Increased Generation of Dendritic Cells from Myeloid Progenitors in Autoimmune-Prone Nonobese Diabetic Mice

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Increased Generation of Dendritic Cells from Myeloid Progenitors in Autoimmune-Prone Nonobese Diabetic Mice

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Aberrant dendritic cell (DC) development and function may contribute to autoimmune disease susceptibility. To address this hypothesis at the level of myeloid lineage-derived DC we compared the development of DC from bone marrow progenitors in vitro and DC populations in vivo in autoimmune diabetes-prone nonobese diabetic (NOD) mice, recombinant congenic nonobese diabetes-resistant (NOR) mice, and unrelated BALB/c and C57BL/6 (BL/6) mice. In GM-CSF/IL-4-supplemented bone marrow cultures, DC developed in significantly greater numbers from NOD than from NOR, BALB/c, and BL/6 mice. Likewise, DC developed in greater numbers from sorted (lineage-"IL-7Rα—SCA-1—c-kit") NOD myeloid progenitors in either GM-CSF/IL-4 or GM-CSF/stem cell factor (SCF)/TNF-α. [3H]TdR incorporation indicated that the increased generation of NOD DC was due to higher levels of myeloid progenitor proliferation. Generation of DC with the early-acting hematopoietic growth factor, flt3 ligand, revealed that while the increased DC-generative capacity of myeloid-committed progenitors was restricted to NOD cells, early lineage-uncommitted progenitors from both NOD and NOR had increased DC-generative capacity relative to BALB/c and BL/6. Consistent with these findings, NOD and NOR mice had increased numbers of DC in blood and thymus and NOR had an increased proportion of the putative myeloid DC (CD11c+CD11b+) subset within spleen. These findings demonstrate that diabetes-prone NOD mice exhibit a myeloid lineage-specific increase in DC generative capacity relative to diabetes-resistant recombinant congenic nonobese NOR mice. We propose that an imbalance favoring development of DC from myeloid-committed progenitors predisposes to autoimmune disease in NOD mice. The Journal of Immunology, 2002, 168: 5032–5041.

Insulin-dependent or type 1 diabetes (T1D) results from autoimmune destruction of insulin-secreting β cells in the pancreatic islets of Langerhans. The nature of immune dysregulation leading to β cell destruction remains poorly understood. However, phenotypic and functional abnormalities of dendritic cells (DC) and myeloid-lineage development have previously been identified in humans at risk for or with T1D (1, 2), as well as in the most widely used animal model of T1D, the nonobese diabetic (NOD) mouse (3, 4).

While many subpopulations of DC have been described based on their anatomical location and phenotypic or functional characteristics (reviewed in Ref. 5), two broad DC subtypes have been proposed. Myeloid DC express high levels of myeloid lineage-associated markers such as CD11b, whereas the other proposed subtype expresses low levels of myeloid markers and high levels of several lymphoid-associated markers. In mice, the latter subtype is CD8α+DEC-205+CD11b- (6) and was termed lymphoid-related DC after being shown to develop in vivo from early lymphoid precursors (7). Despite uncertainty regarding the developmental origins of these DC subtypes (8, 9), they exhibit quite distinct functional characteristics in vivo. For example, CD8α- myeloid DC traffic to regional lymphoid tissues from peripheral tissue, whereas CD8α+ lymphoid DC appear to develop in situ in lymphoid tissues (10, 11). Administration of flt3 ligand (flt3-L) expands both DC subtypes, but GM-CSF expands only the myeloid subtype (12). While CD8α- DC present soluble Ag to CD4+ T cells, CD8α+ DC preferentially present cell-associated or soluble blood-borne Ags to CD8+ T cells (13, 14). Adoptive transfer studies indicate that CD8- DC favor induction of Th2-like responses whereas CD8+ DC favor Th1-like responses (12, 15). Therefore, any perturbation of DC development that impacts on the relative abundance or function of either DC subtype may have profound effects on immune homeostasis.

Alterations in myeloid lineage development in NOD mice (3, 4) could influence the development and function of myeloid-derived DC and contribute to diabetes development. To address this issue, we examined the development of myeloid DC from bone marrow (BM) progenitors in vitro and DC populations in vivo in autoimmune diabetes-prone NOD mice, compared with recombinant congenic nonobese diabetes-resistant (NOR) mice and unrelated BALB/c and C57BL/6 (BL/6) strains.

Materials and Methods

Mice

Female NOD (NOD Lt/Jax, H-2k), NOR (NOR Lt, H-2k), NOD.scid (H-2k), BALB/c (H-2a), CB17.scid (H-2k), C57BL/6 (H-2b), and CBA (H-2b) mice were obtained from the Walter and Eliza Hall Institute central breeding facilities (Parkville, Victoria, Australia) and used at 6–10 wk of age. All studies were performed within institutional animal care guidelines.

Culture medium and reagents

Culture medium (R-10) was RPMI 1640 (Life Technologies, Rockville, MD) with 10-3 M sodium pyruvate, 10-4 M nonessential amino acids (Life Technologies), 2 × 10-5 M glutamine, 5 × 10-8 M 2-ME (Sigma-Aldrich, St. Louis, MO), and 10% v/v FCS (JRH Biosciences, Lenexa, KS).
KS). Recombinant murine (rm)GM-CSF, rmIL-4, rmSCF, and rmTNF-α were from PeproTech (Rocky Hill, NJ). Recombinant human flt-3L was provided by Immunex (Seattle, WA). FITC-dextran (40,000 mol. w.) and LPS (serotype 11F/14) were from Sigma-Aldrich.

