could not be detected in young disease-free control mice.

**Microarray analysis of immunocytes from mice with SLE.** In an attempt to identify gene expression profiles characteristic of SLE and to gain an insight into the modified profile induced by hCDR1, we used the DNA microarray technology on RNA preparations from splenocytes of (NZB × NZW)F₁ mice with SLE treated with hCDR1 or with vehicle alone (as described above and in Table 1). For a control, RNA samples were prepared from the spleen cells of 2-month-old mice, which do not exhibit any of the clinical manifestations typical of SLE. Three independent in vivo treatment experiments were performed, and the clinical manifestations in all of them were significantly ameliorated. The RNA obtained from each experimental group of each of the 3 in vivo experiments was hybridized independently on a separate Affymetrix chip (a total of 3 chips for each experimental group).

Of the ~22,000 genes tested by the microarray experiment, the expression of 348 genes in the vehicle-treated mice with SLE differed significantly from that in healthy young controls. It should be noted that changes in gene expression in the mice with SLE might be partially attributable to the effect of age and not to the disease pathology. However, it is not possible to obtain a perfectly matched control group for these mice, because all old (NZB × NZW)F₁ mice have SLE, and old mice of other strains differ in their genetic makeup. The differently expressed genes are graphically represented in a heat diagram (Figure 1A). In block a, red lines represent the 183 genes that were up-regulated in diseased mice relative to 2-month-old, disease-free (NZB × NZW)F₁ mice, and green lines represent the 165 genes that were down-regulated.

To gain insight into the modified gene expression profile involved in the ameliorating effect of hCDR1, we focused on 76 genes (22% of the 348 genes with altered expression in the vehicle-treated diseased mice) that were affected by treatment with hCDR1 and whose RNA expression was restored to levels similar to those observed in the disease-free controls. In block b of Figure 1A, these 76 genes are indicated by red lines or green lines representing, respectively, genes that were up-regulated or down-regulated in hCDR1-treated mice relative to vehicle-treated diseased mice. White lines represent genes that were not affected by hCDR1 treatment. The results in block b show that the effect of hCDR1 on transcript level was reciprocal to the disease, and many genes (76 genes, ~22%) were regulated as a result of hCDR1 treatment and showed a trend opposite to that seen in diseased mice.

![Figure 1. Heat diagrams of differentially expressed genes in mice with systemic lupus erythematosus (SLE) treated with hCDR1 (a peptide synthesized on the basis of the sequence of the first complementarity-determining region [CDR1] of an autoantibody) or with vehicle alone. A, Genes affected by SLE. Up-regulation is indicated in red; down-regulation is indicated in green. White lines represent genes whose expression was unchanged by treatment with hCDR1. a, Genes whose expression was changed in vehicle-treated mice with SLE relative to young disease-free mice. b, Effect of hCDR1 on the genes presented (for a full list of genes, see Supplementary Table 1, available on the Arthritis & Rheumatism Web site at http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/). B, Genes that were specifically up- or down-regulated ≥2-fold in SLE and oppositely affected by hCDR1. Up-regulation is indicated in red; down-regulation is indicated in green. c, Genes that were up-regulated ≥2-fold in vehicle-treated mice with SLE relative to young disease-free mice. d, Genes that were down-regulated ≥2-fold in vehicle-treated mice with SLE relative to young disease-free mice. Gene symbols and titles are listed in Tables 2 and 3. Results presented were obtained from 3 independent in vivo experiments using 1 chip per experimental group in each experiment. A scale of color intensity is presented for each heat diagram.](image-url)
The heat diagram in Figure 1B shows the genes that were up-regulated or down-regulated in mice with SLE/H11350 2-fold relative to young mice, and that were oppositely affected by hCDR1. Block c shows the genes (in red) that were up-regulated by the disease. Treatment with hCDR1 down-regulated these genes (indicated by green). Block d shows genes (in green) that were down-regulated by the disease and that were up-regulated after treatment with hCDR1 (indicated by red).

