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Expansion of Bone Marrow IFN-α-Producing Dendritic Cells in New Zealand Black (NZB) Mice: High Level Expression of TLR9 and Secretion of IFN-α in NZB Bone Marrow

Zhe-Xiong Lian,† Kentaro Kikuchi,* Guo-Xiang Yang,* Aftab A. Ansari,† Susumu Ikehara,‡ and M. Eric Gershwin*†

Patients with systemic lupus erythematosus have elevated IFN-α production. Furthermore, sera IFN-α levels correlate with disease activity. We have focused our attention on whether this phenotype is also seen in the New Zealand Black (NZB) mice and simultaneously addressed the underlying mechanisms. Specifically, we analyzed: 1) levels of sera IFN-α after type A CpG ODN 2216 injection in autoimmunity-prone NZB and control mice, and 2) levels of IFN-α synthesized by IFN-α-producing dendritic cells (IPDCs) using highly enriched populations of CD11c+ B220+ IPDCs derived from NZB and control mice; IPDCs are divided into two subpopulations (CD4+ CD11c+ B220+ and CD4− CD11c+ B220+). Our data demonstrate that NZB mice produced higher levels of sera IFN-α after type A CpG ODN 2216 injection when compared with control mice (p < 0.01). In addition, the cell numbers, frequency, and TLR9 mRNA levels of CD4+ and CD4− IPDC were markedly increased in the bone marrow (BM) of NZB mice. Upon in vitro stimulation with TLR9 ligand-CpG ODN 2216, higher levels of IFN-α were synthesized by IPDCs from the BM of NZB. The major contributor of IFN-α was the CD4− CD11c+ B220+ IPDC subpopulation. Furthermore, NZB BM IPDCs manifest impaired expression of homing chemokine CCR7 and CD62L, and IL-12 production. These data on the functional characteristics of the IPDC lineages explain in part the mechanism of hyper-IFN-α production and help clarify the mechanism for the expansion of NZB BM IPDCs. The Journal of Immunology, 2004, 173: 5283–5289.

Patients with systemic lupus erythematosus (SLE)2 have ongoing IFN-α production, and sera IFN-α levels correlate with disease severity (1, 2). Moreover, clinical use of IFN-α treatment for either viral infections or neoplasia occasionally results in SLE-like manifestations (3–5). Studies have demonstrated endogenous IFN-α inducers, which consist of small immune complexes (ICs) containing IgG and DNA in patients with SLE; these ICs act specifically on IFN-α-producing dendritic cells (IPDCs) (6). Given the fact that IPDCs play a key role in both innate and adaptive immune response, as well as being integral to the immunoregulatory effects by IFN-α, these observations may be important for the etiopathogenesis of SLE.

Although patients with SLE have reduced numbers of functionally circulating IPDCs in blood (7), they have increased numbers of IPDCs in cutaneous lesions (8), including active IPDCs in both cutaneous lesions and unaffected skin (9). More importantly, there is evidence that IFN-α in the serum of lupus patients is responsible for monocyte differentiation into Ag-presenting dendritic cells (DC) (10), suggesting a major initiating mechanism of the autoimmune process. However, low cell recovery and lymphoid tissue limitation have hampered detailed studies of human IPDCs in SLE. Experimental murine models of lupus provide a unique resource to study the role of IFN-α in disease, and early data demonstrated that the IFN-αβ inducer poly(I:C) accelerated autoimmunity in (New Zealand Black (NZB) × New Zealand White)F1 (NZBWF1) mice (11). Interestingly, renal disease induced in control mice by viral infection was inhibited with anti-IFN Abs (12). The IFN-αβ genes are located on chromosome 4 within the boundaries of several NZ lupus susceptibility loci (13), and the IFN-inducible gene (Ifh202), which is localized to the lupus contribution gene Nba2 interval, has been implicated as a new candidate lupus susceptibility gene for NZB mice (14). More recently, Santiago-Raber et al. (15) demonstrated that IFN-αR-deleted mice had significantly reduced serologic, cellular, and histologic disease characteristics, further emphasizing that type I IFN are important mediators in this disease. In this study, we report significantly increased bone marrow (BM) IPDCs in NZB mice compared with other lymphoid tissue and as contrasted with control mice. Upon in vitro stimulation with the TLR9 ligand-CpG ODN 2216, high levels of IFN-α were synthesized by NZB BM IPDCs. The major contributor of IFN-α was the CD4− CD11c+ B220+ IPDC subpopulation. Furthermore, NZB BM IPDCs demonstrated an impairment in the synthesis of proinflammatory cytokines IL-12 and in the cell surface expression of the homing chemokine CCR7 and CD62L. These functional characteristics of the NZB IPDC lineages explain the mechanisms involved in the abnormal pattern of BM IPDC development as well as the relative increased production of IFN-α in NZB mice.