Abs and flow cytometry

Abs directed against CD3 (KT3), B220 (RA3-6B2), Ly-6G (Gr-1); RB6-8C5, Ly-76 (TERR-19), F4/80 (F4/80), CD11b (M1/70), CD11c (N418), MHC class II (OX-6) (directed against a nonpolymorphic determinant on rat Ia, but also I-A<sup>β</sup>), 10.2.16 (I-A<sup>β</sup>), and M5/114 (I-A<sup>β</sup>), I-E<sup>α</sup>)), DEC-205 (NLCD-145), MHC class II (R-FS9 (AFS-98)), and c-kit (ACK-2) were purchased from hybridoma supernatants and used as purified mAb or conjugated to FITC, PE, or biotin-conjugated primary Abs at 4 °C for 30 min in blocking wash, washed in PBS/1.5% FCS, and incubated with streptavidin-FITC, streptavidin-PE, or streptavidin-Tricolor (Caltag Laboratories (Burlingame, CA). Other mAb directed to CD54 (3E2), IL-7R (4A3, 53G3), anti-CD16/32 (2.4G2) tissue culture supernatant) at 4 °C and incubation in blocking mix (10% v/v normal mouse serum and 10% v/v of goat serum) as necessary before a final wash. Samples were analyzed on a FACSkan (BD Biosciences, Mountain View, CA). Live cells were selected by forward/side scatter gating and/or propidium iodide exclusion. In most instances, 1 × 10<sup>5</sup>–1 × 10<sup>6</sup> cells were collected for analysis. For flow cytometric analysis of murine BM, spleen, and thymus, 5 × 10<sup>7</sup>, 5 × 10<sup>6</sup>, or 10<sup>5</sup> live events, respectively, were collected. Staining intensities were expressed as arithmetic mean fluorescence intensity (MFI) calculated using CellQuest 3.1 (BD Biosciences).

Generation of DC in vitro

To propagate BM-derived DC, mice were euthanized by CO<sub>2</sub> narcosis and femurs and tibiae were collected into cold mouse tonicity PBS. Cells were flushed from the marrow cavity with PBS/2.5% FCS and erythrocytes were lysed with distilled water. After washing in R-10, cells were plated in six-well plates (Nunc, Rochester, NY) at 6 × 10<sup>5</sup> cells/well in 3 ml of R-10 supplemented with GM-CSF/IL-4 (both 1 ng/ml) and IL-7 (1 ng/ml). Cultures were supplemented with GM-CSF (1 ng/ml) and IL-4 (1 ng/ml) and maintained in 5% CO<sub>2</sub> at 37 °C. In general, cultures were established from BM pooled from three mice. Nonadherent cells were removed by gentle washing after 2 days and half the medium was replaced. Cells were collected for analysis at day 5. For analyses, BM, spleen, and thymus, 5 × 10<sup>7</sup>, 5 × 10<sup>6</sup>, or 10<sup>5</sup> live events, respectively, were collected. Staining intensities were expressed as arithmetic mean fluorescence intensity (MFI) calculated using CellQuest 3.1 (BD Biosciences).

Quantitation of endocytosis

Nonadherent cells were harvested from GM-CSF/IL-4–supplemented BM cultures, washed, and equilibrated to 4 °C or 37 °C in R-10 (10<sup>5</sup> cells/50 μl). 0.2 volumes of FITC-dextran (5 mg/ml) was added, and cells were incubated for 2 h at the required temperature. Endocytosis was stopped by washing twice with ice-cold PBS containing 2.5% FCS and 0.02% sodium azide, and samples were maintained at 4 °C for further steps. Controls were cells incubated at 4 °C with or without FITC-dextran and cells incubated at 37 °C without FITC-dextran. Cells were then stained as described with anti-CD11c-biotin–conjugated streptavidin-PE and streptavidin-Tricolor (Caltag Laboratories). For measurement of endocytosis, BM-derived DC populations were gated on the basis of CD11c and CD86 expression and the proportion of endocytically active cells calculated by subtracting background (cells incubated with FITC-dextran at 4 °C) for each of the defined populations.

<sup>[3H]</sup>Thymidine incorporation in BM cultures

BM suspensions were prepared as described and added in triplicate to 96-well flat-bottom plates at 6 × 10<sup>5</sup> cells/well in 300 μl R-10. Cultures were supplemented with GM-CSF (1 ng/ml) and IL-4 (1 ng/ml) and maintained in 5% CO<sub>2</sub> at 37 °C. At day 2, nonadherent cells were removed and cultures were replenished with 300 μl fresh R-10 containing GM-CSF (1 ng/ml) and IL-4 (1 ng/ml). <sup>[3H]</sup>Thymidine (1 μCi) was added for the respective 24-h periods. Cells were harvested onto glass filter mats and proliferation was assessed by <sup>[3H]</sup>thymidine incorporation measured with a scintillation counter (Topcount; Packard, Groningen, The Netherlands). Results were expressed as gross cpm ± SD.

Preparation of single-cell suspensions of lymphoid tissues

Spleens or thymi were excised and placed in ice-cold PBS. Individual organs were disrupted by gentle crushing with the end of a syringe plunger and single-cell suspensions were collected by gentle pipetting. Cells were counted and 1 × 10<sup>6</sup> cells were collected for analysis. For flow cytometric analysis, BM suspensions were prepared as described and added in triplicate to 96-well flat-bottom plates at 6 × 10<sup>5</sup> cells/well in 300 μl R-10. Cultures were supplemented with GM-CSF (1 ng/ml) and IL-4 (1 ng/ml) and maintained in 5% CO<sub>2</sub> at 37 °C. At day 2, nonadherent cells were removed and cultures were replenished with 300 μl fresh R-10 containing GM-CSF (1 ng/ml) and IL-4 (1 ng/ml). Cells were collected by centrifugation and washed (R-10), and erythrocytes were lysed (lens only; NH<sub>4</sub>C1/Tris buffer), washed, and finally resuspended in R-10.

