

# Flt3-Ligand Treatment Prevents Diabetes in NOD Mice

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**The mechanism by which mixed chimerism reverses autoimmunity in type 1 diabetes has not been defined. NOD mice have a well-characterized defect in the production of myeloid progenitors that is believed to contribute significantly to the autoimmune process. We therefore investigated whether chimerism induces a correction of this defect. Mixed chimerism restored production of myeloid progenitors in NOD mice to normal levels. Notably, NOD bone marrow cells as well as donor bone marrow cells produced the mature myeloid progeny, and the level of donor chimerism was not correlated with the degree of restoration of the defect. Moreover, NOD bone marrow cells cultured with Flt3-ligand developed a heat-stable antigen-positive/Ly6C<sup>+</sup> population comprised primarily of mature myeloid dendritic cells, suggesting that the underlying abnormality is not cell intrinsic but rather due to a block in development of mature myeloid progeny, including myeloid dendritic cells. Strikingly, treatment of NOD mice with Flt3-ligand significantly decreased insulinitis and progression to diabetes and was associated with a significant increase in myeloid dendritic cells and in vivo induction of CD4<sup>+</sup>/CD25<sup>+</sup> cells in the pancreatic lymph node. Therefore, Flt3-ligand treatment and/or the establishment of mixed chimerism in prediabetic candidates may provide a benign and novel approach to treat diabetes. *Diabetes* 53:1995–2002, 2004**

**N**OD mice have well-characterized abnormalities that affect antigen presentation (1–4), including defects in the protein kinase C activation pathway (3) and low expression levels of CD80 and CD86, which affect costimulation (5). The decreased expression of CD86 impairs T-cell activation and the upregulation of CD152, which is important in the induction of T regulatory cells (6). Langmuir et al. (7) demonstrated defective production of myeloid progeny in NOD bone marrow cells, most notably cells that coexpress Ly6C and heat-stable antigen (HSA), resulting in impaired responses

to cytokines, including interleukin (IL)-3, granulocyte macrophage–colony stimulating factor (GM-CSF), and IL-5.

NOD mice do not produce normal myeloid dendritic cells in vivo (5,8,9). Until recently this was believed to be caused by a cell-intrinsic defect. However, when bone marrow cells from NOD mice are cultured in vitro, mature myeloid dendritic cells are produced (10). The significance of this paucity of myeloid dendritic cells in NOD mice was demonstrated when diabetes progression was delayed in naive NOD mice after the adoptive transfer of mature myeloid dendritic cells that were either incubated in vitro with islet cells or obtained from the pancreatic lymph nodes (11,12). In contrast, immature dendritic cells had no effect (12,13). Transfer of  $\gamma$ -interferon-treated NOD dendritic cells also provided long-lived protection against autoimmunity (14). Taken together, these studies indicate that NOD mice have defective myeloid dendritic cells in vivo and that these cells are important in the maintenance of self-tolerance to the antigens that mediate autoimmunity and diabetes. The fact that normal levels of myeloid dendritic cells are generated in vitro indicates that the defect is extrinsic to the myeloid dendritic cells or their progenitors.

Mixed allogeneic chimerism eliminates autoimmunity in NOD mice (15). Prediabetic female NOD mice do not become diabetic after bone marrow transplantation (15). No insulinitis is present after transplant, indicating control of the preexisting autoimmunity (15,16). We hypothesized that mixed chimerism supplies missing factors that correct the differentiation of myeloid cells. Notably, bone marrow cells from the chimeras have a distinct population of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells, whereas naive NOD bone marrow cells do not. Moreover, NOD cells contribute to the HSA<sup>+</sup>/Ly6C<sup>+</sup> population, demonstrating that the defect is not an inability to produce myeloid precursor cells, but rather a lack of some factor or cellular interaction that instructs precursor cells to this lineage. In vitro culture of NOD bone marrow cells with Flt3-ligand resulted in production of myeloid cells similar to that observed in control bone marrow cells. Strikingly, Flt3-ligand treatment of NOD mice restored the production of HSA<sup>+</sup>/Ly6C<sup>+</sup> myeloid progenitors and increased numbers of mature myeloid dendritic cells and plasmacytoid dendritic cells in the spleen and pancreatic lymph nodes. A significant increase in regulatory T-cells in pancreatic lymph nodes was also seen. Importantly, insulinitis and diabetes progression were both significantly delayed in the Flt3-ligand-treated NOD mice. These data provide in vivo evidence that peripheral tolerance mediated by mature myeloid dendritic cells is critical to the control of autoimmune diabetes.

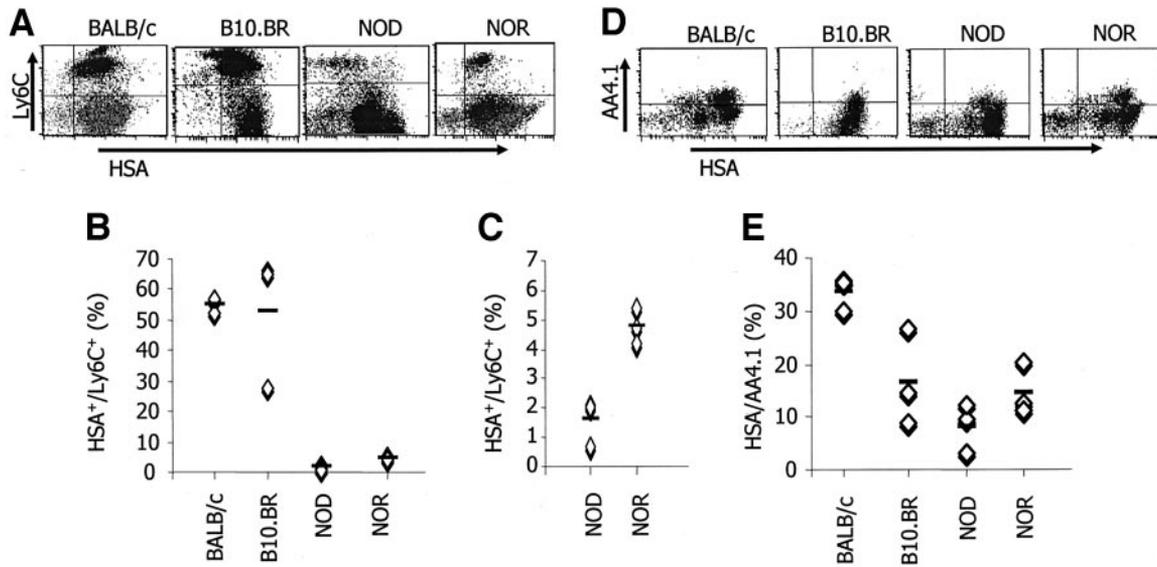
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FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage–colony stimulating factor; HSA, heat-stable antigen; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; PE, phycoerythrin; SCF, stem cell factor; TBI, total body irradiation.