Materials and Methods

Mice

NZB/Bln1, BALB/Cj, C3H/HeJ, and C57BL/6J mice, aged 1–8 mo, were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently

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‡ Abbreviations used in this paper: SLE, systemic lupus erythematosus; BM, bone marrow; DC, dendritic cell; HSA, heat-stable Ag; IC, immune complex; IPDC, IFN-α-producing DC; LN, lymph node; MFI, mean fluorescence intensity; NZB, New Zealand Black; ODN, oligodeoxynucleotide; RT, reverse transcriptase.
maintained by the Animal Resource Service of the University of California under pathogen-free conditions.

Cell preparation

BM cells were obtained by flushing two femurs and tibiae with PBS containing 0.2% BSA using a 23-gauge needle. Single cell suspensions were washed, and viable cells were quantitated and confirmed using trypan blue exclusion. The data presented for each experiment were replicated in three separate experiments using three to four mice per group, unless otherwise noted.

Flow cytometry staining and analysis

A total of 1–2 × 10⁶ whole BM, spleen, hepatic mononuclear cells, thymocytes, lymph node (LN) cells, lung lymphocytes, or PBMC was first incubated with a predetermined optimal amount of anti-CD16/CD32 (except for CD16/CD32 staining) at 4°C for 5 min to block the FcR, and then stained with various combinations of fluorochrome- or biotin-conjugated mAbs. Following washing, PE Cy5.5-conjugated streptavidin (Caltag Laboratories, Burlingame, CA) was used for the development of the biotin-conjugated Ab-stained cells. FACS data were acquired on a dual laser FACS Calibur (BD Biosciences, San Jose, CA). The frequency of cells expressing individual and/or sets of cell surface markers and the mean density of expression of such markers were determined by analyzing a minimum of 50,000 cells after gating out PI-positive dead cells, using CellQuest software (BD Biosciences). The following un conjugated or directly conjugated mAbs were used: purified anti-CD16/CD32 (FcyRII/III, 93) (e-Bioscience, San Diego, CA); FITC-labeled anti-CD4 (GK1.4), CD11b (Mac-1, M1/70), and CD19 (B29) (IgM, H9251); FITC-labeled anti-CD11b (M1/70), and CD43 (S7) (BD Pharmingen, San Diego, CA); MHC class II (M5/114.15.2) (Miltenyi Biotec, Auburn, CA); PE-labeled anti-CD11c (HL3) (BD Pharmingen) and CD127 (IL-7R, A7R34) (e-Bioscience); biotin-labeled anti-CD4 (GK1.1), CD11b (Mac-1, M1/70), CD19 (1D3), CD24 (heat-stable Ag (HSA), M1/69), CD16/32 (FcyRIII/II, 2.4G2), CD40 (23/23), CD80 (B7.1, 16-10A1), CD86 (B7.2, GL1), CD117 (c-kit, 2B8), CD123 (IL-3R, SB11), TCR β-chain (H82-710), and Ly-6G (Gr-1, RB6-8C5) (BD Pharmingen); NK1.1 (PK136) (e-Bioscience); and allopurinol-labeled anti-B220 (B220, RA3-6B2) (Caltag Laboratories). All isotype controls were obtained from BD Pharmingen.

