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Myeloid Dendritic Cells from B6.NZM Sle1/Sle2/Sle3 Lupus-Prone Mice Express an IFN Signature That Precedes Disease Onset

Uma Sriram,* Linda Varghese,* Heather L. Bennett,† Neelakshi R. Jog,‡ Debra K. Shivers,† Yue Ning,† Edward M. Behrens,‡ Roberto Caricchio,‡ and Stefania Gallucci*

Patients with systemic lupus erythematosus show an overexpression of type I IFN-responsive genes that is referred to as “IFN signature.” We found that B6.NZM Sle1/Sle2/Sle3 (Sle1,2,3) lupus-prone mice also express an IFN signature compared with non-autoimmune C57BL/6 mice. In vitro, myeloid dendritic cells (mDCs) (GM-CSF bone marrow-derived dendritic cells; BMDCs) from Sle1,2,3 mice constitutively overexpressed IFN-responsive genes such as IFN-β, Oas-3, Mx-1, ISG-15, and CXCL10 and members of the IFN signaling pathway STAT1, STAT2, and IRF7. The IFN signature was similar in Sle1,2,3 BMDCs from young, pre-autoimmune mice and from mice with high titers of autoantibodies, suggesting that the IFN signature in mDCs precedes disease onset and is independent from the autoantibodies. Sle1,2,3 BMDCs hyperresponded to stimulation with IFN-α and the TLR7 and TLR9 agonists R848 and CpGs. We propose that this hyperresponse is induced by the IFN signature and only partially contributes to the signature, as oligonucleotides inhibitory for TLR7 and TLR9 only partially suppressed the constitutive IFN signature, and pre-exposure to IFN-α induced the same hyperresponse in wild-type BMDCs as in Sle1,2,3 BMDCs. In vivo, mDCs and to a lesser extent T and B cells from young pre-disease Sle1,2,3 mice also expressed the IFN signature, although they lacked the strength that BMDCs showed in vitro. Sle1,2,3 plasmacytoid DCs expressed the IFN signature in vitro but not in vivo, suggesting that mDCs may be more relevant before disease onset. We propose that Sle1,2,3 mice are useful tools to study the role of the IFN signature in lupus pathogenesis. The Journal of Immunology, 2012, 189: 000–000.

Several reports support an important role for type I IFNs (1) in the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) (2). Type I IFNs are a family of cytokines that are pivotal in the activation of the innate (3) and adaptive immune systems (4–6) and in the antiviral response (7).

It has been known for a long time that many SLE patients have high serum levels of IFN-α (8–10). Several groups have found a pattern of elevated expression of IFN-responsive genes in peripheral blood cells of SLE patients, as measured by microarray analysis and now known as “IFN signature” (11–13). Because this IFN signature is frequent in pediatric patients (12) and in adults with CNS involvement and nephritis (13), it has been associated with the early and most acute phases of the disease (14, 15).

Animal models also support a prominent role for type I IFNs in the initiation of autoimmunity in lupus. The administration of IFN-α or inducers of type I IFNs like polyinosinic:polycytidylic acid accelerated disease onset in polygenic lupus-prone mice (16–19), mirroring the sporadic development of lupus in patients treated with IFN-α (20). Lupus-prone mice, made resistant to type I IFNs by the genetic deletion of the receptor common to type I IFNs (IFNAR), had milder disease in four out of five reports. This was true for the lupus-prone mice NZB (21), C57BL/lpr (22), (B6.Nba2 × NZW)F1 (23), and NZM2328 (17). In contrast, the MRL/lpr mice deficient in IFNAR showed an aggravation of the disease (24), although Behrens’s group (25) reported that MRL/lpr mice have an increased expression of type I IFN-responsive genes. In a model of chemically induced lupus, after administration of pristane, IFNAR-deficient mice failed to develop autoimmunity (26), and wild-type mice exhibited an IFN signature just before disease onset (27), indicating the importance of type I IFNs in the initiation of chemically induced lupus. It remains to be demonstrated whether an IFN signature is expressed by all the genetically determined models of murine lupus.

The IFN signature in SLE patients has a complex cause in which genetic and environmental factors play a role (28). The current view considers the high levels of type I IFNs a complex inheritable disease risk factor that can be triggered/amplified by the lupus-associated autoantibodies (29). Indeed, genetic studies in SLE patients have identified gene variants in pathways connected to type I IFNs that result in the excessive release of IFN-α (30). For example, polymorphisms in IRF5, IRF7, and STAT4, which are part of the signaling pathway of type I IFNs, are associated with a higher risk of development of SLE (31–36). In animal models, knocking out the genes STAT4 and IRAK-1,
which were highlighted by GWAS in SLE patients, decreased dis-
ease severity in lupus-prone mice (37, 38), and some IFN-related
genes, such as Ifi202 (39), are located in susceptibility loci of
polygenic lupus-prone mice.

The levels of type I IFNs determined by this genetic makeup
can be upregulated by the activation of TLR7 and TLR9 (40, 41)
triggered by nucleic acids coming from recurrent viral infections
(42), endogenous retroviruses (43), or from badly scavenged apo-
optic cells (44). A duplication of TLR7 accelerated lupus in
BXXSB-Yaa male mice (45), treatment with TLR7 and TLR9 in-
hibitors ameliorated severity (46), and TLR7 deficiency inhibited
disease development in lupus-prone mice (47). These reports sug-
gest that TLR7/9 are involved in lupus development, possibly as
an inducer of type I IFNs.