Preparation of peripheral blood leukocytes and BM for flow cytometry

Single-cell suspensions of BM were prepared as described above and erythrocytes were removed by lysis. For analysis of peripheral blood leukocytes, blood was obtained by cardiac puncture using heparinized syringes and needlles and collected into Alsever’s anticoagulant. Erythrocytes were removed by two rounds of lysis, cells were washed (PBS/2.5% FCS), and peripheral blood leukocytes were collected by centrifugation.

Cytospins

Cytospins were prepared in a Cytospin 3 (Thermo Shandon, Pittsburgh, PA). Preparations were air-dried overnight and stained using Diff-Quik (Lab Aids, Narabeen, Australia) for morphological analysis.
Mixed leukocyte reactions

MLRs were established using nylon wool-enriched splenic T cells (2 × 10^6/well) and graded doses of gamma-irradiated (2000 rad, 60Co source) stimulator cells in R-10 (200 μl final volume in 96-well round-bottom plates). Syngeneic and allogeneic bulk spleen cells were routinely included as controls. MLR were maintained for 3 days and [3H]thymidine (1 μCi/well) was added during the final 18 h of culture. [3H]Thymidine incorporation was assessed as above.

Statistical analysis

Group data were compared by ANOVA followed by Newman-Keuls post-test. Where indicated, Student’s t test was used for comparison of means. Flow cytometric data were corrected for non-normality by log transformation as required before analysis.

Results

Increased yield of CD11c^+ DC from GM-CSF/IL-4-supplemented NOD mouse BM cultures

We first established conditions for comparing DC generation among strains that minimized manipulation of progenitor populations. Erythrocyte-lysed BM was cultured in the presence of GM-CSF/IL-4 for 2 days followed by removal of nonadherent cells. DC subsequently generated from the remaining adherent myeloid progenitors, as described by Inaba et al. (17), were harvested after another 3 days. In keeping with the findings of others (18, 19), IL-4 was required for efficient generation of mature DC (data not shown). With GM-CSF held at 1 ng/ml, little effect of IL-4 was observed at concentrations of 0.1 ng/ml or less. However, IL-4 at 1 ng/ml markedly reduced the proportion of Gr-1^+ myeloid progenitors/granulocytes and resulted in an increase in the proportion of CD11c^+ DC, particularly in NOD and NOR cultures. Further increasing the IL-4 concentration 10-fold to 10 ng/ml slightly reduced the scale of differences among strains, but CD11c^+ DC number remained highest in NOD cultures. As the GM-CSF concentration may be a key determinant of the relative number of DC generated across strains, we titrated the addition of GM-CSF in the presence of IL-4 (1 ng/ml). The combination of 1 ng/ml GM-CSF and 1 ng/ml IL-4 resulted in the highest percentage of CD11c^+ DC (Fig. 1A) and proportion of mature (CD11c^+ CD86^high^) DC (Fig. 1B) in each strain. As expected, the total number of DC was increased by higher GM-CSF concentrations, but this also increased the outgrowth of other cell types such as granulocytes and immature myeloid cells and favored proliferation at the expense of differentiation/maturation. Importantly, the relative yield of CD11c^+ DC in the presence of IL-4 was similar across strains over a wide range of GM-CSF concentrations (Fig. 1C). We next compared a range of GM-CSF/IL-4 combinations that encompassed those commonly used by various investigators (19–23). At all combinations tested DC generation from NOD exceeded that of other strains (Fig. 1D). We then used a combination of GM-CSF and IL-4 at 1 ng/ml each, the combination giving the highest percentage of CD11c^+ DC and mature DC while still producing fully functional DC. This combination avoided excessive outgrowth of non-DC myeloid cells and potential artifacts induced by high cytokine concentrations. In cultures supplemented with 1 ng/ml GM-CSF and 1 ng/ml IL-4 the yield of nonadherent cells, which were mainly CD11c^− DC, immature myeloid cells, and occasional granulocytes, did not differ significantly among strains at day 5 (Table I). The total number of CD11c^+ DC recovered was significantly greater from NOD than from NOR, BALB, or BL/6 cultures. The proportion of CD11c^+ DC was greatest in NOD and NOR cultures, exceeding that in BALB/c and BL/6 cultures by ~50%, indicating that, despite differing yields of DC, myeloid progenitors of both NOD and NOR mice shared an increased commitment to terminal differentiation to DC. Very few T or B lymphocytes were present in day 5 GM-CSF/IL-4-supplemented BM cultures. Moderately adherent cells that could be removed by vigorous washing with cold PBS/10 mM EDTA were almost exclusively CD11c^−CD86^−CD45^low^ immature DC (data not shown). Adherent macrophages, readily discernible by their extensively flattened morphology, comprised only a minor proportion of cells.

Increased costimulatory and adhesion molecule expression by mature NOD and NOR DC in GM-CSF/IL-4-supplemented BM cultures

In GM-CSF/IL-4-supplemented BM cultures we found mixed populations of both phenotypically mature and immature DC bearing high or low levels, respectively, of the costimulatory molecule CD86 (Fig. 2), as reported by others (18–20). We used anti-CD11c to define DC for quantitative analysis due to the lack of anti-MHC class II mAb that react across the MHC haplotypes of all strains tested. The proportion of DC that exhibited the mature CD11c^+CD86^high phenotype was greatest in BL/6 cultures but did not differ significantly among other strains (NOD, 35.1 ± 12.6
(n = 18); NOR, 36.5 ± 14.6 (n = 12); BALB/c, 28 ± 14.2 (n = 15); BL/6, 59 ± 13.1 (n = 8); BL/6 significantly greater than NOD (p < 0.05) and BALB/c (p < 0.001). Mature DC could alternatively be identified as cells coexpressing high levels of MHC class II and CD86, whereas immature DC expressed only moderate levels of both (Fig. 2). Qualitative analysis indicated that MHC class II and CD86 coexpression defined a similar pattern of DC development across strains to that observed using CD11c and CD86. It is noteworthy that expression of MHC class II was not uniformly high on CD86high cells, particularly those from NOD and NOR cultures (Fig. 2). In addition to CD86 expression, mature and immature DC could also be distinguished on the basis of intermediate or high expression of CD11b, respectively. There was concordance across strains of CD11bint and CD11bhigh DC with CD86high and CD86low populations, respectively, which confirmed the findings based on CD86 discrimination of mature and immature populations.