Sorting and culturing of IPDCs

CD4+ and CD4− IPDCs isolated from the BM of 10 mice were pooled, stained with FITC anti-CD4, PE anti-CD11c, TRI-COLOR anti-NK1.1, and allophycocyanin anti-B220. After washing twice, cells were sorted using a 10-parameter MoFlo cell sorter (DakoCytomation, Fort Collins, CO) to obtain enriched populations of the NK1.1(DX5)/CD11c+B220+CD4+ and NK1.1(DX5)/CD11c+B220−CD4− IPDCs. The purity of sorted cells, based on the above phenotypic expression of cell surface markers, was always >97% and consistently CD19 negative. The purity of the sorted cells was analyzed by flow cytometry. Aliquots of 10⁶ sorted IPDCs were cultured in 200 μl of complete RPMI 1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) containing 10% FCS, 20 mM HEPES, 2-ME, penicillin, and streptomycin in U-bottom 96-well plates with or without the following supplements: phosphorothiolated CpG oligodeoxynucleotide (ODN) type A 2216 (μgG GGA CGA TCG TCG 3′ μgG gG) and type B 2006 (tcg tct tgt ctt ggt tgt gcc ggt) and type A CpG ODN 2216, or type B CpG ODN 2006, in complete RPMI 1640 for 48 h at 37°C. Supernatants were collected and analyzed for the following cytokines, using appropriate ELISA kits: IFN-α (Performance Biomedical Laboratory, New Brunswick, NJ), IL-6, TNF-α, and IL-12 p40 (R&D Systems, Minneapolis, MN).

Histological analysis

Sorted BM CD4+ and CD4− IPDCs were incubated in the presence of 2 μM CpG2006 ODN for 48 h. Cytospins of freshly or cultured BM CD4+ and CD4− IPDCs were prepared (300 × g, 5 min), stained with May-Grünwald-Giemsa (Invitrogen Life Technologies), and analyzed for cell morphology on a Provis microscope (Olympus, Tokyo, Japan).

Cell cycle analysis

Cell cycling was detected by the BrdU flow kit (BD Pharmingen). Mice were injected i.p. with 1 mg of BrdU dissolved in PBS, and were thereafter fed drinking water containing 1 mg/ml BrdU for different periods of time. The drinking water was light protected and replaced with fresh BrdU-containing water every 2 days. Briefly, purified IgM/HSA/Cd19− BM cells were stained with PE anti-B220, PE Cy5.5 anti-CD11c, and allophycocyanin anti-NK1.1 mAb, fixed with Cytofix/Cytoperm buffer, and permeabilized with Cytoperm Plus buffer. Cells were then incubated again with Cytofix/Cytoperm buffer, followed by treatment with DNase to expose the BrdU epitopes. Finally, immunofluorescent staining was performed with FITC-conjugated anti-BrdU and analyzed by FACS Calibur.

Detection of apoptotic cells

The frequency of IPDCs undergoing apoptosis was detected by annexin V staining (BD Pharmingen). Sorted BM CD4+ and CD4− IPDCs, 10⁶ fresh or cultured cells were resuspended in binding buffer (10 nM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 nM CaCl2). Thence, the cells were incubated with FITC-conjugated annexin V for 15 min at room temperature in the dark, washed, and analyzed using the FACSscan.

RNA isolation and RT-PCR

Total RNA for cdDNA synthesis was prepared from freshly enriched age-matched NZB and BALB/c BM CD4+ and CD4− IPDCs. RNA was extracted using the RNAeasy Mini kit (Qiagen, Santa Clarita, CA), eluted into diethyl pyrocarbonate-treated H2O, and stored at −70°C. To eliminate DNA contamination, the RNA samples were incubated with DNase I (Invitrogen Life Technologies) at room temperature for 15 min. This RNA was used to synthesize first strand cDNA using Superscript II reverse transcriptase (RT, Invitrogen Life Technologies), 1 μM dNTPs, 1 μg of random hexameric oligonucleotides, and the supplied RT buffer (Invitrogen Life Technologies). The primers used for the RT reaction are listed in Table 1.