TLR7/9 can also be triggered by immune complexes con-
taining nucleic acids and anti-DNA and anti-ribonucleoprotein
Abs, the immunological hallmarks of lupus. Indeed, immune com-
plexes can bind FcγRIIa on the surface of immune cells and
shuttle the nucleic acids to the endosomal compartment contain-
ing TLR7/9, leading to induction of type I IFNs (41, 48, 49). A novel
mechanism of type I IFN production involves the release of neu-
trophil extracellular traps: normally a way to kill and trap patho-
gen (50), neutrophil extracellular traps in lupus patients stimulate
IFN-α production by plasmacytoid dendritic cells (pDCs) via
TLR9 triggering (49, 51).

Although current advances in lupus pathogenesis have pro-
vided many insights into the genetic aspects and progression of
the disease, there is yet an incomplete understanding of the
acellular sources and the triggers of the IFN signature in SLE. The
identification of a mouse model of polygenic lupus that expresses
the IFN signature would be an important tool to perform mecha-
nistic studies and test novel therapeutic approaches. With this
goal, we investigated the lupus-prone B6.NZM Sle1/Sle2/Sle3
(Sle1,2,3) mice (52). Sle1,2,3 mice are congenic mice in which three
susceptibility loci from NZM2410 lupus-prone mice were introgressed into the non-autoimmune mouse C57BL/6. These mice spontaneously develop a form of lupus characterized by high
titers of autoantibodies against dsDNA and chromatin and by
the development of glomerulonephritis (53, 54). We found that Sle1,2,3 mice express an IFN signature in vivo before the onset of the
disease compared with gender- and age-matched non-
autoimmune C57BL/6 mice. To determine the original cellular
source of this IFN response, we investigated dendritic cells (DCs)
because of their pivotal role in lupus (55, 56), in which they are
abnormally activated (57) and are able to induce autoimmunity
(58), and also their ability to activate upon type I IFNs (3) and
produce large amounts of IFN-α/β (59). We investigated the expression of type I IFNs and IFN-responsive genes in Sle1,2,3
bone marrow-derived myeloid dendritic cells (mDCs) and pDCs
in young prediseased mice, in absence of autoantibodies, and we
measured the response of Sle1,2,3 bone marrow-derived dendritic
cells (BMDCs) to stimulation with IFN-α and TLR7 and TLR9
agonists and inhibitors. In summary, our results demonstrate that
Sle1,2,3 lupus-prone mice express an IFN signature, similar to
SLE patients, and mDCs are one important source of the IFN
signature. We propose that Sle1,2,3 mice are useful tools to
study the role of the IFN signature in lupus pathogenesis.

Materials and Methods
Mice
C57BL/6 (B6) mice (The Jackson Laboratory) and B6.NZM Sle1/Sle2/Sle3
(Sle1,2,3) mice (a kind gift from Laurence Morel and later purchased from
The Jackson Laboratory) were bred and maintained in our colony in ac-
cordance with the guidelines of the Institutional Animal Care and Use
Committees of the Children’s Hospital of Philadelphia and Temple Uni-
versity, both of which are American Association for the Accreditation of
Laboratory Animal Care-accredited facilities. Female mice were used
between 7 and 14 wk of age with the exception of the experiments in
which 1-wk-old pups were used.

Measurement of anti-DNA and anti-chromatin Abs
Sera of young mice were tested for anti-DNA and anti-chromatin Abs
by ELISA as described previously (60). Briefly, the plates were coated
with chicken erythrocyte-derived chromatin at 3 μg/ml or with calf
thymus-derived dsDNA at 2.5 μg/ml in borate-buffered saline (BBS).
After addition of blocking buffer (3% BSA and 1% Tween 80 in 1×
BBS), serum samples diluted 1/250 in BBT (BBS, 0.4% Tween 80, 0.5% BSA)
were added in duplicate and incubated overnight at 4˚C. For the anti-
dsDNA ELISA, plates were coated with polystyrene (1 μg/ml; Sigma-
Aldrich) before coating with Ag. Alkaline phosphatase-conjugated goat
for mouse IgG (Fc-specific; Jackson ImmunoResearch Laboratories) was
used as secondary Ab. The plates were developed using 1 mg/ml para-
nitrophenyl phosphate substrate (Sigma-Aldrich) in 0.01 M diethanol-
amine (pH 9.8). To generate a standard curve for these assays, serum
from an older MRL/lpr mouse with high titer of autoantibodies was
also assayed at serial 2-fold dilutions from 1/250 to 1/128,000 (60).
Pooled sera from five B6 mice that were individually negative
for autoantibodies were used as negative controls.

In vitro mDC cultures
As a model of mDCs, BMDCs were generated as previously described
(61). Briefly, bone marrow precursors from young (between 7 and 14 wk)
agarose-separated female Sle1,2,3 and B6 mice were negatively selected
using anti-CD19, Thy1.2, and MHC class II magnetic beads (Miltenyi Biotech)
and stained and sorted for B220+CD11c+ pDCs. RNA was extracted from
bone marrow precursors and plated at 5 × 106/ml in complete IMDM (10% FBS,
penicillin/streptomycin, gentamicin, and 2-mercaptoethanol) enriched
with 3.3 ng/ml GM-CSF (BD Biosciences) in 24-well plates. One milliliter
of medium was added on day 2 and 1 ml of the medium was replaced
on day 5 and subsequently each day until the culture was used (day 6
or 7). We also cultured BMDCs from B6-dfed female mouse pups. The
gender of these very young mice was determined by PCR using primers
for the male-specific gene Sry as described elsewhere (62). Bone marrow
was flushed and cells were plated as 1 × 106 cells per milliliter per well
in a 24-well plate. Resting DC cultures were stimulated at day 6 or 7 of
culture with each of the following: 100 ng/ml LPS (Sigma-Aldrich), 2500
U/ml IFN-α (Hyculit Biotechnology), 10 μg/ml CpG-B 1826 (synthesized
by IDT Biotechnologies), and 1 μg/ml R848 (Invivogen). When TLR7/9
inhibitors (ODN 2088, ODN 954, ODN 661 (20 μg/ml) (46), INH1, INH18
(10 μg/ml) (63) were used (synthesized by IDT Biotechnologies), cells
were first treated with the inhibitors for 30 min and then stimulated
with CpG, R848, or medium alone. BMDCs were harvested after 5 or
24 h of stimulation for RNA analysis and after 24 h for FACS analysis
of surface activation markers. Culture supernatants were collected at the
same time points for cytokine analysis by ELISA.

