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Pivotal Role of Stat4 and Stat6 in the Pathogenesis of the Lupus-Like Disease in the New Zealand Mixed 2328 Mice

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We have developed novel genetically lupus-prone (NZB × NZW)F1-derived congenic New Zealand mixed (NZM) 2328 lines, which are either Stat4- or Stat6-deficient. Our studies show that the deficiency of Stat4 and Stat6 significantly alters the phenotype of the lupus-like disease in NZM 2328 congenic mice. Specifically, Stat4-deficient NZM mice develop accelerated nephritis and increased autoantibodies and kidney disease. Furthermore, these results also question the apparent equation of the effect of Stat deficiency with loss of secretion or response to particular cytokines.


Abbreviations used in this paper: SLE, systemic lupus erythematosus; KO, knockout; GN, glomerulonephritis; NZM, New Zealand mixed; PAS, periodic acid Schiff; HMR 705, Los Angeles, CA 90089. E-mail address: Jacob@usc.edu

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the University of Southern California Laboratory Animal Resource Center (Los Angeles, CA). The Stat4- and Stat6-deficient mice were crossed onto the NZM2328 background, using a marker-assisted selection strategy. Each backcross generation was screened with polymorphic microsatellite markers at a density of ~25 cM. In each generation 25–50 males were screened, and the progeny carrying the target gene in combination with the lowest content of donor genes (129/C57BL/6) was chosen for breeding with NZM 2328 females to obtain the next backcross generation. For identification of Stat4 and Stat6 null mutations, PCR-based assays were used. One assay amplified the linked neomycin resistance (neo) gene using the primers 5′-GTC GAT ATC GCA AGA GAT C-3′ and 5′-GAC TGG GCA CAC CCC ACC-3′. The other assay amplified a segment of the respective Stat gene spanning the null-generating mutation using the following primers: 5′-GTA CAT GAG GAA GGT AGC TC-3′ and 5′-CTG CCA GAG AGT TTT TTC C-3′ (for Stat4) and 5′-CTC AGA GTC GCT AAA GCG G-3′ and 5′-GGC AGT GGT TTG ATG GTG-3′ (for Stat6).

**In vitro culture**

Pooled spleen cells from control and Stat4- or Stat6-deficient mice were activated with 1 μg/ml plate-bound anti-CD3 (145-2C11). After 1 wk in culture, cells were washed and restimulated with anti-CD3 for 24 h. Where indicated, IL-12 was added at 200 U/ml. Supernatants were harvested and quantified by using commercial ELISA kits per the manufacturer’s instructions.

For measuring NK cell cytolytic activity, splenocytes were enriched for NK cells (stained with biotin-anti-CD3 and rat anti-mouse Ig and depleted of T, B and lymphocytes using magnetic beads), and incubated with IL-12 (200 U/ml) overnight. In control experiments, NK-enriched splenocytes were incubated with 250 units based on the standard curve produced by serial dilutions of pooled lpr/lpr from MRL-1pr/lpr mice, pressed in arbitrary units relative to a standard positive sample derived (16). Samples were screened at a 1/50 dilution and the results were expressed in arbitrary units as the percentage of lysis. The development of proteinuria was assessed weekly using Albustix assay strips (Bayer, Elkhart, IN) using a scale of 0 to 3.

Anti-dsDNA Abs were assayed in two steps. First, a semiquantitative, ELISA. Representative results are shown in Fig. 1. Under neutral conditions, Streptococcus pneumoniae IgG and IgG subclasses (IgG1, 2a, 2b, or 3) or IgM (Southern Biotechnology Associates) or with rabbit anti-mouse C3 (Cappel Laboratories, Durham, NC). Glomerular staining was graded by intensity (0, no staining; 1+, maximum staining) and pattern (predominantly mesangial or capillary plus mesangial).

Statistical analyses

The data are presented as mean ± SEM. The Mann-Whitney U test was used to analyze nonparametric data for statistical significance. Values of *p* ≤ 0.05 were considered significant.