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>TdT</td>
<td>GTC TGA ATT CCT CCA GAC GGT TCG AAG</td>
</tr>
<tr>
<td>mυ1</td>
<td>AAC ATC TGA GTT TCT GAC TGG TGG</td>
</tr>
<tr>
<td>mb-1 (Igα)</td>
<td>GCC AGG GGG TCT AGA AGA</td>
</tr>
<tr>
<td>B29 (Igβ)</td>
<td>CTC GGT CTT TCT TGC ATG C</td>
</tr>
<tr>
<td>TLR9</td>
<td>ACT GAC CAG CCC TGC TAT TA</td>
</tr>
<tr>
<td>hTLR2</td>
<td>CAA GCC TCT CTT GCA CCT AA</td>
</tr>
<tr>
<td>CCR5</td>
<td>CTG TGG GTC GTG GGG TTT</td>
</tr>
<tr>
<td>CCR6</td>
<td>CTG CAG TCT GAA GTC ATC</td>
</tr>
<tr>
<td>CCR7</td>
<td>AGC ACC ATG GCA CCA GGG AAA CC</td>
</tr>
</tbody>
</table>

Statistical analysis

Values were determined to be statistically significant using an unpaired Student’s t test; p < 0.05 was considered to be statistically significant.

Results

Production of sera IFN-α

To begin evaluating the role of IFN-α, levels were determined in the sera from NZB and control BALB/c mice; sera IFN-α was undetectable in both NZB and control mice. However, after type A CpG ODN 2216 injection, while significant sera IFN-α production...
CD4/H11002 higher IFN-α mice mount faster and stronger response to microbial, resulting in control BALB/c mice (Fig. 1). These results suggest that NZB nODN 2216. Age-matched female NZB (n/H11005) BM, spleen, LN, liver, thymus, and PBMC were resolved by staining for NK1.1, CD11c, B220, and CD4. The NK1.1 negative controls include lymphoid and myeloid DC cells. This cell lineage functions as an innate defense against viral, microbial, and other stimuli (17). We have previously found that IPDCs exist both in control murine liver (18) and thymus (19); express high levels of Ly-6C, CD45RA, and CD16/32; have low levels of MHC class II and IL-7R expression; and are negative for the costimulator molecules, CD80 and CD86. In the experiment in this study, we also found a similar IPDC population in NZB mice. Isolated BM cells were stained with CD4-FITC, CD11c-PE, and B220-APC, followed by staining with biotin-labeled Abs for CD80, CD86, MHC class II, or CD45RA, and then PE Cy5.5-conjugated streptavidin.

was detected in NZB mice, sera IFN-α remained undetectable in control BALB/c mice (Fig. 1). These results suggest that NZB mice mount faster and stronger response to microbial, resulting in higher IFN-α production.

FIGURE 1. Serum concentrations of IFN-α induced by type A CpG ODN 2216. Age-matched female NZB (n = 4) and control BALB/c (n = 4) mice were sampled before and, at different time points, after i.p. injection of CpG ODN 2216, and IFN-α levels were determined by ELISA.

Unusual expansion of BM IPDCs in NZB mice

To evaluate the role of IFN-α, the absolute number and the frequency of CD4+ and CD4- IPDCs in the BM, liver, spleen, LN, and PBMC were determined in NZB and control BALB/c, C57BL/6, and C3H mice. Liver and splenic CD4+ and CD4- IPDCs were slightly increased in NZB mice compared with control mice, but not to a level of significance; the LN and PBMC CD4+ and CD4- IPDCs were equivalent (data not shown). Unexpectedly, an unusual expansion of BM CD4+ and CD4- IPDCs was detected in NZB mice (Fig. 4); the CD4+ IPDCs count (0.93 × 106–1.43 × 106) and frequency (1.88–3.59%) peaked at 2 mo of age. Thereafter, the CD4+ IPDCs demonstrated an age-associated decline, but still remained higher than levels at 1 mo of age. In addition, there was a marked increase in absolute numbers (0.87 × 106 to 1.54 × 106) and the frequency (from 1.69 to 3.17%) of CD4+ IPDCs in the BM from 1- to 4-mo-old mice. From 4 to 8 mo, there was an age-correlated decline, but the data remained significantly higher than at 1 mo of age. Absolute cell numbers and frequencies of CD4- IPDCs remained relatively constant across all age groups.