While extending across an almost identical range in all strains, CD86 expression was consistently higher on mature and immature DC populations from NOD and NOR mice (Fig. 2, Table II). CD80 expression showed a similar trend (Table II), but the proportion of CD11c+CD80hi DC was similar across strains (29.2 ± 7.5, 32.3 ± 11, 26.1 ± 14, and 32.4 ± 20.3%; NOD, NOR, BALB/c, and BL/6, respectively). CD40 expression was elevated on NOD and NOR DC when relative levels within experiments were compared (p < 0.001), but not when MFI were pooled (Table II) before comparison. The adhesion molecule CD54 was expressed at moderate to high levels on DC from all strains, but a subpopulation of NOD DC had increased expression, resulting in a higher overall MFI (Table II). In contrast to the costimulatory molecules examined, CD11b expression was equivalent on mature or immature DC populations across strains. Expression of the myeloid/granulocyte- and macrophage-associated molecules Ly-6G (Gr-1) and F4/80 (average MFI, ~50 and 20, respectively) and CD43 (average MFI, ~50) was equivalent across strains, and DC from all strains were negative for MAC-3 expression. M-CSF receptor (c-fms) was expressed at equivalent levels on similar proportions (41–50%) of immature DC across strains. As expected, the proportion of mature DC that expressed M-CSF receptor was reduced but similar (18–30%) across strains. Thus, the phenotypic differences identified were selective and restricted to costimulatory and adhesion molecules.

DC from GM-CSF/IL-4-supplemented BM cultures correspond functionally across strains

We sought to determine whether the altered pattern of DC development among strains was reflected in altered function. Endocytic activity, a marker of Ag uptake and processing by immature DC that is down-regulated upon maturation, was measured as FITC-dextran uptake. The majority of immature DC in each strain examined (70 ± 1, 67 ± 2, and 59 ± 1%, NOD, NOR, and BALB/c, respectively) were endocytically active. By comparison, activity was significantly reduced in mature DC (39 ± 2, 26 ± 7, and 24 ± 7.4%, respectively; all p < 0.05, t test; n = 3). We also tested the ability of GM-CSF/IL-4-derived DC to respond to inflammatory stimuli by adding LPS during the final 20 h of culture. In response to LPS, CD86 and MHC class II expression was up-regulated on both mature and immature DC populations in NOD, NOR, BALB/c, and BL/6 cultures; furthermore, LPS increased the numbers of CD11c+CD86hi (or MHC class IIhiCD86hi) DC in each strain (data not shown).

We next determined whether developmental differences among strains affected T cell stimulatory capacity. Mature DC were metrizamide-enriched from GM-CSF/IL-4-supplemented BM cultures and used in allogeneic and syngeneic MLR cultures to assess their ability to provide costimulatory signals. DC from NOD, BALB/c, and BL/6 elicited similar proliferative responses in parallel allogeneic MLR assays (Fig. 3A). Despite a small degree of interassay variability, no consistent differences were observed in the allostimulatory capacity of NOD and NOR DC (Fig. 3B). While it is difficult to make direct comparisons among strains due

### Table I. Cell recovery from GM-CSF/IL-4-supplemented BM cultures

<table>
<thead>
<tr>
<th></th>
<th>NOD</th>
<th>NOR</th>
<th>BALB/c</th>
<th>C57BL/6</th>
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<tbody>
<tr>
<td>Cell recovery (% of starting BM)</td>
<td>13.2 ± 4.8 (19)</td>
<td>9.8 ± 2.2 (13)</td>
<td>12.8 ± 4.5 (13)</td>
<td>12.1 ± 3.5 (9)</td>
</tr>
<tr>
<td>Proportion CD11c+ (%)</td>
<td>46.3 ± 15.1 (18)</td>
<td>46.9 ± 16.0 (12)</td>
<td>32.9 ± 13.9 (15)</td>
<td>25.0 ± 10.0 (11)</td>
</tr>
<tr>
<td>CD11c+ DC yield (10⁴/10⁶ BM)</td>
<td>6.3 ± 1.9 (15)</td>
<td>4.5 ± 0.8 (9)</td>
<td>4.6 ± 1.6 (10)</td>
<td>4.1 ± 1.3 (8)</td>
</tr>
</tbody>
</table>

* Data presented are mean ± SD; the number of experiments appears in parentheses.
* Significantly greater than BALB/c (p < 0.05) and C57BL/6 (p < 0.01).
* Significantly greater than NOR, BALB/c (p < 0.05), and BL/6 (p < 0.01).
to MHC disparities, NOD DC stimulated similar levels of proliferation in syngeneic MLR cultures compared with NOR, BALB/c, and BL/6 DC. This was particularly evident when bulk allogeneic spleen cells were used as internal controls (Fig. 4). No consistent differences were observed between the stimulatory capacity of MHC-identical NOD- or NOR-derived DC when examined in crossover syngeneic MLR cultures (Fig. 4, A and B).