In vitro pDC cultures
Bone marrow precursors were differentiated into pDCs in medium con-
taining FLTL3 (64). Briefly, bone marrow cells were flushed, and cells were
plated at 5 × 105 cells/well in a 6-well plate in complete RPMI medium
containing 10% FBS, l-glutamine, penicillin/streptomycin, 2-mercaptoethanol,
and 7.5% supernatant from FLTL3 cell line (a kind gift from Dr. Terri
Lauffer, University of Pennsylvania). Cells were harvested on day 8 or 9
and stained and sorted for B220+CD11c+ pDCs. RNA was extracted from
the sorted cells and analyzed for the IFN signature.

Flow cytometry
BMDCs were harvested from the wells, washed in cold PBS, incubated
with rat anti-mouse CD16/CD32 (clone 2.4G2) mAb for 10 min to block
FcRs, and then stained for 30 min on ice with allophycocyanin-conjugated
hamster anti-mouse CD11c, PE-conjugated rat anti-mouse CD86, FITC-
conjugated mouse anti-mouse H2Kb, Alexa 488-conjugated rat anti-mouse
B220, and PE-conjugated rat anti-mouse CD86 Abs (BD Biosciences).
Cells were fixed and stained with 1% formaldehyde and analyzed on a FACScan
flow cytometer (BD Biosciences). FlowJo software was used for data analysis.

Ex vivo immune populations sorted from lymphoid organs
Bone marrow and spleen of young prediseased (negative for auto-
antibodies) Sle1,2,3 lupus-prone female mice and age- and gender-matched
B6 mice were processed for sorting by FACS to analyze the IFN gene
signature in the different immune populations ex vivo. Briefly, spleens were
incubated in medium containing 8 mg/ml collagenase and 1000 U/ml
DNase for 45 min, and single-cell suspensions were prepared by passing
through a 100-μm mesh filter. Bone marrow cells were flushed from
femur and tibia. Cells were stained for sorting with the following Abs: rat
anti-mouse CD19 PerCP5.5, CD3 FITC, CD11b PE-Cy7, B220 PE, and
hamster anti-mouse CD11c allophycocyanin (BD Pharmingen and eBio-
science). The immune populations were sorted as B cells (CD19+), T cells
(CD3+), macrophages (CD19-CD11b+CD11c+), mDCs (CD19-CD3-CD11c+CD11b+),
and pDCs (CD19-CD3+CD20+CD11c+). RNA was extracted from these samples and analyzed by real-time RT-PCR
for the IFN signature.

Quantitative RT-PCR
Gene expression in BMDCs was analyzed by real-time RT-PCR using
TaqMan probes. Briefly, RNA was extracted by Trizol method and
repurified using Qiagen columns (Qiagen). cDNA was synthesized using
the cDNA archive kit followed by a preamplification reaction (Applied
Biosystems). Premade TaqMan primers and probes from Applied Bio-
systems were used to study the expression of 17 IFN-responsive genes
(IFN-β, IRF7, ISG-15, CXCL10, Oas-3, Mx-1, STAT1, STAT2, IFN-α,
IL-6, STAT4, IL-15, IFNAR1, IFNAR2, IFN3, IFN5, PKR). Cyclophilin
was used as the reference gene for normalization. In each independent
experiment, one BMDC culture generated from one Sle1,2,3 mouse was
compared with one BMDC culture generated from one B6 mouse. The
Ct method of relative quantification of gene expression was used for
these TaqMan PCR, and the normalized Ct values (against cyclophilin)
calibrated against the control sample (untreated B6 BMDCs) in
each experiment (61). TLR7, TLR9, and TNF-α were analyzed by the
same method.

For the analysis of the BMDCs generated from the 7-d-old pups, six
BMDC cultures were analyzed that had been generated from three
Sle1,2,3 mice and three B6 mice: the average of the normalized Ct values
against cyclophilin for three BMDCs from B6 pups was used to calculate
the results of three BMDCs from three Sle1,2,3 pups. For the analysis
of the IFN signature in the populations sorted ex vivo from bone marrow
and spleen, normalized Ct values (against cyclophilin housekeeping gene)
were averaged and used for normalization against each
Sle1,2,3 mouse tested.

ELISA
We used ELISA kits to measure the levels of TNF-α and CXCL10 (R&D
Systems) in the supernatants of BMDC cultures stimulated for 24 h with
IFN-α, TLR ligands, or medium alone.

Western blot analysis
We performed Western blotting using 30–50 μg total DC cell protein.
In brief, protein samples were denatured by boiling for 5 min and loaded
onto 10% Bis-Tris gels. After electrophoresis, proteins were transferred
to nitrocellulose membranes. Membranes were blocked for 1 h with
blocking buffer (2% nonfat milk in PBS), then incubated overnight at 4˚C with
the primary Abs diluted in blocking buffer with 0.1% Tween 20 simulta-
neously. We used rabbit polyclonal anti-STAT1 and STAT2 (Upstate
Biotechnology). Mouse anti-GAPDH (Santa Cruz Biotechnology) was
used as a loading control. After incubating with primary Abs, the mem-
branes were washed with PBS containing 0.1% Tween 20 (PBST) three
times. Then, the membranes were incubated for 1 h with IR Dye 800 goat
anti-rabbit and IR Dye 680 goat anti-mouse (LI-COR Biosciences) diluted
in blocking buffer plus 0.1% Tween 20. The blots were then washed three
times with PBST and rinsed with PBS. Proteins were visualized by scan-
ing the membrane on an Odyssey Infrared Imaging System (LI-COR
Biosciences) in both 700-nm and 800-nm channels.