**Results**

**Establishment of Stat4 and Stat6 null NZM2328 strains**

We have transferred the Stat4 and Stat6 null mutations onto the lupus background of NZB × NZW. However, rather then using the original parental strains, we used the (NZB × NZWF1)F1-derived inbred mouse strains NZM 2328 which most closely resembled the (NZB × NZWF1)F1. This eliminated the need to backcross the null mice separately to NZB and NZW background.

A marker-assisted selection protocol was used, which accelerates the introgression of genes by using genetic markers to facilitate this process (17–19). By specifically selecting progeny at each backcross generation that carry the target gene and have the lowest content of heterozygosity throughout the genome, the rate of donor genomic segment elimination is significantly accelerated. We screened each generation with microsatellite markers at a density of ~25 cM. At least 25 males were screened each generation. In all, six backcrosses were completed in each of the lines. The final backcross mice carried 76 of 76 (100%) NZM 2328-specific markers but none (0%) of the C57BL/6 or 129 strain-specific marker alleles. These mice were intercrossed to produce homozygous null mice used for all the experiments described below. To follow the Stat4 and Stat6 null mutations in the mice, we used PCR-based assays: one that amplified the neomycin-resistance (neo) gene and the other that amplified a part of the respective Stat gene containing the eliminated portion. The defect in the resulting mice was conferred by the absence of these Stat proteins in Western blot analysis using polyclonal anti-Stat4 and anti-Stat6 (data not shown).

**Characterization of the mice lines produced**

The first step in characterizing these newly created mice models was to verify the effect of Stat-4 and Stat-6 null mutations on the production and regulation of Th1 and Th2 cytokines.

Spleen cells from “wild-type” NZM 2328 (control) and mutant NZM.Stat4−/− and NZM.Stat6−/− mice were activated in vitro with plate-bound anti-CD3 in the absence of exogenous cytokines to determine their developmental potential into Th1 or Th2, as described (20, 21). IFN-γ and IL-4 production was quantified by ELISA. Representative results are shown in Fig. 1. Under neutral conditions, Streptococcus pneumoniae IgG and IgG subclasses (IgG1, 2a, 2b, or 3) or IgM (Southern Biotechnology Associates) or with rabbit anti-mouse C3 (Cappel Laboratories, Durham, NC). Glomerular staining was graded by intensity (0, no staining; 1+, maximum staining) and pattern (predominantly mesangial or capillary plus mesangial).
null mice, there was a significant reduction in the level of IgG1 in IgG levels were not significantly different between Stat4 and Stat6 samples from the congenic mouse lines produced. Although total IL-12-mediated NK activity, had readily detectable cytolytic ac-
tivity against YAC-1 cells (data not shown).

Small resting B cells, obtained after treatment of splenocytes with anti-Thy 1.2 and complement followed by Percoll gradient cen-
trifugation were cultured with IL-4 (30 U/ml). After 48 h, cells were stained with FITC-anti-CD23 and biotinylated 10.2,16 anti-
I-A\(^{a}\) Ab. As shown (Fig. 5), IL-4-induced CD23 and I-A\(^{a}\) expres-
sion was observed in wild-type NZM 2328 and NZM 2328.Stat4, but not in NZM 2328.Stat6 mice.

There was no apparent effect of the Stat4 or Stat6 deletion in these mice as far as the total number of spleen lymphocytes, T cells and B cells or, CD4\(^{+}\) and CD8\(^{+}\) T cell populations, as determined by FACS analysis (data not shown).

Effects of Stat4 and Stat6 KO on autoimmune disease development in the NZM mouse lines

In comparison to wild-type NZM 2328 Stat4-sufficient mice, NZM 2328 Stat4-deficient female mice develop accelerated kidney disease, as measured by proteinuria and mortality (Fig. 6). The fact that proteinuria levels closely correlated with animal mortality supports the notion that these mice die as a consequence of kidney disease. In contrast, NZM2328.Stat6-deficient female mice showed a significant delay in development of kidney disease (p < 0.001).