Of the 15 genes with an identified product that were up-regulated 2-fold in the diseased mice (Table 2) encode for membrane-associated proteins. Six of these gene products are located in the plasma membrane, and 1 (cytochrome P450) is located in the mitochondrial membrane. Of the 12 genes that were down-regulated 2-fold in the diseased mice (Table 3), the gene products of 9 are known to be extracellular proteins. Of the 10 gene products with known activity, 3 are enzymes and 2 are protease inhibitors. Moreover, of the 12 genes that were down-regulated 2-fold, all but 2 (Schlafen 4 and thrombospondin) were identified as genes that encode proteins known to be associated with monocytes.

**Results of real-time RT-PCR analysis.** To confirm the microarray results, we carried out independent testing by real-time RT-PCR of the expression patterns of 6 representative genes that were significantly up-

### Table 2. Genes that were up-regulated in (NZB × NZW)F1 mice with SLE*

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Fold difference</th>
<th>Cellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frizzled homolog 6 (Drosophila)</td>
<td>Fzd6</td>
<td>2.6</td>
<td>Membrane</td>
</tr>
<tr>
<td>Nidogen 1</td>
<td>Nid1</td>
<td>2.4</td>
<td>ECM</td>
</tr>
<tr>
<td>RIKEN cDNA 5830484A20</td>
<td>5830484A20Rik</td>
<td>2.4</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Similar to RIKEN cDNA</td>
<td>5830484A20 LOC 545340</td>
<td>2.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 4</td>
<td>Tnfsf4</td>
<td>2.3</td>
<td>Membrane</td>
</tr>
<tr>
<td>Proline-serine-threonine phosphatase-interacting protein 2</td>
<td>Pstpip2†</td>
<td>2.2</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Polymorphic immunoglobulin receptor</td>
<td>Pigr</td>
<td>2.2</td>
<td>Membrane</td>
</tr>
<tr>
<td>RIKEN cDNA 2700022B06 gene</td>
<td>2700022B06Rik</td>
<td>2.1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Interleukin-5 receptor α</td>
<td>Il5ra</td>
<td>2.1</td>
<td>Membrane</td>
</tr>
<tr>
<td>RIKEN cDNA A130040M12</td>
<td>A130040M12Rik</td>
<td>2.1</td>
<td>Unknown</td>
</tr>
<tr>
<td>G protein–coupled receptor 132</td>
<td>Gpr132</td>
<td>2.1</td>
<td>Membrane</td>
</tr>
<tr>
<td>CD8 antigen, beta chain 1</td>
<td>Cd8b1</td>
<td>2.1</td>
<td>Membrane</td>
</tr>
<tr>
<td>DEAH (Asp-Glu-Ala-His) box polypeptide 9</td>
<td>Dhtx9</td>
<td>2.0</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily a, polypeptide 1</td>
<td>Cyp11a1</td>
<td>2.0</td>
<td>Mitochondrial membrane</td>
</tr>
<tr>
<td>LIM domain only 7</td>
<td>Lmo7</td>
<td>2.0</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ring finger protein 184</td>
<td>Rnf184</td>
<td>2.0</td>
<td>Unknown</td>
</tr>
<tr>
<td>Proline-serine-threonine phosphatase-interacting protein 2</td>
<td>Pstpip2†</td>
<td>2.0</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Unknown</td>
<td>Unknown</td>
<td>1.9</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hepatoma-derived growth factor, related protein 3</td>
<td>Hdgfrp3</td>
<td>1.9</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Zinc finger and BTB domain containing 20</td>
<td>Zbtb20</td>
<td>1.9</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Argininosuccinate synthetase 1</td>
<td>Ass1</td>
<td>1.9</td>
<td>Mitochondrion</td>
</tr>
</tbody>
</table>

* Results are presented as the fold difference between control mice and mice with SLE treated with vehicle alone (designated “SLE”) and between mice with SLE treated with vehicle alone and hCDR1-treated mice with SLE (designated “hCDR1”). Listed are genes that were up-regulated 1.9-fold based on the results obtained from 3 independent in vivo experiments using a total of 3 chips for each experimental group. A negative fold change represents a reduction in signal. ECM = extracellular matrix (see Table 1 for other definitions).

† Replicate probe sets.