FIGURE 2. Identification of NZB IPDC subsets. A. Freshly isolated BM, spleen, LN, liver, thymus, and lung lymphocytes were resolved by staining for NK1.1, CD11c, B220, and CD4. The NK1.1-CD11c+ cell population can be resolved into two subpopulations, CD4+ IPDC and CD4- IPDC cells. B. Levels of mRNA transcripts for TdT, μ, mb-1, B29, and the internal control gene β-actin in the NZB two BM IPDC subsets were analyzed by RT-PCR. Primers used are listed in Table I. Our negative controls include lymphoid and myeloid DC cells. C. Morphology of the NZB BM two IPDC subsets. Cytospin preparations of sorted BM IPDCs were stained with May-Giemsa and photographed at ×100. Upper panel, Shows freshly isolated and uncultured cells. Lower panel, Shows IPDC cells cultured for 48 h with type II CpG ODN 2006 (2 μM). D. The comparison of the surface phenotypes of BM IPDC subsets between NZB and BALB/c mice. Isolated BM cells were stained with CD4-FITC, CD11c-PE, and B220-APC, followed by staining with biotin-labeled Abs for CD80, CD86, MHC class II, or CD45RA, and then PE Cy5.5-conjugated streptavidin.

Characterization of NZB mice IPDCs

IPDCs are the major IFN-α-synthesizing cells. Type A CpG ODN 2216 exclusively induces high amounts of IFN-α by IPDCs (16). This cell lineage functions as an innate defense against viral, microbial, and other stimuli (17). We have previously found that IPDCs exist both in control murine liver (18) and thymus (19); express high levels of Ly-6C, CD45RA, and CD16/32; have low levels of MHC class II and IL-7R expression; and are negative for the costimulator molecules, CD80 and CD86. In the experiment in this study, we also found a similar IPDC population in NZB mice. Isolated BM cells were stained with CD4-FITC, CD11c-PE, and B220-APC, followed by staining with biotin-labeled Abs for CD80, CD86, MHC class II, or CD45RA, and then PE Cy5.5-conjugated streptavidin.

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frequency of both BM CD4⁺ and CD4⁻ IPDC populations were almost 4-fold higher in NZB than controls.

The viability, BrdU labeling, and adhesion molecule expression of IPDCs

The increased frequency of BM IPDCs in NZB mice could also be due to an increase in the survival of cells at this stage (slow turnover rate), resulting in an accumulation of cells in this compartment relative to control mice. To address this issue, the frequency of annexin V-negative cells was quantitated. Because apoptotic cells are rapidly removed from BM, the number of such cells detectable at any one time is low (21); furthermore, the viability of fresh isolated BM IPDCs was comparable between NZB and control mice (data not shown). Therefore, in the studies reported in this work, a short-term culture system that allows for the accumulation and enumeration of apoptotic cells was used. The data in Fig. 5A reflect the viability of IPDCs determined after culturing in vitro for 18 h without stimulation. Highly enriched populations of IPDCs from adult NZB mice exhibited a much higher level of viability with CD4⁺ IPDCs, giving values of 5.09 ± 0.18% as compared with similar preparations from similarly aged BALB/c (0.61 ± 0.31%) and C57BL/6 mice (0.28 ± 0.18%). NZB CD4⁻ IPDCs gave values of 21.02 ± 0.43%, which were higher than those from BALB/c (4.98 ± 1.18%) and C57BL/6 mice (0.78 ± 0.51%). These results support the view that IPDCs from NZB mice turn over much slower, which may be secondary to their resistance to undergo spontaneous apoptosis.