**Increased proliferation in GM-CSF/IL-4-supplemented NOD BM cultures**

While increased commitment to DC generation was observed in both NOD and NOR BM cultures, the number of DC generated was higher only in NOD. We set out to determine whether increased myeloid progenitor proliferation contributed to the greater yield of NOD DC. As lymphocytes and other nonmyeloid cells are removed from cultures in the nonadherent cell fraction at day 2 (17), proliferation of the remaining myeloid progenitor pool could be assessed by [3H]thymidine incorporation. Proliferation was greatest in the 2 days following nonadherent cell removal (Table III) and, notably, in the initial 24 h was significantly greater in NOD than in NOR and BALB/c cultures. DC are terminally differentiated and develop progressively 2–3 days after myeloid progenitor division (17). Therefore, our results are consistent with increased proliferation of myeloid progenitors in NOD cultures around day 2–3, leading to an increased number of DC generated by day 5 in NOD relative to NOR cultures.

The pattern of myeloid DC development reflects myeloid progenitor programming

The differences in the yield of DC among strains could reflect differences in the relative number of myeloid progenitors or their

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**Table II. Costimulatory and adhesion molecule expression on DC from GM-CSF/IL-4-supplemented BM cultures**

<table>
<thead>
<tr>
<th>CD86</th>
<th>CD11c&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD80&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD86&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD40&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD54&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>NOD</td>
<td>342 ± 192&lt;sup&gt;d&lt;/sup&gt;</td>
<td>968 ± 475&lt;sup&gt;e&lt;/sup&gt;</td>
<td>132 ± 29&lt;sup&gt;f&lt;/sup&gt;</td>
<td>343 ± 128&lt;sup&gt;g&lt;/sup&gt;</td>
<td>45 ± 31&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>NOR</td>
<td>433 ± 252&lt;sup&gt;d&lt;/sup&gt;</td>
<td>906 ± 379&lt;sup&gt;e&lt;/sup&gt;</td>
<td>122 ± 22&lt;sup&gt;f&lt;/sup&gt;</td>
<td>269 ± 72&lt;sup&gt;g&lt;/sup&gt;</td>
<td>50 ± 31&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>BALB/c</td>
<td>197 ± 120</td>
<td>567 ± 235</td>
<td>82 ± 26</td>
<td>246 ± 70</td>
<td>21 ± 14</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>174 ± 118</td>
<td>444 ± 177</td>
<td>74 ± 23</td>
<td>189 ± 58</td>
<td>18 ± 10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are mean ± SD MFI from 10–14 experiments depending on strain.

<sup>b</sup>MFI of CD11c<sup>+</sup>-gated population.

<sup>c</sup>MFI of CD80<sup>+</sup> CD86<sup>+</sup> or CD11c<sup>+</sup> CD80<sup>+</sup> CD86<sup>+</sup> gated population as noted.

<sup>d</sup>Significantly greater than BALB/c, BL/6 (p < 0.05).

<sup>e</sup>Significantly greater than BALB/c (p < 0.01), BL/6 (p < 0.001).

<sup>f</sup>Significantly greater than BALB/c (p < 0.05).

<sup>g</sup>Significantly greater than NOR (p < 0.01), BALB/c (p < 0.001), BL/6 (p < 0.01).

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**FIGURE 3.** Allostimulatory activity of mature BM-derived DC. DC were propagated in GM-CSF and IL-4 and purified by density separation over metrizamide columns. Allogeneic MLR were established with 2 × 10<sup>6</sup> nylon wool-passaged CBA T cells and graded doses of DC or bulk splenocytes as stimulators. Stimulators in A were DC from NOD (A), BALB/c (B), and C57BL/6 (C), or splenocytes from NOD (D), BALB/c (E), or CBA (F) mice; stimulators in B were DC from NOD (A) and NOR (B) or splenocytes from BALB/c (C) and CBA (D) mice. Results are representative of at least six experiments.

**FIGURE 4.** Ability of mature BM-derived DC to stimulate syngeneic MLR assays. DC were propagated in GM-CSF and IL-4 and purified by density separation over metrizamide columns. Syngeneic MLR were established with 2 × 10<sup>6</sup> nylon wool-passaged T cells and graded doses of DC or bulk splenocytes as stimulators. A, NOD T cells stimulated with NOD DC (A), NOR DC (B), or NOD CBA (C), or syngeneic NOD (D) splenocytes. B, NOR T cells stimulated with NOD DC (A), NOR DC (B), or syngeneic NOD (C) splenocytes. C, BALB/c T cells stimulated with BALB/c DC (A), or syngeneic BALB/c (B) splenocytes. D, C57BL/6 T cells stimulated with C57BL/6 DC (A), or syngeneic C57BL/6 (B) splenocytes. Results are representative of four to six experiments, depending on strain.
adhesive properties; therefore, we investigated whether the pattern of DC development observed in bulk BM cultures was determined at the level of myeloid-committed progenitors. The recent description of early myeloid progenitors (24) as lineage IL-7Rα+ SCA-1+c-kit− cells allowed us to compare DC development from these cells. BALB/c mice were not analyzed, as they carry the Ly6.1 allotype and do not express SCA-1 (Ly6A-E) on hematopoietic progenitors. Myeloid progenitors obtained by cell sorting were cultured in GM-CSF/IL-4 or GM-CSF/SCF/TNF-α, a second cytokine combination commonly used to generate myeloid DC in vitro. Expansion of cell number (70- to 150-fold) was greatest from NOD myeloid progenitors (Table IV). Likewise, the number of CD11c+ DC generated from NOD myeloid progenitors was ~1.5- to 2-fold that from NOR or BL/6 (Table IV). In GM-CSF/IL-4-supplemented myeloid progenitor cultures, the proportion of mature CD86high DC was greater from BL/6 than from NOD and NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/NOR, mirroring the pattern seen in bulk BM cultures.