Statistical analysis
We analyzed the data using Prism software (GraphPad, San Diego, CA)
and used as statistical tests one sample t test, two-tailed Student t test, or
non-parametric Mann–Whitney U test, as appropriate, for the different
sets of experiments and considered significant values of p < 0.05 (noted
in the figures as *p < 0.05, **p < 0.01, ***p < 0.001).

Results
Constitutive expression of the IFN signature in Sle1,2,3
BMDCs
Previous studies have demonstrated hyperactivated DCs from
bone marrow of lupus patients (65). To determine whether DCs
from lupus-prone mice have an IFN signature, we cultured bone
marrow precursors, depleted of mature T and B cells, macroph-
ages, and DCs, in the presence of GM-CSF to obtain mDCs in
6–7 d of culture (61). We grew BMDCs from young (7–14 wk old)
female Sle1,2,3 mice and compared them with BMDCs from
bone- and age-matched B6 mice. We found that Sle1,2,3 BMDCs
were not different from B6 BMDCs in terms of absolute numbers
of cells and percentages of CD11c+ cells (60–80% of the culture)
(Supplemental Fig. 1A, 1B). The analysis by flow cytometry of
the major differentiation markers revealed that more than 95% of
the BMDCs were CD11c+CD11b+ mDCs, whereas no CD8a or
pDCs (CD11c-CD8a+ and CD11cintB220+), respectively) could be
detected in the cultures from both strains (Supplemental Fig 1C),
as expected because of the culture conditions.

We analyzed by real-time quantitative RT-PCR a repertoire of
17 type I IFN-responsive genes selected because they represent
the different kinds of responses triggered by type I IFNs: the
amplification of the IFN response itself, the anti-viral response
and the immune activation. We found that the expression levels of
some genes (IFN-β, IRF7, ISG-15, CXCL10, and Oas-3 genes)
(Fig. 1A) were very high in Sle1,2,3 BMDCs (>10-fold compared
with B6): these are genes that are induced by type I IFNs as an
autocrine activation (IFN-β and IRF7) and are implicated in the
innate anti-viral response and immune activation (ISG-15, CXCL10,
and Oas-3) (66). Other genes were moderately increased (Fig. 1B),
like Mx-1, which is also part of the anti-viral response, and STAT1
and STAT2, which belong to the signaling pathway downstream of
IFNAR and are upregulated by type I IFNs as part of their posi-
tive feedback loop. IL-15 and IRF5 showed only a very small
but statistically significant increase (<2-fold) (Fig. 1C). There
were no differences in IFNAR1, IFNAR2, IRF3, and PKR in the
Sle1,2,3 versus B6 BMDC cultures (Fig. 1C); these are important
negative results to ensure that the differences we are seeing are not
due to nonspecific alterations of the levels of RNAs but rather
are specific for some IFN-responsive genes. IFN-α, IL-6, and
STAT4 showed a trend toward increased levels in Sle1,2,3
BMDCs compared with B6 BMDCs, but their differences did not
reach statistical significance. These results indicate that Sle1,2,3
mDCs express an IFN signature similar to SLE patients. Further-
more, as the signature occurred in BMDCs differentiated in vitro,
severed from any pro-autoimmune environment in vivo, there is
likely a causative mechanism intrinsic to the DCs driving this
signature.

Bone marrow precursors do not express the IFN signature
We also analyzed the bone marrow precursors, depleted of mature
T and B cells, macrophages, and DCs, that we had put in culture
at day 0 to determine whether these cells already express the
IFN signature. We analyzed the expression of a selected number
of IFN-responsive genes from the original 17 genes shown in
Fig. 1 (IFN-β, IRF7, ISG-15, Mx-1, and CXCL10) and found that
Sle1,2,3 precursors expressed these genes at the same levels as
the B6 precursors (Fig. 1D), suggesting that the IFN abnormalities
develop together with DC differentiation.

Sle1,2,3 bone marrow-derived pDCs express the IFN signature
pDCs were originally called “natural IFN-producing cells” be-
cause they are the most potent producers of type I IFNs (59). In
the context of SLE, pDCs can activate and secrete IFN-α upon
FcR-mediated uptake of autoantibodies complexed to chromatin
and ribonucleoproteins (48). We asked whether pDCs from young
Sle1,2,3 mice express an IFN signature. We generated bone
marrow-derived pDCs in medium containing FLT3L from young
B6 and Sle1,2,3 mice. The cultures yielded similar percentages

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FIGURE 1. Constitutive overexpression of IFN-responsive genes in Sle1,2,3 BMDCs. We analyzed by real-time quantitative RT-PCR a panel of 17 genes as representative of the IFN signature in BMDCs at day 6–7 of culture in absence of any stimulation. The results are expressed as fold differences in RNA expression compared with normal B6 BMDCs. (A) The gene expression levels were >10-fold for some genes; (B) >5-fold for some; and (C) some showed very modest difference or no change. We calculated the statistical significance with the one-sample t test. Mean and SE are from a minimum of four experiments for some genes to a maximum of 14 experiments for other genes. (D) We analyzed by real-time RT-PCR bone marrow precursors at day 0 of culture. The results are expressed as fold differences in gene expression from B6 controls; results are mean and SE of three independent experiments. None of the values was significant.