To examine the question of turnover rate, we analyzed the percentages of BrdU⁺ IPDCs in BM from NZB, and for comparison, BALB/c and C57BL/6 mice. The low density IgM⁺/HSA⁻/CD19⁺ BM cells were depleted using magnetic beads, as described above. As shown in Fig. 5B, the frequency of BrdU⁺ cells

TABLE II. Phenotypic expression of BM IPDC cells

<table>
<thead>
<tr>
<th>MFI (Mean ± SD)</th>
<th>Isotype</th>
<th>CD80</th>
<th>CD86</th>
<th>CD45RA</th>
<th>Class II</th>
<th>CD62L</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/c CD4⁺ IPDC</td>
<td>2.8 ± 0.1</td>
<td>4.3 ± 0.4</td>
<td>8.9 ± 0.8</td>
<td>420.8 ± 25.7</td>
<td>249.3 ± 28.4</td>
<td>22.7 ± 2.2</td>
</tr>
<tr>
<td>NZB CD4⁺ IPDC</td>
<td>2.3 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>8.2 ± 0.5</td>
<td>384.5 ± 39.0</td>
<td>351.1 ± 23.0</td>
<td>10.1 ± 1.1</td>
</tr>
<tr>
<td>B/c CD4⁻ IPDC</td>
<td>2.8 ± 0.1</td>
<td>5.2 ± 0.5</td>
<td>10.4 ± 0.7</td>
<td>248.7 ± 15.0</td>
<td>113.4 ± 10.0</td>
<td>24.3 ± 1.8</td>
</tr>
<tr>
<td>NZB CD4⁻ IPDC</td>
<td>2.3 ± 0.1</td>
<td>4.0 ± 0.3</td>
<td>8.7 ± 0.7</td>
<td>257.1 ± 33.8</td>
<td>239.2 ± 28.0</td>
<td>14.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Results are shown as mean score ± SD from four mice in each group, representative of three independent experiments.

** p < 0.05 as compared with control BALB/c mice.

FIGURE 4. Comparison of proportion (upper panel) and absolute cell numbers (lower panel) of BM two IPDC subsets between NZB and control mice. The average BM IPDC subset proportions and absolute cell number per femur and tibiae reflect the mean ± SD of three separate experiments; each experiment includes three to four mice. * p < 0.01, compared with age-matched control inbred mice.

FIGURE 5. A, Viability of IPDCs from NZB, BALB/c, and C57BL/6 mice BM following in vitro culture. Isolated IPDC cells, as described above, were stained with FITC annexin V after 18 h following in vitro culture. Viability is shown as annexin V negative (mean ± SD) of three independent experiments, and each experiment includes three to four mice. * p < 0.01, compared with control mice. B, Cell cycle and turnover analysis of the BM IPDCs that have incorporated BrdU and total DNA. Percentage of BrdU⁺ cells included the mean ± SD of three independent experiments; each experiment includes three to four mice. * p < 0.05, compared with control mice. C, Both of NZB BM CD4⁺ and CD4⁻ IPDCs expressed low levels of CD62L as compared with age-matched BALB/c mice. Data are representative of one of four experiments. D, Expression of mRNA for the chemokine receptor 5, 6, and 7 in sorted CD4⁺ and CD4⁻ IPDC cells from BM of NZB and BALB/c mice. Total RNA isolation and RT-PCR assays were performed, as described above. Dilutions of cDNA were subjected to PCR amplification specific for CCR5, CCR6, and CCR7, and the resulting products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized by UV light illumination. Two independent experiments show similar results.
in NZB NK1.1<sup>−</sup>CD11c<sup>−</sup>B220<sup>−</sup> IPDCs was lower than in control mice 2 h following injection of BrdU. Only 0.88% of the NK1.1<sup>−</sup>CD11c<sup>−</sup>B220<sup>−</sup> IPDCs were BrdU<sup>+</sup> in NZB mice, while 1.98% of the DX5<sup>−</sup>CD11c<sup>−</sup>B220<sup>−</sup> IPDCs from BALB/c mice were BrdU<sup>+</sup>. In addition, there was a rapid turnover of control BM IPDCs with >80% of cells positive for BrdU uptake at 7 days. In contrast, the turnover in NZB cells was <60% incorporation label by 7 days (Fig. 5B, right panel). NZB cells required 10 days of continuous labeling before 80% of the cells were BrdU positive. Although additional confirmation can be considered with pulse-chase experiments, we believe the conclusion in this study is that the increased frequency and absolute numbers of IPDCs in the BM of NZB mice are secondary to the resistance to spontaneous apoptosis. We also note that NZB BM IPDCs expressed relatively low levels of the homing marker termed L-selectin (CD62L). Thus, while 10.1 ± 1.1 of NZB CD4<sup>+</sup> IPDC and 14.4 ± 1.0 of CD4<sup>+</sup> IPDCs expressed CD62L, similar populations of cells from BALB/c mice gave values of 22.7 ± 2.2 for the CD4<sup>+</sup> IPDC and 24.3 ± 1.8 for the CD4<sup>+</sup> IPDC subsets. These findings, in concert with the finding of the expression of CCR7 by NZB BM IPDCs, suggest that NZB BM IPDCs impair the ability to traffic from BM to the periphery (Fig. 5, C and D, and Table II).