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**FIG. 1.** Ly6C and AA4.1 expression on HSA<sup>+</sup> bone marrow cells. Bone marrow cells from naive BALB/c, B10.BR, NOD, and NOR mice were stained and analyzed for the expression of HSA and Ly6C or AA4.1. Quadrant markers were based on individual isotype control staining. **A:** Representative dot plots from each of the strains tested are shown. **B** and **C:** Data from individual animals (◇) and the means (lines) from one experiment, where **B** compares the percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> in the total bone marrow cells of all strains tested and **C** compares NOD and NOR bone marrow cells on a 10-fold-lower scale than that of **B**. **D:** Representative HSA/AA4.1 plots from each of the strains tested are shown, and **E** shows results from three individual animals (◇) and means (lines) from one experiment.

**RESEARCH DESIGN AND METHODS**

We purchased 4- to 6-week-old female NOD/Lt mice from Taconic Laboratories (Germantown, NY). Also, 4- to 5-week-old female BALB/cByJ, NOR/Lt, B10.BR/SgSnJ, and C57BL/10SnJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a barrier animal facility at the Institute for Cellular Therapeutics, University of Louisville (Louisville, KY), and cared for according to National Institutes of Health animal care guidelines.

**Antibodies.** All monoclonal antibodies (mAbs) used in this study were purchased from BD/Pharmingen (San Diego, CA) and included mAbs against HSA-phycoerythrin (PE), AA4.1-fluorescein isothiocyanate (FITC), Ly6C-biotin or -FITC, H-2K<sup>d</sup>-FITC, H-2K<sup>k</sup>-PE, CD11c-allophycocyanin or -FITC, CD11b-APC or -FITC, B220-peridinin-chlorophyll-protein, CD80-FITC, and CD86-FITC.

**Chimera preparation.** Bone marrow cells were harvested and resuspended to 100 × 10<sup>6</sup> cells/ml in chimera media (medium 199 and 50 μg/ml gentamicin; Gibco/BRL, Grand Island, NY). Fully allogeneic NOD chimeras were prepared by irradiating NOD mice (H2<sup>d</sup>) with 1,000 cGy total body irradiation (TBI) (γ-cell 40; Nordion, Ottawa, ON, Canada) and administering 30 × 10<sup>6</sup> B10.BR bone marrow cells in chimera media as previously described (15). For mixed allogeneic chimeras, NOD mice were given either 750 cGy TBI 4–6 h before infusion with untreated 30 × 10<sup>6</sup> B10.BR bone marrow cells or 950 cGy TBI plus 40 × 10<sup>6</sup> T-cell-depleted B10.BR bone marrow cells.

**Assessment of chimerism.** Recipients were characterized for allogeneic engraftment using two-color flow cytometry 30 days posttransplantation as previously described (17). Briefly, whole blood was collected, and 100 μl aliquots were stained with anti-H-2K<sup>d</sup>-FITC and anti-H-2K<sup>k</sup>-PE for 30 min and either analyzed fresh or fixed in 1% formaldehyde (Polysciences, Warrington, PA) until analysis. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA) and analyzed using CellQuest software (Becton Dickinson).

**In vitro culture of bone marrow cells.** Bone marrow cells from NOD, B10.BR, and C57BL/10 mice were harvested and placed in culture at 33°C at 5% CO<sub>2</sub>. Cells were cultured at 1 × 10<sup>5</sup> cells/ml in long-term bone marrow cell media (Iscove's modified Dulbecco's medium [Gibco] supplemented with 20% horse serum [Gibco], 10<sup>-4</sup> mol/l β-mercaptoethanol [Sigma], 10<sup>-5</sup> mol/l hydrocortisone [Sigma], 100 units/ml penicillin, 100 mg/ml streptomycin [Gibco], and 2 mmol/l L-glutamine [Gibco]) in the absence and presence of the following cytokines used singly or in combination: GM-CSF (10<sup>3</sup> units/ml; Genzyme, Cambridge, MA), stem cell factor (SCF) (1.2 units/ml; Genzyme), and Flt3-ligand (10 ng/ml; generously provided by Amgen, Thousand Oaks, CA). Cells were harvested at selected time points and stained for the coexpression of HSA and Ly6C as well as dendritic cell subsets: myeloid (CD11c<sup>+</sup>/CD11b<sup>+</sup>/B220<sup>-</sup>) and plasmacytoid (CD11c<sup>+</sup>/CD11b<sup>-</sup>/B220<sup>+</sup>) dendritic cells.

**In vivo Flt3-ligand treatment of NOD mice.** Prediabetic (7- to 9-week-old) female NOD mice were treated daily for 10 days with 10 μg of Flt3-ligand subcutaneously. On day 11, peripheral blood and collagenase-digested spleen and pancreatic lymph nodes were analyzed for the presence of dendritic, T-, and natural killer cells and compared with untreated age-matched control NOD mice. Other groups were treated with the 10-day course of Flt3-ligand (10 μg/day) or saline and followed for diabetes progression or insulinitis. Progression of diabetes was monitored by urine glucose testing using Chemstrip uGK test strips (Roche, Indianapolis, IN). From the fixed pancreatic samples, 0.7-μm sections were obtained from control and Flt3-ligand-treated mice, stained with hematoxylin and eosin, and assessed for insulinitis as previously described (18). A minimum of 120 islets were scored per sample on a blinded basis.

**Statistical analysis.** Diabetes progression curves of the Flt3-ligand-treated or saline controls were compared using SPSS for Windows 11.0.1 statistical software package. A Cox-regression analysis was performed with the age at diabetes set as the timed event. Each treatment group consisted of 16 mice followed until at least 40 weeks of age. Log-rank results indicate that the *P* value comparison between the two curves was 0.0084 or *P* < 0.01.