To determine whether the pattern of DC maturation observed in bulk BM cultures may have been influenced by soluble factors secreted into the medium, CM was harvested from day 5 cultures and added to freshly prepared GM-CSF/IL-4-supplemented BM cultures. CM in the culture did not alter the pattern of DC development in any combination, regardless of the strain from which CM or BM was prepared (data not shown). In addition, by comparing GM-CSF/IL-4-supplemented BM cultures from NOD.scid and CB.17 SCID mice with cultures from control immunocompetent NOD and BALB/c mice, we determined that lymphocytes present in BM did not contribute to the development patterns observed (data not shown). These findings provide further evidence that the pattern of DC development was determined in a cell-intrinsic manner.

**Increased yield of DC in flt3-L-supplemented NOD and NOR BM cultures**

To determine whether the altered expansion potential observed for NOD myeloid progenitors extended to early uncommitted progenitors cells we generated DC using the early-acting hematopoietic growth factor flt3-L. In flt3-L-supplemented BM cultures, DC develop as either CD11c−CD11b+ or CD11c−CD11b− populations (Fig. 5), proposed to correspond to CD8α− (myeloid) DC and CD8α+ (lymphoid-related) DC, respectively (16). Nonadherent cell recovery from day 10 flt3-L-supplemented BM cultures was significantly greater (p < 0.01) from NOD and NOR compared with BALB/c and BL/6 (50 ± 12 and 49 ± 13% vs 23 ± 10 and 23 ± 7%, respectively; n = 4 experiments, all strains tested in parallel). As nonadherent cells recovered were primarily CD11c− DC (range 80–95%), the DC yield was closely related to overall cell recovery, and the total number of CD11c− cells recovered was consistently greater from NOD and NOR (Fig. 5). Furthermore, recovery of DC with the CD11c−CD11b− myeloid phenotype from flt3-L-supplemented BM was greatest from NOD and NOR cultures. As the proportion of lineage flt3− cells in BM was similar across strains (<1%) the increased recovery of DC from NOD and NOR cultures reflected greater expansion and DC development from flt3-L-responsive progenitor cells in these strains.

**In NOD and NOR mice, DC are skewed toward the myeloid CD11b− subset in spleen and increased in blood and thymus**

To determine whether the increased proliferative capacity and number of DC precursors in NOD BM were mirrored in vivo we first examined the frequency of DC in peripheral blood leukocytes. CD11c+ cells, most likely comprising mature DC as well as DC precursors, were more abundant in peripheral blood leukocytes recovered from NOD and NOR mice (Table V). We then examined DC in thymus (10 wk old) and spleen (3 and 10 wk old) of NOD, NOR, BALB/c, and BL/6 mice. Single-cell suspensions freshly prepared from individual animals were analyzed to obtain a representative estimate and avoid the nonspecific cell losses and potential artifacts associated with extensive cell depletion and/or enrichment steps commonly used in DC characterization. Thymic DC were readily distinguished as a CD11c−CD8α+ population (Fig. 6), and expression of CD11c and CD8α was consistent with that reported (6, 25). NOD and NOR thymi contained a significantly

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**Table III. Proliferation in GM-CSF/IL-4-supplemented BM cultures**

<table>
<thead>
<tr>
<th>Culture Period (days)</th>
<th>NOD</th>
<th>NOR</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–3</td>
<td>115 ± 28b</td>
<td>74 ± 18</td>
<td>59 ± 19</td>
</tr>
<tr>
<td>3–4</td>
<td>104 ± 59</td>
<td>79 ± 39</td>
<td>74 ± 23</td>
</tr>
<tr>
<td>4–5</td>
<td>57 ± 42</td>
<td>63 ± 42</td>
<td>63 ± 41</td>
</tr>
</tbody>
</table>

*Data are from four (NOD and NOR) or three (BALB/c) experiments in which cultures were performed in parallel.

**Table IV. Generation of DC from myeloid progenitors**

<table>
<thead>
<tr>
<th></th>
<th>NOD</th>
<th>NOR</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>Expt. 1</td>
</tr>
<tr>
<td>GM-CSF + IL-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell recovery (× 10^−4)</td>
<td>97.5</td>
<td>79.4</td>
<td></td>
</tr>
<tr>
<td>Total CD11c+ (× 10^−4)</td>
<td>75.1</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>Proportion CD86^high (%)</td>
<td>11.1</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>CD86 (MFI)^a</td>
<td>719</td>
<td>802</td>
<td></td>
</tr>
<tr>
<td>GM-CSF + SCF + TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell recovery (× 10^−4)</td>
<td>159.0</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>Total CD11c+ (× 10^−4)</td>
<td>80.1</td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>Proportion CD86^high (%)</td>
<td>4.4</td>
<td>4.9</td>
<td></td>
</tr>
</tbody>
</table>

*Data presented are from two separate experiments.

b Proportion of all CD11c+ cells was determined.

a Proportion of all CD11c+ cells was determined.

Proliferation in GM-CSF/IL-4-supplemented BM cultures

Increased yield of DC in flt3-L-supplemented NOD and NOR BM cultures

In NOD and NOR mice, DC are skewed toward the myeloid CD11b− subset in spleen and increased in blood and thymus

To determine whether the increased proliferative capacity and number of DC precursors in NOD BM were mirrored in vivo we first examined the frequency of DC in peripheral blood leukocytes. CD11c+ cells, most likely comprising mature DC as well as DC precursors, were more abundant in peripheral blood leukocytes recovered from NOD and NOR mice (Table V). We then examined DC in thymus (10 wk old) and spleen (3 and 10 wk old) of NOD, NOR, BALB/c, and BL/6 mice. Single-cell suspensions freshly prepared from individual animals were analyzed to obtain a representative estimate and avoid the nonspecific cell losses and potential artifacts associated with extensive cell depletion and/or enrichment steps commonly used in DC characterization. Thymic DC were readily distinguished as a CD11c^high population (Fig. 6), and expression of CD11c and CD8α was consistent with that reported (6, 25). NOD and NOR thymi contained a significantly
greater proportion of CD11c⁺ DC (Table V). This was not due to a relative enrichment of DC, as total cellularity did not differ significantly across strains. To determine whether the increased generation of DC from NOD and NOR progenitors influenced the normally dominant CD8α⁺ DC subtype in the thymus, CD8α and CD11b expression was also examined. In all strains, the majority (65–71%) of thymic DC expressed CD8α (Fig. 6) and the proportion expressing either high or intermediate/low levels of CD8α did not differ. In all strains, CD11b was present on only a minority of thymic DC (12–17%). Therefore, while the relative abundance of DC in the thymus varied among strains, the balance of phenotypically defined DC subtypes did not.