 Constitutive activation of the IFN response in Sle1,2,3 BMDCs

To confirm at the protein level some of the results found with real-time quantitative RT-PCR, we measured by Western blot the constitutive expression of STAT1 and STAT2, the pivotal signaling molecules that form IRF9, the transcription factor complex that is responsible for the transcription of IFN-responsive genes. We found that the protein levels of STAT1 and STAT2 were higher in the Sle1,2,3 BMDCs than in the B6 BMDCs as measured by Western blot (Fig. 2A), indicating that Sle1,2,3 BMDCs have the IFN signaling pathway preactivated in the absence of autoantibodies.

Because type I IFNs upregulate MHC class I expression (67), we tested the constitutive expression of MHC class I by flow cytometry as an indicator of an active response to type I IFN (61) and found that the expression of MHC class I was higher in the Sle1,2,3 BMDCs compared with B6 BMDCs (Fig. 2B). The fact that the expression of the costimulatory molecules CD80 and CD86, which depends on many types of stimuli, was instead similar in Sle1,2,3 versus B6 BMDCs (data not shown) suggests an IFN-specific stimulation of the lupus BMDCs.

In summary, these results indicate that BMDCs from young Sle1,2,3 mice have the transcriptional and translational IFN response constitutively activated in absence of any apparent stimulation.

FIGURE 2. Constitutive overexpression of IFN-responsive proteins in Sle1,2,3 BMDCs. (A) We analyzed the total cell lysates from BMDCs by Western blot for STAT1 and STAT2 proteins using GAPDH as loading control; numbers represent the intensity values normalized with GAPDH. Representative of one of four experiments. (B) We analyzed by flow cytometry the Median fluorescence intensity (MFI) of MHC class I expression on CD11c+ cells. Results are mean and SE from six experiments.
mice were categorized as autoantibody negative (anti-DNA autoantibodies, mice at the time of the harvest of bone marrow to grow BMDCs. The levels of anti-DNA and anti-chromatin Abs in the sera of Sle1,2,3 were present in the mice that the BMDCs were generated from. We measured of the IFN signature in BMDCs according to the levels of autoantibodies study reveals that the IFN signature in BMDCs precedes the first autoantibodies start to appear in the serum (Fig. 3B). Similar analyses of other IFN-responsive genes, like IRF7, ISG-15, Mx-1, and so forth, led to similar conclusions (data not shown). Our study reveals that the IFN signature in BMDCs precedes the onset of the autoimmune process, but it does not depend on the presence of autoantibodies and is not affected by autoantibody levels.

**The IFN signature is present in BMDCs from 1-wk-old Sle1,2,3 pups**

To support the evidence for a constitutive IFN signature that precedes the onset of the disease, we grew BMDCs from 7-d-old pups. This age corresponds in immunological terms to the time of birth in humans, in which the adaptive immune cells are still very immature and they are beginning to colonize the spleen and lymph nodes. We determined the gender of the pups by PCR genotyping for Sry male gene (62) and used only female pups as we have used female adult mice for the rest of the experiments shown in this study. Because at 7 d of age there are not many mature T and B cells in the bone marrow, and also because of the paucity of cells available from one single mouse, we did not perform any cell depletion, and we plated the total population of bone marrow cells from individual pups at 1 million per milliliter per well, as described in Materials and Methods. When we analyzed the BMDCs on day 7 of culture in vitro, we found that >98% were myeloid CD11c þ CD11b þ DCs in both the strains (data not shown). The analysis by real-time quantitative RT-PCR of the same 17 genes shown in Fig. 1 revealed the presence of the beginning of an IFN signature (Fig. 3C and data not shown). Indeed, IRF7, the master regulator of the response to type I IFNs, showed a 3-fold increase in the BMDCs from Sle1,2,3 pups compared with BMDCs from B6 pups; Oas-3, one of the most upregulated genes in the constitutive IFN signature in adult mice, was highly expressed in Sle1,2,3 (9-fold difference) compared with B6 pups; the rest of the 17 genes did not show any important difference from the controls (Fig. 3C and data not shown). These results indicate that an IFN signature is already present in BMDCs generated from lupus-prone mice very early in life and definitively precedes the onset of any sign of disease. Because the IFN signature is not as complete, in terms of number of genes upregulated and levels of expression, as in the 7- to 14-wk-old mice, these results suggest the existence of a missing step occurring during the differentiation of DCs both in vitro and in vivo that is required for a full development of the DC IFN response.

**Sle1,2,3 BMDCs hyperrespond to stimulation with IFN-α**

Type I IFNs are a family of cytokines that can be produced by almost any cell in the body and share the same receptor (IFNAR) (7). mDCs produce mostly IFN-β whereas pDCs produce mostly IFN-α (1), and these two cytokines are important messengers in the cross talk between mDCs and pDCs. To reproduce in vitro the in vivo situation in which mDCs are stimulated by the IFNs produced by pDCs, we determined how mDCs responded to exogenous IFN-α. We stimulated BMDCs from young prediseased Sle1,2,3 mice with murine recombinant IFN-α (2500 U/ml) and tested the same selected number of IFN-responsive genes as shown in Fig. 3A (IFN-β, IFR7, ISG-15, Mx-1, and CXCL10): we found that Sle1,2,3 BMDCs had an increased response to type I IFN stimulation and expressed the five IFN-responsive genes at higher levels than B6 BMDCs (Fig. 4A). Because it has been shown in cell types other than DCs that type I IFNs induce a positive feedback loop and strengthen the response to a second challenge with type I IFNs (69), the overresponse of Sle1,2,3 BMDCs to IFN-α functionally confirms the results that Sle1,2,3 BMDCs have a constitutive active IFN response and suggests that a cross talk between myeloid and pDCs could amplify such activation.