**RESULTS**

**Bone marrow cells from NOD mice express decreased numbers of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells compared with B10.BR, BALB/c, or NOR mice.** Bone marrow cells from age-matched NOD, B10.BR, and BALB/c mice were analyzed for the coexpression of Ly6C or AA4.1 with HSA. As previously described (7), NOD bone marrow cells lack a distinct population of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells (Fig. 1A). The HSA<sup>+</sup>/Ly6C<sup>+</sup> population represents 1.6 ± 0.8% of the NOD bone marrow cells, in contrast with 52.8 ± 22.0% of the B10.BR (*P* < 0.05) and 56.8 ± 2.5% of the BALB/c (*P* < 0.001) bone marrow cells. Although the HSA<sup>+</sup>/Ly6C<sup>+</sup> population in bone marrow cells from congenic diabetes-resistant NOR mice was significantly lower compared with either BALB/c or B10.BR strains (*P* < 0.05 each), this value was nevertheless threefold greater than in NOD bone marrow cells (4.8 ± 0.6%, *P* < 0.01) (Fig. 1C). Furthermore, when NOD bone marrow cells were stained for the myeloid dendritic cell markers CD11b and CD11c, a defi-

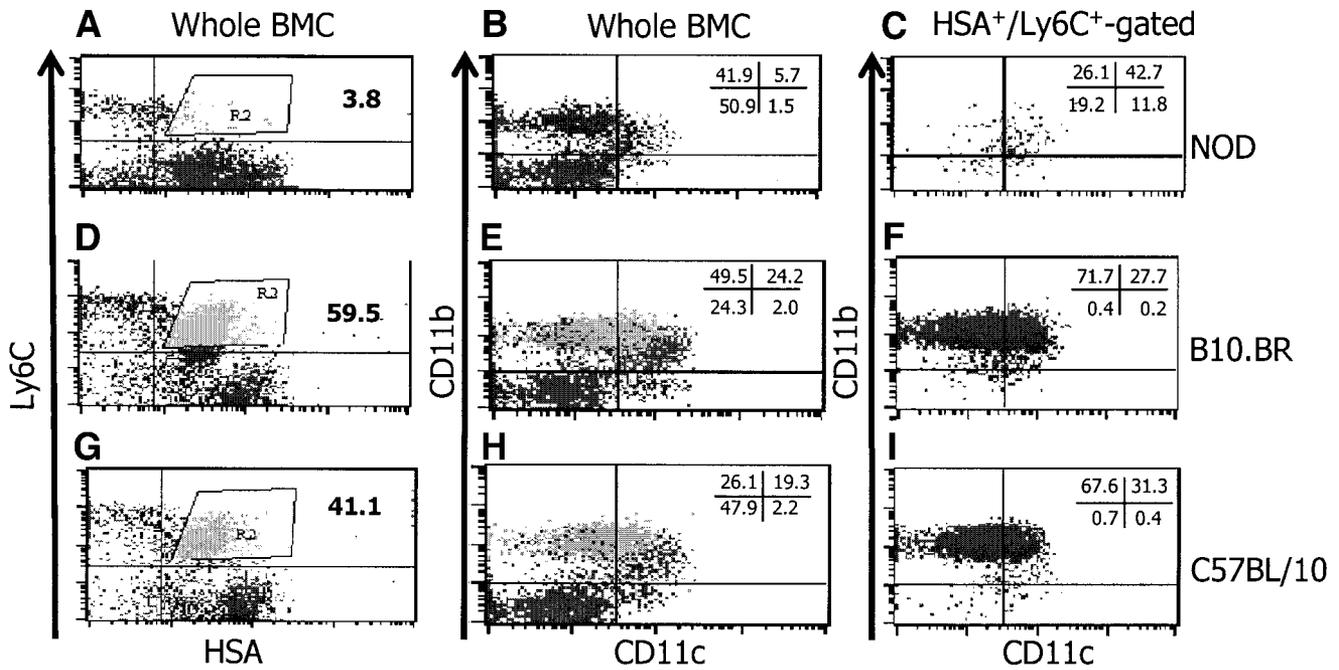


FIG. 2. The HSA<sup>+</sup>/Ly6C<sup>+</sup> population is composed of myeloid cells (CD11b<sup>+</sup>) and myeloid dendritic cells (CD11b<sup>+</sup>/CD11c<sup>+</sup>). Bone marrow cells from NOD (A, B, and C), B10.BR (D, E, and F), and C57BL/10 (G, H, and I) mice stained for the HSA<sup>+</sup>/Ly6C<sup>+</sup> population (A, D, and G). Whole bone marrow cells (B, E, and H) and HSA<sup>+</sup>/Ly6C<sup>+</sup>-gated cells (C, F, and I) were analyzed for the expression of CD11b and CD11c. Plots are from one of three representative experiments. Numbers indicate the average percent of cells per quadrant from 3–4 individual mice. BMC, bone marrow cells.

ciency in the CD11c<sup>+</sup> population was noted (Fig. 2). The NOD CD11c<sup>+</sup> cells stain only dimly compared with controls (Fig. 2A–C), indicative of an immature myeloid dendritic cell population. Notably, the more brightly stained population of mature myeloid dendritic cells is lacking.

In contrast, no difference was detected in the HSA<sup>+</sup>/AA4.1<sup>+</sup> cell population between NOD and B10.BR mice ( $P > 0.05$ ) (Fig. 1D and E). We suspected that the difference between our data and that from the Langmuir group was caused by strain differences. When we analyzed BALB/c bone marrow cells, we determined that BALB/c bone marrow cells have significantly more HSA<sup>+</sup>/AA4.1<sup>+</sup> cells compared with bone marrow cells from B10.BR, NOR, or NOD mice ( $P < 0.05$  each). Because the differences in the HSA<sup>+</sup>/AA4.1<sup>+</sup> cell population did not correlate with the diabetic phenotype of the NOD mouse, the remaining studies focused on the myeloid HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population.

**The bone marrow HSA<sup>+</sup>/Ly6C<sup>+</sup> population in B10.BR and C57BL/10 mice is myeloid.** To determine the basis

for the cellular deficiency in the HSA<sup>+</sup>/Ly6C<sup>+</sup> population in NOD, bone marrow cells were harvested from NOD, B10.BR, and C57BL/10 mice and analyzed for other lineage markers (Fig. 2A). When the HSA<sup>+</sup>/Ly6C<sup>+</sup> cells were gated and analyzed for the expression of CD11b and CD11c, nearly 100% of the HSA<sup>+</sup>/Ly6C<sup>+</sup> population was CD11b<sup>+</sup> (Fig. 2). Of these cells, ~30% also expressed CD11c, a phenotype typical of myeloid dendritic cells (Fig. 2C vs. F and I).