Interestingly, the NZM 2328.Stat4 KO mice with accelerated kidney disease displayed much lower levels of anti-dsDNA Ab and anti-chromatin Abs compared with the wild-type NZM 2328 mice. Fig. 7 shows results for anti-chromatin and anti-dsDNA Abs tested in 4- to 7-mo-old mice of the different genotypes. Stat4\(^{-/-}\) animals had significantly lower levels of total IgG autoantibodies com-
pared with Stat4\(^{+/+}\) NZM 2328 control mice (p < 0.05). In contrast, Stat6-deficient mice produced as high (or somewhat higher) levels of IgG autoantibodies as the wild-type NZM 2328 animals (differences are not statistically significant).

The NZM 2328.Stat4\(^{-/-}\) mice show a panisotype elevation of auto-

FIGURE 2. IL-12-induced production of IFN-γ in Stat4- and Stat6-de-
cient NZM2328 mice. Spleen lymphocytes from 9- to 12-wk-old mice from the indicated strains and genotypes were activated with or without IL-12 as described in Materials and Methods and levels of IFN-γ were measured in supernatants.

FIGURE 1. IFN-γ and IL-4 levels in Stat4- and Stat6-deficient NZM 2328 mice. Spleen lymphocytes from 9- to 12-wk-old NZM 2328 mice with the indicated genotype (wild-type, Stat4\(^{-/-}\), and Stat6\(^{-/-}\)) were tested for cytokine production, as de-
scribed in Materials and Methods. Results are expressed as the mean ± SE values of 10–14 mice per group.
at 10–12 mo of age \((p < 0.05)\), an age when all Stat4-deficient mice and most control NZM 2328 mice were already dead. The Stat4 null mice could not be differentiated from Stat6 null mice by the extent of involvement of interstitium and blood vessels or on the level of evidence for glomerulosclerosis vs glomerular infiltration (data not shown).

A trivial explanation for why severe disease is seen in the absence of high levels of circulating autoantibodies might be that the Abs are bound up as immune complexes in the kidneys and the kidneys are serving as a sink for formation of immune complexes. To determine whether these Abs actually deposit in the glomeruli in vivo, we have performed immunohistochemistry analysis of kidneys of the different congenic mice. For immunofluorescence, kidneys were embedded in OCT and 3-μm frozen sections were stained with 1/20 goat anti-mouse IgM, IgG1, 2a, 2b, 3 or with rabbit anti-mouse C3. Glomerular staining was graded by intensity. NZM 2328 \(^{-/-}\) mice show high levels of panisotype deposition of Ig and complement (Fig. 9G) compared with the NZM 2328 mice. The Stat6 mice show also reduced immune deposits. However, although in the Stat4 \(^{-/-}\) mice the deposits shown are mostly IgM and IgG1 and no IgG2a or IgG2b, the Stat6 \(^{-/-}\) mice deposits are mainly IgM, IgG2a and IgG2b and almost no IgG1 (Fig. 9H).

**Discussion**

In this study, we explore the development and progression of mouse SLE as a consequence of deficiency in Stat genes (Stat4 and Stat6). The generation of these new mice has provided useful experimental models to explore cytokine regulation, as signaling by a specific cytokine using a specific Stat pathway has been abolished. Among Stat family members, Stat4 and Stat6 are key regulators of T cell differentiation involved in immune responses (12).

The Stat4 and Stat6 genes encode transcription factors that, when phosphorylated by Janus kinases, are activated and transported to the nucleus where they regulate cytokine-induced gene expression (22). Until very recently, production of IL-4, an essential cytokine for differentiation of Th2-responsive cells, was thought to be completely dependent on signaling via Stat6. Indeed, original studies with Stat6 \(^{-/-}\) mice suggested that these KO mice were completely deficient in Th2 production (20, 21, 23). However, more recent studies indicate there is an alternative pathway for differentiation of Th2 cells that is independent of Stat6 (24–27). Despite this alternative pathway, CD4 \(^{+}\) T cell differentiation in Stat6 \(^{-/-}\) mice defaults to a Th1-like response and a deficiency of functional Th2 cells (26, 28, 29).