![Figure 3](http://www.jimmunol.org/)
Sle1,2,3 BMDCs hyperrespond to TLR7 and TLR9 ligands

The role for TLRs in stimulating DCs in lupus was suggested by the finding that DCs secrete inflammatory cytokines via a TLR9- or TLR7-dependent mechanism upon stimulation with nucleic acid-containing immune complexes (41, 45, 47, 48, 70). Santiago-Raber et al. (43, 71) have also shown that TLR7 and TLR9 are important regulators of disease severity and B cell reactivity in B6.Nba2.Yaa lupus-prone mice. These results validated the notion that TLRs, presumably recognizing endogenous ligands, play a key role in systemic autoimmunity. However, the constitutive sensitivity of lupus DCs to these stimulators remains undetermined. We found that the response of BMDCs from young prediseased Sle1,2,3 mice to TLR7 (R848) and TLR9 (CpG-B 1826) ligands was significantly higher compared with the response of B6 BMDCs (Fig. 4B, 4C). This hyperreactivity was specific for TLR7 and TLR9 because the response to LPS, ligand of TLR4, was not different in B6 and Sle1,2,3 BMDCs (Fig. 4D).

The chemokine IP-10/CXCL10 is overexpressed in lupus patients and correlates with disease activity (72, 73). We have found that Sle1,2,3 BMDCs constitutively have a very high gene expression of CXCL10 (Fig. 2A) that was greatly increased upon TLR7 and TLR9 stimulation compared with the unstimulated B6 control (Fig. 4B, 4C). This hyperreactivity was specific for TLR7 and TLR9 because the response to LPS, ligand of TLR4, was not different in B6 and Sle1,2,3 BMDCs (Fig. 4D). The chemokine IP-10/CXCL10 is overexpressed in lupus patients and correlates with disease activity (72, 73). We have found that Sle1,2,3 BMDCs constitutively have a very high gene expression of CXCL10 (Fig. 2A) that was greatly increased upon TLR7 and TLR9 stimulation compared with the unstimulated B6 BMDCs and with the B6 BMDCs upon stimulation (Fig. 4B, 4C). We also measured CXCL10 protein levels by ELISA and found that Sle1,2,3 BMDCs secreted significantly more CXCL10 in the culture supernatants than B6 BMDCs in response to R848 and CpG stimulation (Fig. 4E). These results confirm that Sle1,2,3 BMDCs hyperactivate upon TLR7/9 stimulation and suggest that DCs can be an important source of CXCL10 in lupus.

TNF-α production is normal in Sle1,2,3 BMDCs

TNF-α is a pleiotropic cytokine that has both proinflammatory and immunoregulatory effects. Its involvement in lupus is ambiguous as it was reported to be increased in SLE patients and correlated with disease activity, but anti-TNF blockers occasionally trigger a latent disease (74, 75). The lupus-prone mice (NZB × NZW)F1 and Sle1,2,3 mice are low producers and respond less than wild-type mice to TNF-α (76), but the same cytokine has been found to accelerate nephritis (77). It has been also suggested that TNF-α could be beneficial in lupus because it is able to antagonize type I IFNs (78). We investigated the ability of Sle1,2,3 BMDCs to produce TNF-α. There were no detectable constitutive levels of TNF-α in the culture supernatants of B6 and Sle1,2,3 BMDCs (Fig. 4F), whereas the levels of TNF-α after TLR stimulation were highly upregulated by the three TLR ligands used (R848, CpGs, and LPS) with no differences between B6 and Sle1,2,3 BMDCs (Fig. 4F). Gene expression analysis confirmed these results at the RNA level (Fig. 4G). These data indicate that Sle1,2,3 BMDCs do not have a constitutive activation of the signaling pathway leading to TNF-α production (NF-κB dependent) as they have for type I IFNs (STAT1-2 dependent). These data also suggest that the IFN signature in Sle1,2,3 DCs is the result of a specific activation of the type I IFN pathway rather than a sign of a generalized hyperactivation of the Sle1,2,3 DCs.
Inhibitors of nucleic acids or nucleic acid sensing receptors partially block the constitutive IFN signature of Sle1,2,3 BMDCs

The finding that Sle1,2,3 BMDCs overproduce IFN-responsive genes upon TLR7 and TLR9 stimulation (Fig. 4B, 4C) prompted us to investigate whether the constitutive IFN signature is due to an overresponse to DNA/RNA fragments present in the culture supernatants and possibly released in vitro by apoptotic cells naturally occurring in the culture. These nucleic acids would trigger TLR7 and TLR9 to which Sle1,2,3 BMDCs respond by overproducing IFN-responsive genes (Fig. 4B, 4C). We used inhibitory oligonucleotides that have been reported in the literature to block the response to TLR7 and TLR9 stimulation (46, 63): the oligonucleotide 661 specifically inhibits TLR7-induced responses, and the oligonucleotide 2088 inhibits TLR9-induced responses, and 954, INH1, and INH18 oligonucleotides have been reported to suppress both TLR7- and TLR9-induced responses (48, 63). We found that none of these inhibitors decreased the constitutive IFN signature by any significant measure after 5 h of incubation (Fig. 5A); after 24 h of incubation 2088, 954, and INH18 reduced the IFN signature by roughly 50%, whereas 661 and INH1 increased the constitutive IFN signature in Sle1,2,3 BMDCs (Fig. 5B). Extending the time of incubation with the inhibitors to 48 h did not change the extent or pattern of the suppression observed at 24 h (data not shown). At the same doses, all the inhibitors, with the exclusion of one, efficiently inhibited the responses of both B6 and Sle1,2,3 BMDCs to the exogenous ligands of TLR7 or TLR9, R848 and CpG, when added respectively to the culture (Fig. 5C, 5D). We have found that the oligonucleotide 954 inhibited the induction of IFN-β by CpGs but much less so by R848, with even less inhibition in Sle1,2,3 than in B6, as previously suggested in other studies (63). The results of the five inhibitors together suggest that the constitutive IFN signature of Sle1,2,3 BMDCs, which is partially decreased by TLR9 inhibitors but not by TLR7 inhibitors, may be in part induced by TLR9 triggering.