**Fully chimeric NOD mice express the HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population similar to donor strain levels.** To examine whether chimerism corrected the myeloid defect in the NOD marrow, B10.BR→NOD chimeras were prepared, and 1 month after transplantation, the recipients were typed for donor chimerism. All mice ( $n = 9$ ) exhibited >99% donor B10.BR-derived cells in peripheral blood. Bone marrow cells from chimeras were harvested and examined for the coexpression of HSA and Ly6C. The HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population in the chimeric NOD bone marrow ( $68.5 \pm 19.0\%$ ) was significantly greater compared with naive NOD mice ( $2.0 \pm 1.2\%$ ,  $P < 0.05$ ) (Fig. 3A), and

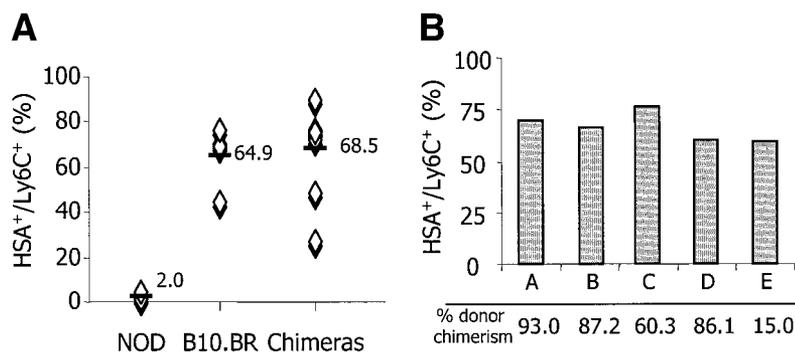
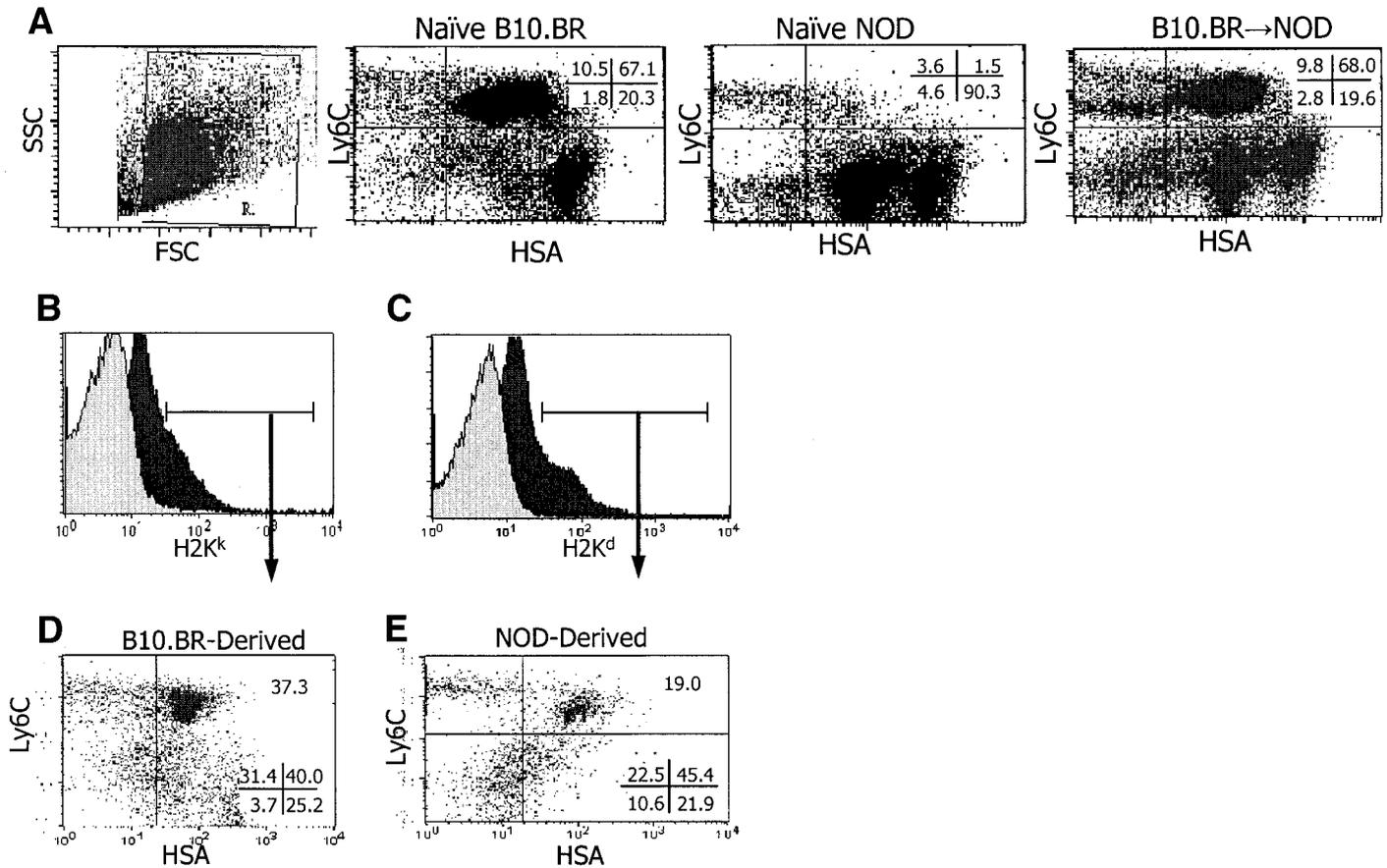


FIG. 3. The HSA<sup>+</sup>/Ly6C<sup>+</sup> population is restored in the bone marrow of fully allogeneic and mixed B10.BR→NOD chimeras. A: Percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> bone marrow cells from naive B10.BR and NOD mice and from fully allogeneic NOD chimeras is shown ( $n = 5$  animals/group). B: Bone marrow cells from five B10.BR→NOD mixed allogeneic chimeras (A–E) were analyzed for HSA/Ly6C coexpression. % donor chimerism, level of donor-derived cells in the peripheral blood lymphocytes at the time of analysis.



**FIG. 4.** The HSA<sup>+</sup>/Ly6C<sup>+</sup> population present in mixed NOD chimeras is composed of donor- and recipient-derived cells. **A:** Bone marrow cells from naive B10.BR and NOD mice and from B10.BR→NOD mixed chimeras were analyzed using the forward scatter (FSC)/side scatter (SSC) profile (left panel), and total cells (R1) were analyzed for the coexpression of HSA and Ly6C. Plots represent data from individual animals, whereas the numbers in the right upper quadrant are the average results from three mice. **B** and **C:** Strain-specific MHC class I H2-K<sup>k</sup> (**B**) and H2-K<sup>d</sup> (**C**) cells in the total bone marrow cell gate of a mixed allogeneic B10.BR→NOD chimeric mouse. Black histogram, MHC class I mAbs; gray histogram, isotype control. Positive MHC class I events were gated, and the HSA<sup>+</sup>/Ly6C<sup>+</sup> population within each specific population was analyzed (**D** and **E**). The dot plot is from a representative chimera. Numbers in the upper right of each plot are the average from three (H2-K<sup>k</sup>) or five (H2K<sup>d</sup>) individual mixed chimeras.

was similar to the HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population in naive B10.BR mice (64.9 ± 13.9%, *P* = 0.35).