‡ Not statistically significant.
regulated (Tnfsf4, Il5ra, Zbtb20, and Nid1) or downregulated (Tfpi and S100a8) in mice with SLE and oppositely affected by hCDR1 treatment. We chose these genes because they reportedly meet at least 1 of the following 3 criteria: involvement in SLE, functioning in lymphocytes, and expression in the kidney. Figures 2A and B show the mean ± SD values from 3 treatment experiments performed on pooled RNA samples (n = 8–10 per group) of the various groups, using real-time RT-PCR. A marked increase was shown for Tnfsf4, Il5ra, Zbtb20, and Nid1 transcripts in diseased mice relative to the young, disease-free controls (Figure 2A). Treatment with hCDR1 reduced the expression of these genes to levels similar to those seen in the young mice. Likewise, transcripts of Tfpi and S100a8, which were down-regulated in vehicle-treated diseased mice, were up-regulated after hCDR1 treatment to levels similar to those in the young controls (Figure 2B). In order to determine interindividual variability in each group, real-time RT-PCR was performed on RNA samples from individual mice (n = 5 per group), and the mean ± SEM values for RNA expression for each gene are presented in Figures 2C and D. The results were similar to those shown for pooled RNA samples.

**Immunostaining of kidneys of (NZB × NZW)F$_1$ mice for OX40L expression.** In view of the reported presence of OX40L (26) in kidney biopsy specimens from all tested lupus patients with proliferative lupus nephritis, it was of interest to further determine whether this ligand is also up-regulated in the kidneys of diseased (NZB × NZW)F$_1$ mice, and whether, as shown for the transcripts in spleen cells, its expression is affected by treatment with hCDR1. We performed immunohistologic analysis of kidney sections from 9 vehicle-treated mice with SLE, 8 hCDR1-treated mice with SLE, and 5 disease-free young mice. Figure 3 shows representative sections and mean ± SD intensity values for all individuals in the group. A significant expression of OX40L was determined in the glomeruli, the interstitial tissue, and the tubuli of kidneys from the diseased mice that received vehicle. The expression of OX40L in the glomerular and interstitial tissues was significantly down-regulated in kidneys of mice that were treated with hCDR1. The effect of hCDR1 on the tubuli was less prominent. Expression of OX40L could not be detected in kidneys of young disease-free mice.

**DISCUSSION**

The main finding of this study was that the expression of many genes is up-regulated or down-regulated in (NZB × NZW)F$_1$ mice with SLE relative to young healthy controls. Moreover, treatment with the disease-ameliorating hCDR1 restored the expression of some of these genes to levels similar to those observed in the disease-free control mice. These results were verified

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**Table 3. Genes that were down-regulated in (NZB × NZW)F$_1$ mice with SLE**

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Fold difference</th>
<th>Cellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>Mpo</td>
<td>−3.2</td>
<td>ES</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>Ltf</td>
<td>−2.8</td>
<td>ES</td>
</tr>
<tr>
<td>Lipocalin 2</td>
<td>Lcn</td>
<td>−2.7</td>
<td>ES</td>
</tr>
<tr>
<td>Cathelicidin antimicrobial peptide</td>
<td>Camp</td>
<td>−2.6</td>
<td>ES</td>
</tr>
<tr>
<td>Neutrophilic granule protein</td>
<td>Ngp</td>
<td>−2.5</td>
<td>ES</td>
</tr>
<tr>
<td>Schlafen 4</td>
<td>Slfn</td>
<td>−2.4</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>Ctsg</td>
<td>−2.4</td>
<td>ES</td>
</tr>
<tr>
<td>Thrombospondin 1</td>
<td>Thbs1</td>
<td>−2.3</td>
<td>ES</td>
</tr>
<tr>
<td>S100 calcium-binding protein A8 (calgranulin A)</td>
<td>S100a8</td>
<td>−2.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>RIKEN cDNA 11900003K14</td>
<td>1190003K14Rik‡</td>
<td>−2.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>Prtn3</td>
<td>−2.2</td>
<td>ES</td>
</tr>
<tr>
<td>S100 calcium-binding protein A9 (calgranulin B)</td>
<td>S100a9</td>
<td>−2.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor</td>
<td>Tfpi</td>
<td>−2.0</td>
<td>ES</td>
</tr>
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</table>
for 6 of the genes by real-time RT-PCR. We also showed that expression of Tnfsf4, which encodes for OX40L, was induced in the kidneys of mice with SLE and was inhibited by treatment with hCDR1.