**Gene expression and production of cytokines stimulated with TLR9 ligand**

In efforts to further clarify the mechanisms responsible for differences within the IPDC lineage in NZB mice, we examined the expression of the lupus gene Ifi202 and TLR9 mRNA expression using highly enriched populations of CD4<sup>+</sup> and CD4<sup>−</sup> IPDCs from the BM of 2-mo-old NZB and age-matched control BALB/c mice. As seen in Fig. 6, A and B, both CD4<sup>+</sup> and CD4<sup>−</sup> BM IPDCs from NZB mice had higher levels of Ifi202 and TLR9 mRNA expression than age-matched control BALB/c mice.

To determine whether this increased level of TLR9 mRNA correlated with functional differences between NZB and BALB/c IPDCs, we stimulated cell sorter-purified enriched populations of NZB and BALB/c BM CD4<sup>+</sup> and CD4<sup>−</sup> IPDCs and incubated them with the TLR9 ligand and examined the production of the cytokines IFN-α, IL-12 (p40), TNF-α, and IL-6. As shown in Fig. 6C, the TLR9 ligand type A CpG ODN 2216 was the only ligand that induced IFN-α, and caused higher levels of IFN-α production from NZB IPDCs than BALB/c mice. These data are consistent with our in vivo studies (Fig. 1). These data also support the view that the CD4<sup>+</sup> IPDCs are the major IFN-α-induced cells following CpG ODN 2216 stimulation. We did not detect IL-12p40 production either by the NZB CD4<sup>+</sup> nor CD4<sup>−</sup> IPDC cells following stimulation with type B CpG ODN 2006 and type A CpG ODN 2216 (Fig. 6C).

**Discussion**

The data presented in this work describe detailed characteristics of IPDCs from NZB mice with a focus on the potential mechanisms of increase of BM IPDCs in NZB mice and the ability of IPDCs to produce IFN-α in vitro following incubation with type A CpG ODN 2216 as compared with control mice. The results include the observation that NZB IPDCs demonstrate higher levels of Ifi202, TLR9 expression, and reduced CCR7 and CD62L expression.

Our data demonstrate that NZB mice produce higher levels of sera IFN-α after type A CpG ODN 2216 treatment. More recently, it has been reported that the poly(I:C) produced high levels of sera IFN-α in NZB mice; IFN-α receptor-depleted NZB mice had significantly reduced anti-erythrocyte autoantibodies, erythroblastosis, hemolytic anemia, anti-DNA autoantibodies, kidney disease, and mortality (15).

**FIGURE 6.** A, Ifi202 and TLR9 profiles of BM IPDCs. cDNA derived from sorted NZB or BALB/c BM, and IPDC transcription levels were examined by RT-PCR. B, BM IPDCs from NZB or BALB/c mice were amplified using Ifi202, TLR9, or β-actin-specific primer as shown in Table I, and visualized with SYBR Green by real-time PCR. Standard curves were generated using diluted cDNA of NZB BM IPDCs. To measure relative intensity between NZB and control mice, the ratio of Ifi202 or TLR9 mRNA level was calculated as Ifi202 or TLR9 intensity divided by β-actin intensity. Data are representative of at least two experiments. C, Comparison of IFN-α, IL-12p40, TNF-α, or IL-6 production by IPDC subsets between NZB and BALB/c stimulated with TLR9 ligand, CpG ODN 2006, or 2216. Sorted IPDC subsets were cultured for 48 h in the presence or absence of CpG ODN 2006 (2 μM) or 2216 (2 μM). Media was harvested and examined for IFN-α, IL-12p40, TNF-α, or IL-6 by ELISA. Similar results were obtained from two independent experiments.