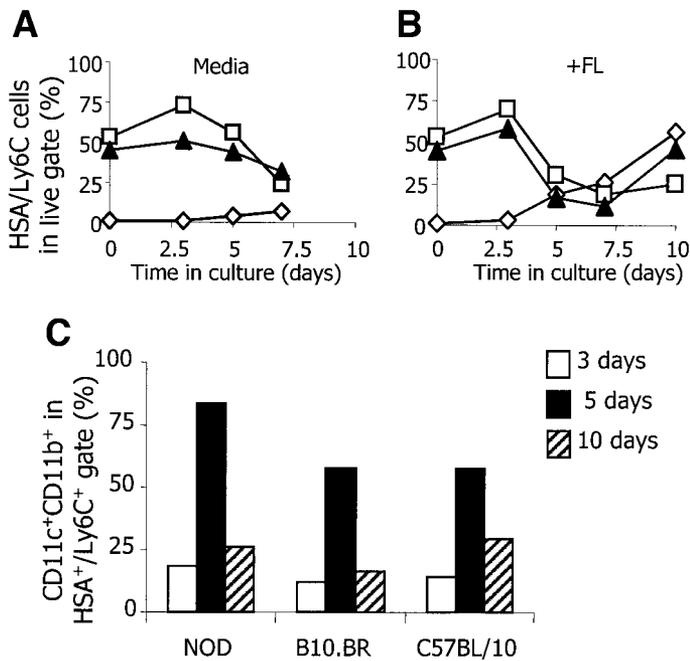
**Myeloid bone marrow cell expression in B10.BR→NOD mixed chimeric mice is similar to donor bone marrow.** We next evaluated whether the restored expression was attributable solely to the donor cells, or if NOD-derived cells contributed to the HSA<sup>+</sup>/Ly6C<sup>+</sup> population. Mixed B10.BR→NOD chimeras were prepared and typed for chimerism at 1 month. The percentage of B10.BR-derived cells in peripheral blood of these mice at the time of analysis ranged from 15.0 to 93.0% (*n* = 5). The HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population in the bone marrow from the mixed chimeras (Fig. 3B) was significantly increased over that of unmanipulated NOD mice (Figs. 1 and 3A). Additionally, the proportion of donor chimerism did not correlate with the increase in the HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population. All chimeras had levels of HSA<sup>+</sup>/Ly6C<sup>+</sup> comparable to B10.BR mice (Figs. 1B and 3A).

The HSA<sup>+</sup>/Ly6C<sup>+</sup> population was further analyzed for the relative contribution by NOD versus B10.BR bone marrow cells using flow cytometry. Figure 4A depicts a representative HSA/Ly6C stain of the total bone marrow cell population from a B10.BR→NOD mixed chimera compared with controls. Both B10.BR-derived H2-K<sup>k</sup> cells and NOD-derived H2-K<sup>d</sup> cells from chimeric mice

contributed to the HSA<sup>+</sup>/Ly6C<sup>+</sup> population (Fig. 4B–E). These data indicate that the lack of the HSA<sup>+</sup>/Ly6C<sup>+</sup> population in naive NOD mice is not due to an intrinsic inability to produce myeloid precursors, but rather due to a lack of signal(s) that would instruct precursor cells to this lineage.

The level of expression of major histocompatibility complex (MHC) class I antigens on the cells in the myeloid gate is relatively low, especially for the HSA<sup>+</sup>/Ly6C<sup>+</sup> population (Fig. 4A, left panel). To assure that the cells were of both donor and recipient origin, the analysis in Fig. 4D and E included only those cells that stained the brightest for MHC class I. There was a distinct population of NOD-derived HSA<sup>+</sup>/Ly6C<sup>+</sup> cells (Fig. 4B and C).

**In vitro culture of NOD bone marrow cells results in the expression of an HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population.** We next determined whether NOD bone marrow cells could be induced to produce the HSA<sup>+</sup>/Ly6C<sup>+</sup> population in vitro. Bone marrow cells from NOD, B10.BR, and C57BL/10 mice were harvested and placed in culture with the following hematopoietic growth factors used singly or in combination: GM-CSF, SCF, and Flt3-ligand. Interestingly, culture of NOD bone marrow cells in long-term bone marrow cell media alone resulted in a slight increase in the HSA<sup>+</sup>/Ly6C<sup>+</sup> population, from 0.6% in fresh cells to 6.8%



**FIG. 5.** NOD bone marrow cells acquire an HSA<sup>+</sup>/Ly6C<sup>+</sup> phenotype after in vitro culture with Flt3-ligand. Bone marrow cells from NOD, B10.BR, and C57BL/10 mice were cultured in the absence (A) and presence (B) of Flt3-ligand for up to 10 days. Cells harvested at the indicated time points were analyzed for HSA<sup>+</sup>/Ly6C<sup>+</sup> coexpression. One of four experiments is shown.  $\diamond$ , NOD;  $\square$ , B10.BR;  $\blacktriangle$ , C57BL/10. C: Flt3-ligand-cultured cells from NOD, B10.BR, and C57BL/10 mice were harvested at the indicated times, and the HSA<sup>+</sup>/Ly6C<sup>+</sup> events were analyzed for myeloid dendritic cells as defined by CD11c and CD11b coexpression at 3 days ( $\square$ ), 5 days ( $\blacksquare$ ), and 10 days ( $\hatched$ ) of culture. Data are from one of two experiments.

after 7 days in culture (Fig. 5A). The HSA<sup>+</sup>/Ly6C<sup>+</sup> population decreased in the B10.BR and C57BL/10 cultures with time. The HSA<sup>+</sup>/Ly6C<sup>+</sup> populations in the 7-day cultures were lower than in fresh bone marrow cells, ranging from 52.8 to 24% for B10.BR cells and from 44.7 to 32% for C57BL/10 cells. However, the percentage of cells in these cultures was always higher than that in the NOD cultures (Fig. 5A). Viable cells were not obtained on day 10, when the cells were cultured in media alone; therefore, no day 10 results are shown.