In the present study we identified genes that distinguish (NZB × NZW)F1 mice with SLE treated with vehicle alone from those treated with hCDR1. In most of the microarray studies performed to date, the subjects were SLE patients (15–19; for review, see ref. 21), a highly heterogeneous group in whom clinical manifestations vary widely. In the only reported study carried out using (NZB × NZW)F1 mice, nephritic kidneys of these mice were compared with kidneys of healthy NZW controls (22). Because SLE is a systemic autoimmune disease, we were interested in the gene expression profile of immune cells, and our analyses of the SLE-prone mouse model were therefore carried out in spleens. To the best of our knowledge, this is the first study to investigate cells derived from an immune organ of (NZB × NZW)F1 mice. It is also the first study in which microarray analysis was used to compare immune cells derived from mice with SLE that were either treated or not treated with an immunomodulatory synthetic peptide that ameliorates SLE manifestations.

On the basis of previous reports in connection with gene involvement in lupus, in other autoimmune
diseases, or in lymphocyte functions, we chose to use real-time RT-PCR for further testing of mice with SLE for the expression of 6 genes, 2 of which were down-regulated and 4 of which were up-regulated. Of the genes that were both down-regulated by the disease and up-regulated by treatment with hCDR1, we selected 2, Tfpi and S100a8, for testing of RNA levels. Tfpi, which is expressed by monocytes (among other cells), encodes for tissue factor pathway inhibitor (TFPI), which is a serine protease inhibitor and an anticoagulant that modulates the tissue factor pathway by acting on the factor VIIa–TF complex, factor Xa, and thrombin (for review, see ref. 27). Ertenli et al (28) reported that baseline TFPI concentrations in the plasma (but not in the vascular endothelium) of patients with SLE were 6–7-fold lower than in the plasma of the control group. This is concordant with our finding of down-regulation of this gene in mice with SLE relative to controls. Treatment with hCDR1 up-regulated the expression of this gene to levels comparable with those in control mice (Figure 1B and Table 3).

The second gene chosen for testing for RNA levels was S100a8, which was found by microarray to be down-regulated by the disease and up-regulated after hCDR1 treatment. S100a8 encodes for the S100 calcium-binding protein A8 (calgranulin A). The S100 protein family comprises small calcium-binding proteins, some of which reportedly have intracellular and extracellular functions associated with inflammation and autoimmune diseases (for review, see ref. 29). The S100 calcium-binding protein A10 is reportedly up-regulated in T lymphocytes of SLE patients treated with cyclo-
phosphamidie (30). In line with the latter finding is the observed up-regulation of S100a8 and S100a9 after treatment with the disease-ameliorating peptide hCDR1 (Figure 1B and Table 3).

Real-time RT-PCR was also carried out for 4 genes that were up-regulated by the disease and subsequently down-regulated by hCDR1. Two of them, Il5ra and “zinc finger and BTB domain containing 20” (Zbtb20), were not previously reported to be related to SLE. The IL-5 receptor α (IL-5Rα) protein specifically binds IL-5 and stimulates the proliferation and differentiation of B cells and eosinophils (for review, see ref. 31). Although IL-5Rα has not been reported to play a role in lupus, its participation is not unlikely, given the role of B cells in this disease. The second gene, Zbtb20, contains the BTB domain (also known as the POZ domain), a versatile protein–protein interaction motif that can be found in proteins containing the zinc finger domain and that participates in a wide range of cellular functions (for review, see ref. 32). In addition, one of the Zbtb proteins is cKrox, which mediates the CD4 lineage differentiation during intrathymic positive selection of T cells (33).

The third gene tested by real-time RT-PCR was Nid1, which encodes for nidogen (also termed entactin), a major glycoprotein component of basement membranes. Autoantibodies against nidogen were identified in the sera of B6.Sle1.lpr lupus mice as well as in that of lupus patients (34).