Splenic DC were also readily distinguished as a CD11chigh population, and DC subtypes were clearly defined by the presence or absence of CD8α or CD11b expression (Fig. 6). At 3 but not 10 wk of age, splenic DC were more abundant in NOD mice (Table V). At both ages, the CD11c⁺CD11b⁺ (or the corresponding CD11c⁺CD8α⁺) subtype predominated, and was most abundant in NOD mice (Table VI).

Discussion
Based on the premise that altered development of myeloid-derived DC could contribute to the pathogenesis of T1D, we examined development of DC in vitro from the autoimmune-prone, spontaneously diabetic NOD mouse, the related non-diabetes-prone NOR mouse, and unrelated non-diabetes-prone strains. In GM-CSF/IL-4-supplemented conditions, DC commitment and expression of co-stimulatory and adhesion molecules on DC was greater in NOD and NOR mice relative to unrelated strains. However, DC were generated in greater numbers from NOD compared with NOR mice due to increased proliferation and expansion of NOD myeloid progenitors. Our findings of increased expansion potential of NOD myeloid progenitors are consistent with previous observations of increased cycling of stem cells and greater numbers of GM-CFC in NOD BM (26). In contrast, two recent studies using GM-CSF in the absence of IL-4 concluded that DC development from NOD BM is impaired (27, 28). When we used the combination of GM-CSF and IL-4, superior for generating mature DC (18), we found that DC development from NOD myeloid progenitors was increased rather than reduced. Another report suggesting that the yield of DC from GM-CSF/IL-4-supplemented NOD BM cultures is reduced (22) enumerated only mature (low buoyant density) DC separated on density gradients, thereby excluding the large number of less buoyant immature DC we also enumerate. Additionally, our cultures were established with whole BM to avoid manipulating progenitor populations. Others have used complement-depleted BM (22, 27), which could inadvertently alter the relative proportions of progenitor populations. Significantly, the elevated expression of CD86, CD80, and CD40 we observed is consistent with previous reports of GM-CSF/IL-4-generated NOD BMDC (22, 30). While DC from NOD and other strains were functionally similar in the assays we used, this does not preclude other as yet undefined functional differences.

The exact nature of the cell type(s) that give rise to DC in flt3-L-supplemented BM cultures is yet to be determined. Loss of flt3 expression from hematopoietic progenitor cells (HPC) as differentiation proceeds (reviewed in Ref. 31) and generation of DC from flt3⁺ cells (16) suggest that flt3-L drives DC development from early HPC. Consistent with this, lineage-committed lymphoid or myeloid progenitors retain some responsiveness to flt3-L.
but DC development from these cells in vitro depends primarily on other growth factors (IL-7 and GM-CSF, respectively) (32, 33). Our findings show that early uncommitted HPC from both NOD and NOR share an increased expansion and DC-generative capacity in response to flt3-L relative to unrelated strains. In contrast, myeloid-committed progenitors in BM exhibit increased expansion capacity only in NOD mice. The exact mechanism underlying the increased proliferation of myeloid progenitors in NOD mice is unclear but could be either dysregulation of cell cycle control or enhanced cytokine responsiveness.

Members of the NF-κB family are important regulators of DC development and maturation (34, 35). The presence of NF-κB binding sites in the promoter regions of CD86, CD80, and CD40 (36–38) suggests that increased activation and nuclear translocation of NF-κB molecules could contribute to the selective up-regulation of these molecules in NOD and NOR DC. Increased commitment to DC development within the myeloid lineage of NOD and NOR is also consistent with increased NF-κB activation. The dependence of myeloid DC on RelB activation and nuclear translocation for differentiation (35, 39), and in particular terminal activation (40), indicates it may be a key mediator of enhanced myeloid DC development in NOD mice. Alternatively, other transcription factors, such as early growth response 1, which enhances monocyte development at the expense of granulocyte and erythroid differentiation (41), could also be important. Altered kinetics of NF-κB activation could also underlie the differences in maturation between strains, because GM-CSF/IL-4-supplemented NOD BM cultures eventually become more enriched for mature DC if maintained until day 7. The suggestion that NF-κB may be important in altered development of NOD DC is supported by a recent report showing hyperactivation of NF-κB in NOD DC in response to LPS stimulation (23). The similarity of DC stimulatory capacity among strains was unexpected in light of increased co-stimulatory molecule expression on NOD and NOR DC; however, it is possible that following DC-T cell interaction (and NF-κB activation) little further up-regulation of co-stimulatory molecules occurs on NOD and NOR DC, whereas expression on BALB/c and BL6 DC is up-regulated to levels equivalent to NOD and NOR.