To confirm this interpretation, we added DNase or RNase, alone or in combination, to the culture supernatant of the BMDCs to degrade DNA and RNA, and we found that these treatments did not affect the IFN signature of Sle1,2,3 BMDCs after 5 h of stimulation, but DNase and RNase combined reduced the IFN signature only by 30% after 24 h of incubation (Fig. 5E). These results indicate that the IFN signature of Sle1,2,3 BMDCs is only partially due to an overresponse of TLR7 and TLR9 to a hypothetical chronic stimulation by endogenous nucleic acids. Because at least half of the IFN signature remained unaffected by TLR7/9 inhibitors, these results suggest a second TLR-independent mechanism mediating the induction of the IFN signature in Sle1,2,3 BMDCs.

Pretreatment with type I IFN increases the response of BMDCs to TLR7 and TLR9 ligands

Although the TLR7/9 inhibitors had limited effects on the IFN signature, it is still important to understand why the Sle1,2,3 BMDCs overrespond to these TLR stimuli because it could be

then we added R848 (C) or CpG (D) and harvested BMDCs after a total of 5 h. The results are shown as mean and SE of the percentages of the expression of IFN-β RNA in Sle1,2,3 BMDCs after the indicated stimulations, calculated from two to four experiments. (E) We added DNase I (6.8 U/ml) or RNase (25 μg/ml), alone or in combination and harvested BMDCs 5 or 24 h later. Representative of one of two independent experiments is shown. *p < 0.05. IFNβ, IFN-β.
an important mechanism for fueling the excessive production of type I IFNs in lupus (43). First, we hypothesized that Sle1,2,3 BMDCs express more TLR7 and TLR9. We analyzed the expression of TLR7 and TLR9 genes in BMDCs by real-time quantitative RT-PCR and found a 2- to 3-fold increase in the expression of both genes in the Sle1,2,3 compared with the B6 BMDCs (Fig. 6A). Furthermore, type I IFNs have been shown to increase TLR-induced responses in macrophages (79); therefore, we investigated whether exposure to type I IFNs during the culture, as it occurs in Sle1,2,3 BMDCs due to their own IFN signature, could increase the response to TLR stimulation in wild-type BMDCs. Pretreatment of B6 BMDCs for 12 h with rIFN-α dramatically increased the expression of IFN-β upon TLR7 and TLR9 stimulation as it did in Sle1,2,3 BMDCs and eliminated the big difference in the response of BMDCs from the two strains (Fig. 6B). Also TLR-induced IRF7 was strongly upregulated by pretreatment with rIFN-α in BMDCs from both strains of mice (Fig. 6C), indicating that the TLR-dependent induction of the master regulator of type I IFN responses is also sensitive to IFNs. These results indicate that type I IFNs increase the response of mDCs to TLR7/9 stimulation and suggest that the chronic exposure to type I IFNs, due to the constitutive IFN signature, could be responsible for the overresponse to TLR7/9 stimulation in Sle1,2,3 BMDCs.

**Sle1,2,3 mice express the IFN signature in vivo**

We investigated whether the IFN signature that we have observed in lupus BMDCs in vitro is expressed by Sle1,2,3 mice in vivo and also whether it is specific to DCs or it is expressed by other immune populations. We sorted five immune subsets (B cells, T cells, macrophages, mDCs, and pDCs) from bone marrow and spleen of young prediseased Sle1,2,3 mice and gender- and age-matched B6 controls. Although none of the results reached statistical significance due to large variabilities between individual mice, we have found that ex vivo mDCs from spleen and bone marrow of young prediseased Sle1,2,3 mice express an IFN signature, which is also in part shared by other immune cells such as B and T cells, whereas macrophages are less involved and pDCs seem to be refractory to the hyperexpression of IFN-responsive genes.

**Discussion**

We have shown that Sle1,2,3 lupus-prone mice have an IFN signature similar to that discovered in SLE patients. Because this signature was expressed by bone marrow-derived mDCs in vitro, severed from the rest of the pro-autoimmune immune system, we propose that the IFN signature is intrinsic to the DCs. In vivo, T and B cells expressed the IFN signature like the mDCs, suggesting that it may be intrinsic to the lymphocytes as well; alternatively, lymphocytes could express the IFN signature in response to the type I IFNs produced by the adjacent mDCs. Because we have found the IFN signature in young Sle1,2,3 mice that were negative for autoantibodies, we propose that Sle1,2,3 DCs have a primary defect that predates the disease and participates in the initiation of the autoimmune process. Compared to the strong, statistically significant overexpression of IFN-responsive genes by the Sle1,2,3 mDCs in vitro, the IFN signature in vivo was incomplete. These results can be explained in the context of the “two hit model” for the role of IFNs in lupus pathogenesis: this model proposes that a genetically determined threshold of type I IFN production is a primary risk factor in human disease (80) that is then triggered/amplified by lupus autoantibodies that form immune complexes with DNA and ribonucleoproteins and activate TLR7 and TLR9 in DCs (63, 80). The incomplete IFN signature in vivo in Sle1,2,3 mice