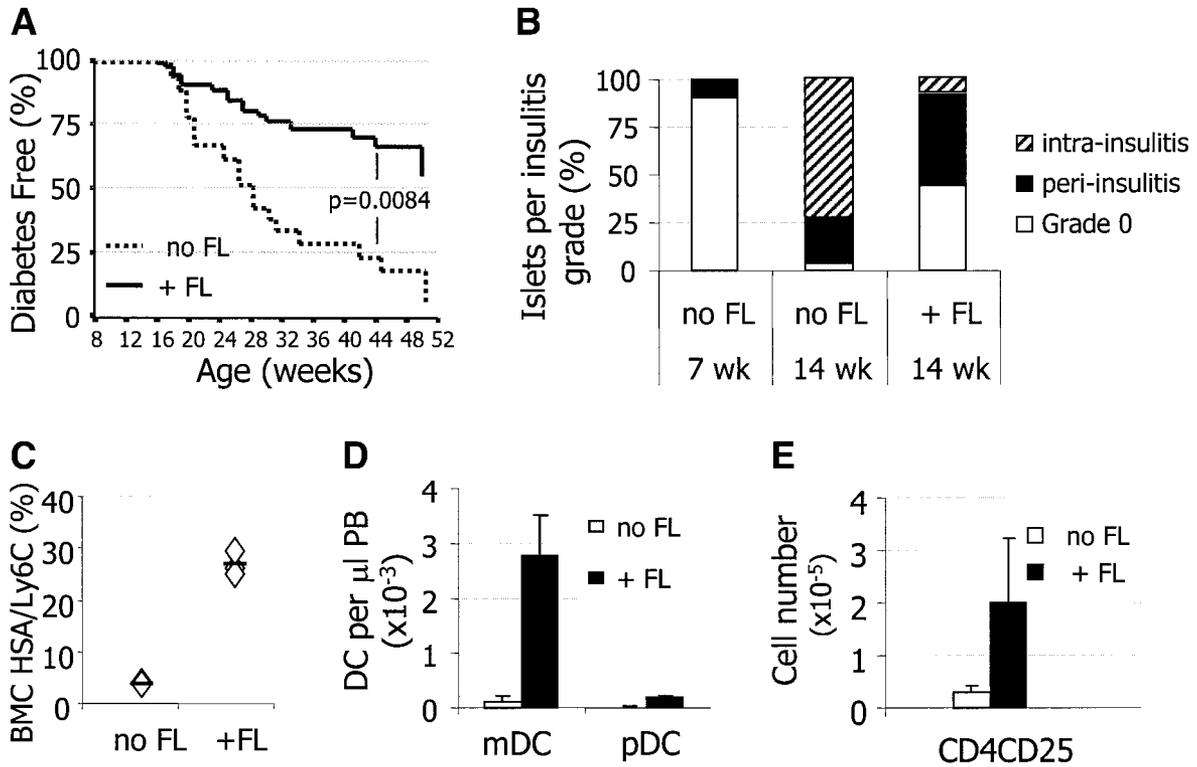
Coculture of NOD bone marrow cells with SCF or GM-CSF did not result in a significant increase in the HSA<sup>+</sup>/Ly6C<sup>+</sup> population compared with media alone. However, when Flt3-ligand was added to the bone marrow cell cultures, the HSA<sup>+</sup>/Ly6C<sup>+</sup> population was significantly increased as early as day 5 (Fig. 5B). The percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells in the culture continued to increase over time to levels similar to or greater than the control strains. The combination of Flt3-ligand, SCF, and GM-CSF was not different from Flt3-ligand alone (data not shown).

All of the HSA<sup>+</sup>/Ly6C<sup>+</sup> cells are CD11b<sup>+</sup>, with many of the cells coexpressing CD11c. When NOD bone marrow cells were cultured with Flt3-ligand, the percentage of CD11b<sup>+</sup>/CD11c<sup>+</sup> myeloid dendritic cells present within the HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population increased to a level similar to that in B10.BR and C57BL/10 cultures, with each culture reaching the highest percentage of myeloid dendritic cells on day 5 (Fig. 5C). In addition, the HSA<sup>+</sup>/Ly6C<sup>+</sup>/CD11c<sup>+</sup> population also stained brightly for MHC class II (data not shown), indicative of mature myeloid

dendritic cells. Taken together, these data indicated that culture of NOD bone marrow cells with Flt3-ligand was sufficient for differentiation of the myeloid precursor cells into mature myeloid dendritic cells, confirming the in vivo observation that precursor cells are present in NOD marrow but need the proper signal for differentiation to occur. **In vivo treatment of NOD mice with Flt3-ligand decreases insulinitis and delays onset of diabetes.** We therefore hypothesized that treatment of NOD mice with Flt3-ligand in vivo would restore myeloid cell differentiation and lead to the generation of mature myeloid dendritic cells, which would help to control the peripheral autoimmune processes that lead to diabetes. To test this hypothesis, 7- to 9-week-old prediabetic NOD mice were treated with either Flt3-ligand (10  $\mu$ g/day) or saline for 10 consecutive days and monitored for diabetes progression. The first of the untreated NOD mice became diabetic at 16 weeks of age (Fig. 6A). At 40 weeks, 75% of untreated mice had developed diabetes. In the Flt3-ligand-treated group disease progression was significantly delayed or prevented ( $P < 0.01$ ). The first conversion to diabetes in the Flt3-ligand-treated group did not occur until 24 weeks of age, and 70% of the animals remained disease-free at 44 weeks.

Insulinitis dramatically increases between the ages of 7 and 14 weeks in NOD mice (Fig. 6B). Strikingly, a significant decrease in insulinitis was detected in the 14-week-old NOD mice treated with a single 10-day course of Flt3-ligand starting at 9 weeks of age. The percentage of islets with no lymphocytic infiltration at 14 weeks was increased with Flt3-ligand treatment (4% untreated to 44.2% Flt3-ligand-treated). Although the islets exhibiting peri-insulinitis increased more than twofold in the Flt3-ligand-treated mice (22.8 vs. 47.8%, respectively), the percentage of islets exhibiting more aggressive intra-insulinitis was significantly decreased compared with untreated age-matched controls (73.2 to 8.1%,  $P < 0.05$ ).

**Flt3-ligand treatment of NOD mice increases HSA<sup>+</sup>/Ly6C<sup>+</sup> cells in bone marrow and mobilizes predominantly myeloid dendritic cells.** We evaluated Flt3-ligand-treated NOD mice for an increase in HSA<sup>+</sup>/Ly6C<sup>+</sup> cells in the bone marrow and mobilization of myeloid dendritic cells and pre-dendritic cells into peripheral blood. The HSA<sup>+</sup>/Ly6C<sup>+</sup> population is indeed significantly increased in the NOD bone marrow after Flt3-ligand treatment ( $26.8 \pm 2.2\%$ ) compared with untreated NOD bone marrow cells ( $3.7 \pm 0.3\%$ ;  $P < 0.01$ ). Although both dendritic cell subsets were detected in the peripheral blood, the majority were of myeloid dendritic cell phenotype (Fig. 6D), paralleling the in vitro data for culture of NOD bone marrow cells with Flt3-ligand. There was also a significant increase in both myeloid dendritic cells and pre-dendritic cells in the spleen and pancreatic lymph nodes after treatment with Flt3-ligand as long as 5 weeks after treatment (Tables 1 and 2, respectively). Notably, the myeloid dendritic cells obtained from the pancreatic lymph nodes after Flt3-ligand treatment were significantly increased in number and had increased cell surface expression of both CD80 and CD86. However, CD80 and CD86 expression was not increased in the myeloid dendritic cells found in the spleen (Tables 1 and 2). Increased costimulatory molecule expression is indicative of mature myeloid dendritic cells, which have been shown to delay