Similarly, Stat4 was originally thought to be critical for development of Th1 cells (30) because it is a transcriptional regulatory molecule for IL-12, which is a potent differentiation agent for Th1 cells (31). However, recent studies demonstrate that there are both Stat4-dependent and Stat4-independent pathways for the development of Th1 cells. Although the Stat4-dependent pathway involves induction of IL-12, the Stat4-independent pathway does not, and thereby represents a default pathway that occurs in the absence of Stat4 signaling (32). Even though Stat4 \(^{-/-}\) mice contain an alternative pathway for generating Th1 cells, the CD4 \(^{+}\) T cell response...
in these mice is strongly skewed toward a Th2 response, and Stat4−/− mice are deficient in Th1-type cells.

There is great interest in the possibility that a skewing toward a Th2-type response plays a major role in murine lupus. Such skewing has been suggested because CD4+ T lymphocytes from (NZB × NZW)F1 mice secrete less and less IL-2 as the mice age and the disease becomes more clinically evident (33). Further, IL-4 mRNA expression increases with age and disease development in (NZB × NZW)F1, while IL-12 mRNA expression decreases (34). Cytokine profiles in mitogen-stimulated T cells in these mice showed high Th2-type cytokine production (IL-4, IL-5, and IL-10), while Th1 cytokine (IL-2, IFN-γ) production appears to be low (10). Moreover, the administration of anti-IL-10 to (NZB × NZW)F1 mice delays disease onset (35), while treatment with IL-4 antagonists decreases anti-DNA Ab and associated renal disease (36). Also, transgenic expression of IL-4 under a MHC class I promoter induces autoimmunity in a nonautoimmune background (37).

Although the above studies suggest a pivotal role for Th2 cytokines, other findings suggest an important role for Th1 responses, as well. For example, the major isotype eluted from glomerular lesions of lupus mice is IgG2a (38), which depend on IFN-γ for its synthesis. Furthermore, lupus mice are protected from disease development by inhibition of IFN-γ signaling. Thus, it has been shown that neutralization of IFN-γ by mAb (8) or soluble IFN-γ receptor (9) prevent GN in (NZB × NZW)F1 mice, while administration of exogenous IFN-γ accelerates renal disease. Interestingly, deficiency in IFN-γ receptor crossed onto NZB and NZW mice causes a marked reduction in anti-DNA Ab, including IgM and IgG1 as well as IgG2a (39). Further, IFN-γ receptor null (NZB × NZW)F1 mice show significant reduction in kidney disease development (39).

Our results presented in this study suggest that the validity of the Th1/Th2 dichotomy in explaining the pathogenesis of lupus appears too simplistic. Some recent studies have raised doubts about the validity of the Th1/Th2 paradigm in other systems as well (40, 41).

The results raise questions regarding the accepted paradigm that autoantibodies (especially anti-dsDNA Ab) play a major, direct, and culpable role in inducing some of the disease manifestations of SLE (especially nephritis). It is commonly believed that if Ab-Ag interaction occurs and complement is activated, tissue damage will result. The mechanism by which anti-DNA Ab is believed to fix to tissue and cause damage is either by forming immune complexes
of the correct size and charge to be trapped in the basement membranes of glomeruli, or by binding directly to DNA, DNA-histone, or to non-DNA Ag present in the basement membranes. Our results suggest that the association between the presence of autoantibody and kidney disease is not a simple and direct cause-effect relationship—any explanation reduced to such a simple cause-effect relationship is precarious, at best.