We found that the populations of DC subtypes in vivo in NOD mice reflected the differences in DC development in vitro in GM-CSF/IL-4 and flt3-L. GM-CSF and flt3-L are both important mediators of DC development in vitro (16, 17, 32, 33) but have different effects in vivo. flt3-L mobilizes and expands HPC (42) and results in accumulation of DC of both proposed subtypes in mice and humans (43–46). Conversely, mice lacking flt3-L have a substantial reduction in both DC subtypes (47). In contrast, GM-CSF is redundant in regulating DC numbers (48) or myeloid cells in the steady state (49) but is required to sustain myeloid inflammatory responses (50). It expands myeloid DC in mice (12) and most likely regulates inflammatory site DC derived from monocytes (51). The increased DC frequency shared by NOD and NOR mice in vivo is consistent with increased DC generation from early flt3-responsive HPC, as suggested by the increased generation of DC in flt3-L-supplemented NOD and NOR cultures. We show that

### Table VI. Frequency of CD11c+CD11b+ and CD11c+CD8− DC in spleen

<table>
<thead>
<tr>
<th></th>
<th>NOD</th>
<th>NOR</th>
<th>BALB/c</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c+</td>
<td>68.2 ± 3.7b</td>
<td>60.7 ± 3.6c</td>
<td>62.3 ± 2.2c</td>
<td>56.7 ± 2.2</td>
</tr>
<tr>
<td>CD11c−</td>
<td>69.1 ± 1.6d</td>
<td>63.6 ± 2.4</td>
<td>70.1 ± 3.0</td>
<td>64.9 ± 2.5</td>
</tr>
<tr>
<td>10 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c+</td>
<td>77.3 ± 2.2e</td>
<td>72.4 ± 1.0f</td>
<td>69.5 ± 2.7</td>
<td>66.4 ± 0.9</td>
</tr>
<tr>
<td>CD11c−</td>
<td>75.2 ± 0.6g</td>
<td>71.6 ± 3.1h</td>
<td>67.6 ± 1.7</td>
<td>64.0 ± 2.3</td>
</tr>
</tbody>
</table>

*a* Data are mean ± SD; *n* = 6 for 3 wk; *n* = 3 for 10 wk.
*b* Significantly greater than NOR, BALB/c (p < 0.01), and BL6 (p < 0.001).
*c* Significantly greater than BL6 (p < 0.05).
*d* Significantly greater than NOR (p < 0.01), and BL6 (p < 0.05).
*e* Significantly greater than NOR and BL6 (p < 0.01).
*f* Significantly greater than NOR (p < 0.05), BALB/c, and BL6 (p < 0.01).
*g* Significantly greater than BL6 (p < 0.05).
*h* Significantly greater than BALB/c (p < 0.01) and BL6 (p < 0.001).
differential sensitivities to hematopoietic growth factors that drive the different DC development streams exist among strains. It is possible, in fact, that differential responsiveness to flt3-L and GM-CSF in vivo determines the differences observed in DC population subtypes among strains. Mobilization and expansion of DC in vivo by administration of flt3-L and/or GM-CSF is likely to reflect the patterns observed in vitro. Both myeloid- and lymphoid-committed progenitors have been shown to give rise to CD8α− and CD8α+ DC subtypes following adoptive transfer (8, 9, 33). While the spleen is permissive for both CD8α− and CD8α+ DC development, the latter subtype predominates. However, the relative contribution of the myeloid and lymphoid lineages to each DC development stream within the spleen under steady-state conditions is currently unclear. Despite these uncertainties, the increased proportion of CD8α− (myeloid) DC in NOD spleen reflects the increased generation of myeloid-derived DC observed in vitro.

Our findings indicate that the NOD genetic background confers broad alterations in hemopoiesis and DC generation that can be detected as increased progenitor proliferation, commitment to DC terminal differentiation within the myeloid lineage, and increased expression of costimulatory molecules on mature DC. These alterations could predispose to autoimmunity in the NOD mouse. While MHC class II molecule I-A^B^ is the major susceptibility allele (Iddl) for T1D in NOD mice, at least 16 other susceptibility alleles also contribute (reviewed in Ref. 52). NOR mice are recombining congenic NOD mice carrying portions (11–12%) of the C57BL/KsJ genome that impart resistance to pancreatic islet inflammation (insulinitis) and protection from diabetes (53, 54), while retaining NOD-derived Iddl, 2, 3, 6, 7, 8, 10, 12, and 14 alleles (55). We found that myeloid-committed progenitors of NOR mice lack the increased proliferative capacity characteristic of NOD mice. This suggests the latter trait may be an important element in the development of spontaneous diabetes in NOD mice, and that the locus controlling the latter trait may have been replaced in NOR mice. Further investigation of mice congenic for single Iddl alleles may help identify the genetic loci determining these effects. The alterations in DC development described in this work could have an impact on the development of T1D by, on the one hand promoting pathogenic immunity or, on the other, impairing protective immunity. The thymus preferentially supports development of CD8α− DC, even from myeloid progenitors (9), which are estimated to contribute 50% of thymus DC in C57BL/Ka mice (33). From our findings it is plausible to extrapolate that myeloid progenitors make an even greater contribution to thymic DC development in NOD mice. If thymic DC derived from myeloid progenitors were impaired in their ability to mediate negative selection, this could lead to escape of high-affinity autoreactive T cells from NOD thymus. Infiltration of the islets in female NOD mice by macrophages and TNF-α-producing DC precedes T cell infiltration (56, 57) and is an essential initial step in disease progression (58). The expanded pool of immediate DC precursors in BM and blood of NOD mice may be readily recruited to pancreatic islets in response to an as yet unknown environmental or developmental signal and precipitate disease by local production of TNF-α and/or transfer of Ag to regional lymphoid tissues. Conversely, reduced numbers of CD8α− DC that cross-present Ag to CTL (13) and may be responsible for induction of cross-tolerance (59) could impair peripheral deletion of autoreactive CTL. In summary, our findings demonstrate that NOD mice have increased development of DC from myeloid progenitors in vitro, reflected in the homeostasis of DC populations in vivo. This shift toward myeloid-derived DC could predispose to autoimmune disease.

Acknowledgments
We thank Immunex for supplying flt3-L, Drs. Charlie Maliszewski and Ken Brasel for assistance in establishing the generation flt3-L-propagated DC, and Dr. S.-I. Nishikawa for the AFS-98 and ACK-2 hybridomas.

References