**FIG. 6.** In vivo treatment of NOD mice with Flt3-ligand delays progression of diabetes and significantly decreases insulinitis at 14 weeks. **A:** NOD mice were given saline ( $n = 16$ ) or Flt3-ligand ( $n = 16$ ) and monitored for diabetes. **B:** Pancreata from 7- and 14-week-old NOD mice that had received either saline or Flt3-ligand starting at 9 weeks of age were scored for insulinitis (15). □, grade 0 insulinitis; ■, grade 1–2 periinsulinitis; ▨, grade 3–4 intrainsulinitis. Data are from two combined experiments, between 120 and 300 individual islets were scored in a blinded fashion per pancreas. Individual numbers for data shown are: 7 weeks old, no Flt3-ligand ( $n = 3$ ); 14 weeks old, no Flt3-ligand ( $n = 5$ ); 14 weeks old + Flt3-ligand ( $n = 6$ ). **C:** Total bone marrow cells from age-matched untreated and Flt3-ligand-treated (+Flt3-ligand) NOD mice were stained for HSA and Ly6C immediately after therapy. Data are from individual mice (◇) and the mean (line) from one experiment.  $P < 0.01$ . **D:** The number of myeloid ( $CD11c^+/CD11b^+/B220^-$ ) and plasmacytoid ( $CD11c^+/CD11b^-/B220^+$ ) dendritic cells per microliter of peripheral blood was determined in NOD mice given either saline (no Flt3-ligand,  $n = 6$ ) or Flt3-ligand (+Flt3-ligand,  $n = 6$ ) daily for 10 days. Data presented are averages  $\pm$  SD from six individual mice from two experiments. **E:** The number of  $CD4^+CD25^+$  cells obtained from the pancreatic lymph nodes in untreated (□) and Flt3-ligand-treated NOD (■) mice. Data are the average of three individual mice from one of two experiments. BMC, bone marrow cells; DC, dendritic cells; FL, Flt3-ligand; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; wk, weeks.

diabetes onset upon adoptive transfer into naive NOD mice (13). Concomitant with the increase in mature myeloid dendritic cells in the pancreatic lymph nodes is a sixfold increase in the numbers of  $CD4^+/CD25^+$  T-cells in the Flt3-ligand-treated NOD mice compared with untreated controls ( $P < 0.05$ ), indicative of the generation of T regulatory cells (Fig. 6E).

**DISCUSSION**

We previously reported that mixed chimerism, even at low levels, prevented the development of diabetes in prediabetic NOD mice (15). The mechanism by which this occurs

has not been defined. The  $HSA^+/Ly6C^+$  myeloid precursor cell population is readily detected in the bone marrow of disease-resistant mouse strains BALB/c, B10.BR, and C57BL/10, and even in the NOD-related strain NOR, but it is nearly absent in NOD mice. The impaired production of these progenitors and their progeny has been hypothesized to contribute to the development of autoimmunity (1,3,7,12). Therefore, we evaluated whether restored production of  $HSA^+/Ly6C^+$  myeloid progenitor cells occurred in these chimeras. The results shown herein demonstrate that the NOD cells are capable of producing myeloid precursors when provided with a suitable microenviron-

**TABLE 1**

Expansion of dendritic cell subsets in the spleen and their expression of maturation markers after one 10-day course of Flt3-ligand in vivo

	Cell number ( $\times 10^{-6}$ )	Fold increase	I-A <sup>+</sup> (%)	CD80 <sup>+</sup> (%)	CD86 <sup>+</sup> (%)
mDC					
No Flt3-ligand	8.84 $\pm$ 0.8	—	81.9 $\pm$ 0.81	64.8 $\pm$ 2.8	41.7 $\pm$ 1.3
+Flt3-ligand	114 $\pm$ 28.9	12.9	51.9 $\pm$ 1.04	66.7 $\pm$ 4.7	51.5 $\pm$ 5.9
pDC					
No Flt3-ligand	4.96 $\pm$ 1.37	—	95.8 $\pm$ 0.98	34.8 $\pm$ 6.3	52.2 $\pm$ 2.4
+Flt3-ligand	29.0 $\pm$ 5.27	5.8	91.0 $\pm$ 1.39	52.9 $\pm$ 1.4	27.2 $\pm$ 0.8

Data are average  $\pm$  SD.

TABLE 2

Expansion of dendritic cell subsets in the pancreatic lymph nodes and their expression of maturation markers after one 10-day course of Flt3-ligand in vivo

	Cell number ( $\times 10^{-5}$ )	Fold increase	I-A <sup>+</sup> (%)	CD80 <sup>+</sup> (%)	CD86 <sup>+</sup> (%)
mDC					
no FL	0.01 $\pm$ 0.006	—	96.4 $\pm$ 1.1	37.0 $\pm$ 5.26	53.6 $\pm$ 5.1
+FL	13.8 $\pm$ 14.2	1380	98.5 $\pm$ 1.3	77.6 $\pm$ 9.7	61.1 $\pm$ 13.8
pDC					
no FL	0.12 $\pm$ 0.02	—	100 $\pm$ 0.0	33.9 $\pm$ 6.8	56.0 $\pm$ 6.7
+FL	1.63 $\pm$ 1.05	13.6	99.0 $\pm$ 0.6	57.8 $\pm$ 8.8	34.2 $\pm$ 9.5

Data are average  $\pm$  SD.

ment, either in vitro through culture with Flt3-ligand or in vivo after Flt3-ligand treatment or bone marrow transplantation. HSA<sup>+</sup>/Ly6C<sup>+</sup> cells are restored to normal levels in B10.BR $\rightarrow$ NOD chimeric mice and in Flt3-ligand-treated NOD mice. Treatment of NOD mice with Flt3-ligand restores production of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells in naive NOD mice. Therefore, the defect in the ability of NOD bone marrow cells to produce the HSA<sup>+</sup>/Ly6C<sup>+</sup> population is not an intrinsic defect, but rather reflects a missing signal to promote production of this lineage. Most importantly, Flt3-ligand treatment is associated with a significant reduction in the development of diabetes, reduced insulinitis, and increased production of both myeloid and plasmacytoid dendritic cell subsets in vivo.

We first reproduced the observation that bone marrow from NOD mice contained a significantly lower percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells compared with BALB/c (7). Although a significant difference between NOD and B10.BR mice in the lymphocyte-gated HSA<sup>+</sup>/Ly6C<sup>+</sup> population was detected, an even more striking difference was observed when myeloid cells were included in the analysis (Fig. 4A). Hence, total bone marrow cells were used to analyze all data reported. The HSA<sup>+</sup>/Ly6C<sup>+</sup> population detected in naive B10.BR mice has the same mean fluorescence intensity as the HSA<sup>+</sup>/Ly6C<sup>-</sup> population in the naive NOD bone marrow cells (Fig. 4), possibly representing a precursor stage where the myeloid cells accumulate due to the lack of instruction for further development.