Recently, Shlomchik and colleagues (42) have demonstrated an Ab-independent mechanism for renal and vascular disease in the MRL-\(lpr/lpr\) mouse model using a mutant transgene encoding membrane Ig, but which did not permit the secretion of circulating Ab. Thus, a possible explanation of the accelerated nephritis obtained in Stat4-deficient lupus mice might be that autoantibodies are not critical for the development of lupus nephritis. However, while the membrane only Ig transgenic MRL-\(lpr/lpr\) mice showed preferential involvement of the interstitium and blood vessels rather than proliferative GN, there seems to be no such preferential involvement in the Stat4-deficient NZM2328 mice. In contrast, the genetic background of the mice developing the Ab-independent lupus nephritis is very different from the background of the Stat 4 null mice. Thus, the differences in the nature of the nephritis between these mice might be a consequence of their different genetic background. An alternative mechanistic explanation might be that for developing lupus nephritis a certain threshold of insult to the kidney is necessary to initiate the process. Presumably, such an insult can be delivered by autoantibody complement complexes. If the threshold of the insult is achieved, the additional excess of autoantibody might make no difference in terms of the overall outcome of the kidney disease. We are currently testing these possibilities.

Our results in Stat4-deficient NZM 2328 mice also substantiate the importance of Th1-type cytokines in autoantibody production. These results also raise questions regarding the claimed crucial role of IFN-\(\gamma\) in the development of lupus pathology because we found that the nephritis in Stat4 null mice is worse than in wild-type NZM 2328 mice despite reduced production of IFN-\(\gamma\). Furthermore, the data might suggest a protective role for IFN-\(\gamma\) in disease initiation, possibly by a mechanism similar to what has been shown in transplantation studies (43, 44) and in some cases of autoimmunity (45–47). Further studies will attempt to verify these possibilities.

It is also possible that the observed effects of the Stat4 or Stat6 KO reflect the removal of lupus susceptibility or resistance genes during backcrossing of the mutated locus from the 129/C57BL6 background on to the NZM.2328 genetic background. Stat4 and
Stat6 genes, however, are on mouse chromosome 1 (25.9 cM) and mouse chromosome 10 (70.0 cM), respectively; thus, both genes are clearly outside any known lupus susceptibility or resistance region in NZM 2328 mice (5). Therefore, it is unlikely that the phenotypes presented by these mice are due to any interference of the mutated locus with potential lupus susceptibility genes. More importantly, these findings may be relevant to human SLE because there is abundant clinical experience demonstrating that

*FIGURE 9.* Relative distribution of Ig isotype and complement deposits in glomeruli of Stat-deficient and control NZM 2328 mice. The severity of GN in the kidneys was first graded according to the World Health Organization classification before the immunofluorescence studies. A–F, Representative Ig isotype deposits in the various lines using immunofluorescence. A, Granular IgG1 deposits in wild-type NZM2328 mouse with diffuse proliferative GN class IV; B, diffuse IgG2a deposits in wild-type NZM 2328 mouse with diffuse proliferative and sclerosing GN; C, IgG2a focal glomerular deposits in a Stat6<sup>−/−</sup> mouse with focal proliferative GN class II; D, IgM deposits in Stat4-deficient mouse with diffuse proliferative GN class IV; E, minimal IgG1 deposits in a Stat4<sup>−/−</sup> mouse with diffuse proliferative GN class IV; F, segmental IgG1 deposits in Stat4-deficient mouse with diffuse proliferative and sclerosing GN class IV; G, comparison of relative deposition of complement C3 in renal glomeruli of the congenic mouse strains tested at 4–4.5 and 5.5–6 mo of age, respectively. Values are the mean of seven to nine mice per group. *, p < 0.05 compared with the control NZM2328 mice. H, Comparison of distribution of Ig isotype deposits in glomeruli of individual Stat-deficient and control mice tested at 5–5.5 mo of age.
severe GN can arise even in the absence of elevated anti-dsDNA Ab. In addition, in our lupus clinic we are following several patients that have stable high titer of anti-dsDNA Ab in their serum, without any evidence of kidney disease and a very mild SLE, an occurrence that is reminiscent of our Stat6-deficient NZM2328 mice.

Finally, our results suggest that development of drugs that specifically regulate Stat4 and Stat6 activity may pave the way for novel therapeutic intervention in SLE.

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