IFN-α is produced by a wide variety of cells and secreted in response to viral and bacterial infection. IPDCs stand out as a major source of IFN-α possibility because of their capacity to produce unusually large amounts of IFN-α (17, 22); immature IPDCs normally migrate from the BM to peripheral tissue. Our results demonstrate a 4-fold higher proportion and absolute cell numbers of CD4<sup>+</sup> and CD4<sup>−</sup> IPDCs in the BM of NZB mice as compared with age-matched control mice. In contrast, the frequency and absolute numbers of IPDCs in the spleen, LN, liver, thymus, lung, and PBMC IPDCs are comparable to control mice. There are several mechanisms that either individually or in concert could account for the accumulation of the IPDCs in the BM of NZB mice. These include prolonged persistence and long-lived IPDCs, which could be due to decreasing sensitivity to undergo spontaneous apoptosis. As shown in this work, the IPDCs of NZB mice exhibited a much slower turnover rate and prolonged persistence, and resistance to apoptosis as compared with control mice. The trafficking of lymphocytes through endothelium requires a sequence of events involving adhesion molecules (such as CD62L), chemokine receptors (such as CCR7), and integrins; lymphoid chemokines are critical for trafficking into lymph nodes and within lymphoid compartments (23). Defective expression of CD62L and CCR7 will
lead to decreased IPDC output from the BM. Similarly, the increased numbers of IPDCs in cutaneous lesions (8) and active IPDCs in cutaneous lesion and unaffected skin (9) can lead to low numbers of IPDCs in the blood of SLE patients.

NZB mice, as well as several other models of murine lupus models, manifest abnormal patterns of B lineage cell development (24, 25). The frequency and absolute numbers of pre-B and immature B cells are markedly reduced when compared with age-matched control murine strains. A significant reduction of B cells in the periphery was also detected in patients with SLE (26). However, although the mechanism remains unclear, one may postulate that the unusual expansion of BM IPDCs, with high expression of TLR9, can release large amounts of IFN-α (Fig. 7). Similarly, IgM from NZB serum can inhibit pre-B cell growth to IL-7 (25). IFN-α and -β, natural regulators of cell growth and differentiation, have been shown to severely inhibit IL-7-induced growth and survival of B cells at comparable early stages (27), and IFN-α induced by viral infection leads to transient BM aplasia (28). In NZB mice BM, IPDCs release large amounts of IFN-α and inhibit B precursors cell growth and differentiation, leading to apoptosis. Further NZB IPDCs produce factors that suppress growth and survival of B cell precursors on injection into control mice (29). The IFN-inducible If202 is thought to affect cell-signaling pathways by binding to several transcription factors and inhibiting cell proliferation and apoptosis. High expression of If202 was detected in NZB B cells (14) and IPDC (Fig. 6). In control circumstances, apoptotic cells are cleared by macrophages in the early phase of apoptotic cell death. Thus, no Ag presentation to T cells occurs as well as in the clearance of apoptotic cells (34).

Release of IFN-α by IPDCs induces monocytes to differentiate into DC. These cells efficiently capture apoptotic cells and nucleosomes, which form ICs that may sustain IFN-α production (33). Thus, IFN-α is an important influence on the differentiation of T cells, inhibition of apoptosis associated with activation, and induction of Fas ligand-mediated apoptosis (36–38). IFN-α can also promote the survival and differentiation of B cells and enhance BCR-dependent responses by lowering their threshold of activation (39, 40). We are now focusing on the characteristics of other lymphoid organ IPDCs, including relationships with apoptosis, cycling, and TLR9 expression. Understanding the differences in the autoimmune disease-modifying activities of IFNs and related cytokines will impact our ability not only to dissect etiologies, but also to develop new therapy.

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References