The establishment of chimerism in NOD mice restored the production of the HSA<sup>+</sup>/Ly6C<sup>+</sup> population to normal levels. Bone marrow cells from fully allogeneic B10.BR $\rightarrow$ NOD chimeras have a distinct HSA<sup>+</sup>/Ly6C<sup>+</sup> population, significantly increased over that seen in naive NOD mice and similar to that in B10.BR bone marrow cells. This result was expected because fully allogeneic NOD chimeras have, in essence, the hematopoietic compartment of a B10.BR mouse. Strikingly, NOD mixed chimeras also developed the HSA<sup>+</sup>/Ly6C<sup>+</sup> cell population. The percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells did not correlate with the percentage of donor chimerism, as would be expected if the cells were only of donor origin. When the HSA<sup>+</sup>/Ly6C<sup>+</sup> population was stained for donor versus recipient, we indeed confirmed that NOD-derived cells contributed to the restored HSA<sup>+</sup>/Ly6C<sup>+</sup> myeloid population.

To confirm that NOD bone marrow cells could produce myeloid precursor cells, NOD bone marrow cells were cultured in the presence of hematopoietic growth factors in vitro. Steptoe et al. (10) recently demonstrated that

culturing NOD bone marrow cells in the presence of IL-4 and GM-CSF not only induced the production of myeloid dendritic cells, but the NOD cultures had a greater percentage of dendritic cells when compared with cultures obtained from other mouse strains. Here we show that when NOD bone marrow cells were cultured in vitro with long-term bone marrow cell media alone, a small increase in the HSA<sup>+</sup>/Ly6C<sup>+</sup> cells was detected (1% of the cells to  $\geq 6\%$ ). When NOD bone marrow cells were cultured with Flt3-ligand, the percentage of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells was significantly increased by day 5 in culture and approached control strain levels on days 7 and 10. Although the numbers of NOD HSA<sup>+</sup>/Ly6C<sup>+</sup> cells were low on day 3 of culture, by day 5 the levels were near to that of the control strains, reflecting normal differentiation of these cells. The decrease in the HSA<sup>+</sup>/Ly6C<sup>+</sup> population detected in the B10.BR and C57BL/10 bone marrow cell cultures in the presence of Flt3-ligand may represent a normal maturation that occurs in the HSA<sup>+</sup>/Ly6C<sup>+</sup> precursor population in response to Flt3-ligand, whereas the 5- to 10-day expression in the cultures are indicative of newly developed precursor cells (Fig. 5). Moreover, HSA<sup>+</sup>/Ly6C<sup>+</sup> expression in bone marrow cells from all three strains exhibited similar kinetics after 3 days in culture with Flt3-ligand.

Notably, culture of NOD bone marrow cells with Flt3-ligand resulted in the production of HSA<sup>+</sup>/Ly6C<sup>+</sup> myeloid dendritic cells. Interestingly, the CD11b<sup>+</sup>/CD11c<sup>+</sup> population exhibited a higher peak of expression than that of the other mouse strains on day 5 of culture. These results are reminiscent of previous reports in that the dendritic cell population absent in NOD bone marrow cells can be generated and in fact is increased over other strains once placed in culture with differentiating cytokines (10) or under competitive reconstitution conditions (19).

We next asked whether the restoration of the HSA<sup>+</sup>/Ly6C<sup>+</sup> population in vivo would delay diabetes progression. Several lines of evidence indicate that myeloid dendritic cells are important in the maintenance of peripheral tolerance in NOD mice. The induction of low-level apoptosis that leads to increased peripheral tolerance through T regulatory cells was mediated by increased numbers of myeloid dendritic cells in the pancreatic lymph nodes (20). Moreover, the transfer of ex vivo differentiated mature NOD myeloid dendritic cells decreases the incidence and progression of diabetes (12,13,21). Therefore, we hypothesized that treatment of NOD mice in vivo with Flt3-ligand would induce endogenous differentiation of myeloid dendritic cells needed to maintain peripheral tolerance, thus delaying diabetes progression. When we

treated prediabetic NOD mice with a single 10-day course of Flt3-ligand, the incidence of diabetes was significantly decreased and the time of onset also significantly delayed. In addition, there was a marked reduction of insulinitis in the NOD mice treated with Flt3-ligand. The increase of myeloid dendritic cells, as well as the sixfold increase in CD4<sup>+</sup>/CD25<sup>+</sup> T-cells in the pancreatic lymph nodes, was striking and supports the hypothesis that Flt3-ligand treatment of NOD mice enhances mechanisms of peripheral tolerance. Moreover, the observation that the myeloid dendritic cells generated in the spleen had a lower level of expression of CD80 and CD86 would further support this mechanism of disease progression because this phenotype induced a delay in disease onset after adoptive transfer in NOD mice (13).

CD4<sup>+</sup>/CD25<sup>+</sup> T regulatory cells have been shown to play an important role in the maintenance of tolerance and prevention of autoimmunity (13). NOD mice exhibit impaired production of T regulatory cells (13). It is of note that Flt3-ligand-treated NOD mice contained significantly greater numbers of CD4<sup>+</sup>/CD25<sup>+</sup> cells in their pancreatic lymph node compartment compared with untreated controls. Although the CD4<sup>+</sup>/CD25<sup>+</sup> T-cell phenotype can be indicative of newly activated T-cells, and is not strictly demonstrative of T regulatory cells, the significant increase in CD4<sup>+</sup>/CD25<sup>+</sup> T-cells in the pancreatic lymph nodes of Flt3-ligand-treated mice is suggestive of an increase in regulatory cells because it occurred in mice demonstrated to have reversal of insulinitis and a decreased incidence in overall diabetes. In addition, the increase in cells of this phenotype is reminiscent of the increase of regulatory cells in NOD mice observed after adoptive transfer of myeloid dendritic cells (13) or after the induction of low-level  $\beta$ -cell apoptosis (20), both of which were associated with diabetes prevention.

Together, the data presented here demonstrate that NOD mice are lacking a key component for the differentiation of myeloid cells, and that the induction of mixed chimerism provides a needed factor that results in the production of myeloid precursors in the bone marrow. Whether Flt3-ligand initiates a cascade of events or is the key cytokine restored by chimerism is under evaluation. Notably, Flt3-ligand culture in vitro restores the production of HSA<sup>+</sup>/Ly6C<sup>+</sup> cells and myeloid dendritic cells by NOD bone marrow cells. The in vivo treatment of NOD mice with Flt3-ligand increases the endogenous production of both myeloid and plasmacytoid dendritic cells in the pancreatic lymph nodes and the spleen and significantly decreases the incidence and delays the onset of diabetes. Further treatment with Flt3-ligand may increase the efficacy in controlling autoimmune diabetes and provide a relatively benign therapeutic approach for inducing and maintaining peripheral tolerance in autoimmune disease-prone patients.

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