Another gene that was up-regulated by the disease and then down-regulated by hCDR1 was Tnfsf4. This gene encodes OX40L (also known as CD134L), a type II membrane protein with homology to TNF (35). OX40L is expressed on activated B cells (36), endothelial cells (37), macrophages, dendritic cells, and some activated T cells. It was shown to participate in costimulation of T lymphocytes (35), activation of B lymphocytes (36), and adhesion of T cells to activated endothelium (37). Tnfsf4 also enhances the proliferation and differentiation of T lymphocytes and the development and survival of memory CD4+ T cells (38). Wang et al (39) showed that mice overexpressing OX40L have significantly larger atherosclerotic lesions than those in control mice. The role of OX40L in lupus was demonstrated by Aten et al (26), who reported the abundant presence of this protein, localized predominantly along the epithelial side of the glomerular capillary wall, in patients with proliferative lupus nephritis. Our confirmation by real-time RT-PCR of the up-regulated expression of Tnfsf4 and its down-regulation by hCDR1 is consistent with those findings. In addition, we demonstrated the expression of this protein in kidneys of mice with SLE and its down-regulation after treatment with hCDR1.

Other members of the Tnfsf family were reported to play a role in lupus. Rus et al (15) showed, for example, that transcripts of Tnfsf10 (encoding TRAIL) as well as transcripts of Tnfsf10C and Tnfsf10D (both encoding TRAIL receptors) were increased in PBMCs from lupus patients relative to controls. Also, Tnfsf9 was one of the genes shown to differentiate PBMCs of SLE patients from those of healthy controls (40).

Treatment of mice with SLE with hCDR1, which specifically affects T cells, initiates a cascade of events that affects numerous cell types and several systems, leading to disease amelioration. The present study demonstrates the final effect of hCDR1 on genes derived from immune cells such as neutrophils (e.g., lipocalin 2) and B cells (e.g., Il5ra). Among the genes that were down-regulated ≥2-fold in splenocytes from mice with SLE were 4 genes that encode proteins known to be derived from neutrophils. Although ~60% of SLE patients have neutropenia (41), this is not among the main manifestations observed in (NZB × NZW)F1 mice. However, the reduction in transcript levels of the above-mentioned genes, which are crucial for the effective functioning of neutrophils, might reflect a down-regulation in the state of functionality or activity of these cells.

In the present study, we used microarray analysis to identify genes whose involvement in SLE has not been previously described. We therefore employed a stringent filtration process that does not permit the inclusion of genes whose expression was not significantly modulated (P ≥ 0.05) between hCDR1-treated mice with SLE and vehicle-treated mice with SLE, although some of the genes in that category (such as IFNγ, IL-10, apoptosis-related genes, etc.) were shown by ELISA and/or real-time RT-PCR to be differentially expressed (5–7).

A number of possible reasons might account for the fact that most of the genes with the greatest changes (up-regulation or down-regulation) were not reported by other investigators. First, our study was conducted using cells from mice, while in most of the other studies the cells were of human origin. Second, substantially more genes were screened in the present study (22,000) than in other studies, which have used smaller chips. In addition, we did not restrict ourselves to specific pathways (e.g., cytokines, apoptosis) as in some of the studies. The origins of the genes tested, namely, spleen cells and PBMCs, might also contribute to the observed differences.

Recently, microarray analysis in the case of sev-
eral autoimmune diseases (SLE, rheumatoid arthritis, multiple sclerosis, and type I diabetes) was found to yield a common expression signature (17,40). The genes that we identified might be unique to SLE, or they might be genes with potential relevance for other autoimmune diseases as well.

To summarize, SLE is a complex disease in which multiple genes influence the clinical phenotype. In the present study, we were able to identify gene expression profiles of the disease process and gain an insight into the modified profile induced by treatment with hCDR1. Elucidation of the genes that are modified (up-regulated or down-regulated) by hCDR1 in association with disease amelioration might shed light on genes that play an important role in the development and progression of SLE as well as on the mechanism of action of hCDR1.

**AUTHOR CONTRIBUTIONS**

Dr. Mozes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.  

**Study design.** Elmann, Mozes.  

**Acquisition of data.** Elmann, Sharabi, Dayan, Zinger.  

**Analysis and interpretation of data.** Elmann, Mozes.  

**Manuscript preparation.** Elmann, Sharabi, Ophir, Mozes.  

**Statistical analysis.** Elmann, Sharabi, Dayan, Ophir.

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**REFERENCES**