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**FIGURE 6.** Exposure to type I IFN increases the response of DCs to TLR7 and TLR9 stimulation. (A) We analyzed TLR7 and TLR9 RNA expression in BMDCs from young female age-matched B6 and Sle1,2,3 mice by quantitative RT-PCR. Results are fold difference in gene expression from B6 control; mean and SE from three independent experiments. We calculated the statistical significance with the one-sample t test. (B and C) We treated B6 and Sle1,2,3 BMDCs overnight with IFN-α (2500 U/ml), then stimulated them with either R848 (1 μg/ml) or Cpg (10 μg/ml) for 5 h and then processed RNA to measure IFN-β (B) and IRF7 (C) gene expression by quantitative RT-PCR as described above. Results are mean and SE from three independent experiments. *p < 0.05. p, Pretreated; IFNa, IFN-α; IFNb, IFN-β.
Bar graphs are mean and SEM of at least three sets of mice. The normalization from B6 is indicated by the black line in the graph. IFNb, IFN-β indicating that the IRF7 allele is from the non-autoimmune B6 side the Sle3 locus, at the opposite end of the chromosome, in that also harbors the Sle3 susceptibility locus, but IRF7 is out-

Accession No. U73037; http://www.ncbi.nlm.nih.gov/genbank/) The mouse IRF7 gene is located on chromosome 7 (GenBank Accession No. U73037; http://www.ncbi.nlm.nih.gov/genbank/) that also harbors the Sle3 susceptibility locus, but IRF7 is outside the Sle3 locus, at the opposite end of the chromosome, indicating that the IRF7 allele is from the non-autoimmune B6 genome and its higher expression is not directly due to a lupus-related polymorphism in these mice. Because pretreatment with IFN-α strongly upregulated IRF7 both in Sle1,2,3 and in B6 BMDCs (Fig. 6C), but IRF7 was also upregulated in BMDCs from lupus-prone pups (Fig. 3C) in which there is little or no overexpression of IFN-α/β, we do not know yet whether IRF7 is the cause or an effect of the IFN signature.

The IFN signature was absent in lineage-negative bone marrow precursors (Fig. 1D) and was only partial in DCs from 1-wk-old pups (Fig. 3C) in which the innate immune system is still acquiring its ability to differentiate into effector cells. These results suggest that the IFN signature in these mice depends on a defect that expands as the DCs differentiate and that a full differentiation to DCs is necessary to develop the complete IFN signature.

The finding that Sle1,2,3 BMDCs express constitutively high levels of STAT1 and STAT2 (Fig. 2A), together with the high expression of IRF7, indicates that Sle1,2,3 mDCs have already upregulated the molecular apparatus required to respond to type I IFNs and to stimuli that are dependent on type I IFNs such as TLR ligands. This leads them to express IFN-responsive genes at high levels. This molecular setting is normally not present in mDCs whereas it is characteristic of pDCs (84), suggesting the highly speculative hypothesis that in Sle1,2,3 mice, mDCs behave more like pDCs in their heightened IFN production. The analysis of the pDCs from the young Sle1,2,3 mice yielded puzzling results. Indeed, pDCs generated in vitro showed an IFN signature whereas pDCs sorted ex vivo did not. It will be important to determine the cause of this dichotomy in vitro–in vivo to under-

The hyperactivation of the molecular machinery to produce type I IFNs can explain the hyperresponse that we found in Sle1,2,3 BMDCs to the stimulation with recombinant IFN-α (Fig. 4A). The phenomenon that IFN induces a positive feedback
loop that potentiates subsequent responses to IFN was already known in other types of cells (69) and we have confirmed it in BMDCs, both from control B6 and Sle1,2,3 mice, in which it is heightened.

We propose that the hyperactivation of the IFN machinery also mediates the hyperresponse to R848 and CpGs as the result of the positive feedback loop of the IFN response induced by exposure to the IFN signature. Indeed, the pretreatment with type I IFNs strongly increased the expression of IFN-β and IRF7 both in B6 and Sle1,2,3 BMDCs, indicating that type I IFNs increase the response of mDCs to TLR7/9 stimulation. Furthermore, the results that B6 and Sle1,2,3 BMDCs responded equally to TLR7/9 stimulation after pretreatment with high doses of rIFN-α suggest that we had saturated the response of the BMDCs of both strains to TLR7/9 and that Sle1,2,3 BMDCs do not have an intrinsic hyperresponse to TLR7/9 but rather they are pre-primed to higher IFN responses (Fig. 6B, 6C). The DC sensitization by the IFN signature to overrespond to TLR7 and TLR9 would create a vicious circle because higher responses to TLR stimulation amplify the IFN signature (Fig. 4B, 4C). Moreover, the result that TNF-α expression is similar in Sle1,2,3 and B6 BMDCs upon TLR stimulation suggests that the hyperresponse to R848 and CpGs is not actually a direct hyperactivity of the TLR7/9 pathway but rather a hyperactivated response to the autocrine IFN-β induced by TLR stimulation. It remains to be explained why this mechanism is not acting upon LPS stimulation. It has been previously proposed that type I IFNs and TNF-α antagonize their reciprocal expression (78). Our results that Sle1,2,3 BMDCs and B6 BMDCs produce the same amounts of TNF-α although they are exposed to different amounts of type I IFNs suggest that the type I IFNs do not inhibit TNF-α production in mDCs. It remains to be demonstrated whether the opposite is true, and these results would justify the anecdotal triggerings of SLE upon TNF blockers (74).

Accumulating evidence supports the role for TLR7 and TLR9 in the development of murine lupus (36, 40, 41, 43, 45–48, 70). TLR7 and TLR9 are abundant in the pDCs, but also mouse mDCs in the development of murine lupus (36, 40, 41, 43, 45–48, 70). Recently, TLRs triggerings of SLE upon TNF blockers (74).

In conclusion, we present evidence that Sle1,2,3 mice are a useful model to study the role of type I IFNs in lupus pathogenesis and understand the dynamics between genetic threshold and immunologic and environmental stimuli in determining the IFN signature. These mice will also be useful to test novel therapeutic approaches aimed to target the IFN signature and determine the impact on the onset and severity of the disease.

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Disclosures
The authors have no financial conflicts of interest.


